Assessment of complement activation in human disease
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Chapter 7

Summarizing Discussion
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Complement activation plays a pivotal role in human health and disease. On the one hand, it protects against invading pathogens and is involved in the clearance of apoptotic cells and debris. Deficiencies of complement may therefore lead to increased susceptibility for microbial infections or autoimmune diseases, depending on the nature of the complement component lacking. On the other hand, excessive complement activation can contribute to inflammation and tissue injury and has been described to play a role in the pathogenesis of many diseases. The degree of complement activation reflects the intensity of inflammation, which provides objective information about disease activity and response to therapy. Assessment of complement activation may therefore be helpful in the diagnosis of several diseases.

In this thesis various aspects of complement activation as diagnostic tool were investigated. Most complement activation products are easily generated due to in vitro activation. This makes it difficult to use complement activation products as diagnostic tool. To circumvent this problem, we set up a novel assay to measure classical complement pathway activation, which assay is not influenced by in vitro artefacts. We applied this assay in different clinical conditions and studied the exact composition of the activation products. Furthermore, we discuss the complex genetics of C4 and developed various methods to study the C4 polymorphism both on genetic and protein level.

Complement in diagnostics
In general, total levels of complement proteins such as C3 and C4 are measured as a diagnostic parameter for complement activation. Decreased C3 and C4 serum levels point to consumption. However, the serum concentration of complement proteins is always the result of a balance between production and consumption. Moreover, low protein levels may also reflect a genetic deficiency.

C4 polymorphism
The difficulty of measuring total protein levels as indication for complement activation is best illustrated by the C4 protein. C4 serum concentration is highly influenced by genotype, as described in Chapter 6. The C4 protein exists as two main isotypes: C4A and C4B, which are encoded by separate genes and may occur in multiple copies (copy number variation). Serum levels of both C4A and C4B are closely related to their gene copy number. Furthermore, C4 serum levels are influenced by the presence of null alleles and a retroviral insertion (HERV-
K(C4)) in a large proportion of C4 genes. This HERV-K(C4) insertion negatively influences protein expression, leading to lower serum levels. Total C4 serum levels consist of both C4A and C4B. Associations with disease however are usually found with deficiency in one of the isotypes, because they differ in function. Furthermore, it may be possible that either C4A or C4B are preferentially consumed in some conditions. Therefore, it might be helpful to measure C4A and C4B levels separately next to (or instead of) total C4 serum levels.

For accurate determination of C4A or C4B deficiencies, phenotyping should always be accompanied by genotyping. Current techniques to study C4 geno-or phenotyping are not suited for studying large patient cohorts, since they are very laborious and difficult to interpret. Therefore we developed novel techniques to facilitate a high throughput analysis for the C4 polymorphism. To this end, an MLPA assay and isotype-specific ELISA’s were set up which are described in Chapter 6. We observed a highly significant correlation between the results of these assays.

**Detection of complement activation products**

Instead of detecting a drop in total protein levels, measurement of specific activation products is more informative to assess complement activation in vivo. Complement activation products are only present in trace amounts in normal serum and are quickly elevated during inflammation. In the past decades, several assays have been described to measure complement activation products to establish the degree of inflammation. In Chapter 3 we give an overview of these assays and show that it is very important to carefully consider which parameters should be measured to assess complement activation in different situations. Characteristics of complement activation products such as clearance rate and sensitivity for in vitro artefacts should guide the choice of the correct parameter. For example, activation products with a short half-life (such as C3a) are more suitable to monitor acute activation processes and activation products with a long half-life (such as sC5b-9) better can be measured in case of chronic conditions.

