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Complement and Disease:
Activation and Control

Tom W.L. Groeneveld

Complement and Disease: Activation and Control

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Fulget amica dies,
iam fugere et nubila et procellae.
W.A. Mozart 1773

voor mijn ouders

Contents

Abbreviations:

AP	Alternative Pathway
AP50	Alternative pathway hemolytic assay
ATCC	American Type Culture Collection
C1 INH	C1 Inhibitor
C1q-HA	C1q-dependent hemolytic assay
C4BP	C4b-Binding Protein
CH50	Classical pathway hemolytic assay
CLR	Collagen-Like Region
CP	Classical Pathway
CR1	Complement Receptor-1
CRD	Carbohydrate Recognition Domain
CRT	Calreticulin
DAF	Decay Accelerating Factor
DMEM	Dulbecco's Modified Eagle Medium
FcRγ	Fc Receptor γ-chain
GAG	Glycosaminoglycan
GBM	Globular Basement Membrane
HNP-1	Human Neutrophil Peptide 1
HUS	Hemolytic Uremic Syndrome
HUVS	Hypocomplementemic Urticarial Vasculitis
IC50	50% Inhibiting Concentration
IL-8	Interleukine-8
LP	Lectin Pathway
LPS	Lipopolysaccharide
MAC	Membrane Attack Complex
MASP	MBL-Associated Serine Protease
MBL	Mannose Binding Lectin
MCP	Membrane Cofactor Protein
MCP-1	Monocyte Chemoattractant Protein 1
MPO	Myeloperoxidase
PMN	Polymorphonuclear
SLE	Systemic Lupus Erythematosis
SLRP	Small Leucine Repeat Proteoglycans
SP-A	Surfactant Protein A
SP-D	Surfactant Protein D

Introduction

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Contents Chapter 1

1. Introduction

The complement system plays a dual role in human disease, being on the one hand a key player in innate host defense and on the other hand a frequent cause of unwanted and harmful inflammation. Therefore, appropriate control of complement activation is necessary. The current thesis deals with the role of complement, especially the classical pathway of the complement system, in inflammatory disease, and with various options for modulation of complement activation by the host. These topics will be introduced in the present chapter.

1.1 The complement system

The complement system is part of the innate immune system and is an element of the first-line defense of the host against pathogens. It is made up of over 30 proteins that take either part in the complement activation cascade or act as regulators of activation. Four pathways of complement activation are currently known to exist; the classical- (CP), the lectin- (LP), the alternative pathway (AP) and the pathway known as the C2 bypass (See *Figure 1*).

All pathways converge after activation at the level of C3 and follow a common route thereafter, though they differ in the way they arrive at this point. The CP and LP pathways are activated by binding of pathway-specific recognition molecules to their ligands. The recognition molecule of the classical pathway, C1q is an abundant molecule that consists of a head domain that recognizes ligands and a collagen-like tail domain that is involved in receptor binding and signaling. C1q binds via its head domains e.g. to IgG and IgM that are attached to their antigen, but also binds for example LPS.

In the presence of calcium, i.e. in circulation, C1q is associated with a serine protease quartet of two C1r and two C1s molecules that together form the C1 complex. Upon binding to its ligand, the C1q molecule activates the C1r molecule that in turn cuts and activates C1s. C1s then cleaves subsequently the C4 molecule into the small C4a and the bigger C4b. C4b binds covalently via an exposed reactive thioester to the activating surface of e.g. a pathogen. Next, a C2 molecule is cleaved by C1s into C2a and C2b. C2a associates with C4b to form the C4bC2a (the classical C3 convertase). This convertase is able to cleave C3 into C3a and C3b. The lectin pathway is activated by several recognition molecules; Mannose Binding Lectin (MBL) and L-, M-, and H-ficolin. MBL has a similar structure as C1q, and recognizes specific hydroxyl groups in carbohydrates like e.g. mannose, fucose and N-acetyl-glucosamine (GlcNac) [1] [2] via its calcium-dependent carbohydrate recognition domains (CRD) located at the head part of the molecule and also possesses a collagen-like tail.

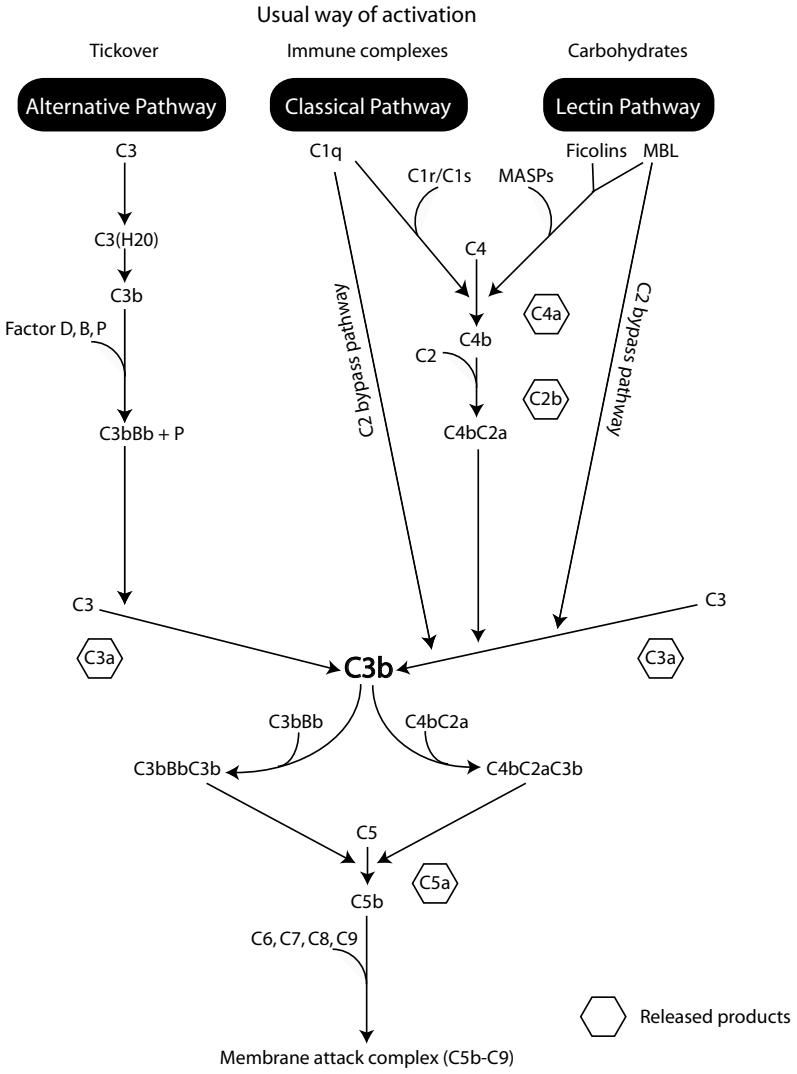

Figure 1. The four pathways of complement ativation.
The activation routes of the classical-, lectin-, alternative- and the C2 bypass pathway are depicted. Complement cleavage products that are released but do not play a role in the activation cascade are depicted in hexagonals

MBL is not associated with C1r and C1s but with other serine proteases specific for the LP, the so-called MBL-associated serine proteases (MASPs).

These molecules subsequently activate C4 and C2 in similar way as C1r/C1s for the CP. The C4b2a complex that is formed is identical to the CP convertase. The more recently discovered H and L -ficolins have a similar action via the MASPs as MBL, while M-ficolin is considered a membrane-bound molecule [3]. The ficolins also recognize carbohydrates like e.g. GlcNac, but unlike MBL bind to patterns of acetyl groups [2].

The C2 bypass [4] [5], makes use of the same starting molecules of the lectin and classical pathway, namely C1q and MBL. However, when these molecules are bound to their ligands they are shown to be able to directly activate C3 without the use of the C1r/s, MASPs, C4 and C2 molecules. The contribution of the bypass mechanism is considered to be minor compared to the activation via the classical convertase route, though it is believed it can play a more important role in situations of complement deficiency by providing rudimentary protection.

In contrast to the other pathways, the alternative pathway has no actual recognition molecule. C3, the 'start' molecule of the AP undergoes continuous hydrolysis into C3(H_2O) in the circulation, generating ultimately C3b and C3a. C3b attaches covalently to surfaces and then binds factor B. When factor B is cleaved to Bb by factor D, the C3bBb complex is formed which is stabilized by the presence of properdin (P). This complex, the alternative pathway C3 convertase, is able to cleave successive C3 molecules.

Both the classical and alternative C3 convertases result into more binding of C3b to activating surfaces [6, 7]. Surfaces not equipped with inhibitors that remove or convert the bound C3b, e.g. pathogens and immune complexes (IC), activate the subsequent steps in the complement activation cascade that lead to the generation of the C5b-9 membrane attack complex (MAC). Eventually, continuous C3 activation leads to both convertases binding an extra C3b molecule and thus generating the alternative and classical C5 convertases, i.e. C3bBbC3b and C4bC2bC3b. C5 is then cleaved by these convertase into C5b and C5a. C5b binds subsequently C6, C7 and C8, which makes it possible for C9 molecules to bind and insert in a pore-like configuration into the membrane of a pathogen.

1.2 Effects of complement activation

Activation of the complement system results in formation of activation products that have effects like opsonisation, chemotaxis and formation of the membrane attack complex, leading to clearing of pathogens and immune complexes.

The coating of pathogens or ICs by proteins of the complement system termed 'opsonisation' marks them as 'targets to be removed' for the immune system. Already the recognition molecules C1q and MBL serve as marker molecules. They facilitate uptake of their bound targets by effector cells like macrophages and dendritic cells, by binding to receptors present on the surface of these cells. Furthermore, C4b and C3b present on targets can bind to the Complement Receptors (CR)1-3 and will be taken up by effector cells [8].

The major complement components with chemo-attracting capability (anaphylatoxins) that are known are the C3a and C5a activation products. Release of these molecules leads to influx of effector cells, like neutrophils and macrophages, to the site of complement activation. The most potent of the anaphylatoxins, C5a binds to the C5a-receptor (C5aR) on these cells, gives a survival signal to neutrophils, induces upregulation of complement receptors on the membrane, elicits an oxidative burst and increases the ability of the neutrophils to interact with endothelial and epithelial cells. [9, 10]

The Membrane Attack Complex (MAC) consists of a complex of the complement proteins C5b-C9, resulting in pore formation by single or multiple C9 molecules in the membrane of the target. The effects that these pores can have depend on the density of the pores and the type of the cellular target: lower amounts do not kill a nucleated cell directly but lead to alterations of the homeostasis [11], e.g. de-regulation of the Ca^{2+} homeostasis of the target cell, which ultimately can lead to killing of the cells by necrosis. Nauta et al. have also shown that MAC activation can lead to apoptosis [12]. Higher concentrations of the MAC in the cell membrane can lead to direct lysis of the target [11].

1.3 Regulation of complement activation

Activation of the complement system can potentially lead to an uncontrolled inflammatory reaction with associated tissue-damaging effects. At the same time the complement system is continuously activated by numerous ligands, each requiring different levels of response by the host. Control of the amount of activation by complement regulators ensures that this response is appropriate in a typical individual.

Several inhibitory proteins control activation at different levels in the cascade by regulating e.g. the amount of C3 and C5 convertases and the presence of activation products like C3b. Currently, a number of naturally occurring regulators of the complement system have been discovered in humans: Factor I (fI), Factor H (fH), C4b Binding Protein (C4BP) and C1-inhibitor (C1 INH) are circulating molecules, while CD59, Decay Accelerating Factor (DAF, CD55), Complement Receptor type 1 (CR1, CD35) and membrane cofactor protein (MCP, CD46) are present on the cell surface. (*Figure 2*)

C1-inhibitor is a serpine-type proteinase inhibitor that inhibits complement activation via both the C1 complex and the MBL-MASP complex by acting as a pseudo-substrate for these complexes. After binding it traps and inactivates them [13].

C4b-Binding Protein (C4BP) acts on the CP and LP pathway by directly binding C4b, thus preventing activation of the successive proteins. Furthermore, C4BP has decay accelerating effects on the classical C3 convertase and acts as a cofactor for Factor I [14] .

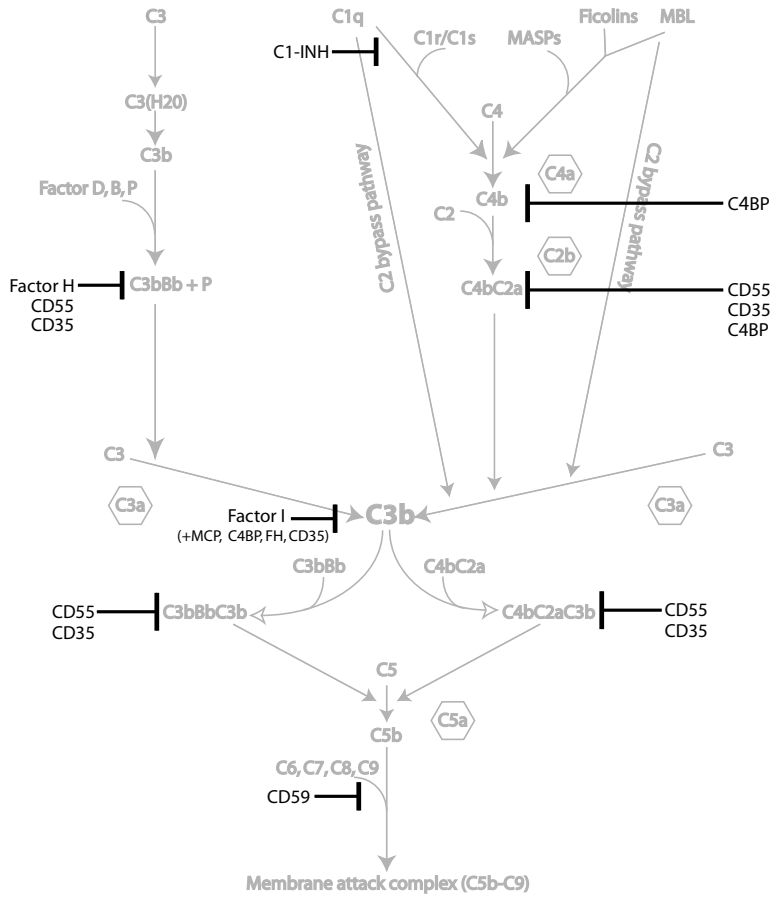

Figure 2. Control of the complement cascade
Points where inhibitors associated with the complement system are known to interact with the classical, lectin and alternative activation routes. C1-Inhibitor (C1-INH), Decay Accelerating Factor (DAF, CD55), Complement receptor 1 (CR1, CD35), Membrane Cofactor Protein (MCP, CD46) (⊣ Inhibition)

Factor I is a complement inhibitor that can inactivate C3b molecules by cleaving them to iC3b and subsequently to C3dg. These degradation products are not able to participate in the C3 convertase anymore, but still function as opsonins [15]. However, the inactivation of C3b by Factor I requires the presence of either factor H, MCP or C4BP as cofactors.

Factor H has been shown to promote the decay of the alternative C3 convertase and acts as a cofactor for factor I [16] . The membrane-bound CD59 binds the C8 and C9 molecules present on an cell membrane and prevents additional C9 molecules to bind, thereby interfering with formation of the membrane attack complex. DAF accelerates the degradation of the C3 and C5 convertases and prevents the formation of new convertases. MCP acts as a cofactor of the factor I- mediated cleavage of C3b and CR1 has shown similar actions as DAF and MCP [17].

1.4 Complement activation in the glomerulonephritides

Complement activation is considered to have beneficial as well as deleterious effects [18]. Controlled complement activation is required to clear pathogens, cell debris and immune complexes and to interact with the adaptive immune system. However, uncontrolled 'over'-activation can lead to extensive inflammation, tissue damage, organ failure and death. Several diseases are known where complement activation plays a role in the etiology or the propagation of the disease.

Several forms of glomerulonephritis are associated with deposition of immunoglobulins in the glomerulus. Immunoglobulin deposition can lead to the interaction of C1q with its ligands, e.g. IgG- en IgM-containing immune complexes.

The pathological effect of antibody-mediated damage depends on the site of complement activation. In membranous nephropathy immunoglobulin deposition occurs subepithelially. The resulting co-deposition of C3 does not result in the influx of inflammatory cells presumably because this site is not accessible for cells residing in the blood compartment. The complement products produced in this disease are most probably released in the pre-urine. If on the other hand immune complexes accumulate in a subendothelial location, products of complement activation like C5a will be released into the bloodstream and lead to chemotaxis and activation of inflammatory cells. Examples of this kind of damage include post-infectious nephritis, anti-GBM nephritis and certain forms of lupus nephritis. The third site of immune complex deposition is the mesangium. The mesangial area is accessible for cells and molecules without the need of crossing the GBM. Immune complex deposition at this site can lead to mesangial proliferation and occasionally to extracapillary proliferation.

1.4.1 C1q deposition patterns in human glomerulonephritis

C1q deposition is only found in a limited number of glomerulonephritides associated with immunoglobulin deposition. The "full house" pattern of immunofluorescence including IgG,

IgM, IgA, C3 and C1q is the hallmark of glomerulonephritis in patients with SLE. The role of C1q and specifically that of autoantibodies against C1q in this disease will be discussed later. In idiopathic membranous nephritis, granular staining for IgG and C3 along the glomerular basement membrane is seen, whereas C1q deposition is usually not found. If C1q is detected in the presence of a morphological and immunofluorescence pattern otherwise compatible with membranous nephritis this will lead to the suggestion of WHO class V lupus nephritis or C1q-nephropathy [19, 20].

Anti-glomerular basement membrane disease, an inflammatory form of glomerulonephritis caused by antibodies directed against the α-3 chain of collagen type VI, is characterized by a linear deposition of IgG and C3 along the basement membrane. C1q-deposition is an unusual finding in anti-GBM glomerulonephritis. In membranoproliferative glomerulonephritis type I, complement deposition is dominated by C3, though C1q deposition is regularly found.

Disease	Glomerular deposition
Focal segmental glomerulosclerosis (FSGS)	Focal IgM and C3
Membrano proliferative glomerulonephritis I (MPGN I)	Granular C3, IgG, C1q along capillary wall and in mesangium
Membrano proliferative glomerulonephritis II (MPGN II)	C3 along capillary wall and in mesangium
IgA Nephropathy	IgA, IgG, IgM, C3 and MBL in mesangium
Membranous glomerulonephropathy	Granular IgG and C3 along GBM
Post-streptococcal glomerulonephritis	Granular IgG and C3 along capillary wall and in mesangium
Goodpasture syndrome	Linear IgG and C3 and occasionally C1q in GBM pattern
Lupus Nephritis	'Full house pattern ' of IgG, IgM, IgA, C1q, C4 and C3 along capillary wall and in mesangium

Table 1. Distribution of complement and immunoglobulins in glomerulonephritides**.**

An overview of site and character of complement deposition in various forms of glomerulonephritis is summarized in *table 1*. The absence of detectable C1q in the presence of glomerular staining for IgG and C3 does not exclude that the classical pathway is responsible for the complement deposition in these kidneys. Since C1q does not undergo

covalent binding to its ligands, in contrast to C4b and C3b, it may have a short half-life and can be difficult to detect. In line with this explanation, the frequent detection of C1q in lupus nephritis may be related to a local stabilizing effect of anti-C1q antibodies (see *chapter 2*).

