Assessment of complement activation in human disease

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Chapter 3

Assessment of complement activation \textit{in vivo}: which assay when? (review)

Diana Wouters and C Erik Hack
Chapter 3

Abstract
The complement system plays a major role in host defense against infection and in inflammatory processes. The degree of complement activation reflects the intensity of inflammation and may be an objective index of disease activity and response to therapy. Assessment of complement activation is largely based on measurement of residual complement factors such as C3 and C4 or the determination of complement haemolytic function. These parameters reflect complement activation only indirectly and are quite insensitive. A more reliable approach is to detect activation products that are specifically produced during the activation process. Complement activation in vivo results in the generation of activation fragments, multimolecular complexes and the appearance of neo-epitopes on these fragments and complexes. In this review we give an overview of the various assays that have been described to measure specific complement activation products. Furthermore we discuss some important aspects of these activation products: Stability in vitro, half-life in vivo, acute phase reactivity, influence of renal function and the diagnostic value should be considered when choosing a parameter for a given condition.
Introduction

The complement system is part of the innate host defense system and contributes to the inflammatory reactions against invading pathogens. Complement activation may also occur in pathological conditions and contribute to disease symptoms. Therefore, a variety of new complement inhibitors are presently developed for clinical application that can be used to alleviate symptoms of complement-mediated diseases. In this review we will shortly discuss the complement system, in particular the assays that can be used to assess activation of the system in patients.

Complement pathways

Complement can be activated via three pathways, which all result in activation of C3, which in its turn leads to activation of a common terminal pathway (Fig. 1). The classical pathway is initiated by C1 binding to an appropriate activator such as immune complexes containing IgG or IgM antibodies. After ligand binding by the C1 subcomponent C1q, the pro-enzymes C1r and C1s, which are associated to C1q, are activated. This (auto)activation results from a fixed position of the C1r dimer by the arms of the C1q molecule in a way that the cleavage site of one C1r molecule completes the enzymatic pocket of the other C1r molecule via an induced-fit mechanism, and subsequently becomes cleaved. The enzymatic pocket of the cleaved C1r

Figure 1. Schematic overview of the complement system
molecule now becomes fixed in an active configuration and cleaves the remaining intact C1r molecule, which also becomes activated. Active C1r then cleaves C1s, which in its turn becomes activated and subsequently cleaves C4 as well as C2. The combined C4b and C2a fragments then form the classical C3-convertase.

The lectin pathway differs from the classical pathway only in that mannan binding lectin (MBL) or ficolins are the recognition molecules and MBL-associated protease (MASP)-1, 2- and 3 constitute the associated enzymes. C1q, MBL and ficolins are structurally related and are members of the collectin family (1). Similarly, C1r, C1s and MASPs are structurally related (2, 3). Apparently, these pathways have been evolved from one archetypical pathway by gene-duplication. The lectin pathway is activated upon MBL binding to carbohydrate-rich microbial structures. The alternative pathway provides an antibody-independent activation pathway and can be spontaneously initiated via a “tick-over” mechanism. This mechanism implies hydrolysis of an internal thio-ester bond in native C3, which subsequently gets a conformation that resembles that of C3b, and which can interact with factors B and D. This interaction generates another C3-convertase, C3(H2O)Bb, which cleaves small amounts of fluid-phase native C3 to give rise to low amounts of fluid phase C3b. Excessive fluid phase activation of C3 by this mechanism is prevented by the action of factors H and I. When small amounts of C3b fix onto a surface of for example microbes, where it is protected against the inhibitory action of factors H and I, it can interact with factors B and D to generate another C3-convertase, C3bBb. This activation triggered by C3b fixed on a surface where it is protected against factors I and H, is called alternative pathway activation. Whether a surface is able to activate the alternative pathway is determined by the carbohydrate environment of bound C3b. Negatively charged molecules, such as sialic acid on host cells, promote binding of fH, which abrogates further activation. In contrast, activating surfaces such as bacterial cell wall components allow binding of fB which leads to further activation of the alternative pathway. Properdin can enhance alternative pathway activation by stabilizing the C3bBb complex. All three pathways converge at the level of C3, eventually leading to the formation of the terminal pathway membrane attack complex (MAC), consisting of C5b, C6, C7, C8 and multiple molecules of C9.
Effects of complement activation