**C1q-C4 complexes**

Most assays that detect complement activation products are very sensitive for in vitro artefacts, due to the fragility of complement proteins. Nevertheless, samples are often collected and stored under suboptimal conditions leading to these artefacts. In Chapter 2 we describe a novel ELISA assay to detect activation products of the classical pathway, which is not influenced by in vitro artefacts. During classical pathway activation, C4 binds covalently to the C1q molecule via its
thio-ester. The so-formed C1q-C4 complexes are exclusively formed during classical pathway activation. In contrast to most other complement activation products, levels of these complexes remain constant upon repeated freeze-thaw cycles and during several storage conditions. This makes C1q-C4 complexes a robust parameter to assess complement activation in case of suboptimal storage conditions.

**Levels of C1q-C4 complexes in disease**

Using this newly developed assay, we assessed classical complement pathway activation in various clinical conditions. Significantly elevated levels of C1q-C4 complexes were observed in patients undergoing cardiopulmonary bypass (CPB) surgery and in patients with rheumatoid arthritis (RA), which are both conditions with known classical pathway activation. In CPB patients, much higher serum levels of C1q-C4 complexes were observed than in RA patients (Chapter 2). During CPB surgery, protamine is administered to the patients to neutralize heparin. It has been demonstrated before that protamine/heparin complexes induce CRP-mediated classical pathway activation (1). A surgical procedure such as CPB is an acute event of classical pathway activation. Therefore, we may assume that the majority of C1q-C4 complexes that were formed upon activation were not yet cleared at the time of sampling. In contrast to CPB surgery, RA is a chronic condition in which classical pathway activation is constantly ongoing. C1q-C4 levels are significantly elevated in RA patients as compared to healthy controls, but probably only trace amounts of the originally formed C1q-C4 complexes are detected. Presumably, most of the complexes are cleared from the circulation at the time of blood collection. C1q with C3/C4 fragments attached to it gets most likely cleared at a faster rate than uncomplexed C1q, because of the contribution of complement receptors on phagocytes.

In RA, we observed a positive correlation between the severity of clinical symptoms and the level of circulating C1q-C4 complexes, indicating this might be a helpful tool in assessing disease activity (Chapter 4). It has been shown before that at least part of classical pathway activation in RA patients is CRP-mediated. The other part may be due to immune complexes (2), although little evidence has been found to support this (3). It is not clear whether classical pathway activation plays a role in the pathogenesis of RA or is an epi-phenomenon during disease. Recently, it has been shown in antibody-induced animal models for RA that the classical pathway is totally dispensable in the pathogenesis of disease, but the alternative pathway is absolutely required (4, 5). In these disease models, antibodies are capable of inducing the alternative pathway of complement, while bypassing C1q.
In humans, the presence of rheumatoid factors (RF) and anti-CCP antibodies is predictive for the development of RA. The pathogenic role of these antibodies is not completely clear yet. In line with the published results in animal models, it would be interesting to study whether these antibodies are capable of activating the alternative pathway of complement without requirement of C1q. However, assays to monitor alternative pathway activation don’t exclude involvement of either the classical or lectin pathway, since the amplification loop is initiated by both these pathways and cannot be distinguished from direct alternative pathway activation. Instead of being pathogenically important, it may well be that classical pathway activation as detected in RA patients is a reflection of ongoing inflammation in the synovium. Apoptotic and damaged cells are constantly removed from the circulation. Since CRP is known to play an important role in the clearance of apoptotic cells, it is not very surprising to find CRP-mediated classical pathway activation in RA patients which correlates with disease activity.

**Functional activity of C1q after deposition of C3 and C4 fragments**

Deposition of C3 and C4 activation fragments on C1q not only allows us to develop an assay to analyze classical pathway activation; it could as well affect C1q activity. In Chapter 5 we show that the formation of C1q-C3/C4 complexes results in diminished haemolytic activity of the C1q molecule. C1q-C3 and C1q-C4 complexes were isolated by affinity chromatography from a C1q preparation that was purified from healthy donor plasma. The majority of the complexes present in this C1q preparation were artificially generated during the purification procedure. Upon incubation of recalcified plasma with 4% PEG, which is the first step in the isolation of C1q, activation of the classical pathway of complement is induced. This results in artificially high levels of C1q-C3/C4 complexes, consisting of C1q with either the larger cleavage fragments (C3b/bi and C4b/bi) or their degradation products (C3d and C4d). This is in contrast to the complexes that can be detected in human plasma, that only consist of C1q with the late cleavage fragments C3d or C4d attached to it. Presumably, factor I in human serum, together with its cofactors, cleaves C3b and C4b into the smaller fragments C3d and C4d. This has also been shown for CRP-C3 and CRP-C4 complexes (6).