1.4.2 Complement-mediated damage in animal models of glomerulonephritis

Numerous animal models have been studied to explore the role of complement in renal damage. Early studies of antibody-induced glomerular injury in rats linked complement-associated damage to the influx of neutrophils induced by C5a [21]. Complement depletion by administration of human IgG prior to the treatment with nephrotoxic serum markedly diminished neutrophil influx and renal damage in this model. Later it was shown that depletion of the complement system is beneficial in the non-inflammatory passive Heymann nephritis model, which morphologically resembles membranous nephropathy in humans [22]. These findings suggested neutrophil-independent mechanisms of complement-mediated damage.

Studies utilizing C6-depleted or C6-deficient rats more specifically demonstrated the importance of the terminal pathway of complement in various models of renal disease. In the anti-thymocyte serum model of mesangioproliferative glomerulonephritis the absence of C6 was associated with a marked reduction in various parameters of renal damage [23]. The effect was comparable to the administration of cobra venom factor. C6 depletion using an anti-C6 antibody abolished proteinuria in the passive Heyman nephritis model [24]. These data established the relative importance of C5b-9 in complement-mediated renal damage. C5b-9 has also been linked to interstitial fibrosis and inflammation in a non-immune mediated model of proteinuric renal disease induced with the aminonucleoside puromycine [25].

C5b-9 may exert its effect in the Heymann nephritis model by increasing the formation of oxygen radicals by podocytes [26, 27]. Similarly C5b-9 has been shown to activate endothelial and mesangial cells [28, 29]. These reactive oxygen species may then cause damage to matrix proteins and lipid peroxidation. Activation of complement in proteinuric urine has been proposed as an important mediator of chronic progressive renal damage irrespective of the underlying glomerular disease [30].

Taken together, there is clear evidence for a pro-inflammatory and pathogenic role of complement activation products in renal disease. However, the pathway that is responsible for complement activation is in most cases not established. Recent data suggested a role for the lectin pathway in complement activation in IgA nephropathy [31, 32] and in lupus nephritis

[33]. However, direct identification of mechanisms of complement activation has been hampered by the lack of appropriate animal models for complement-dependent renal disease.

1.4.3 C1q in glomerulonephritis

Few tools are available to study the relative importance of C1q in complement-mediated renal damage. As described earlier C1q deposition is regularly found in lupus nephritis but not in most other forms of glomerulonephritis. In humans there is a strong association between the homozygous deficiency of the early components of complement activation (C1q, C4 and C2) and the development of SLE [34]. It should be noted that the effect of C1q and C4 deficiency is much stronger than the effect of C2 deficiency. Gene-targeted C1q-deficient mice have been recently used to study the role of the classical pathway in SLE.

In mice the effect of C1q-deficiency strongly depends on the genetic background. Both C1q- and C4-deficient mice with a 129 x C57BL/6 genetic background develop glomerulonephritis associated with autoantibodies and accumulation of apoptotic cells [35, 36]. No evidence of glomerulonephritis was found in C1q-deficient C57BL/6 mice. Similarly C1q deficiency does not lead to a significant change in the severity of glomerulonephritis in the MLR/lpr mouse [37]. Both the high incidence of SLE with severe glomerulonephritis in C1q-deficient humans and the aggravation of lupus nephritis in certain C1q-deficient mouse strains show that activation of the classical pathway is not an essential component in the development of renal damage in lupus nephritis.

In fact C1q seems to play a protective role in the setting of SLE. This may be explained by the role of C1q in the clearance of immune complexes [38] and apoptotic cells [39-41]. Absence of C1q may lead to defective clearance of apoptotic cells leading to increased exposure of the immune system to autoantigens. This concept is underscored by the finding that high concentrations of the autoantigens identified in SLE are found in apoptotic blebs [42]. This beneficial role of C1q may not only apply to SLE. Robson et al. studied the effect of C1q deficiency in the accelerated nephrotoxic serum model of glomerulonephritis in mice, using injection of heterologous rabbit anti-GBM antibodies in mice that were immunized with rabbit IgG [43]. C1q-deficient mice developed more severe glomerular thrombosis compared to wildtype mice. This exacerbation of disease was associated with increased IgG deposits, enhanced influx of neutrophils and increased numbers of apoptotic cells. Defective processing of immune complexes seems to result in increased influx of neutrophils with Fc-receptors being more important mediators of damage than complement in this model. Using a different model, in which primary injury was studied following injection of rabbit anti-mouse GBM

antibodies in mice, Sheerin et al. [44] showed that the development of prompt renal injury in this model was C3-dependent. Although the classical pathway is very likely to be responsible for complement activation in this model, the role of C1q in such a model has not yet been studied.

1.4.4 Anti-C1q antibodies

Autoantibodies reacting with the collagenous portion of the C1q molecule [45] have been described in patients with various autoimmune diseases. In SLE, anti-C1q antibodies are detectable in 30-40% of the patients whereas this incidence is almost 100% in the hypocomplementemic urticarial vasculitis syndrome. In patients with SLE the presence of anti-C1q correlates with active renal disease. Anti C1q-antibodies have been reported to correlate with lupus nephritis with a sensitivity of 87% and a specificity of 92% [46] and a rise in the anti-C1q antibody titer has been suggested to predict renal flares [46, 47]. A number of studies have shown that patients with a negative anti-C1q titer generally do not develop lupus nephritis [48].

Obviously these data suggest an important role of anti-C1q antibodies in the development of lupus nephritis. Several animal models have been developed to study whether C1q antibodies actually are pathogenic.

Treatment of naïve mice with rabbit polyclonal antibodies directed against mouse C1q resulted in glomerular deposition of C1q and anti-C1q antibodies. Although this treatment resulted in stable deposition of C1q and anti-C1q antibodies these mice did not develop overt glomerulonephritis [49]. The injection of anti-C1q antibodies did not result in the glomerular deposition of C1q and anti-C1q in IgG-deficient Rag2-/- mice [50], suggesting that minor amounts of IgG associated with the GBM are necessary for the deposition of C1q and anti-C1q in the glomerulus.

Antibodies directed against C1q have also been described in murine models of SLE. To study whether the disease associations of anti-C1q antibodies in mice are comparable with human SLE the course of the anti-C1q titer in relation to renal and non-renal manifestations of SLE in MLR-lpr mouse has been described [51]. With increasing age of the mice a rise in the anti-C1q titer and a decrease of the C1q concentration was observed. Worsening glomerulonephritis with signs of complement activation, cell influx and loss of renal function paralleled the increase in anti-C1q.

1.5 Complement inhibitors

Since complement appears to be involved in either the etiology and/or the propagation of a number of diseases, including the above mentioned glomerulonephritides, complement is an interesting target for therapeutic intervention. Intervention may not only be used to improve the outcome of these diseases but also provides a tool to study more in-dept the contribution of complement to the development of these diseases. Several angles of approach have been used to develop inhibitors of the complement system. For instance the complement system can be inhibited at different points in the activation cascade.

Furthermore, there is a choice between using in vivo occurring natural complement inhibitors, inhibitors based on sequences of natural proteins/peptides, antibodies, and synthetic non-protein chemical agents. Much research has been focused on inhibitors that target C3, as this is the common and pivotal step in the activation of all four pathways. However, the actions of the individual pathways can also be targeted separately, which has the advantage of leaving the other pathways intact, and thus still supporting the host in the defense against pathogens and partially leaving the contribution of complement to the debris/immune complex-clearance intact. An overview of the discussed CP and LP inhibitors is shown in *figure 3.*

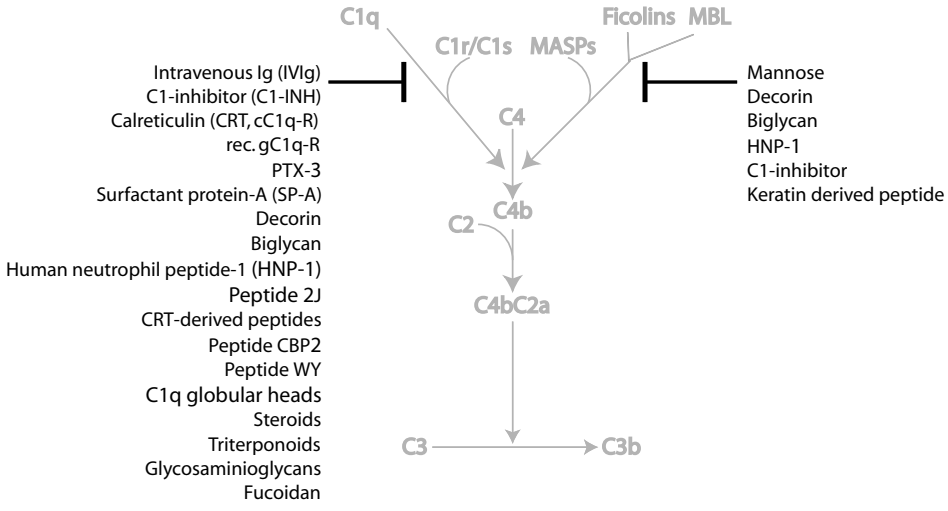

Figure 3. Classical and lectin pathway inhibitors.
Overview of the discussed inhibitors that have been shown to interact with the classical and/or lectin pathway. (⊣ Inhibition)

1.5.1 Inhibitors of the recognition molecule of the classical pathway

The molecules that are unique for the CP are C1q, C1r, C1s and the complex of these three molecules, C1. The C1q molecule is considered to consist of two important domains with distinct functions, the recognition domain, comprised out of six globular heads, and the collagen-like tail. Either domain can be a target for inhibitors that work by either preventing the recognition of C1q ligands or blocking the effects of target recognition, i.e. activation of C1r/s and the following complement activating steps or binding to receptors and uptake by cells. Both the use of natural occurring inhibitors and the application of synthetic classical pathway inhibitors are under investigation.

Intravenous Ig (IVIg) is a preparation of purified Ig obtained from plasma, and has been shown to have complement inactivating effects on several levels, including C1q. The circulating Ig molecules compete for the available C1q molecules and thereby prevent local activation [52, 53].

As described before, C1 inhibitor is a naturally occurring inhibitor of C1 and acts by binding and inactivating the C1 complex. C1-INH has a normal value of 0.25-0.45 mg/ml in blood. C1-inhibitor of therapeutic grade has been isolated from human serum and has also been produced recombinantly using a baculovirus system [54]. C1-Inhibitor is already used as treatment in hereditary angioedema where circulating or functional C1-INH is reduced, leading to uncontrolled complement activation [55], and has been shown to improve the outcome in inflammatory diseases like sepsis, Gram negative endotoxin shock, hyperacute transplant rejection, and ischeamia-reperfusion injury [56].

Calreticulin (CRT, cC1q-R) is a ubiquitous protein present on the cell surface in the cytosol and the ER. It has been shown that the N-terminal half of CRT contains a binding site for C1q [57]. The s-domain of CRT is able to prevent the formation of C1 and prevents C1q-dependent hemolysis [58] .

In 1995 Ghebrehiwet et al.[59] showed that a recombinant soluble form of the globular C1q receptor (gC1q-R) and a synthesized 18-mer peptide that is part of the gC1q-R, could inhibit the C1q hemolytic activity by binding to the globular head domain.

PTX-3 is a family member of the long pentraxins and is an acute phase protein. It has functions in the innate response against microbes and in the regulation of cellular debris scavenging. Nauta et al. [60] have shown that fluid phase PTX-3 can prevent C1q from binding to its ligand and thus prevent complement activation. However, if PTX is immobilized it acts as an activator of complement.

Surfactant Protein-A, a collectin family member present in lung surfactant, has been shown to bind to C1q but less efficiently to the C1 complex. By preventing C1r and C1s to associate with the C1q molecule it can inhibit complement activation [61].

There is also a category of described C1q inhibitors that are not closely related to the complement system. Decorin is a protein present in the extracellular matrix and has a role in the alignment of collagens. It consists of a 44 kD leucine-rich (a member of the Small Leucin-Rich Proteoglycans, SLRPs) core with an attached dermatan or chondroitin glycosaminoglycan chain. Bovine forms of decorin have been shown to bind C1q and inhibit the activation of the CP [62]. In this thesis (*chapter 3*) human recombinant decorin as well as the closely related matrix protein biglycan are shown not only to inhibit the CP by binding the C1q via head and tail interactions, but also to prevent the inflammatory effects of the C1q molecule on cells, i.e. the release of the inflammatory molecules IL-8 and MCP-1 [63]

Human Neutrophil peptide-1 (HNP-1) is a 30 amino acid large peptide and a member of the α-defensins family. It is produced mainly in neutrophils and has anti-microbial actions, presumably by MAC-like activities. HNP-1 has also been shown to bind to the C1-complex and prevents activation of the classical complement pathway as described by Van den Berg *et al*. [64] and in *chapter 5* of this thesis [65].

Furthermore, there are peptides and sequences that are either based on parts of inhibiting molecules or novel sequences that can inhibit C1q. Lauvrak et al. [66] have discovered C1q binding peptides using the phage display technique. Later, these peptides were produced and assessed for their complement inhibiting properties [67]. Several of these peptides bound readily to C1q, however one 15-mer peptide (2J) bound C1q via the globular head domain and specifically inhibited the classical pathway in different species (including *homo sapiens*) while at the same time leaving the alternative and MBL pathways intact. Unfortunately, the IC_{50} of the 2J peptide is too high to be useful in vivo.

CRT was analyzed for the C1q binding site using overlapping peptides [68]. A number of these peptides were able to inhibit the classical pathway activation as well as preventing the interaction between C1q and IgG. These peptides contain the ExKxKx motif, which is similar to a binding site of C1q on the IgG CH2 domain.

Fryer et al. [69] have investigated peptides that interfere with the interaction between C1q and antibodies. These peptides were called CBP2 (derived from the B globular head of C1q) and WY (a di-aromatic peptide derived from the binding site of C1q on IgG). These peptides

inhibit the binding of aggregated IgG to immobilized C1q, classical pathway activation in a hemolytic assay and prolong survival of guinea pigs and rats in a hart transplant model.

Other groups have also used sequences of C1q itself as a means to inhibit the classical pathway activation. Kishore et al [70] have expressed a recombinant homotrimer of the B globular head of C1q and were able to inhibit the classical pathway activation.

Another angle of approach is to make use of non-protein/peptide based inhibitors. Recently Bureeva et al. [71] have shown that modified steroids and triterpenoids can effectively and almost selectively inhibit the classical pathway.

Glycosaminoglycans (GAG) are polysaccharides that have been shown to interfere with the binding of C1q to β-ameloid [72]. Binding of C1q to β-ameloid is able to activate the classical pathway [73]. Also fucoidan, a sulfated polysaccharide derived from brown algae that possesses anti-inflammatory properties has been shown by Tissot et al. [74] to inhibit both the classical and alternative pathway. The effects on the CP pathway are mediated not only via prevention of consumption of C2, C4, and C3 but also by interfering with the association of the tetramer of C1r and C1s with C1q.

1.5.2 Lectin pathway inhibition

In comparison to C1q, the recognition molecules MBL and the ficolins are present in relatively low concentrations: 1 μg/ml for MBL and 5 and ~ 4 μg/ml for Ficolin H and L respectively [3], compared to 100 μg/ml for C1q [75]. The third ficolin, M-ficolin is not detected in serum and is thought to be a membrane-bound molecule, although recently M-ficolin was discovered to be present in the secretory vesicles of neutrophils and macrophages [3].

The main target investigated for lectin pathway inhibition is the activation via MBL, as the role of the ficolins in the complement-mediated damage in disease is not yet clear. MBL however, has been indicated to be involved in complement-mediated damage in a number of models and human diseases [76] .

MBL binding can be inhibited by using carbohydrates that bind the CRD domains and interfere with binding of these domains to their natural targets. In particular mannose is effective in inhibiting activation of the lectin pathway. However, a carbohydrate-based inhibitor that can be used in vivo has not yet been reported. In vivo, most inhibitory studies focused on MBL have used anti-MBL antibodies.

Furthermore, because of the similarity between the collagen-like tail of MBL and the collagen-like tails of the other members of the collectin family and the classical pathway recognition molecule C1q, inhibitors have been reported that work on multiple molecules. In the present thesis, for example, Human Neutrophil Peptide-1 (*chapter 5*), decorin and biglycan (*chapter 4*) are shown to share the capability to bind MBL and C1q and to inhibit activation of the complement cascades.

Interestingly, C1-inhibitor has been shown to inhibit the lectin pathway even more effectively than the classical pathway at low doses [77], via a similar mechanism.

Although MBL is a carbohydrate-binding molecule, Montalto et al. JI 2001 [78] described a keratin-derived peptide that can inhibit the LP by interfering in the binding of MBL to a carbohydrate-ligand, GLcNAc.

1.6 Scope of the thesis

Activation of the complement system provides an important mechanism of defense of an organism against invading pathogens. In the healthy individual this defense is finely regulated to prevent attack of the complement system against cells and tissues of the host, however in abnormal situations this regulation can be out of balance. In the present thesis we look at a mouse model where the classical pathway of complement, in conjunction with anti-C1q autoantibodies, is shown to be involved in the development of renal disease (*Chapter 2*). In *Chapter 3*, a novel mouse model of complement-mediated glomerulonephritis is described. This model seems to be dependent on the alternative pathway of complement activation as well as on Fc receptors, and provides a novel tool to dissect the contribution of different effector systems in renal inflammation.

Then the natural complement-inhibitory properties of the defensin Human Neutrophil Peptide-1 and the extracellular matrix molecules decorin and biglycan are investigated, which are presented to play a role in the regulation of complement in vitro, which hopefully can be extended to in vivo situations of health and disease in the near future (*Chapter 4 & 5*). The thesis is concluded with a general discussion in *Chapter 6*.

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Anti-C1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular C1q-containing immune complexes

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Abstract

Anti-C1q autoantibodies are present in sera of patients with several autoimmune diseases, including systemic lupus erythematosus (SLE). Strikingly, in SLE the presence of anti-C1q is associated with the occurrence of nephritis. We have generated mouse anti-mouse C1q mAb's and used murine models to investigate whether anti-C1q autoantibodies actually contribute to renal pathology in glomerular immune complex disease. Administration of anti-C1q mAb JL-1, which recognizes the collagen-like region of C1q, resulted in glomerular deposition of C1q and anti-C1q autoantibodies and mild granulocyte influx, but no overt renal damage. However, combination of JL-1 with a subnephritogenic dose of C1q-fixing anti-glomerular basement membrane (anti-GBM) antibodies enhanced renal damage characterized by persistently increased levels of infiltrating granulocytes, major histological changes, and increased albuminuria. This was not observed when a non-C1q-fixing anti-GBM preparation was used. Experiments with different knockout mice showed that renal damage was dependent not only on glomerular C1q and complement activation but also on Fcγ receptors. In conclusion, anti-C1q autoantibodies deposit in glomeruli together with C1q but induce overt renal disease only in the context of glomerular immune complex disease. This provides an explanation why anti-C1q antibodies are especially pathogenic in patients with SLE.