Complement activation leads to direct and indirect effector mechanisms that all contribute to protection of the host from invading organisms, often at the expense of inflammation. The MAC complex leads to pore formation and target cell lysis. During complement activation, the anaphylatoxins C3a and C5a are generated. In particular C5a is chemoattractive and can attract and activate leukocytes upon binding to specific C5a receptors on these cells. C5a and C3a have other pro-inflammatory effects as well, including enhancement of vasopermeability, smooth muscle cell contraction, mast cell degranulation and other. Furthermore, the larger fragments of activated C3 and C4 (C3b and C4b, respectively) act as opsonins, and facilitate phagocytosis which is mediated by complement receptors on phagocytic cells (CR1, CR2, CR3) and erythrocytes (CR1). In order to control excessive complement activation and to minimize damage to host cells, there are multiple soluble and membrane bound complement regulators. Soluble complement regulators such as C1-inhibitor (C1-inh), C4 binding protein (C4bp), factors H and I, S-protein (vitronectin) and clusterin restrict the action of complement in the fluid phase. Host cells are protected by membrane-bound complement regulators such as CR1, MCP (CD46), decay accelerating factor (DAF (CD55)) and CD59.

Complement and disease

Complement plays a pivotal role in humans; on the one hand complement deficiencies lead to increased susceptibility to autoimmune disease and infections. On the other hand, excessive complement activation may cause host tissue damage and inflammation. Complement activation leads to cell lysis, influx of inflammatory cells, degranulation of phagocytic cells, mast cells and basophils, smooth muscle contraction and increase of vascular permeability. Because of these effects, complement activation has been implicated in the pathogenesis of several diseases. Usually, activation of complement is not the sole cause of disease, but rather exacerbates or sustains clinical problems. Table I gives an overview of inflammatory diseases in which complement activation is thought to be involved. The degree of complement activation reflects the intensity of inflammation, and potentially provides an objective index of disease activity and response to therapy. Moreover, by determining the specific pathway(s) involved, additional information may be obtained on the pathogenic mechanisms of the disease.

The complement system may be a target for therapy. Indeed several complement inhibitors have been or are being developed such as plasma-derived or recombinant C1-inhibitor (19), soluble CR1 (20), anti-C5 monoclonal antibody (21) and others. Approved indications for complement inhibitors include hereditary angio-edema (22) and paroxysmal nocturnal hemoglobinuria (23,
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24). In addition, complement inhibitors are evaluated in diseases such as myocardial infarction, rheumatoid arthritis, and age related macular degeneration. In order to monitor pharmacodynamics of complement inhibitors, convenient and reliable assays to monitor complement activation are required.

<table>
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<tr>
<th>Disease</th>
<th>Involvement</th>
<th>Refs</th>
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<tr>
<td>Rheumatoid arthritis (RA)</td>
<td>Consumption of plasma complement</td>
<td>4-6</td>
</tr>
<tr>
<td></td>
<td>Complement activation products in synovial fluid and plasma</td>
<td></td>
</tr>
<tr>
<td>Systemic Lupus erythematosus (SLE)</td>
<td>Consumption of plasma complement</td>
<td>7, 8</td>
</tr>
<tr>
<td></td>
<td>Complement activation products in circulation</td>
<td></td>
</tr>
<tr>
<td>Ischaemia-reperfusion injury (myocardial infarction)</td>
<td>Deposition of complement in infarcted area</td>
<td>9, 10</td>
</tr>
<tr>
<td>Membranoproliferative glomerulonephritis (MPGN)</td>
<td>Deposits of complement activation products in glomeruli</td>
<td>11, 12</td>
</tr>
<tr>
<td>Multiple Sclerosis (MS)</td>
<td>Complement activation products in CSF and areas of demyelination</td>
<td>13, 14</td>
</tr>
<tr>
<td>Alzheimer's disease (AD)</td>
<td>Complement activation products in senile plaques</td>
<td>15, 16</td>
</tr>
<tr>
<td>Cardiopulmonary bypass</td>
<td>Complement activation products in circulation</td>
<td>58</td>
</tr>
<tr>
<td>Age related macular degeneration (AMD)</td>
<td>Deposits of complement in affected eye</td>
<td>17, 18</td>
</tr>
</tbody>
</table>