C1q-C3 and C1q-C4 complexes isolated from healthy donor plasma are less effective in restoring the haemolytic activity of C1q-deficient plasma as compared to uncomplexed C1q. It would be interesting to study the composition of C1q-C3/C4 complexes in patients with elevated serum levels of these complexes. The inhibiting effect of C3/C4 deposition on C1q may be more pronounced in patients. For example, the density of C3 and C4 deposits on C1q might be higher, which may lead to a more dramatic loss of function. In addition, C1q-complexes that circulate in
patients may carry C3 and C4 fragments on the same C1q molecule in contrast to healthy donors in which C3 and C4 fragments are attached to separate C1q molecules. Finally, next to the smallest activation fragments of C3 and C4 that are bound to C1q from healthy donor plasma (C3d and C4d), larger fragments (C3b/bi and C4b/bi) may be present on C1q in patient plasma, which possibly has a more pronounced effect on C1q activity.

In Chapter 5 we describe a loss of function of C1q as result of deposition of C3 and C4 activation fragments on the molecule upon classical pathway activation. However, we should also consider the possibility that C1q might gain in other functions as a result of C3 and C4 deposition. Some of the biological effects ascribed to C1q may be explained by C3 or C4 deposition on the molecule.

For example, Klickstein et al described that C1q is able to bind to complement receptor 1 (CR1), which is a membrane bound receptor for C3b and C4b, involved in the clearance of immune complexes by erythrocytes (7). C1q binding to CR1 could well be mediated by C3 and C4 activation fragments present on the C1q molecule. A drawback of this hypothesis is that we only detected complexes between C1q and the smaller fragments C3d and C4d in human plasma (Chapter 5), while the ligands for CR1 are C3b and C4b. However, we only studied the composition of C1q-C3/C4 complexes in healthy donor plasma containing low levels of C1q-C3/C4 complexes. This does not exclude the possibility that during massive complement activation, as may occur in several disease states, complexes between C1q and C3b and C4b do exist. This is supported by the fact that upon in vitro activation of the classical pathway by IgG aggregates or incubation with PEG, C1q-C3b and C1q-C4b complexes can be detected.

Furthermore, it has been described by the same group that MBL, the recognition molecule of the lectin pathway of complement, is also able to bind to CR1 (8). Pilot experiments of our group revealed that MBL-C3/C4 complexes are formed upon lectin pathway activation similar to C1q-C3/C4 complexes during classical pathway activation (data not shown). It would be interesting to analyze whether MBL binding to CR1 is mediated by C3 and C4 fragments present on the molecule as we suggest for C1q.
Summary
In summary, assessing complement activation may be helpful to assess the inflammatory status in several diseases. Measuring C3 and C4 consumption as reflection of activation is insensitive and is hampered by the complex genetics of the C4 protein. We set up assays on both protein and genetic level to study the C4 polymorphism. To assess complement activation, it is better to measure activation products that are specifically produced during complement activation. To circumvent in vitro artefacts that influence most complement activation assays, we set up a novel assay to specifically detect classical pathway activation, i.e. complexes between C1q and activated C4 (C1q-C4 complexes). This new complement parameter is very stable and not influenced by in vitro artefacts. We observed elevated C1q-C4 levels during CPB surgery and in RA patients, where C1q-C4 serum levels correlate with disease activity. Furthermore, we described that deposition of C3 or C4 activation fragments results in diminished haemolytic activity of the C1q molecule. This indicates that deposition of C3 or C4 on C1q is not only a bystander effect, but also has a regulatory function.

References