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease immunologically characterized by B cell hyperreactivity, production of a multitude of different autoantibodies, and immune complex formation (1, 2). It affects 0.04% of the general population of developed countries. Since nearly 80% of the cases occur in women in the childbearing years, it may affect as many as 1 in 1,000 young women (3). The etiology of SLE is largely unknown, but it involves genetic, hormonal, and environmental factors (4). The complement system plays an important role in the onset as well as the effector phase of SLE (5, 6) and may also be the target of an autoantibody response (7). In SLE many organs may be affected, including serosa, joints, CNS, skin, and kidney.

Lupus nephritis (LN), the renal disease that accompanies SLE, is present in 25-50% of the cases (8) and is the major cause of morbidity and mortality (9). Understanding the sequence of events leading to full-blown LN in these patients is of major importance. Anti-C1q autoantibodies have been suggested to be closely associated with LN (10). This association is concluded from the correlation between anti-C1q autoantibody positivity and renal involvement (11, 12), the predictive value of anti-C1q autoantibody titers for flares of nephritis (13, 14), and the accumulation of anti-C1q autoantibodies in LN kidneys (15, 16). Conversely, in the absence of anti-C1q autoantibodies, no LN develops (17, 18). However, no causal relationship has been established until now. Anti-C1q autoantibodies may be associated with other immune complex renal diseases as well; however, the total number of patients studied limits firm conclusions (10). Interestingly, anti-C1q autoantibodies can be found in several other conditions as well, such as the hypocomplementemic urticarial vasculitis syndrome (HUVS), and even in some healthy individuals (10), but in these instances they are unrelated to renal pathology. Anti-C1q autoantibodies also occur in murine models of SLE (19, 20). In MRL-lpr mice, rising anti-C1q autoantibody titers parallel a rise in LN, and anti-C1q autoantibodies also accumulate in glomeruli in murine SLE (21) as in human SLE.

In the present study we have investigated how anti-C1q autoantibodies contribute to the development of nephritis in mouse models. We show that administration of anti-C1q mAb's to naive mice results in glomerular deposition of C1q and anti-C1q autoantibodies but not in overt renal disease. However, administration of anti-C1q autoantibodies to mice pretreated with C1q-fixing anti-glomerular basement membrane (anti-GBM) antibodies, as a model for glomerular immune complex disease, resulted in strong synergistic enhancement of renal

disease. Therefore, anti-C1q autoantibodies can be pathogenic to the kidney but only in the context of C1q-containing glomerular immune complexes as found in SLE.

Results

Generation and characterization of anti-C1q mAb's

Following immunization of $C1q^{-/-}$ mice with purified mouse C1q, we obtained several mouse anti-mouse C1q mAb's. The stable clones JL-1, JL-2, and JL-3 were of the IgG2b, IgG2a, and IgM isotypes, respectively.

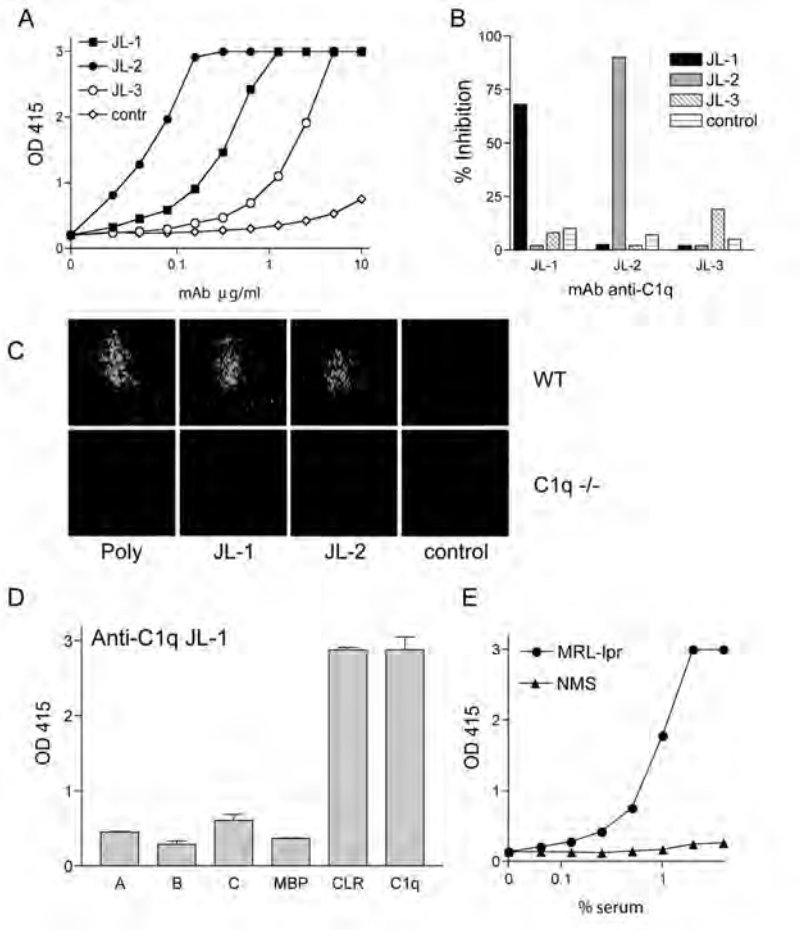

Figure 1. In vitro characterization of mouse anti-mouse C1q mAb. (A) Anti-C1q detection ELISA for anti-C1q mAb's JL-1, JL-2, and JL-3 and control mAb IgG2b under 0.5 M NaCl buffer conditions. OD415, OD at 415 nm. (B) Epitope competition ELISA showing inhibition of binding of DIG-labeled anti-C1q mAb to mouse C1q by unlabeled anti-C1q mAb or control mAb lgG2b. (C) Immunohistochemistry of WT or $C1q^{-/-}$ mouse spleen stained with anti-C1q mAb or controls. Original magnification, x250. Poly, polyclonal antibody. (D) C1q head domains of the human A, B, and C chains or the control maltose-binding protein (MBP), CLRs, and intact human C1q were coated, and mAb JL-1 binding was analyzed. (E) Anti-C1q tail ELISA using human CLRs and serum of autoimmune MRL-lpr mice (MRL-lpr) or nonautoimmune normal mouse serum (NMS) for binding.

Purified Ig's reacted in a dose-dependent fashion with C1q in ELISA (Figure IA). For all our experiments we used an IgG2b control mAb as negative control. All 3 anti-C1q mAb's recognize different epitopes, since digoxigenin-conjugated (DIG-conjugated) mAb's were only competed by the respective unlabeled mAb but not by any of the other mAb's (Figure 1B). Anti-C1q mAb's were used to stain sections of spleens of WT mice and $C1q^{-/-}$ mice as an additional argument for specificity. Both mAb's JL-1 and JL-2 stain mouse C1q specifically on follicles of spleen of WT mice, as do polyclonal antibodies, but not on spleen of $C1q^{-/-}$ mice (Figure 1C). Both mAb JL-3 (data not shown) and control mAb (Figure 1C) did not stain either the WT or the $C1q^{-/-}$ spleen. Western blot analysis showed that whereas the polyclonal antibody recognized all 3 chains of C1q, mAb JL-1 specifically recognized the A/B chain and JL-2 recognized the C chain. Neither JL-3 nor IgG2b recognized C1q in Western blots (data not shown). In humans, anti-C1q autoantibodies react primarily with the collagen-like region (CLR), or "tail," of the C1q molecule (10, 21). Using recombinant heads or tails (CLRs), we found that JL-1, which was the only mAb showing cross-reactivity with human C1q (data not shown), specifically recognized the CLR only (Figure 1D). Similarly, serum from autoimmune MRL-lpr mice, but not normal mouse serum, reacted dose-dependently with CLRs (Figure 1E). Based on these in vitro data we concluded that mAb JL-1 is the most appropriate anti-C1q mAb to test in vivo.

Anti-C1q mAb's deplete circulating C1q and deposit in glomeruli but do not induce overt renal damage

Administration of mAb JL-1 (1 mg intraperitoneally) resulted in complete depletion of circulating C1q levels at 2 hours, which remained low at least up to 24 hours, whereas injection of IgG2b control mAb did not alter circulating C1q levels (Figure 2A). Administration of mAb JL-1 resulted in an increased glomerular deposition of mouse IgG and especially mouse C1q, whereas injection of control mAb did not result in additional IgG or C1q deposition (Figure 2, B and C). Administration of mAb JL-1 to $C1q^{-/-}$ mice did not result in enhanced glomerular IgG deposition, which excludes the possibility that mAb JL-1 is reacting with glomerular antigens other than C1q (Figure 2B). Administration of mAb JL-1 resulted in a mild but significant glomerular granulocyte influx (Figure 2D). However, none of the mice developed albuminuria (Figure 2E).

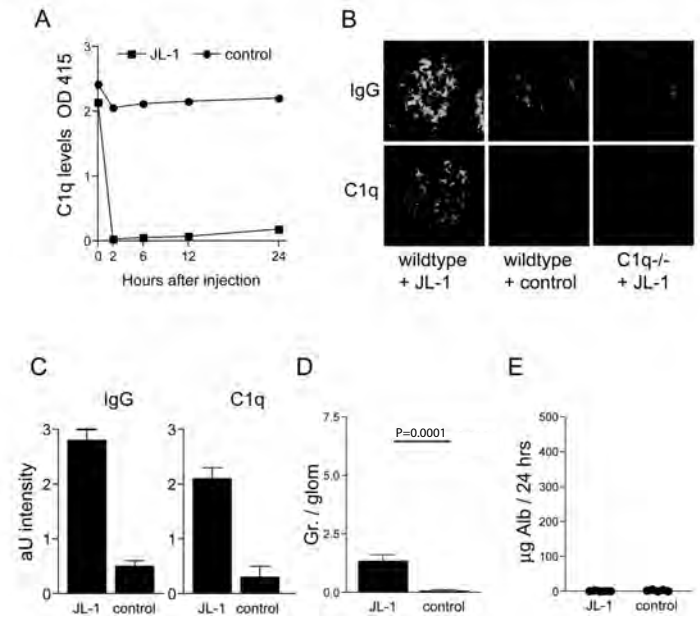

Figure 2. Effects of administration of anti-C1q mAb to naive mice. (A) C1q sandwich ELISA for the detection of serum C1q levels following administration of anti-C1q mAb and control mAb.(B) Immunofluorescence analysis of mouse kidneys stained for the presence of mouse IgG and mouse C1q following administration of JL-1 or control mAb to WT or $C1q^{-/-}$ mice. Original magnification, x400. (C) Quantification of immunofluorescence analysis of the deposition of IgG and C1q in WT mice. Data are expressed as arbitrary units (aU), as described in Methods. (D) Quantification of glomerular granulocyte influx. Depicted on the y axis are granulocytes per glomerular cross section (Gr/glom) at 24 hours after injection. (E) Assessment of albuminuria by ELISA. Alb, albumin.

Anti-C1q mAb JL-1 deposits on to glomerular C1q in a planted antigen-like fashion

Previous studies (22) have demonstrated that Ig-deficient, $Rag2^{-/-}$ mice, when compared with WT mice, not only lack circulating IgG but also lack IgG in glomeruli. As a consequence these Rag $2^{-/-}$ mice also lack glomerular C1q, since there is no glomerular IgG as a focus for glomerular C1q localization. Administration of JL-1 to $Rag2^{-/-}$ mice, which have comparable levels of circulating C1q, resulted in a similar depletion of circulating Cq (Figure 3A). However, this did not lead to glomerular deposition of Cq and anti-C1q autoantibodies as seen in WT mice (Figure 3, B and C).

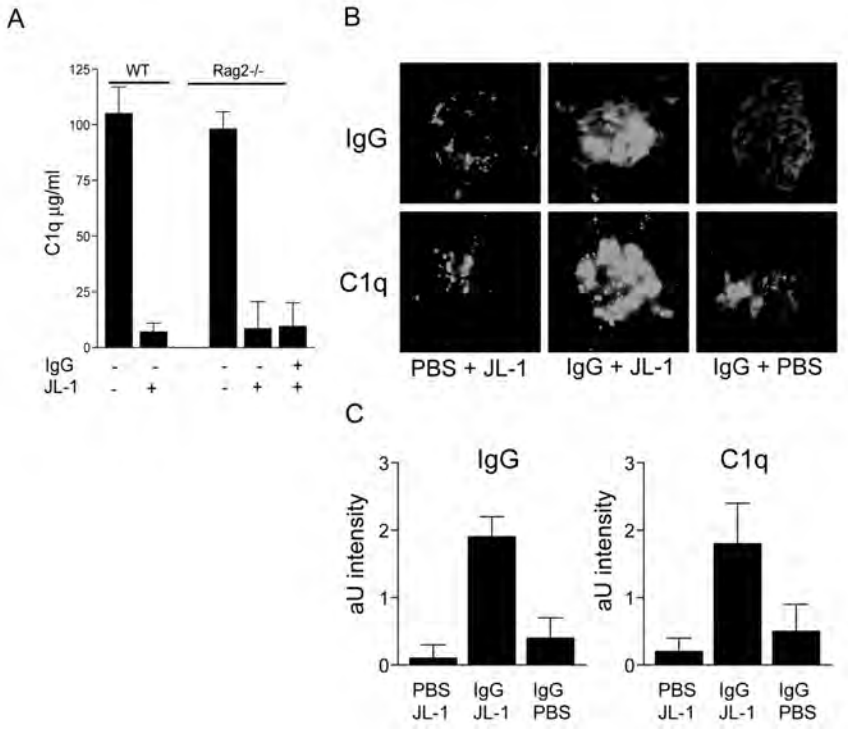

Figure 3. Anti-C1q autoantibodies react with C1q in the glomerulus in a planted antigen-like fashion. (A) C1q sandwich ELISA for serum levels of C1q in naive WT mice, WT mice treated with JL-1, naive Rag2$^{-/-}$ mice, and Rag2$^{-/-}$ mice pretreated with PBS or with mouse IgG and then treated with JL-1. (B) Immunofluorescence analysis of renal sections of Rag2$^{-/-}$ mice, either naive or reconstituted for mouse IgG followed by injection of either JL-1 or PBS. Images show the absence of IgG and C1q in naive Rag2$^{-/-}$ mice and positivity for IgG and C1q, both mesangially and along the GBM, in IgG-reconstituted mice. The positivity for IgG and C1q is highly increased following administration of JL-1. Original magnification, x400. (C) Quantification of Immunofluorescence analysis of the glomerular deposition of IgG and C1q.

Reconstitution of circulating IgG led to the presence of both IgG and C1q in glomeruli (Figure 3, B and C). Subsequent injection of these IgG-reconstituted mice with JL-1 induced glomerular deposition of C1q and anti-C1q autoantibodies as seen in WT mice (Figure 3, B and C). These experiments show that JL-1 does not capture C1q from the circulation and deposit in the glomerulus but that mAb JL-1 deposits on to C1q already present in the glomerulus in a planted antigen-like fashion.

Administration of anti-C1q mAb JL-1 induces overt renal damage when combined with a subnephritogenic dose of anti-GBM antibodies

Since LN is characterized by the glomerular deposition of C1q-containing immune complexes, we tested the effect of JL-1 in a murine model of C1q-containing solid-phase glomerular immune complexes. Administration of a subnephritogenic dose of C1q-fixing

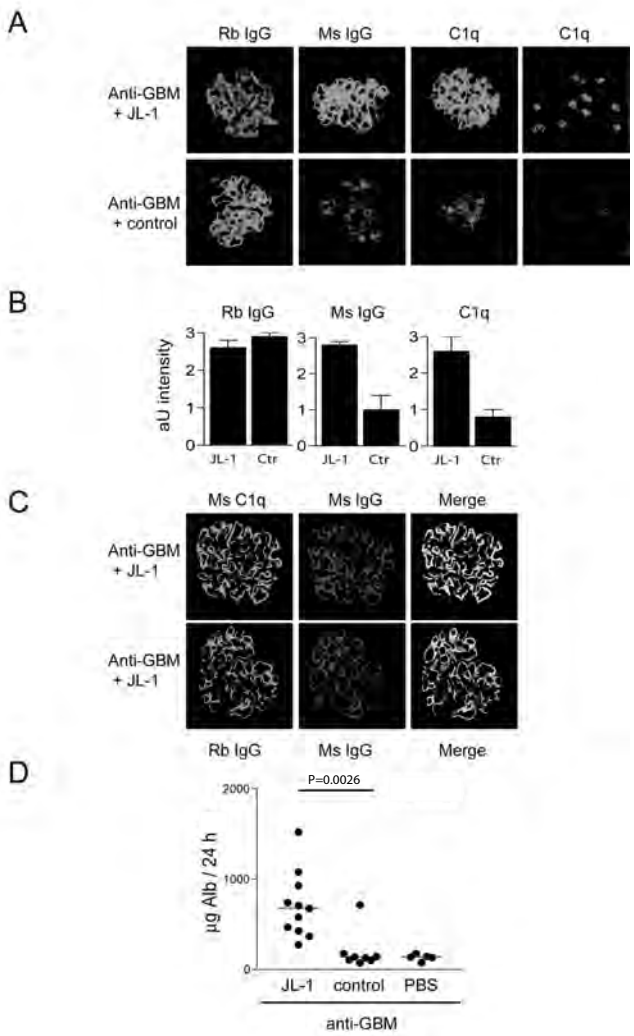

Figure 4. Effects of administration of anti-C1q mAb to mice pretreated with C1q-fixing anti-GBM antibodies. (A) lmmunofluorescence of renal sections obtained from mice pretreated with rabbit (Rb) anti-GBM antibodies combined with either mAb JL-1 or lgG2b control mAb stained for the presence of mouse (Ms) IgG, mouse C1q, or rabbit IgG. Images show linear, GBM-like deposition of anti-GBM antibodies, linear fixation of C1q and anti-C1q in the JL-1-coinjected mice, and only mild mesangial positivity for C1q and anti-C1q in control-coinjected mice. Original magnification, x400. Right panels: Low-power magnifications of renal sections of mice injected with anti-GBM in combination with either JL-1 or control and stained for C1q. Original magnification, x100. (B) Quantification of immunofluorescence analysis of the glomerular deposition of rabbit IgG, mouse IgG, and C1q. Ctr, control. (C) Confocal analysis of kidney sections of mice injected with rabbit anti-GBM and JL-1. Representative pictures are shown for the colocalization (yellow) of mouse C1q (green) and mouse IgG (red) and the colocalization (yellow) of rabbit IgG (green) and mouse IgG (red). They indicate that rabbit IgG, mouse C1q, and mouse IgG do colocalize in these mice, in a linear, GBM-like pattern. (D) Albuminuria of mice injected with anti-GBM antibodies followed by JL-1, control mAb, or PBS.

rabbit anti-mouse GBM antibodies was followed by administration of JL-1 or control mAb. Equal administration of anti-GBM antibodies was demonstrated by staining of renal sections for rabbit IgG (Figure 4, A and B). Administration of control mAb IgG2b directly following anti-GBM administration did not result in glomerular localization of additional mouse IgG or C1q and did not increase the mild albuminuria observed with this subnephritogenic dose of anti-GBM (Figure 4, A and B). In sharp contrast, mAb JL-1 gave a strong glomerular deposition of mouse IgG and also of additional C1q (Figure 4, A and B). This increase in IgG and C1q deposition involved all glomeruli (Figure 4A) and was highly consistent between individual mice (Figure 4, A and B). Confocal analysis following double staining for mouse IgG and mouse C1q and for mouse IgG and rabbit IgG revealed that rabbit IgG was present on the GBM and that both mouse C1q and mouse IgG (anti-C1q mAb) followed the same pattern (Figure 4C). Importantly, the glomerular deposition of mAb JL-1, in this model of immune complex glomerulonephritis, resulted in markedly increased albuminuria ($P = 0.0026$ compared with IgG2b) (Figure 4D).