Table I. Inflammatory diseases with involvement of complement

Assays to measure complement activation in vivo

Generally, immunochemical measurement of total levels of complement components such as C3, C4 and factor B or haemolytic assays to assess total activity levels in biological fluids are used to evaluate complement activation. Marked reduction in serum levels of a single complement protein points to a genetic deficiency in one of the complement components, whereas the decrease of multiple complement components points to an activation process leading to consumption (or decreased synthesis). To make the distinction between acquired and inherited complement deficiency, complement activation products may be measured next to total protein
levels. Secondary complement deficiency as result of consumption is in principle accompanied with elevated levels of activation products. Decreased plasma levels of complement factors as an indication for activation are somewhat insensitive, since complement levels only decrease significantly after massive activation (presumably >10% of a given factor needs to be activated). Most complement proteins exhibit a wide normal range and serum levels are always influenced by the balance between synthesis and catabolism. Some complement proteins are acute phase proteins. So, an acute phase increase of complement proteins may mask consumption during inflammatory conditions, thereby leaving serum levels within the normal range. Altogether, a more reliable approach to assess complement activation is detection of activation products which are specifically produced during activation, in biological fluids. In the rest of the paper, we will review the various assays that have been described over the last decades to measure specific complement activation products (Table II).

Analysis of complement activation products
Complement activation in vivo can be assessed by making use of the unique properties of the complement system, such as the generation of activation fragments, multimolecular protein-protein complexes and the appearance of neo-epitopes on these fragments and complexes. Multimolecular protein-protein complexes may result from either complement deposition or interaction of a complement protease with its inhibitor. Neo-epitopes on activation products are not present on the intact proteins and may be involved in novel functional activities of the protein obtained upon activation. The use of monoclonal antibodies against neo-epitopes exposed on activation products in assays for activated complement components will minimize interference of native components in the assay.

Assays for classical and lectin pathway activation
Upon activation of C1, C1-inh binds to the serine proteases C1r and C1s; thereby releasing these components from activator-bound C1q. Early activation of the classical pathway is indicated by the presence of stable C1-inh/C1rC1s complexes in the circulation. These complexes can be measured either by radioimmunoassay (RIA) (25) or ELISA. In both assays, the C1-inh/C1rC1s complexes were originally captured by antibodies against C1s and subsequently detected by C1-inh specific antibodies. In these assays, native unbound C1s competes with C1s in the complexes, which influences the sensitivity of the assays. To circumvent this, Fure et al developed a modified assay in which a capturing antibody is used that recognizes a neo-epitope on C1-inh when complexed with its substrates (26).
<table>
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<tr>
<th>Split products</th>
<th>Analytical method</th>
<th>Half-life</th>
<th>In vitro activation</th>
<th>Pathway</th>
<th>Recommendations</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4a</td>
<td>RIA</td>
<td>Short</td>
<td>Sensitive</td>
<td>CP, LP</td>
<td>Elevated levels at impaired renal function, not suitable to monitor kidney disease.</td>
<td>28, 29</td>
</tr>
<tr>
<td>C4b/C</td>
<td>ELISA</td>
<td>10-30 min</td>
<td>Sensitive</td>
<td>CP, LP</td>
<td>Appropriate to assess acute complement activation.</td>
<td>30, 59</td>
</tr>
<tr>
<td>C4d</td>
<td>RIE, ELISA, nephelometry</td>
<td></td>
<td>Sensitive</td>
<td>CP, LP</td>
<td>Depicts as ratio to native C4, because of variation in C4 concentration. Polymorphism in C4d fragment.</td>
<td>91-98</td>
</tr>
<tr>
<td>C3a</td>
<td>RIA, ELISA</td>
<td>7 min</td>
<td>Sensitive</td>
<td>CP, LP, AP</td>
<td>In vitro generation in EDTA-containing tubes. Addition of Futhan minimizes in vitro activation.</td>
<td>46-49</td>
</tr>
<tr>
<td>C3b/C</td>
<td>ELISA</td>
<td>10-30 min</td>
<td>Sensitive</td>
<td>CP, LP, AP</td>
<td></td>
<td>30, 59, 51</td>
</tr>
<tr>
<td>C3d</td>
<td>ELISA, nephelometry</td>
<td>4 hrs</td>
<td>Sensitive</td>
<td>CP, LP, AP</td>
<td>Suitable to assess chronic complement activation. Longest half-life of all C3 split products.</td>
<td>45, 52, 53</td>
</tr>
<tr>
<td>C5a</td>
<td>ELISA</td>
<td>1 min</td>
<td>Sensitive</td>
<td>CP, LP, AP</td>
<td>Inappropriate parameter in vivo, because of rapid binding to receptors on granulocytes.</td>
<td>54</td>
</tr>
<tr>
<td>Bα</td>
<td>ELISA, nephelometry</td>
<td>Short</td>
<td>Sensitive</td>
<td>AP</td>
<td>Elevated levels at impaired renal function, not suitable to monitor kidney disease.</td>
<td>41-43</td>
</tr>
<tr>
<td>Bβ</td>
<td>ELISA</td>
<td></td>
<td>Sensitive</td>
<td>AP</td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>Complexes</td>
<td>Analytical method</td>
<td>Half-life</td>
<td>In vitro activation</td>
<td>Pathway</td>
<td>Recommendations</td>
<td>Refs</td>
</tr>
<tr>
<td>------------------</td>
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<td>---------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>C1-inh/C1r/C1s</td>
<td>RIA, ELISA</td>
<td>Short</td>
<td>Slight increase in vitro</td>
<td>CP</td>
<td>Not increased in many clinical conditions, with suspected complement activation, probably due to rapid clearance</td>
<td>25-27</td>
</tr>
<tr>
<td>C1-inh/MASP-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>LP</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C1q-C4</td>
<td>ELISA</td>
<td>Stable</td>
<td></td>
<td>CP</td>
<td>Very stable, no in vitro generation. Also suitable for complement assessment in tropical areas</td>
<td>34</td>
</tr>
<tr>
<td>MBL-C4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>LP</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C5b-9-C9</td>
<td>ELISA</td>
<td>Short</td>
<td></td>
<td>CP, LP</td>
<td>Rapidly inactivated by factor I</td>
<td>408</td>
</tr>
<tr>
<td>C4-C3</td>
<td>ELISA</td>
<td>Sensitive</td>
<td></td>
<td>CP, LP</td>
<td></td>
<td>39</td>
</tr>
<tr>
<td>C3bBb(F)</td>
<td>ELISA</td>
<td>50-60 min in vitro</td>
<td>AP</td>
<td></td>
<td>Complex dissociates in vitro, half life in vivo probably &lt; 30 min</td>
<td>44</td>
</tr>
<tr>
<td>sC5b-9</td>
<td>ELISA</td>
<td>50-60 min</td>
<td>Stable</td>
<td>CP, LP, AP</td>
<td>Very stable, no in vitro generation. Serum levels of S-protein may be limiting factor under inflammatory conditions.</td>
<td>55, 56</td>
</tr>
</tbody>
</table>