Mechanism of deposition of anti-C1q mAb JL-1 in combination with anti-GBM antibodies

We used $C1q^{-/-}$ mice to verify that the observed renal damage was dependent on C1q and not caused by contaminating factors like endotoxin. Administration of the rabbit anti-GBM preparation to WT mice induced linear deposition of both rabbit IgG and mouse C1q in glomeruli. Administration of the same preparation to $C1q^{-/-}$ mice led to the linear deposition of only rabbit IgG, not mouse C1q (Figure 5A). In contrast to WT mice, $C1q^{-/-}$ mice did not display any enhancement of albuminuria upon coinjection of JL-1 (see Figure 8).

We next investigated the significance of serum C1q versus glomerular C1q in this model of glomerulonephritis. We compared a C1q-fixing rabbit anti-GBM preparation and a non-C1q-fixing sheep anti-GBM preparation. Administration of both antibody preparations induced linear deposition of anti-GBM antibodies, but linear deposition of mouse C1q was only observed in the mice injected with C1q-fixing anti-GBM (Figure 5A). Administration of JL-1 to these mice enhanced renal damage only in the mice injected with C1q-fixing anti-GBM (Figure 5B). This points to a dependence on glomerular C1q.

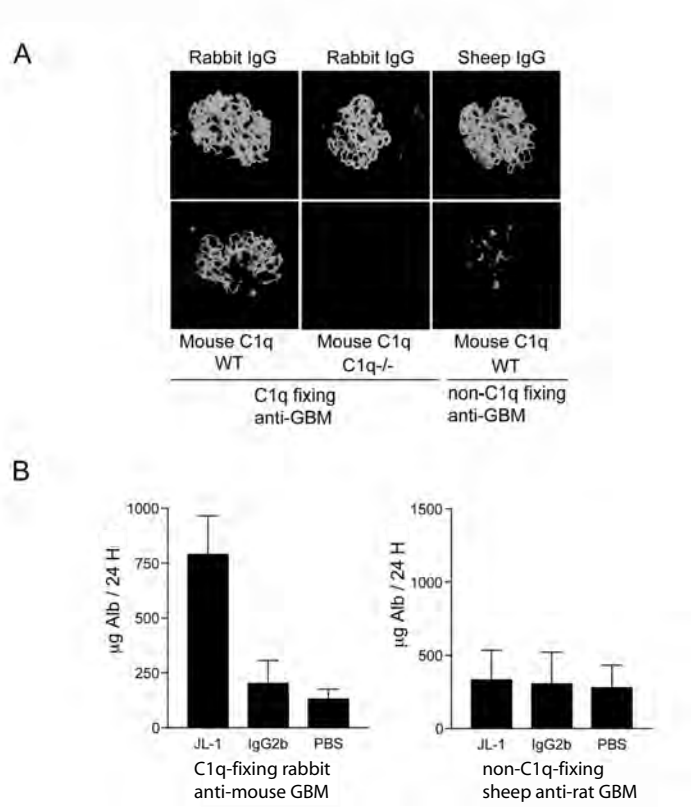

Figure 5. The disease-enhancing effect of JL-1 is dependent on glomerular C1q. (A) Immunofluorescence analysis of anti-GBM antibody deposition and C1q deposition for the C1q-fixing anti-GBM preparation in WT and $C1q^{-/-}$ mice and the non-C1q-fixing anti-GBM preparation in WT mice. The images show the linear IgG fixation for both anti-GBM preparations, but only linear fixation of C1q for the rabbit anti-GBM preparation. Only some mild mesangial C1q positivity can be observed in the mice given sheep anti-GBM. Original magnification, x400. (B) Groups of WT mice were injected with either the C1q-fixing or the non-C1q-fixing anti-GBM preparation and coinjected with JL-1, control, or PBS, and albuminuria was determined.

Glomerular histological changes induced by anti-GBM and anti-C1q mAb JL-1

In addition to causing albuminuria, administration of JL-1 in the model of C1q-containing glomerular immune complexes induced major histological changes in glomeruli analyzed 24 hours after injection, characterized by pronounced inflammatory cell influx, focal capillary tuft occlusion by microthrombi, necrotizing lesions, nuclear debris, and wireloop-like lesions (Figure 6A). We did not observe crescents, extracapillary proliferation, or mesangial proliferation. Glomeruli of mice coinjected with control mAb only displayed limited inflammatory cell influx. Quantification of histological changes was performed using the activity index as described by Austin et al. (23). The JL-1-coinjected mice consistently displayed a much higher activity index than the control-coinjected mice (Figure 6B). Electron

microscopy revealed capillary tuft occlusion by either granulocytes or fibrinlike material in mice injected with anti-GBM and JL-1, whereas in mice coinjected with control mAb IgG2b the capillaries showed normal contours, without alterations of the endothelium or GBM (Figure 6C). At higher magnifications, some subendothelial electron-dense deposits (wireloop-like lesions) were seen in mice injected with anti-GBM and JL-1 but not in mice coinjected with the control mAb IgG2b (Figure 6C).

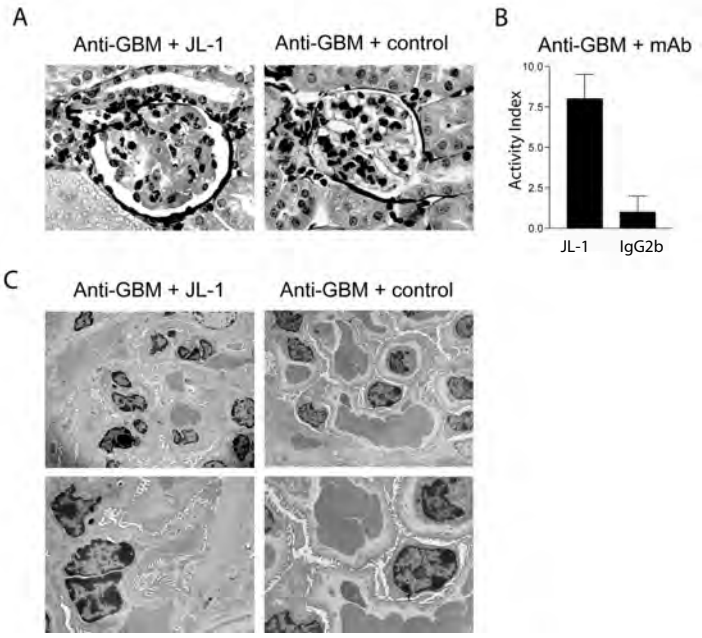

Figure 6. Histological changes induced by JL-1 at both the light microscopic and the electron microscopic levels. (A) Histological analysis of Silver-stained renal sections of mice injected with rabbit anti-mouse GBM and coinjected with either mAb JL-1 or lgG2b control mAb, obtained at 24 hours after injection. For JL-1-coinjected mice, images show pronounced inflammatory cell influx, focal capillary tuft occlusion by microthrombi, necrotizing lesions, nuclear debris, and wireloop-like lesions. Control-coinjected mice only display marginal inflammatory cell influx. Original magnification, x400. (B) Quantification of histological changes using the activity index as described in Methods. (C) Electron microscopic analysis of glomerular lesions of mice injected with rabbit anti-mouse GBM and coinjected with either anti-C1q mAb JL-1 or control mAb lgG2b. At higher magnification, we observed several wireloop-like lesions in the JL-1-coinjected mice, whereas the lgG2b-coinjected mice did not display any abnormalities. Original magnifications, x2,000 (left) and x4,000 (right).

Glomerular granulocyte influx is pronounced and extended in JL-1-injected mice

Analysis of renal sections of mice injected with anti-GBM in combination with either mAb JL-1 or IgG2b for granulocytes revealed significantly more infiltrating granulocytes at both 2 and 24 hours after injection in the JL-1-injected mice compared with the IgG2b-injected mice (Figure 7A).

Granulocyte influx in this model of anti-GBM nephritis is known to peak at 2 hours after injection and then to diminish over time (24). Strikingly, the granulocyte influx at 24 hours was maintained in the JL-1-injected mice and was decreased, as expected, in the IgG2b-injected control mice (Figure 7A). Using confocal microscopy, we confirmed the increased number of infiltrating granulocytes (Figure 7B). Interestingly, the staining for granulocytes occasionally colocalized with the linear deposition of C1q (Figure 7B; colocalization in white; see arrow).

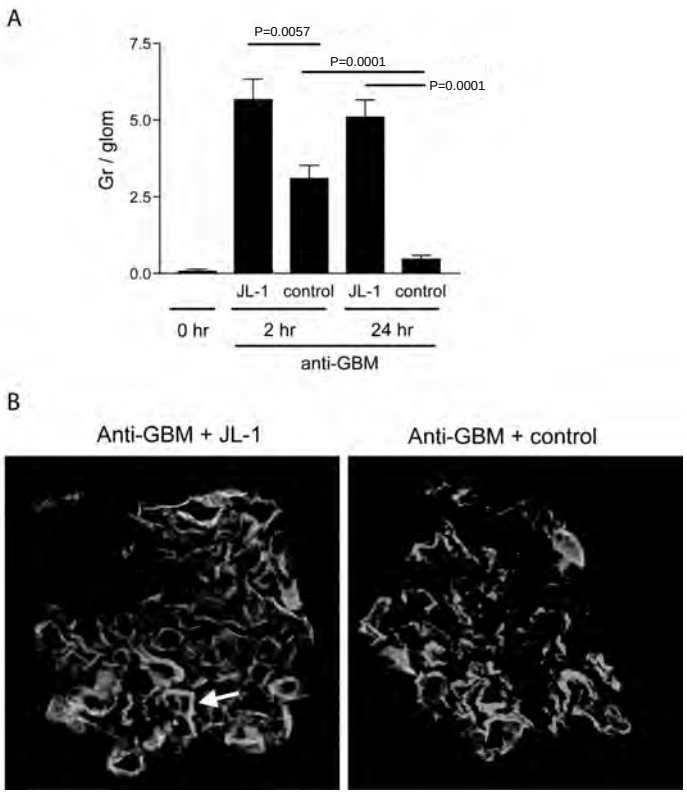

Figure 7 Quantification of glomerular granulocyte influx. (A) Mice were injected with anti-GBM antibodies in combination with either JL-1 or control mAb. Granulocytes per glomerular cross section were scored either at 2 hours or at 24 hours after injection. (B) Confocal analysis of sections stained for mouse C1q (green), mouse IgG (red), and mouse granulocytes (purple). The pictures are merged and show, in yellow, colocalization of green and red, and, in white, colocalization of green and purple. The anti-GBM antibodies induced linear fixation of C1q in both groups, but only in the JL-1-coinjected mice is there colocalization between C1q and IgG and a pronounced influx of granulocytes. Original magnification, x400. The white arrow indicates the white colocalization between C1q and granulocytes.

Requirements for JL-1 in combination with anti-GBM to enhance renal disease

To investigate the requirements for the enhanced renal injury and albuminuria, the combination of the C1q-fixing anti-GBM preparation and JL-1 was administered to various mice genetically deficient for complement components or Fcγ receptors. We found that the enhanced albuminuria was dependent on complement activation, based on the observations in $C3^{-/-}$ mice. Potentially, complement may be activated via 3 pathways, the classical,

alternative, and lectin pathways of complement activation. Since $C4^{-/-}$ mice were also protected, either the classical or the lectin pathway was activated (Figure 8). In addition to complement activation albuminuria in this model is also dependent on Fcγ receptor (FcγR), since injection of anti-GBM in combination with mAb JL-1 did not result in any albuminuria in FcγR triple (CD64, CD32, CD16) deficient mice (Figure 8).

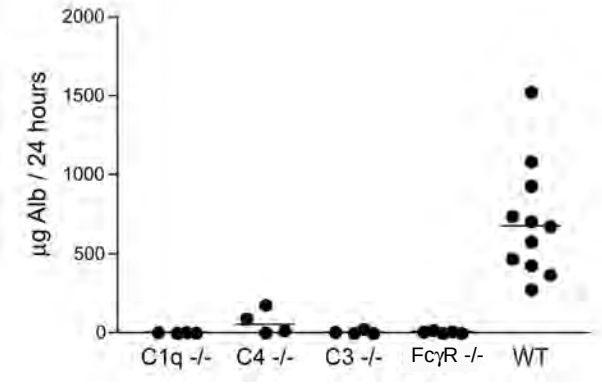

Figure 8 Anti-GBM and JL-1 in various genetically deficient mice. Rabbit anti-mouse GBM in combination with JL-1 was administered to either WT C57BL/6 mice or $C1q^{-/-}$, $C4^{-/-}$, $C3^{-/-}$, or $Fc\gamma R^{-/-}$ mice, and albuminuria was assessed.

Discussion

SLE is immunologically characterized by the production of a wide variety of autoantibodies and the formation and deposition of immune complexes. Renal involvement in this multiorgan disease is a major cause of morbidity and mortality (8, 9). One parameter strongly linked to renal involvement is the presence of anti-C1q autoantibodies (10). In the present study we demonstrate that anti-C1q antibodies are pathogenic to the kidney in conditions of glomerular C1q-containing immune complexes. These data provide essential evidence on how anti-C1q autoantibodies participate in the sequence of events leading to renal inflammation.

Previously, 2 studies have reported the deposition of anti-C1q antibodies in the kidney (25, 26) following the administration of high concentrations of human C1q and anti-human C1q anti-bodies in mice. Since the heterologous nature of such reagents may have been responsible for either the deposition or the lack of overt renal disease, we have generated mouse anti-mouse C1q autoantibodies by immunizing $C1q^{-/-}$ mice with purified mouse C1q. Since $C1q^{-/-}$ mice are reported to be autoimmune-prone if on a mixed genetic background (27), we used non-autoimmune-prone $C1q^{-/-}$ mice on a full C57BL/6 background (28) to avoid the generation of polyreactive autoantibodies. We determined anti-C1q reactivity using an anti-C1q ELISA as described previously (21), under 0.5 M NaCl buffer conditions to

exclude Fc tail interactions with C1q (29). Since IgG2b antibodies can interact with C1q via their Pc tails, we used, in all our in vivo experiments, an isotype-matched IgG2b control mAb that has a similar capacity to interact with C1q via its Fc tail.

Characterization of the mouse anti-mouse C1q mAb revealed reactivity with different epitopes on mouse C1q in ELISA. The mAb's JL-1 and JL-2 displayed reactivity toward C1q in splenic sections of WT but not of $C1q^{-/-}$ mice, confirming their specificity. Since mAb JL-1 also recognized human C1q, we had the unique opportunity to test whether it would be reactive with either the C1q heads or the CLR, since these are only available for human C1q. We found our mAb to be reactive with the CLR only, which is in fact the same region with which the autoantibodies in mice (ref. 19 and current article) and humans (21, 30) are reactive. The CLR of C1q is most likely exposed and accessible after binding of C1q to a ligand, whereas the heads are occupied. Based on these observations, we have chosen mAb JL-1 as the most appropriate mAb for in vivo testing.

Administration of anti-C1q mAb JL-1 to naive mice resulted in glomerular deposition of both C1q and anti-C1q autoantibodies, but without overt signs of renal damage. Previously we reported similar findings using a rabbit anti-mouse C1q polyclonal antibody (31). These results are consistent with the observation that anti-C1q autoantibodies can be found, unrelated to renal problems, in serum of HUVS patients and healthy individuals. Since no renal biopsies are performed without indications of renal problems, to our knowledge there are no data available on glomerular deposition of C1q and IgG in either HUVS or healthy individuals positive for anti-C1q autoantibody.

Injection of JL-1 in mice that had glomerular C1q-containing immune complexes resulted in enhanced renal damage. Several reports describe that the vast majority of SLE patients have glomerular deposits of both IgG and C1q even in the absence of clinically overt renal disease (32-34). This glomerular C1q could potentially serve as a target for anti-C1q autoantibody. Thus, the occurrence of anti-C1q autoantibodies may be highly relevant to the development of nephritis. Although most SLE patients have glomerular immune complexes containing C1q, only those positive for anti-C1q autoantibodies suffer overt renal disease. Conversely, patients who do not have anti-C1q autoantibodies do not develop nephritis (17). We provide causal evidence to suggest that anti-C1q autoantibodies are essential but not sufficient for the development of full-blown renal inflammation (18).

Although anti-C1q autoantibodies are an important determinant for LN, the degree of disease also seems to correlate with the amount of C1q present in the glomerulus. For instance, in glomeruli of $Rag2^{-/-}$ mice, in the absence of glomerular C1q, no deposition takes

place and no renal damage occurs. In naive WT mice, trace amounts of C1q are present in glomeruli, which facilitates the glomerular deposition of anti-C1q autoantibodies; however, this is not sufficient for overt renal damage. Only when there is a relatively high concentration of C1q in the glomerulus, as is the case for glomerular C1q-containing immune complexes, will anti-C1q autoantibodies not only deposit but also induce overt renal disease.

We verified that the enhanced renal damage observed was the effect of anti-C1q reactivity by testing the combination of the C1q-fixing anti-GBM preparation and JL-1 in $C1q^{-/-}$ mice. No enhancement of disease was observed in $C1q^{-/-}$ mice, which indicates that it was indeed the interaction between anti-C1q autoantibodies and C1q, and not cross-reactivity of mAb JL-1 with rabbit IgG or contaminating factors like endotoxin, that enhanced renal disease in this model (35).

The enhanced renal damage as observed with the combination of anti-GBM antibodies and anti-C1q mAb JL-1 can be explained by a "2-hit" model. In this model, binding of anti-GBM antibodies to the GBM and the separate deposition of C1q and anti-C1q autoantibodies may trigger pathological effects. In an alternative synergistic model, events such as binding of anti-GBM to the GBM, fixation of C1q by the Fc parts of the anti-GBM, and deposition of anti-C1q autoantibodies on to this immune complex C1q could converge. We tested the 2 possible mechanisms by using a C1q-fixing and a non-C1q-fixing anti-GBM preparation. In the C1q-fixing model, the enhancing effect of JL-1 was observed only in WT, not in $C1q^{-/-}$ mice. It was also absent in the model using a non-C1q-fixing anti-GBM preparation. This indicates that, in our model, anti-C1q autoantibodies, in the short term, enhances renal disease only in the context of renal C1q-containing immune complexes and, therefore, has a "synergistic" effect.

The damage induced by anti-C1q autoantibodies, in combination with anti-GBM antibodies, involves increased numbers of glomerular granulocytes, capillary tuft occlusion, microthrombi, and wireloop-like lesions. LN is categorized in several WHO classifications, depending on histological abnormalities (8). Many histological criteria of LN are present in the lesions induced by our anti-C1q mAb, such as IgG and C1q deposition, formation of subendothelial immune deposits, influx of leukocytes, and glomerular tuft occlusion. The fact that our experiments lasted only 24 hours may explain the lack of proliferative lesions or crescent formation.

The mechanism by which these anti-C1q autoantibodies enhance immune complex renal disease involves both complement activation and Fc receptors. Several reports have described the anti-GBM model as complement-independent (35-37), although others did report an effect

of complement (24, 38, 39). Most likely, the dose of antibody given will determine the dependence on complement. Low concentrations, as used in the present study, are still dependent on complement, whereas high doses of antibody damage the kidney in a complement-independent way (38). In contrast, both concentrations of antibody work Fc receptor-dependently (37,40). We have shown our model to be indeed dependent on the C1q, the antigen for anti-C1q autoantibodies, by using $C1q^{-/-}$ mice. Using $C3^{-/-}$ mice, we also found that complement activation was essential. Our experiments using $C4^{-/-}$ mice demonstrate that our model was dependent on C4 activation, suggesting involvement of either the classical or the lectin pathway of complement activation. Complement activation itself may damage the kidney or it may attract and activate leukocytes, which, in turn, may cause damage via Fcγ receptor-dependent mechanisms. Therefore, we have analyzed our model in Fcγ receptor-deficient mice and observed normal deposition of C1q and anti-C1q but no enhanced albuminuria, which indicates that Fcγ receptor triggering is also important in this model.

Based on our data and the literature, we postulate that the sequence of events leading to severe immune complex renal disease is deposition of immune complexes on the GBM (Figure 9A), fixation of C1q from the circulation (Figure 9B), binding of anti-C1q autoantibodies (Figure 9C), and massive complement activation and attraction of inflammatory cells (Figure 9D).

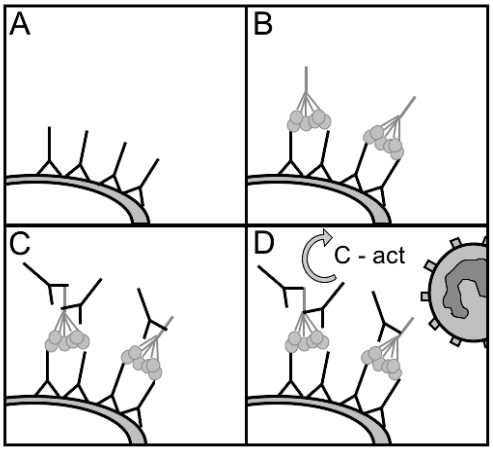

Figure 9 Schematic representation of the pathogenic role of anti-C1q antibodies in immune complex-mediated renal disease. (A) Deposition of immune complexes on the GBM. (B) Fixation of 01 q from the circulation by the immune complexes. (C) Binding of anti-C1q autoantibodies to C1q present on the immune complexes. (D) Activation of complement (C-Act) and attraction of inflammatory cells.

In conclusion, anti-C1q antibodies induce glomerular deposition of C1q and anti-C1q in naive mice but do not cause overt renal disease. However, anti-C1q autoantibodies strongly enhance renal disease when C1q-containing immune complexes are present in the glomerulus, which may explain the relationship between these antibodies and nephritis in SLE patients. These data provide essential insight into the mechanisms involved in immune complex-mediated renal inflammation.

Methods

Animals

Male CS7BL/6 mice (Harlan), $C1q^{-/-}$ C57BL/6 mice (27), $C3^{-/-}$ C57BL/6 mice (41), $C4^{-/-}$ CS7BL/6 mice (41), FcγR triple $^{-/-}$ (CD64, CD32, CD16) mice (42), and $Rag2^{-/-}$ C57BL/6 mice (43) (Taconic) were maintained at our facility. Mice were 8-12 weeks of age and had free access to water and standard chow. Animal care and experimental procedures were performed in accordance with the NIH guidelines for the care and use of laboratory animals.

Purification and detection of mouse C1q

Mouse C1q was purified as described previously (31). Briefly, mouse serum (Harlan) was adjusted to 10 mM EDTA and applied to a rabbit IgG Sepharose column. After extensive washing with PBS containing 10 mM EDTA, bound C1q was eluted using 1 M NaCl containing 10 mM EDTA. The C1q-enriched fractions were identified by ELISA, pooled and dialyzed against PBS, concentrated to 0.5 mg/ml, and stored in aliquots at -80°C. Levels of mouse C1q were detected by sandwich ELISA as described previously (31).

Generation of mouse anti-mouse C1q mAb

Anti-C1q mAb's were generated by immunization of $C1q^{-/-}$ C57BL/6 mice with purified mouse C1q. Mice were injected subcutaneously with 20 µg C1q in 50 µl CFA (Difco), followed by 3 boosts with 20 µg mouse C1q in 50 µl incomplete Freund's adjuvant (Difco) at 2-week intervals. Splenocytes of immunized animals were fused with SP2.0 cells using standard procedures. Cells were grown in IMDM (BioWhittaker Europe) supplemented with 2% PenStrep (Invitrogen Corp.), 0.5 ng/ml human IL-6 (PreproTech Inc.), 0.05 mM [beta]-mercaptoethanol, and 10% heat-inactivated FCS (Invitrogen Corp.). Clones were screened for anti-C1q reactivity using an anti-C1q detection ELISA as previously described (21). Briefly, ELISA plates were coated with C1q-binding peptide 2J (44) at 12.5 µM in coating buffer for 2 hours at 37°C. Plates were washed and incubated with Rag $2^{-/-}$, Ig-deficient serum as a source of C1q at 1:20 in PBS containing 0.05% Tween 20 and 1% BSA (PBS-T-BSA) for 1

hour at 37°C. Serial dilutions of samples were incubated in PBS-T-BSA containing 0.5 M NaCl for 1 hour at 37°C. Bound mouse IgG was detected using goat anti-mouse IgG conjugated to HRP (DAKO A/S). Data are expressed as OD at 415 nm. Isotypes were determined using an Isotype kit (BD Biosciences - Pharmingen).

Positive clones were subjected to 2 rounds of limiting-dilution selection. Large quantities of mAb were generated using in vitro culture. Antibody purification was performed using Protein A-Sepharose HT columns (Pharmacia Corp.) for IgG2a and IgG2b antibodies and by precipitation and gel filtration for IgM antibody. Purity of the mAb preparations was verified by SDS-PAGE and found to be consistently more than 90%. As a control we used IgG2b mAb anti-TNP (kindly provided by L. Aarden, Sanquin Research, Amsterdam, The Netherlands). Three stable clones of mouse anti-mouse C1q were obtained, designated JL-1, JL-2, and JL-3.

Characterization of anti-C1q mAb's

Epitope competition. Fixed amounts of DIG-conjugated anti-C1q mAb or control mAb IgG2b were coincubated with an excess of nonconjugated anti-C1q mAb or IgG2b in the anti-C1q detection ELISA. Binding of DIG-conjugated mAb was detected by anti-DIG HRP (Boehringer Mannheim). Data are expressed as percent inhibition compared with noninhibited mAb binding.

Immunohistochemistry.

Spleen sections of either WT or $C1q^{-/-}$ mice were stained with DIG-conjugated anti-C1q mAb and DIG-conjugated control antibodies at 5 µg/ml in PBS containing 1% BSA for 1 hour at room temperature followed by FITC-conjugated anti-DIG (Boehringer Mannheim) at 5 µg/ml.

Anti-globular head or -collagen-like tail reactivity of mAb's

Recombinant forms of the globular regions of human C1q A, B, and C chains (globular head A [ghA], ghB, and ghC, respectively) were generated as described recently (45). Human CLRs were generated from purified human C1q as described previously (46, 47). The recombinant globular regions, CLRs1 or human C1q were coated in ELISA, and anti-C1q mAb and control mAb were incubated at 2.5 µg/ml in PBS-T-BSA and analyzed for binding. Maltose-binding protein (MBP) was used as a negative control protein, since ghA, ghB, and ghC were expressed in bacteria as fusions to Escherichia coli MBP. Anti-CLR reactivity was

also analyzed in serum from an autoimmune MRL-lpr mouse of 5 months of age and an age-matched nonautoimmune BALB/c mouse, by coating of CLR and incubation of serially diluted serum.

Generation of anti-GBM antibodies

A C1q-fixing anti-GBM polyclonal antibody was generated by immunization of male New Zealand white rabbits (Harlan) with purified mouse GBM as described previously (48). Subcutaneous injection of 50 µl GBM mixed with 100 µl CFA (Difco) was followed by 3 boosts with 50 µl mouse GBM in 100 µl incomplete Freund's adjuvant (Difco) at 2-week intervals. Rabbit immune serum was heat-inactivated at 56°C for 30 minutes and centrifuged for 20 minutes at 2,000 g. γ-Globulin precipitation was performed using $(NH_4)_2SO_4$ at 40% wt/vol saturation, and the precipitate was further purified by anion exchange Chromatograph/ using DEAE-Sephacel (Pharmacia Corp.). Rabbit IgG-containing fractions were pooled, concentrated to 20 mg/ml, dial/zed against 0.15 M NaCl, and stored in aliquots at -20°C.

Alternatively, we have used the γ-1 fraction of a sheep anti-rat GBM polyclonal antiserum that cross-reacts with mouse GBM (49, 50). Although this fraction is known to activate mouse complement (51), we previously observed that this preparation does not fix C1q in vivo.

Experimental procedure

Groups of 2 C57BL/6 mice were injected intraperitoneally with 1 mg of purified anti-C1q mAb or isotype control in 1 ml of sterile PBS. Blood was collected by tail cut at 0, 2, 6, 12, and 24 hours after injection. Serum was prepared and stored at -80°C. Groups of 5 C57BL/6 mice were injected with 1 mg of purified anti-C1q mAb intraperitoneally in 1 ml of sterile PBS. Overnight urine was collected by placement of the mice in metabolic cages. Mice were anesthetized with urethane (Sigma-Aldrich) and sacrificed by heart puncture. To exclude cross-reactivity, an additional group of 3 $Clq^{-/-}$ mice was injected with 1 mg of mAb JL-1.

Three naive $Rag2^{-/-}$ mice were injected with JL-1. Six $Rag2^{-/-}$ mice were reconstituted for mouse IgG by means of mouse IgG injection as described previously (22). One group of reconstituted mice (n = 3) was injected with PBS and the other group (n = 3) with JL-1. Both groups were analyzed for the renal deposition of mouse IgG and mouse C1q by immunofluorescence.

Groups of at least 5 mice were injected intravenously with a subnephritogenic dose of either 2 mg rabbit anti-mouse GBM or 0.5 mg sheep anti-rat GBM polycional antibodies in a total volume of 200 µl sterile PBS via the tail vein, and then injected intraperitoneally 10

minutes later with 1 mg purified mAb JL-1 or IgG2b in 1 ml sterile PBS or by 1 ml PBS. Overnight urine was collected by placement of the mice in metabolic cages. Mice were anesthetized with urethane (Sigma-Aldrich) and sacrificed by heart puncture at 24 hours. Similarly, an additional 2 groups of 4 mice were injected with the C1q-fixing rabbit anti-GBM antibody in combination with JL-1, IgG2b control antibody, or PBS only, sacrificed at 2 hours, and analyzed for glomerular granulocyte influx as a measure of glomerular inflammation.

The C1q-fixing anti-GBM preparation was administered in combination with JL-1 to groups of $C1q^{-/-}$, $C4^{-/-}$, $C3^{-/-}$, and $Fc\gamma R\ triple^{-/-}$ (CD64, CD32, CD16) mice (n = 4 in each group), and urine was collected and analyzed for albuminuria.

Histological analysis

Immediately after sacrifice, 1 kidney from each mouse was frozen in precooled 2-methylbutane for immunohistochemistry, and the other was fixed in methyl Carnoy's solution for light microscopy.

For immunofluorescence, cryostat sections of 3 μm were fixed using acetone, all antibodies were diluted in PBS containing 1% BSA, and sections were incubated in a humid incubator for 1 hour at room temperature. sections were washed 3 times for 5 minutes using PBS. sections were stained for the presence of mouse IgG, rabbit IgG, and mouse C1q using goat anti-mouse IgG Oregon Green (Invittogen Corp.), goat anti-rabbit IgG conjugated to FITC (Nordic Immunological Laboratories), and rabbit anti-mouse C1q DIG, followed by sheep anti-DIG conjugated to HRP, where appropriate. Evaluation of the extent and intensity of immunofluorescence was performed by scoring of the intensity of staining for individual glomeruli as 0 (negative), 1 (positive above background), 2 (positive), and 3 (brightly positive) for at least 20 glomeruli per section. Means and SDs for at least 3 mice per group are shown. For double staining, goat anti-mouse IgG Alexa 546 (Invitrogen Corp.) was used in combination with anti-C1q and anti-rabbit IgG stainings, as described above. For triple staining, the antibodies described above were combined with rat anti-mouse granulocyte mAb GR-1 (a kind gift of G. Kraal, Vrije Universiteit Medisch Centrum, Amsterdam, The Netherlands), and rat IgG was detected using goat anti-rat IgG Alexa 594 (Invitrogen Corp.). The double- and triple-stained sections were mounted in Mowiol 40-88 (Sigma-Aldrich Chemie) and examined using an LSM 510 confocal microscope (Carl Zeiss AG).

For immunohistochemistry, sections were stained for mouse granulocytes using rat anti-mouse granulocyte mAb GR-1, and rat IgG was detected using goat anti-rat IgG conjugated to

HRP (DAKO), preincubated with 10% normal mouse serum to absorb anti-mouse IgG cross-reactivity. Nova-RED (Vector Laboratories Inc.) was used as a substrate. Granulocyte influx was scored by counting of the number of granulocytes per glomerular cross section. Granulocytes were counted by 2 observers on coded sections.

For light microscopy, the other kidney was fixed and embedded in paraffin, and 3-[mu]m sections were stained with H&E, periodic acid-Schiff, and silver. Evaluation of histopathological changes was performed by a pathologist who was blinded to the code of the sections, using the activity index as described by Austin et al. (23). This index scores several parameters of active renal inflammation, such as glomerular proliferation, leukocyte exudation, karyorrhexis/fibrinoid necrosis, and hyaline deposits.

For electron microscopy, small pieces of cortex were fixed in 1% paraformaldehyde/1.5% glutaraldehyde overnight and washed in 0.1 M cacodylate buffer. Pieces of kidney were postfixed in 1% OsO_4, dehydrated, embedded in Epon 812, and cut into ultrathin sections using a Leica UCT ulcramicrotome (Leica Microsystems Inc.). On the grids, sections were poststained with uranyl acetate and lead citrate and examined with a JEM1011 electron microscope (JEOL USA Inc.).

Renal damage

Renal damage was determined by analysis of urinary albumin excretion using an autoanalyzer (Hitachi 911; Roche). Data are expressed as micrograms albumin excreted in urine per 24 hours.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 3.03 software (GraphPad Software Inc.). Differences between parameters were analyzed using unpaired Student's *t* tests. The P values were considered statistically significant at *P* less than 0.05.

Acknowledgments

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Nonstandard abbreviations used: CLR, collagen-like region; DIG, digoxigenin; FcγR, Fcγ receptor; GBM, glomerular basement membrane; gh, globular head; HUVS, hypocomplementemic urticarial vasculitis syndrome; LN, lupus nephritis; SLE, systemic lupus erythematosus.

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The alternative pathway of complement and Fc receptors are both required for the induction of anti-GBM-mediated glomerulonephritis in the mouse.

3

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In preparation

Abstract

Classically, models of antibody-mediated disease are thought to be dependent on activation of the classical pathway of complement. However, more recent studies indicate a significant contribution of the alternative pathway. In the present study, we demonstrate the same principle in an attenuated model of anti-GBM nephritis in the mouse.

The model involved the injection of rabbit anti-mouse GBM antibodies in mice followed 6 days later by a complement-activating mouse mAb directed against rabbit IgG. After 24 hours, C57/BL6 mice treated with both antibodies developed glomerular injury characterized by albuminuria and deposition of complement components C1q, C4 and C3 in a GBM-like pattern. Fc-receptor gamma-chain knockout mice were completely and $C3^{-/-}$ mice were partially protected from damage as determined by albumin excretion. In contrast $C1q^{-/-}$ and $C4^{-/-}$ mice displayed comparable results as wildtype mice.

Wildtype mice followed for 3 weeks after receiving a single shot of both antibodies displayed a rapid increase in albuminuria in the first days after injection, followed by persistent albuminuria at a lower level. No detectable albuminuria was observed in mice injected with only rabbit anti-GBM antibodies. In both treatment groups complement deposition was observed until 3 weeks after injection.

In conclusion, this study demonstrates the combined contribution of Fc receptors and complement activation via the alternative pathway in an attenuated anti-GBM model of glomerulonephritis. The model shows an acute and a chronic phase of glomerular disease characterized by albuminuria and complement activation.

Introduction

The complement system plays an important role in the body's innate immune defense and includes a number of plasma proteins that can be activated in a cascade reaction via three activation pathways, i.e., the classical pathway, the alternative pathway, and the lectin pathway. Next to many beneficial effects of complement, such as microbial opsonisation and lysis, chemotactic and anaphylatoxic functions, augmentation of tissue repair, regulation of apoptosis, and clearance of immune complexes, it can have less beneficial effects as well.

When excessive complement activation occurs at the tissue or cellular level, the inflammatory process induced by complement activation can turn itself towards the host and cause tissue injury. Under healthy conditions most host tissues are protected against host complement attack by membrane-bound complement regulators such as CD46, CD55 and CD59 [1]. In diseases like anti-glomerular basement membrane (GBM) disease (Goodpasture's disease), antibodies are produced against the α3 chain of type IV collagen in the GBM [2]. Subsequent binding of these antibodies to the GBM leads to an autoimmune damage in the glomerulus that is characterized by strong complement activation as evidenced by the deposition of C3, cellular infiltration and proliferation, ultimately leading to crescent formation, scarring and loss of renal function [3].

Earlier experimental studies have mainly used rat models to investigate the underlying mechanisms of this disease. Intravenous injection of heterologous rabbit anti-GBM antibodies resulted in acute proteinuria, influx of polymorphonuclear leukocytes and subsequent influx of monocytes [4]. In this model, deposition of the heterologous antibodies in the kidney results in classical pathway activation, generation of chemotactic and anaphylatoxic fragments, recruitment of inflammatory cells and injury. To further delineate the role of complement in this disease, and to profit from animals that have genetic deletions of components of the complement system, a number of investigators have employed mice in which the disease is induced by heterologous antibodies against mouse GBM [5] [6].