Table II. Overview of complement activation parameters
CP: Classical pathway, LP: Lectin pathway, AP: Alternative pathway
C1-inh/C1rC1s complexes are rapidly cleared from the circulation, which explains why sometimes low circulating levels of these complexes are found despite evidence of complement activation (27). Since MASP-2 is also inhibited by C1-inh, it might be valuable to develop an ELISA to detect complexes between C1-inh and MASP-2 based on the same principle. This may provide the first assay to specifically measure lectin pathway mediated complement activation, since assays that can monitor activation of this pathway specifically, have not been described up until now.

Generation of C4 cleavage products provides evidence of classical or lectin pathway mediated complement activation. At the level of C4, several activation fragments can be measured such as C4a, C4d and C4b/c which are formed upon enzymatic cleavage by C1s or MASP-2. C4a is a small anaphylatoxin, which is released after proteolytic cleavage of C4. In vivo, C4a is inactivated to C4a desArg by serum carboxypeptidase N. Quantification of the C4a desArg fragment comprises a precipitation step prior to a double competitive inhibition RIA performed in the supernatant (28). The reason for this precipitation step is that the antibodies used in the assay for C4a to some extent cross-react with native C4, and hence the latter has to be removed from samples to be tested. One disadvantage of measuring C4a is that as an anaphylatoxin, C4a has a short half life. Moreover, C4a levels may not be appropriate to monitor renal disease, since C4a is cleared by the kidneys and impaired clearance by the kidneys could account for high C4a plasma concentration (29).