Although it is possible to induce significant injury in these so-called direct models, large amounts of antibody are required. Therefore additional models were employed in which mice are pre-immunized with the heterologous immunoglobulins of choice and after a week heterologous anti-GBM antibodies are administered. In this so-called accelerated model, flamboyant inflammation takes place. Several of these studies have shown that the classical pathway of complement is involved in this type of renal injury [7, 8]. Since the degree of renal inflammation in the latter model is strongly dependent on the initial immune response

against the IgG used for immunization, and since this response is variable between individual mice, this model is difficult to control.

Therefore in the present study we have employed a distinct approach where a limiting amount of rabbit anti-GBM is given, followed a week later with a fixed concentration of a mouse monoclonal antibody against rabbit IgG. We assumed that the use of a fixed amount of mouse mAb would induce the disease in a controlled fashion, and should facilitate interaction with mouse effector systems in a more natural manner. Using this approach, we succeeded in induction of attenuated renal injury that is clearly dependent not only on complement but also on the contribution of cellular Fc-receptors. Surprisingly, although this is an antibody-mediated disease, our results indicate involvement of the alternative rather than the classical pathway.

Materials and methods

Mice

Male and female C57/BL6 mice were purchased from Charles River (Maastricht, The Netherlands). $C1q^{-/-}$, $C4^{-/-}$, $C3^{-/-}$ and FcR-γ $chain^{-/-}$ mice were maintained by the Nephrology department, as described before [9]. All experiments were performed in accordance with the national institutes of health guidelines for the care and use of animals.

Antibodies

Rabbit anti-mouse GBM was obtained as described before [9]. The IgG2a mAb anti-rabbit IgG (mAb anti-RbIgG) producing cell line was obtained from the ATCC, (CRL-1753, Mannasis, VA) and was cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% foetal calf serum, 50 μM β-mercaptoethanol and penicillin / streptomycin. The supernatant was concentrated, centrifuged at 10,000 rpm and applied to a commercial protein G column (Amersham, Little Chalfont, UK). After washing with PBS, the mAb was eluted with 0.1 M citric acid, pH 3.0. The fractions were checked for the presence of mAb anti-RbIgG by ELISA. The positive fractions were pooled, concentrated and after dialysing against PBS, aliquotted and stored at -20ºC until further use. The mAb anti-RbIgG was coupled to DIG as described before [10].

Induction of glomerulonephritis

Mice were injected intravenously with 0.5 mg rabbit anti-mouse GBM in PBS at day 0. After 6 days this was followed by an intraperitoneal injection of 1 mg mouse anti-rabbit IgG mAb.

Mice were sacrificed either after 24 hours or after 3 weeks, the kidneys were collected, directly snap frozen in 2-methylisobutanol and stored at -150°C until further use.

Albuminuria and creatinine

Mice were placed in metabolic cages for 18 hrs and urine was collected, centrifuged at 2000 rpm and subsequently stored at -20°C. The concentration of albumin in the urine was quantified by rocket immunoelectrophoresis (modified version of [11]) and calculated back to mg/24 hr. Creatinine levels in urine were determined by a kinetic colorimetric assay using a commercially available kit (Creatinine Jaffé method, Roche diagnostics, Basel, Switzerland) and a Cobas Integra 800 analyser (Roche).

Fluorescence and immunohistochemistry

Sections of the frozen kidneys were cut into 3 µm slides and deposition of complement components and IgG was assessed with immunofluorescence analysis. The presence of rabbit IgG in the glomeruli was detected using a goat anti-rabbit IgG FITC antibody (Nordic immunological laboratories, Tilburg, The Netherlands). Mouse IgG was detected using goat anti-mouse IgG coupled to Oregon Green (Invitrogen, Carlsbad, CA). C1q was detected with polyclonal rabbit anti-mouse C1q coupled to dig as described before [9]. C4 and C3 were detected using rat mAb anti-mouse C4 and C3 (both from Cedarlane Laboratories Ltd, Burlington, Ontario, Canada) followed by mAb mouse anti-rat kappa (His 8, kindly provided by Dr. N.A. Bos, Dept. of Histolology and Cell biology, Groningen University, The Netherlands) coupled to dig. Bound dig-coupled antibodies were detected using sheep Fab anti-dig conjugated to FITC (Roche Diagnostics, Basel, Switzerland). To determine the influx of granulocytes in the glomeruli, slides were stained with mAb GR-1 (a kind gift from Dr. R. Toes, department of Rheumatology, LUMC, The Netherlands), and the influx of leukocytes was determined using an antibody against CD45 (AbD Serotec, Oxford, UK). Both these antibodies were followed by incubation with a secondary goat anti-rat IgG coupled to alexa fluor 488 (Molecular Probes, Leiden, The Netherlands).

Statistics

All statistical analyses were performed using Graphpad software (Graphpad software Inc., San Diego, CA). Differences were evaluated by one way ANOVA using Kruskal Wallis statistics and Dunn's correction for multiple comparison. Differences were considered statistically significant when $P < 0.05$.

Results

A new mouse model for anti-GBM glomerulonephritis

For our studies, an attenuated mouse model for anti-GBM nephritis was set up using a mouse monoclonal antibody, in order to facilitate interaction with effector systems in the mouse. The mAb used for these experiments recognizes the Fc part of rabbit IgG and binds well to the rabbit anti-GBM antibodies used for in vivo experiments, as shown by ELISA (Figure 1).

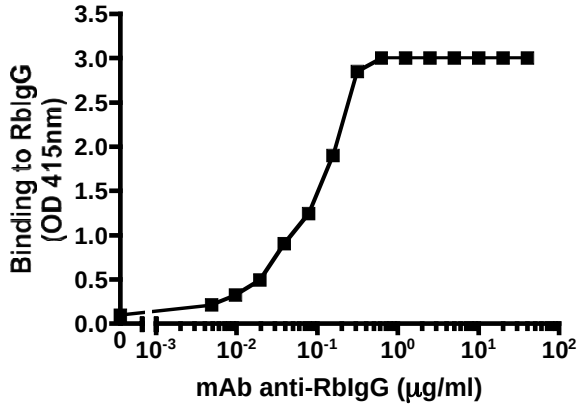

Figure 1. mAb anti-RbIgG binds anti-GBM in vitro. Rabbit IgG anti mouse GBM (1 µg/ml) was coated on a plate, followed by addition of dig-labelled mAb anti-rabbit IgG in different concentrations. Detection was completed using sheep Fab anti-dig conjugated to HRP (Roche Diagnostics, Basel, Switzerland), and ABTS as a substrate.

In initial dose-finding experiments, mice were injected I.V. with 0.5 mg rabbit anti-GBM antibodies followed 6 days later by 0, 1 or 1.5 mg mAb anti-RbIgG. After 24 hrs both the 1 mg and 1.5 mg injected mice developed albuminuria, with a median of respectively 5.06 mg / 24 hr (range 3.63-6.37 mg / 24hr, n = 3) and 12.71 mg / 24 hr (range 3.47-16. 27 mg / 24hr, n = 3) while virtually no albuminuria was seen in the control injected mice (0.3 mg / 24hr) (results not shown). Based on these experiments, in the following studies a working dose of 1 mg mAb anti-RbIgG was chosen.

Induction of glomerulonephritis in wildtype mice

C57/BL6 mice were first injected with either rabbit anti-GBM or PBS on day 0, followed after 6 days by injection with 1 mg mAb anti-RbIgG in PBS or only PBS. Mice that received both antibodies showed overt albuminuria 24 hours after injection of the secondary antibody (Median 6.15 mg / 24 hr (range 2.42-40.66) (Fig 2A). In contrast, mice that received anti-GBM and PBS or PBS alone showed no significant albuminuria (Median 0.068 mg / 24hr

(range 0.037-0.3) and 0.058 mg / 24 hr (range 0.038-0.075) respectively). There was no difference in the levels of urinary creatinine between the treatment groups (not shown).

The presence of rabbit- and mouse IgG and deposition of complement in the kidneys was examined by immunofluorescence at 24 hours following the last injection (Fig 2B). Rabbit IgG was equally present in the glomeruli of all the mice that received anti-GBM antibodies, and was distributed in a GBM-like pattern. No staining for RbIgG could be seen in the mice that received two injections of PBS only. MsIgG was clearly deposited in a GBM-like pattern in mice treated with both antibodies. MsIgG deposition was observed to a lesser extent in mice treated with only anti-GBM antibodies and was absent in mice that were treated with PBS only. This indicates that MAb anti-RbIgG co-localizes with rabbit IgG on the GBM.
Glomerular antibody deposition was clearly associated with complement activation, as evidenced by a co-localization with C1q, C4 and C3. At 24 hours after the last injection, all mice studies showed a low number of leukocytes in the glomeruli (Fig. 2C), consisting of granulocytes and some macrophages. At this time point, we could not observe a significant difference between the different groups (Fig. 2C).

C3 is an important factor in the development of disease

Wildtype mice, C1q-, C4- and C3-deficient mice were injected with both antibodies and the development of glomerulonephritis compared to controls was assessed using albuminuria, complement deposition and cellular influx as readout parameters.

Upon induction of anti-GBM nephritis with both antibodies, wildtype mice C1q-deficient mice and C4-deficient mice developed a very similar level of albuminuria, indicating that deficiency of C1q or C4 was not protective against the disease. In contrast, mice deficient for C3 developed a significantly reduced albuminuria (Median 0.81 mg/24hr (range 0.05-2.93), $P < 0.01$), although this albuminuria was still significantly higher than that in wildtype mice or knockout mice injected with anti-GBM antibodies only ($P < 0.001$; fig. 2A and data not shown). Therefore, C3-deficient mice show a partial protection. (Fig 3A).

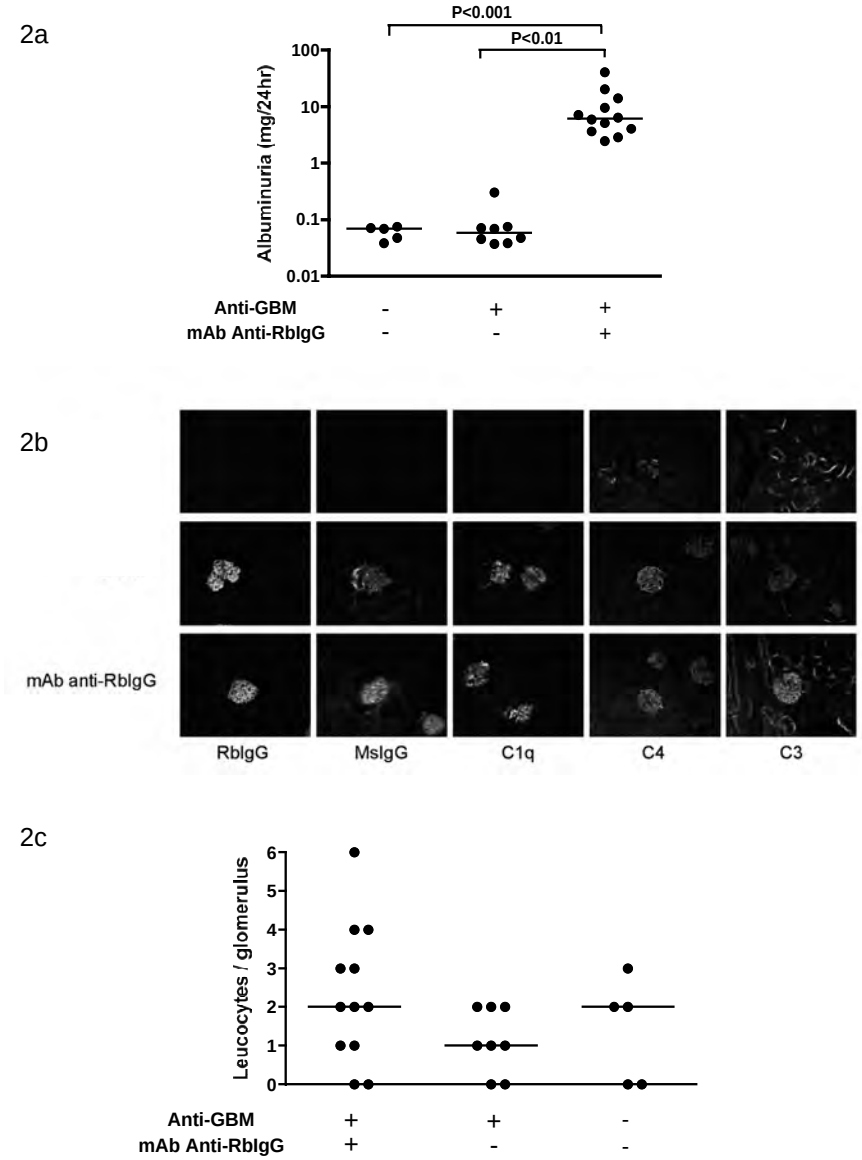

Figure 2. Induction of glomerulonephritis in Wt mice. A) C57/BL6 mice were injected I.V. with either 0.5 mg anti-GBM in PBS or PBS alone. After 6 days, PBS or 1 mg mAb anti-RbIgG antibody was injected I.P. Urine was collected using metabolic cages and mice were sacrificed after 24 hrs. The amount of albuminuria was determined using a rocket immunoelectrophoresis assay. Data are expressed as mg albumin/24 hr. B) Mouse kidneys of PBS, anti-GBM or anti-GBM + mAb anti-RbIgG injected mice were stained for the presence of RbIgG, MsIgG, C1q, C4 and C3 using specific antibodies (magnification 200x). C) Mouse kidneys of PBS, anti-GBM or anti-GBM + mAb anti-RbIgG injected mice were stained for the presence of leukocytes. Data are expressed as number of leukocytes per glomerulus.

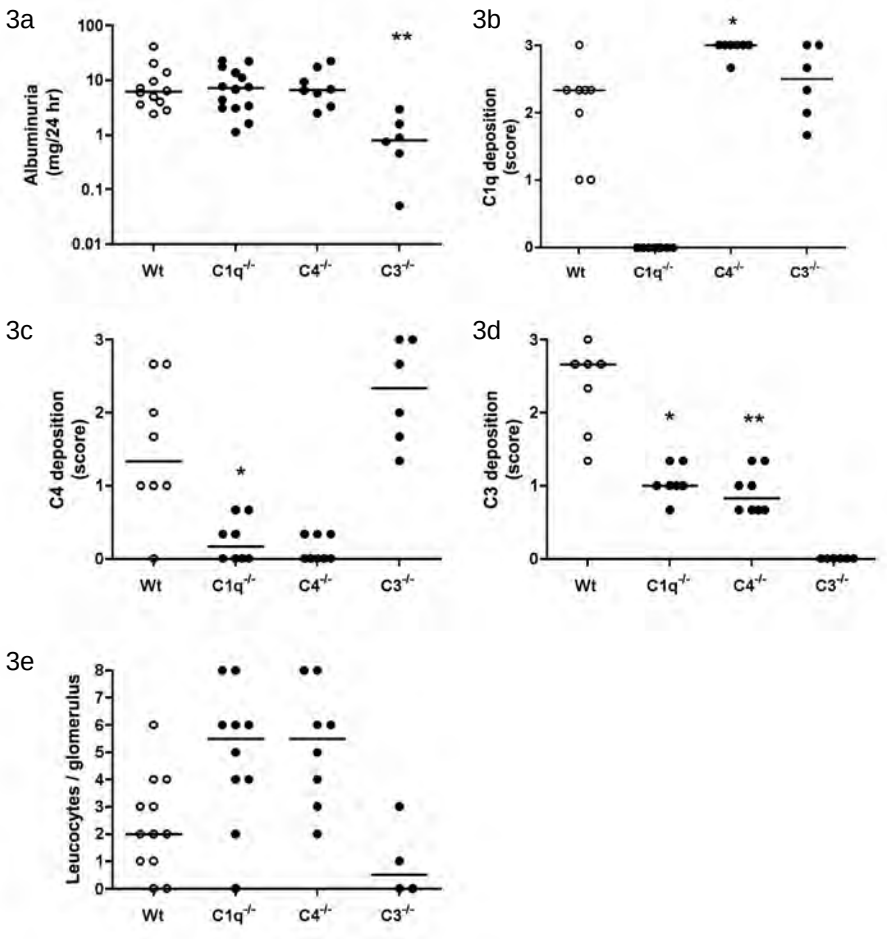

Figure 3. The role of complement activation in renal injury. A) Groups of wildtype, $C1q^{-/-}$, $C4^{-/-}$, $C3^{-/-}$ mice were injected with anti-GBM followed 6 days later by either 1 mg mAb anti-RbIgG or PBS as control. The amount of albuminuria was measured by rocket immunoelectrophoresis assay and expressed as mg / 24 hr. ** P <0.01 vs Wt. B, C, D) Kidneys of Wt, $C1q^{-/-}$, $C4^{-/-}$, $C3^{-/-}$ mice injected with both antibodies were stained for the presence of C1q, C4 and C3. Semi-quantitative analysis of complement deposition in the different strains. Fluorescence intensity (range 0-3) was scored by three blinded observers. *P<0.05 vs Wt, **P<0.01 vs Wt. E) Kidneys of Wt, $C1q^{-/-}$, $C4^{-/-}$, $C3^{-/-}$ mice injected with both antibodies were stained for the presence of leukocytes . Data are expressed as number of leukocytes per glomerulus.

All knockout mice lacked significant staining for the complement component that was knocked out, confirming the staining specificity (Fig 3 B, C, D). Deposition of C1q was increased in C4-deficient but not in C3-deficient mice ($P < 0.05$). Furthermore, $C1q^{-/-}$ but not $C3^{-/-}$ mice showed reduced deposition of C4 ($P < 0.05$), whereas C3 deposition was significantly reduced in the glomeruli of C1q- and C4-deficient mice ($P < 0.05$ and < 0.01, respectively), as compared to Wt mice.

These results indicate that the classical pathway is involved in the glomerular deposition of C3 in this model. Analysis of leukocyte infiltration showed that mice deficient for C1q or C4 have similar numbers of intraglomerular leukocytes than wildtype mice (fig. 3E). C3-deficient mice, however, tend to show less leukocyte infiltration (fig. 3E) and, remarkably, do not show any intraglomerular macrophages (not shown). However, the number of C3-deficient mice available for histological analysis was too low to reach statistical significance.

The presence of activating Fc-receptors is crucial for the development of renal injury

To further examine the role of cellular components in renal injury, mice deficient for the common γ-chain of the Fc-receptors were used. Mice deficient for the FcR-gamma chain did not show any significant albuminuria, in contrast to wildtype mice ($P < 0.001$ (Fig. 4A)).
However, all FcR-gamma chain-deficient mice exhibited glomerular deposition of antibodies and complement (fig. 4B), and showed glomerular leukocyte infiltration that was similar to wildtype mice (not shown). These data suggest that the functional presence of FcR on these cells is crucial to develop albuminuria.

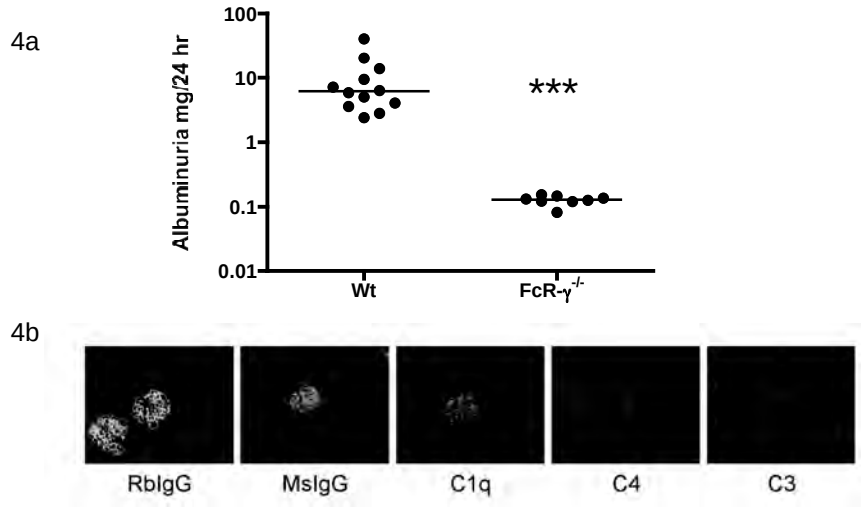

Figure 4. Albuminuria is dependent on Fc-receptors. A) FcR γ-chain$^{-/-}$ or Wt mice were injected with 0.5 mg anti-GBM, after 6 days was followed by an injection of 1 mg mAb anti-RbIgG. Albumin excretion in urine was measured and expressed as mg / 24 hr. ***P<0.001 (B) Kidneys of FcR γ-chain$^{-/-}$ mice were stained for the presence of RbIgG, MsIgG, C1q, C4 and C3. (Representative staining, magnification 200x).

A single injection of both antibodies leads to chronic disease

C57/BL6 mice injected with anti-GBM and after 6 days with mAb anti-RbIgG or PBS were followed for a period of 3 weeks. Mice developed a rapid rise in albuminuria in the first days after injection of mAb anti-RbIgG, which declined thereafter until reaching a relatively stable

level of albuminuria after 2 weeks (median 2.078 mg / 24 hr (range 0.807-5.639), t=18-28), which persisted until the mice were sacrificed after three weeks (Fig 5). Mice injected with anti-GBM alone did not develop any significant albuminuria during the whole period.

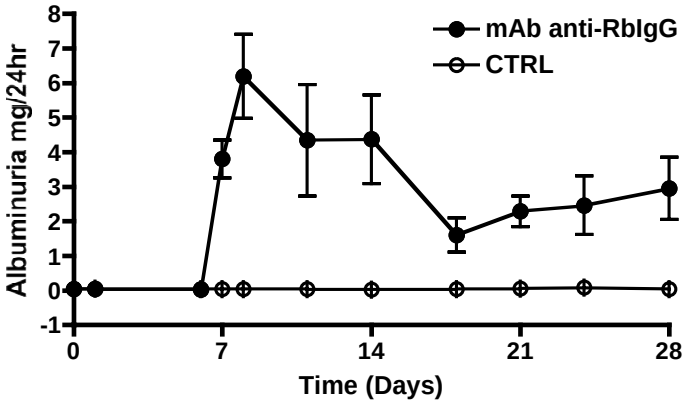

Figure 5. Over time only mice injected with both antibodies develop and maintain albuminuria. Groups of mice were injected with 0.5 mg anti-GBM and after 6 days 1 mg mAb anti-RbIgG (n=4) or with anti-GBM and after 6 days PBS (n=5). Urine was collected at multiple time points and analysed for albuminuria. Mice were sacrificed at day 28.

At day 28 the mice were sacrificed and the kidneys were stained for complement, rabbit IgG and mouse IgG. In all mice, anti-GBM could still be observed in significant amounts in the glomeruli, indicating that the rabbit IgG was not cleared from the GBM. Furthermore, mouse IgG was still present in the glomeruli of mice injected with mAb anti-RbIgG after 3 weeks. However, hardly any mouse IgG could be observed in mice injected with anti-GBM alone.

In both treatment groups, deposition of C1q, C4, and C3 was still observed in the glomeruli at 3 weeks after the second injection, indicating low clearance of deposited complement or continuous complement activation (Fig 6). However, complement deposition was lower in mice treated with anti-GBM alone.

Discussion

In the present study we present a novel model for anti-GBM-mediated glomerulonephritis, characterized by albuminuria and complement deposition. Albuminuria in this model depends on glomerular mouse IgG deposition, complement activation and Fc-receptors. Surprisingly, although we show that complement activation is at least for a significant part mediated by the

classical pathway, the alternative pathway but not the classical pathway is indispensable for full-blown disease.

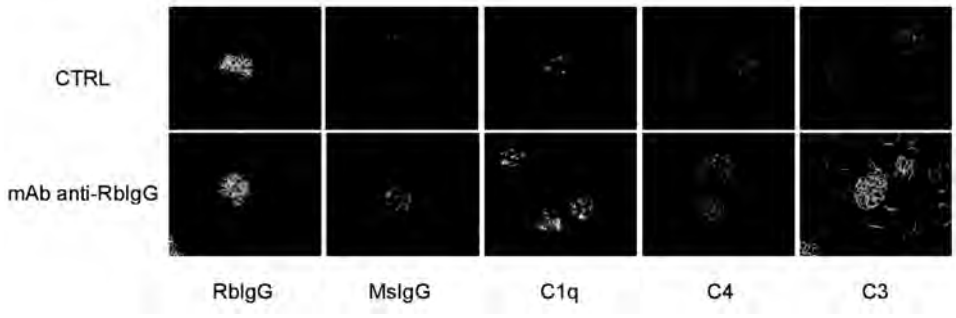

Figure 6. Rabbit IgG, mouse IgG and complement are still present at day 28. Groups of mice injected with anti-GBM alone or both antibodies were sacrificed after 28 days, and the presence of RbIgG, MsIgG, C1q, C4 and C3 was determined by immunofluorescence.

At present the described mouse models for anti-GBM glomerulonephritis show opposite results with respect to the role of complement. C1q was shown to be protective in the accelerated model of anti-GBM disease, presumably via its role in immune complex clearance [7]. In contrast, C1q and C3 were shown to have deleterious functions in the acute phase following injection of heterologous anti-GBM antibody [6] [12], depending on the genetic background, suggesting the involvement of complement in renal injury. In the model presented in our study we inject the primary rabbit IgG anti-GBM antibody in a rather low dose (0.5 mg as compaired to 2 [12] and 4 mg [6]). This elicits complement deposition (C1q) and activation (C4, C3) after 24 hrs and complement is present in the glomeruli even after 3 weeks. However, the amount of antibody is not enough to induce albuminuria. In contrast, injection of higher amounts, e.g. 7.5 mg, of this antibody preparation leads to albuminuria after 24 hr (not shown), indicating the requirement to pass a certain threshold. Using the low dose of rabbit anti-GBM antibodies, we show that hardly any mouse IgG is present in the glomeruli after three weeks, suggesting that the injected amount of rabbit IgG does not cause a significant autologous immune response.

The mouse mAb against rabbit IgG binds to the anchoring antibody in the glomerulus, leading to overt albuminuria accompanied by complement activation and inflammation. Our experiments with $C3^{-/-}$ mice show that complement is responsible for at least part of the glomerular injury, as evidenced by reduced albuminuria, which is consistent with the findings in the direct model [12, 13] but not with the accelerated model [8, 12]. However, the absence of either C1q or C4 showed no effect on the albuminuria compared to wildtype mice, suggesting that the classical and the lectin pathway did not contribute to renal injury.

However, although C1q and C4 were not required for induction of full-blown renal injury, we show that these components significantly contribute to glomerular complement activation as deduced from clearly reduced C3 deposition along the glomerular basement membrane in C1q and C4-deficient mice, respectively. Furthermore, we show that glomerular C4 deposition is largely C1q-dependent. Therefore, these experiments demonstrate that the classical pathway is at least partially responsible for C4 and C3 activation, which is line with the expectations in immune complex-mediated disease.

These experiments gain more interest when compared to experiments in mice deficient for C3, which show strongly reduced albuminuria. Therefore, taken together, our experiments indicate that although the classical pathway and possibly also the lectin pathway contribute significantly to complement activation, activation of C3 via the alternative pathway is sufficient and indispensable for induction of advanced renal injury.

These results are in line with recent studies in an antibody-mediated model of arthritis in the mouse, where the alternative pathway was shown to be both required and sufficient for disease induction [14]. Classically, the alternative pathway is mainly considered as an amplification mechanism of preceding initiation of complement activation via either the classical or the lectin pathway. In this respect, properdin, a key molecule of the alternative pathway, binds efficiently to complexes of C3b with IgG, thus providing excellent amplification [15]. However, recent studies indicate that properdin also can play a more independent role in recognition of complement-activating ligands [16]. In view of the growing list of diseases that turns out to be alternative pathway-dependent, including antibody-mediated arthritis, anti-phospholipid syndrome, ischemia / reperfusion injury [17] and anti-GBM disease, it is worthwhile to further investigate the role of properdin in recognition of autologous ligands for complement activation.

Experiments with Fc-receptor knockout mice indicate that also in this model there is a major role for these receptors in the induction of glomerulonephritis, as has been shown before [18, 19]. Clearly, the present model shows an interplay between humoral mechanisms including antibodies and complement, the latter presumably being partially responsible for tissue injury and for leukocyte attraction, and cellular mechanisms, where Fc receptors are probably crucial for leukocyte adhesion and activation. Together, these mechanisms lead to acute glomerular injury.

Analysis of the late phase of the proposed disease model shows that the renal injury develops into a chronic disease. Injection of the secondary antibody leads to a peak of

albuminuria that declines over time followed by stabilization at a significant level. Complement deposition is still present after 3 weeks. The contribution of antibodies, complement and Fc receptors in this chronic phase of the model is subject to future studies.

In conclusion, our results show the involvement of the alternative pathway of complement, in conjunction with cellular mechanisms, in an attenuated model of anti-GBM-mediated glomerulonephritis. These results offer novel insight into the mechanisms that may play a role in patients with Goodpasture's syndrome, a life-threatening renal disease, and may point towards novel potential approaches for the treatment of acute attacks. Interestingly, renal biopsies from Goodpasture's syndrome usually show linear deposition of IgG and C3 but not of C1q and C4, thus supporting our conclusions.

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Interactions of the Extracellular Matrix Proteoglycans Decorin and Biglycan with C1q and Collectins

4

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Abstract

Decorin and biglycan are closely related abundant extracellular matrix proteoglycans that have been shown to bind to C1q. Given the overall structural similarities between C1q and mannose-binding lectin (MBL), the two key recognition molecules of the classical and the lectin complement pathways, respectively, we have examined functional consequences of the interaction of C1q and MBL with decorin and biglycan. Recombinant forms of human decorin and biglycan bound C1q via both collagen and globular domains and inhibited the classical pathway. Decorin also bound C1 without activating complement. Furthermore, decorin and biglycan bound efficiently to MBL, but only biglycan could inhibit activation of the lectin pathway. Other members of the collectin family, including human surfactant protein D, bovine collectin-43, and conglutinin also showed binding to decorin and biglycan. Decorin and biglycan strongly inhibited C1q binding to human endothelial cells and U937 cells, and biglycan suppressed C1q-induced MCP-1 and IL-8 production by human endothelial cells. In conclusion, decorin and biglycan act as inhibitors of activation of the complement cascade, cellular interactions, and proinflammatory cytokine production mediated by C1q. These two proteoglycans are likely to down-regulate proinflammatory effects mediated by C1q, and possibly also the collectins, at the tissue level.

Introduction

The complement system can be activated via three different pathways: the classical pathway, the lectin pathway, and the alternative pathway. C1q and mannose binding lectin (MBL)3 serve as ligand recognition molecules of the classical and the lectin pathway of the complement system, respectively. Binding of C1q or MBL to their ligands results in activation of the complement cascade and ultimately to opsonization and possibly lysis of pathogens via the membrane attack complex. Bound IgG and IgM serve as principal ligands for C1q, whereas polysaccharides such as yeast-derived mannan are ligands for MBL. C1q and MBL consist of polymers of structurally related trimeric subunits. Ligand recognition is mediated by the heterotrimeric C1q globular head (gC1q) domain composed of A, B, and C chains in the case of C1q and by homotrimeric C-type lectin domains in the case of MBL. Trimeric subunits in both C1q and MBL have a characteristic triple-helical collagen-like region (CLR) at the N terminus. Accordingly, C1q and MBL share the ability to bind to receptors on the cell surface including calreticulin (1) and complement receptor 1 (2) via their CLR domain. Thus, it has been shown that C1q exhibits receptor-mediated binding to endothelial cells, leading to the production and secretion of proinflammatory molecules such as MCP-1 and IL-8 (3).

Undesired activation of the complement pathway is regulated via a number of membrane-bound and soluble complement inhibitors. C1 inhibitor, for instance, is a soluble protein that inhibits both the classical and the lectin pathways via its interactions with the serine proteases associated with the C1 and MBL complexes, respectively. Furthermore, several natural C1q-binding proteins, including proteoglycans, when presented in soluble forms, are able to inhibit the functional activity of C1q (4-9).

Decorin and biglycan are structurally related, abundant extracellular matrix (ECM) proteins, which belong to the family of the small leucine repeat proteoglycans. The 44-kDa core protein of decorin contains long repetitive leucine repeats that are thought to play an important role in the collagen-binding capabilities of decorin (4). Depending on the tissue type, the core protein is either attached to a dermatan or a chondroitin glycosaminoglycan chain (5). Decorin plays a role in the arrangement of collagens and in the regulation of TGF- function (6, 7). Bovine and recombinant human decorin as well as recombinant biglycan have been shown to bind C1q (8, 9, 10). The affinity of human decorin for C1q was much higher than for collagens, suggesting a biologically important interaction between decorin and C1q

(9). Accordingly, bovine decorin was shown to inhibit the classical pathway of complement (8); however, such data concerning human decorin are not available.

Given the overall structural similarities and functional overlaps between C1q and MBL, we have addressed their interaction with decorin and biglycan and examined whether such interactions modulate the classical and the lectin pathways. In this study, we show that decorin and biglycan bind not only to C1q, but also to members of the collectin family including MBL, surfactant protein D (SP-D), collectin 43 (CL-43), and conglutinin. Decorin strongly inhibits the classical pathway but fails to modulate the lectin pathway. However, biglycan inhibits the classical pathway but also binds MBL and prevents activation of the lectin pathway. At the cellular level, decorin and biglycan are able to prevent binding of C1q to U937 cells as well as to endothelial cells. Furthermore, biglycan prevents the C1q-induced production of MCP-1 and IL-8 by endothelial cells. These results suggest that as inhibitors of C1q, decorin, and biglycan can dampen the classical pathway and minimize proinflammatory cellular responses triggered by C1q. Therefore, decorin and biglycan may have an important role in the resolution of C1q-mediated inflammatory processes in the tissues.

Materials and Methods

Purification of human MBL

MBL was purified from human serum, by first precipitating with polyethylene glycol 3350 (7% w/v) (Sigma-Aldrich). The precipitate was resuspended in TBST (pH 7.8) containing 20 mM Ca^{2+}. Subsequently, the solution was rotated overnight with mannan-coupled Sepharose beads at 4°C. After washing with TBST-Ca^{2+} containing 1 M NaCl to remove nonspecifically bound protein, the beads were transferred to a column, and MBL was eluted using TBST containing 10 mM EDTA. Fractions were tested for the presence of MBL by ELISA as described before (11). Peak fractions containing MBL were pooled, concentrated, and dialyzed against PBS. To obtain mannose-binding lectin-associated serine protease (MASP)-free MBL, this preparation was loaded onto a Sepharose 6B fast protein liquid chromatography column using 0.1 M acetic acid containing 0.2 M NaCl and 5 mM EDTA, pH 5, as a running buffer. Fractions were tested for MBL by ELISA and assessed for MASP-2 activity using a C4 consumption assay. Fractions positive for MBL and negative for MASP-2 activity were pooled, dialyzed against PBS, and subsequently stored in aliquots at –20°C.

Preparation of human C1q and its CLR domain

C1q was purified from human plasma as described previously (12). C1q CLR was prepared by digestion of 1 mg of purified C1q with 0.2 mg of pepsin diluted in 0.1 M sodium

acetate/0.15 M NaCl (pH 4.5) for 4.5 h in a shaking water bath at 37°C. The solution was neutralized using 1 M Tris (pH 10), dialyzed against PBS/10 mM EDTA, and subsequently loaded onto a human IgG/rabbit IgG-Sepharose column as described previously by Nauta et al. (12) to remove uncleaved C1q as well as gC1q domain. The flow through of the column was shown to contain C1q CLR but not gC1q domain as revealed by specific mAb. The fractions containing the CLR were pooled, dialyzed against PBS, concentrated, and stored in aliquots at –20°C.

Other proteins

Recombinant forms of human decorin and biglycan were expressed in mammalian cells using a vaccinia virus/T7 bacteriophage expression system and were isolated under nondenaturing conditions as described (9, 10). The purity of recombinant decorin and biglycan was examined on a 10% SDS-PAGE (Fig. 1). Both protein preparations consist of a proteoglycan form as represented by the polydisperse band migrating between 80 and 200 kDa and a core protein form migrating between 50 and 60 kDa. The decorin core protein appears as a doublet and is due to differential asparagine-linked glycosylation (9).

Native human SP-D was purified from pooled amniotic fluid, as described previously (13). A recombinant fragment of human SP-D (rhSP-D) composed of homotrimeric neck region and C-type lectin domains was expressed in Escherichia coli and purified as described recently (14). Bovine conglutinin (15) and CL-43 (16) were produced as described. Recombinant forms of the C-terminal globular regions of human C1q A, B, and C chains (ghA, ghB, and ghC, respectively) were expressed in E. coli as fusion proteins linked to maltose binding protein and purified as described recently (17). The collagen-free and native heterotrimeric gC1q domain was prepared using human C1q, as described previously (18).

Generation of rabbit antisera

Polyclonal antisera against decorin and biglycan were generated in rabbits, using synthetic peptides corresponding to regions near the N terminus of human decorin (GIGPEVPDDRDF-C) and human biglycan (GVLDPDSVTPTYSAM-C). The peptides were synthesized with an additional cysteine at the C terminus, which was used for coupling to keyhole limpit hemocyanin.

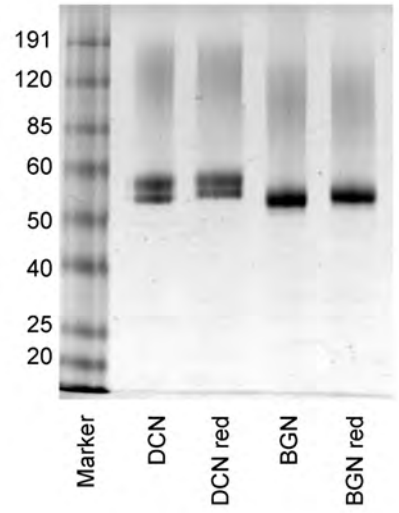

Figure 1. SDS-PAGE analysis of recombinant decorin (DCN) and biglycan (BGN). Two micrograms of DCN or BGN were run on a 10% polyacrylamide gel under nonreduced conditions (*lanes 2* and *4*) or reduced conditions with -mercaptoethanol (*lanes 3* and 5).