Using a monoclonal antibody (mAb) that reacts with a neo-epitope exposed on the activation products C4b, C4bi and C4c (abbreviated as C4b/c) but not on native C4, C4 activation can be quantified using a relatively simple ELISA method. This neo-epitope reflects conformational changes resulting from disruption of an internal thio-ester bond. The neo-epitope specific mAb is used as capturing antibody and a polyclonal rabbit anti human C4 for detection (30). However, this ELISA, as well as other assays that detect C4 cleavage products, is influenced by in vitro generation of the activation product. Amongst others, this is due to the fact that the thio-ester within C4 is fragile in physiological fluids and is easily hydrolysed upon temperature changes. Hydrolysed C4 generated in this way may lead to artificially high responses in the assay for C4 activation fragments.

In the human circulation, C4b is rapidly inactivated by factor I (using CR1, factor H or C4bp as cofactor) resulting in the formation of C4bi. Further degradation of C4bi by factor I yields the fragments C4c and C4d. C4d can be measured in human serum by rocket immunoelectrophoresis (RIE) (31, 32), ELISA or nephelometry (33). The measurement of C4d is complicated by the polymorphic nature of C4; it is well known that the C4 polymorphism lies within the C4d
Assessment of complement activation (review)

fragment. Using monoclonal antibodies to detect C4d, one should make sure that the antibody reacts with all C4 allotypes. In general, measurements of C4 degradation fragments are complicated by the allelic variation of C4 and the high frequency of heterozygous C4 deficiency. Therefore, C4 split products given as ratio compared to levels of intact C4, may be preferred. As C4d (and also C3d) contains the structural region that mediates binding to biological surfaces, one can postulate that C4d levels in biological fluid insufficiently reflect total C4 activation. However, although this cannot be denied, in general the amount of C4 fixed to an activator will be less than 10% of the total amount of activated C4. The majority of activated C4 will be hydrolyzed by surrounding water molecules and hence the effect of underestimation will be limited.

Activated C4 binds covalently to surrounding molecules via its thio-ester, which becomes exposed after proteolytic cleavage. This results in the deposition of C4 to the activator and to proteins in the vicinity. Recently, our group described that C4 not only binds to its activator, but also to C1q, the recognition unit of the classical pathway (34). C1q-C4 complexes can be measured by ELISA and are specific activation products of the classical pathway. A major advantage of measuring C1q-C4 complexes is that unlike many other complement activation products, these complexes are very stable at different storage conditions and are not generated in vitro, at least provided the samples contain EDTA. Complexes between activated C4 and MBL would provide a specific assay for lectin pathway activation. MBL-C4 levels should be depicted as ratio to total MBL, because of the large variation in MBL concentration due to its polymorphism. However, more research is required to develop such an assay.

Elevated levels of C4d deposited on erythrocytes and thrombocytes have been described to be highly specific for SLE and may be a useful diagnostic marker (35, 36).

During classical pathway activation, C3b has been demonstrated to bind covalently to C4b attached to the target (37). Meri and Pangburn found that C3b also bound covalently to C4b in the fluid phase (38). For the detection of circulating C4-C3 complexes, Zwirner described an ELISA in which two mAbs are combined with specificities for C3b/iC3b/C3dg and C4/C4b/C4d (39). These complexes appeared to be specific for the classical pathway. In the above mentioned ELISA no discrepancy is made between direct C4-C3 complexes and larger immune complexes containing both activated C4 and C3.

During classical and lectin pathway activation complexes are formed between C4b and the inhibitor C4bp. An ELISA has been described in which these C4b-C4bp complexes are measured (40). Since C4b bound to C4bp is easily cleaved by factor I in the serum, C4b-C4bp complexes are rapidly lost, which may be the explanation for the limited use of this assay.
Assays for alternative pathway activation

Alternative pathway activation can be measured by nephelometry or ELISA using monoclonal antibodies recognizing neo-antigens on the factor B split products Ba and Bb (41, 42). Comparable to C4a, the Ba fragment is cleared by the kidneys (43), which makes this fragment less suitable for evaluation of complement activation in renal diseases.

During alternative pathway activation, activated C3b binds factor B which results in the cleavage of factor B by factor D. The so formed C3bBb complex is stabilized by properdin, resulting in the alternative pathway C3-convertase. Properdin will only bind to C3 in the presence of factor B. Therefore, C3bBb(P) complexes can be measured in an ELISA with a properdin specific antibody as a catching antibody and anti-C3 antibody as detecting antibody (44). Not only direct activation of the alternative pathway by activators such as gram negative bacteria is measured in this assay. Triggering of the amplification loop as result of initial classical or lectin pathway activation is detected as well.

Assays for terminal pathway activation

C3 is the central protein of all three activation pathways. Thus, measurement of C3 cleavage products provides information about overall complement activation. Various C3 activation products can be measured in plasma (45). C3 is broken down by C3-convertases to the smaller C3a and the larger C3b fragment. C3b is inactivated by factor I to form C3bi. Further degradation leads to C3c and C3d fragments.

The biologically active anaphylatoxin C3a is released upon C3 activation and rapidly inactivated to the more stable C3adesArg. Circulating C3a levels may be measured by RIA (46, 47), which requires a pre-assay precipitation step to separate native C3 from the C3a fragment, since the antibody used in this assay recognizes both C3 and C3a. An ELISA method developed later (48, 49) was not affected by the presence of native C3, since it makes use of a neo-epitope specific antibody against C3a.

C3b, C3bi and C3c (C3b/c) can be measured by ELISA in which the antigen is captured by a mAb that reacts with a neo-epitope exposed on activated C3, but not on native C3 (30, 50, 51). The thio-ester in C3 is fragile and is easily hydrolysed upon temperature changes. Therefore, due to improper handling of the samples, C3b/c levels may be artificially high. The C3d fragment has a long half-life in circulation. To measure C3d in serum, the activation fragment should first be separated from native C3 by PEG precipitation. The C3d fragment remains in supernatant, in which it can be detected by either nephelometry or ELISA (52, 53).
C5a can be used as indicator of terminal pathway activation. C5a levels can be measured by RIA or ELISA. However, C5a levels may not be an appropriate parameter in vivo; C5a is cleared from the circulation very rapidly by binding to high affinity receptors on neutrophils (54).

Increased concentrations of sC5b-9 (soluble terminal complement complex, consisting of S-protein, the components C5b, C6, C7, C8 and polymerized C9), reflect complement activation via each activation pathway. The sC5b-9 complex is only generated when the whole activation route is completed. The sC5b-9 complexes are not detectable in normal serum or plasma, are stable in vitro and have a relatively long half-life in vivo. S-protein levels in serum may be the limiting factor in sC5-9 formation. The complexes can be detected by ELISA with antibodies against the complement components C5 and C9. Particularly a monoclonal antibody against neo-epitopes expressed on polymerized C9 has been used to further optimize assays for MAC (55, 56).

Discussion

Excessive activation of complement may harm the host by mediating inflammatory tissue destruction and likely contributes to the pathogenesis of a number of human diseases. Detection of complement activation is not only important for assessment of disease activity in these diseases but may also help to monitor response to treatment. The preferred approach to evaluate complement activation is detection of complement activation products with assays that are specific for cleavage fragments or multimolecular complexes. Monoclonal antibodies against neo-antigens exposed on these fragments or multimolecular complexes have been shown to be suitable tools in these assays. Before assessing complement activation in vivo, it is important to consider which activation parameter is most appropriate for a given clinical condition. In the next paragraphs we will give some thoughts on aspects of activation products that may help to select an optimal parameter for assessing complement activation in vivo.

In normal situation, most complement activation products are only present in trace amounts in vivo, whereas they are rapidly generated in vitro. Therefore, the conditions of sample collection, processing and storage are critical to get results that reliably reflect in vivo activation processes. For most assays, blood should be collected in EDTA containing tubes, to prevent in vitro activation of both the classical and alternative pathway by chelating Ca^{2+} and Mg^{2+}. The plasma should be processed soon after collection and preferably stored at -70°C until analysis. Preferably, several aliquots of samples are stored since this may avoid repeated freezing and thawing of samples. Repeated freezing and thawing may result in false-positive results since the thio-ester in both C3 and C4 is fragile and gets easily hydrolyzed (57). Disruption of the thio-
ester causes conformational changes in C4 and C3 that are similar to the changes occurring upon activation. Nevertheless, samples are often collected and stored under suboptimal conditions, which may lead to artificially high levels of activation products. In these situations measurement of activation products that are stable in vitro, is preferred. C1q-C4 complexes are very stable activation products that are not generated in vitro in the presence of EDTA (34). However, these complexes only reflect classical pathway activation. To assess total complement activation in samples that were not carefully processed, measurement of sC5b-9 complexes is a reliable indicator (56).

Next to handling samples correctly, one should realize that complement activation products have different clearance rates. This has an effect on circulating levels of these products. Table III shows a calculation model for C3 cleavage products demonstrating to what extent serum levels of these activation products are influenced by their different half-lives in vivo. Theoretically, one would expect comparable molar increases of the individual C3 activation products after induction of complement activation. However, the concentrations of these products may differ markedly, since small fragments are in general more rapidly removed from the circulation than larger fragments. The median normal C3 concentration in serum is 1.5 g/L, so when all C3 is converted, the molar concentration of each C3 cleavage product would be 8283 nM. Table III shows the molar concentration of C3a, C3b/c and C3d after acute activation of 5%, 10% or 25% of total C3. Half-lives of these products are 7 minutes, 20 minutes and 4 hours respectively. In case of acute conversion of 5% of total serum C3, elevated C3d levels can be measured up until 4 hours after induction of complement activation, whereas C3a and C3b/c levels have returned to normal values already after 1 hour. Notably, the formation of C3d does not occur instantaneously following acute activation as the breakdown of C3bi into C3c and C3d by factor I and cofactors takes some time. At 10% C3 conversion, elevated C3b/c can be detected up to one hour after the event. However, increased C3a levels can only be detected very shortly after the event, due to its shorter half-life. Even at 25% C3 activation, C3a levels are again within the normal range one hour after induction of complement activation. Thus, C3d levels stay longer increased after induction of complement activation than levels of C3b/c and C3a values, which rapidly return into the normal range. The comparison of activation products however can be very useful, as a high concentration of C3d with normal C3b/c may indicate acute activation of short duration, whereas high levels of both parameters point to strong ongoing activation. In case of mild ongoing activation, again C3d levels may be elevated.
The effect of different half-lives of C3 cleavage products on the levels that can be measured in patients is illustrated in a study on complement activation after cardiopulmonary bypass surgery (58). In this study, C3a and C3b/c levels were measured as parameters of complement activation. After induction of complement activation during surgery, the molar concentration of C3b/c was almost tenfold higher than that of C3a, whereas initially these products were equally increased. Knowledge of the clearance rates of complement activation products is therefore important to select the most appropriate activation products for clinical and experimental studies. In chronic conditions, complement activation products with a long half-life are preferred like C3d or sC5b-9. On the contrary, for acute activation processes products that are rapidly generated and cleared are more appropriate such as C3a, C3b/c and hence elevated levels of these products point to a recent or still ongoing activation process.

In this review we gave a short overview of the various assays that are currently available to measure complement activation in vivo. In conclusion, for reliable assessment of complement activation in clinical settings the following characteristics are important to consider: in vitro stability of the activation product, half-life in vivo, acute phase reactivity, influence of renal function, pathway specificity and diagnostic or prognostic value.

<table>
<thead>
<tr>
<th>% C3 activation at t = 0 h</th>
<th>Parameter</th>
<th>0 h</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>24 h</th>
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</thead>
<tbody>
<tr>
<td>5%</td>
<td>C3a (nM)*</td>
<td>414</td>
<td>1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>C3b/c (nM)**</td>
<td>414</td>
<td>52</td>
<td>7</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>C3d (nM)***</td>
<td>414</td>
<td>362</td>
<td>311</td>
<td>207</td>
<td>7</td>
</tr>
<tr>
<td>10%</td>
<td>C3a</td>
<td>828</td>
<td>2</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>C3b/c</td>
<td>828</td>
<td>104</td>
<td>13</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>C3d</td>
<td>828</td>
<td>725</td>
<td>621</td>
<td>414</td>
<td>13</td>
</tr>
<tr>
<td>25%</td>
<td>C3a</td>
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<td>5</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>C3b/c</td>
<td>2070</td>
<td>259</td>
<td>32</td>
<td>0.5</td>
<td>nd</td>
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<tr>
<td></td>
<td>C3d</td>
<td>2070</td>
<td>1811</td>
<td>1553</td>
<td>1035</td>
<td>32</td>
</tr>
</tbody>
</table>

**Table III.** Calculation model for C3 cleavage products

*Half-life C3a: 7 min, normal value < 6 nM; **Half-life C3b/c: 20 min, normal value < 57 nM;***Half-life C3d: 4 hrs, normal value 41-257 nM

Total C3 concentration: 1500 μg/ml (8283 nM), nd: not detectable
Chapter 3

References