The keyhole limpit hemocyanin-peptide conjugates were then used to immunize rabbits following a standard immunization protocol. Both the peptide synthesis and Ab production were performed by Alpha Diagnostic International.

General ELISA

In general, ELISA experiments were performed using Maxisorb plates (Nunc). For coating, proteins were diluted in coating buffer (100 mM $Na_2CO_3/NaHCO_3$, pH 9.6) and incubated either overnight at room temperature or for 2 h at 37°C, followed by blocking of nonspecific binding sites with PBS containing 1% w/v BSA for 1 h at 37°C. The secondary Abs were, unless indicated otherwise, diluted in PBS containing 1% w/v BSA/0.05% v/v Tween 20 (PTB) and incubated 1 h at 37°C. Between every incubation step the wells were washed three times with PBS containing 0.05% v/v Tween 20, unless indicated otherwise. Enzyme activity of HRP was detected using ABTS substrate (Sigma-Aldrich). A414 was measured using a microplate biokinetics reader (EL312e; Biotek Instruments).

Interaction of C1q and MBL with decorin and biglycan

Either decorin (5 µg/ml), biglycan (5 µg/ml), purified human IgM (3 µg/ml), or BSA were coated to microtiter wells. After the blocking step, the wells were incubated with different concentrations of purified human C1q or its CLR diluted in PTB, normal human serum (NHS) diluted in BVB^+ buffer (Veronal-buffered saline, 5% BSA, 1 mM $CaCl_2$, 0.25% Tween 20),

or MBL (diluted in BVB buffer with calcium or with 2 mM EDTA but no calcium). Bound C1, C1q, or CLR were detected using a mAb directed against the C1q CLR (mAb 2214) coupled to digoxygenin-3-O-methyl-carbonyl--aminocaproicacid-N-hydroxy-succinimide ester (DIG; Boehringer Mannheim). Bound MBL was detected using a mAb against MBL (mAb 3E7; provided by Dr. T. Fujita, Fukushima Medical University, Fukushima, Japan) coupled to DIG. DIG-conjugated mAb were detected using Fab of sheep IgG directed against DIG, coupled to HRP (Boehringer Mannheim).

C1 binding and complement activation

Decorin, biglycan, IgM, or BSA were coated to wells, followed by incubation with NHS as a complement source, diluted in BVB^{++} buffer (BVB^{+} + 0.5 mM $MgCl_2$). Activation of C4 was assessed as described previously (19). Binding of C1 was assessed using a mAb against C1q (mAb 2214) conjugated to DIG. For functional assessment of C1 binding, wells were incubated with NHS (diluted in BVB^{++}) for 2 h at 4°C, followed by incubation with purified C4 and assessment of C4 binding, as described earlier (19).

Inhibition of C1q binding to decorin

Decorin (5 µg/ml) was coated to microtiter wells, followed by addition of DIG-conjugated C1q in the presence or absence of 5 µg/ml anti-gC1q (mAb 85 or mAb 2204), anti-CLR (mAb 2211), or 100 µg/ml purified mouse Fc tails (provided by Dr. J. Egido (Department of Immunology, Fundación Jiménez Díaz, Autonoma University, Madrid, Spain) and Dr. F. Vivanco (Renal Research Laboratory, Fundación Jiménez Díaz, Autonoma University, Madrid, Spain)) for 1 h at 37°C. After washing, C1q binding was detected using HRP-conjugated Fab of sheep IgG directed against DIG (Boehringer Mannheim).

Binding of decorin and biglycan to immobilized lectins or C1q fragments

Purified C1q, C1q-derived fragments (CLR, gC1q, ghB), collectins (MBL, SP-D, rhSP-D, conglutinin, and CL-43), or BSA were coated on an ELISA plate at 2 µg/ml. After blocking, decorin or biglycan diluted in PTB containing 2 mM EDTA was added to the wells and incubated for 1 h at 37°C. Binding was detected using rabbit antiserum against decorin or biglycan diluted in PTB, followed by detection with a polyclonal goat anti-rabbit Ab coupled to HRP (Jackson ImmunoResearch Laboratories).

Inhibition of the lectin pathway

Mannan (100 µg/ml) was coated to an ELISA plate. C1q-depleted plasma (20) was preincubated on ice for 15 min in the presence of decorin, biglycan, or D-mannose and then

added to the plate for 1 h at 37°C. As a measure for MBL-pathway activation, C5b-9 deposition in the wells was detected using a mAb against C5b-9 coupled to DIG (AE-11; provided by Dr. T. E. Mollnes, Institute of Immunology, Rikshospitalet University Hospital, Oslo, Norway).

Inhibition of MBL binding

Mannan (100 µg/ml) was coated to an ELISA plate. After blocking, MBL (1 µg/ml) was added in the presence or absence of different concentrations of either decorin, biglycan, or D-mannose. Bound MBL was detected using a mAb against MBL coupled to DIG (3E7-DIG).

Hemolytic assays

Inhibition of the classical pathway by decorin or biglycan was assessed using a CH_{50} assay, which measures the total activity of the classical pathway as well as a C1q-dependent hemolytic assay (C1qHA) as previously described (21). Briefly, in the CH_{50}, Ab-sensitized sheep Ab-opsonized erythrocytes (EA; 1 x 10^8 cells) were incubated with NHS in a fixed dilution for 1 h at 37°C. NHS was preincubated for 15 min on ice with or without decorin, biglycan, or human serum albumin (HSA) before addition of the mixture to the EA. Total classical pathway activity was assessed by measuring EA lysis. In the C1qHA, EA were incubated with a fixed dose of C1q (20 ng/ml) and C1q-depleted plasma (1/100). C1q was preincubated for 15 min on ice with or without decorin, biglycan, or HSA before addition of the mixture to the EA. The C1q-dependent classical pathway activity was assessed by measuring EA lysis. The lytic activity of both CH_{50} and C1qHA was expressed as a Z value: $Z = -\ln [1 - ((OD414 \text{ lysis by sample}) - (OD414 \text{ 0\% lysis}))/((OD414 \text{ 100\% lysis}) - (OD414 \text{ 0\% lysis}))]$. The amount of C1q in the C1qHA and the dilution of the NHS in the CH_{50} were chosen in such a way that Z value in the absence of inhibitor was 1 (63% lysis).

Cell culture

HUVEC and U937 cells were used for flow cytometry and cell-stimulation assays. HUVEC were obtained from fresh umbilical cords after collagenase (Sigma-Aldrich) treatment and were cultured in M199 medium (Invitrogen Life Technologies) containing 10% v/v heat-inactivated FCS (Invitrogen Life Technologies), 100 IU/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma-Aldrich), 7.5 IU/ml heparin (Sigma-Aldrich), 2 ng/ml EGF (R&D Systems), and 250 pg/ml -ECGF (BioSource International). Cells were harvested using trypsin and used for flow cytometry analysis. HUVEC were used in the experiments between passages 2 and 4. U937 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-

inactivated FCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin (both from Sigma-Aldrich).

Flow cytometric analysis of C1q binding

The effect of decorin or biglycan on the ability of C1q to bind to cells was examined using flow cytometry. HUVEC and U937 cells were first washed in low-ionic-strength buffer (0.5x PBS, 155 mM glucose, 1% BSA, and 0.1% sodium azide). Cells were incubated in low-ionic-strength buffer for 30 min on ice. C1q (2.5 µg/ml) was preincubated with different concentrations of decorin or biglycan before being added to the cells. After washing, C1q binding was detected by a polyclonal rabbit anti-C1q Ab (20) followed by PE-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates). Dead cells were excluded from the analysis by the use of propidium iodide (Molecular Probes). The cell-associated fluorescence was measured using a FACSCalibur (BD Biosciences) and results were expressed as the mean fluorescence intensity.

Stimulation of endothelial cells with C1q in the presence of biglycan or decorin

The effect of decorin and biglycan on the C1q-induced production of MCP-1 and IL-8 was examined using confluent layers of HUVEC on 0.5% gelatin-coated 96-well plates. C1q (20 µg/ml) was coincubated with or without different concentrations of decorin or biglycan for 1 h at room temperature in AIM-V serum-free medium (Invitrogen Life Technologies) completed with 100 IU/ml penicillin and 100 µg/ml streptomycin (both Sigma-Aldrich). Subsequently, HUVEC were incubated with this mixture for 48 h, then supernatants were harvested and analyzed for IL-8 and MCP-1 levels by a sandwich ELISA, as has been previously described by van den Berg et al. (3).

Results

Binding of C1q to decorin and biglycan and inhibition of the classical complement pathway

In ELISA, human C1q bound strongly to solid-phase decorin and biglycan in a dose-dependent manner (Fig. 2A). The strength of binding between C1q and proteoglycans was comparable to that between C1q and IgM. No binding was observed to wells coated with BSA (Fig. 2A). Native bovine decorin was shown to bind C1q to a similar extent as recombinant human decorin (results not shown).

The ability of decorin and biglycan to inhibit the functional activity of the classical pathway was examined using hemolytic assays. The effect of human decorin and biglycan on the total activity of the classical pathway was examined in a CH_{50} assay, using total human serum as a complement source. Decorin inhibited the hemolytic activity of the classical pathway (IC_{50} ~5 μg/ml) in a dose-dependent manner (Fig. 2B). Although biglycan also inhibited the classical pathway, it was found to be 10 times less effective than decorin. In agreement with a direct interaction of decorin with C1q, decorin was able to dose-dependently inhibit the hemolytic activity of C1q in a C1qHA, with an IC_{50} of ~0.1 μg/ml (Fig. 2C). In addition, biglycan was able to inhibit C1q hemolytic activity but less effectively than decorin (IC_{50} ~1 μg/ml). The control protein HSA did not have any effect on complement-induced lysis of EA.

Solid-phase decorin binds C1 but fails to activate the classical pathway

Because immobilized ligands of C1q are known to activate the classical pathway, we examined whether immobilized decorin and biglycan had similar properties. When coated to microtiter wells and incubated with different serum concentrations as a complement source, both decorin and biglycan failed to activate C4. In contrast, IgM, as a positive control, activated complement, leading to strong deposition of C4 (Fig. 3A). BSA, which was used as a negative control protein, did not activate C4.

Because decorin and biglycan did not activate the classical pathway despite binding C1q, we sought to examine whether decorin and biglycan were able to bind C1 (C1q in association with C1r and C1s). Solid-phase decorin and IgM, but not biglycan, bound C1 in a dose-dependent manner (Fig. 3B). When C1 was allowed to bind human IgM, and then incubated with exogenous C4, a strong activation of C4 was observed (Fig. 3C). However, no activation of exogenous C4 was observed after binding of C1 to decorin. Furthermore, neither biglycan nor BSA did induce activation of exogenous C4.

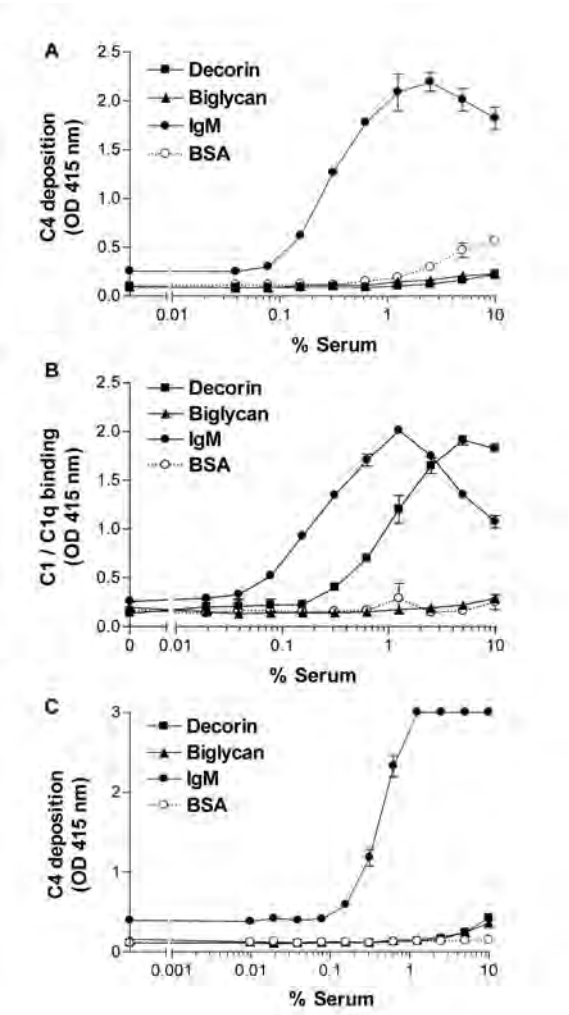

Figure 2. Decorin and biglycan bind C1q and inhibit the classical complement pathway. *A*, Microtiter wells were coated with decorin, biglycan, IgM, or BSA and subsequently incubated with different concentrations of C1q. Binding was detected using a C1q-specific mAb. *B*, NHS was premixed with different concentrations of decorin, biglycan, or HSA, followed by incubation with EA and measurement of hemolysis. *C*, C1q (20 ng/ml) was preincubated with different concentrations of decorin, biglycan, or HSA and then mixed with C1q-depleted serum and EA.

Characterization of the interaction between decorin and C1q

To identify the regions/domains within C1q that interacted with decorin, a competitive ELISA was performed where ligands and Abs directed against gC1q or CLR domains of C1q were allowed to compete for binding of C1q to immobilized decorin. Coincubation of C1q with two mAb directed against the gC1q domain (mAb 2204 and mAb 85) completely abolished C1q binding to decorin (Fig. 4A). Furthermore, purified Fc portions of mouse IgG that bind to

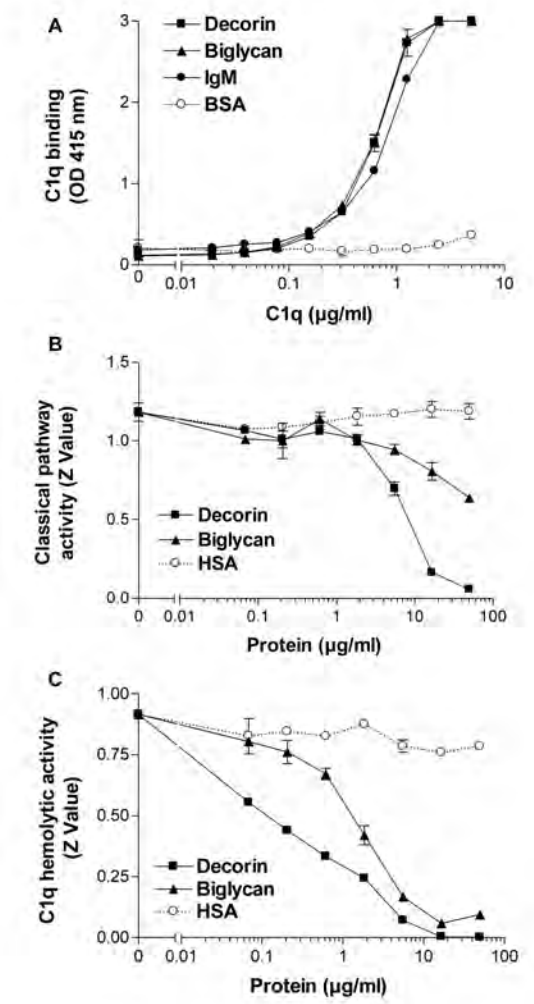

Figure 3. Decorin binds C1 but does not activate the classical pathway. *A*, Decorin, biglycan, IgM, or BSA were coated onto microtiter wells and incubated with NHS for 1 h at 37°C. C4 deposition was measured using a C4-specific mAb. *B*, Decorin, biglycan, IgM, or BSA were coated and then incubated with NHS in a Ca^{2+}-containing buffer for 2 h at 4°C. C1 binding was detected using mAb 2214. *C*, Decorin, biglycan, IgM, or BSA were coated and incubated with NHS for 2 h at 4°C. The wells were washed with buffer containing Ca^{2+} and then incubated with human C4 for 1 h at 37°C, followed by C4 detection.

the gC1q domain efficiently competed with the binding of C1q to decorin. As a negative control for inhibition, we used an Ab (mAb 2211) that recognizes the CLR portion of C1q, which did not inhibit the C1q-decorin interaction. Furthermore, the interaction of decorin and biglycan with the gC1q domain was studied by comparing the ability of decorin or biglycan to directly bind to immobilized intact C1q, gC1q, and the recombinant form of ghB (Fig. 4B). Biglycan and decorin showed strong binding to the immobilized native gC1q domain,

significantly better than to intact C1q. However, both proteoglycans did not bind to the recombinant ghB. In a similar binding experiment, neither decorin nor biglycan showed any detectable binding to immobilized recombinant modules ghA, ghB, or ghC, suggesting a requirement for a heterotrimeric structure of the gC1q domain for interaction with proteoglycans (results not shown).

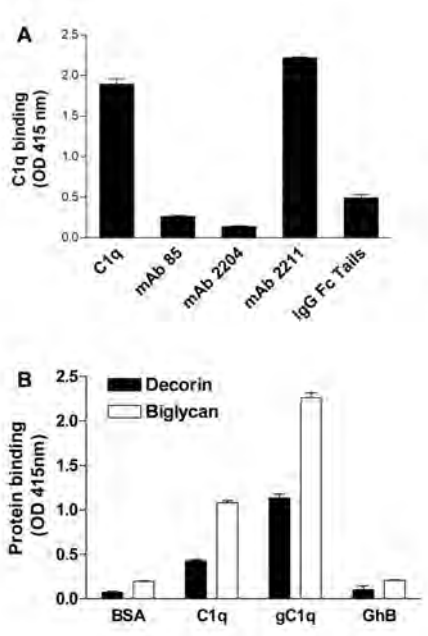

Figure 4. Decorin and biglycan bind the gC1q domain of human C1q. *A*, DIG-conjugated C1q was preincubated in the presence of gC1q-specific mAb (mAb 2204 and mAb 85), a noninhibitory mAb directed against the CLR domain (mAb 2211), or mouse Fc tails. After incubation on a decorin-coated ELISA plate for 1 h at 37°C, C1q binding was detected using anti-DIG Abs. *B*, C1q, native gC1q, ghB, or BSA were coated on a plate (2 μg/ml) followed by incubation with either 10 μg/ml decorin or 1 μg/ml biglycan. Binding of decorin and biglycan was detected using a rabbit antiserum raised against decorin and biglycan, respectively.

In a direct binding ELISA, CLR domain was able to bind decorin, although nearly 15 times less efficiently than intact C1q (Fig. 5A). C1q, but not CLR domain, bound IgM (Fig. 5B), confirming that the CLR preparation did not contain portions of gC1q domain. Furthermore, mAb directed against the gC1q domain (mAb 85 and mAb 2204) did not bind the CLR preparation, whereas a mAb specific to the CLR domain (mAb 2214) showed strong binding (data not shown). Both decorin (Fig. 5C) and biglycan (Fig. 5D) bound immobilized CLR domain in a dose-dependent manner. Detection with an Ab against the CLR domain of C1q confirmed that both C1q as well as CLR were able to bind well to an ELISA plate (Fig. 5E).

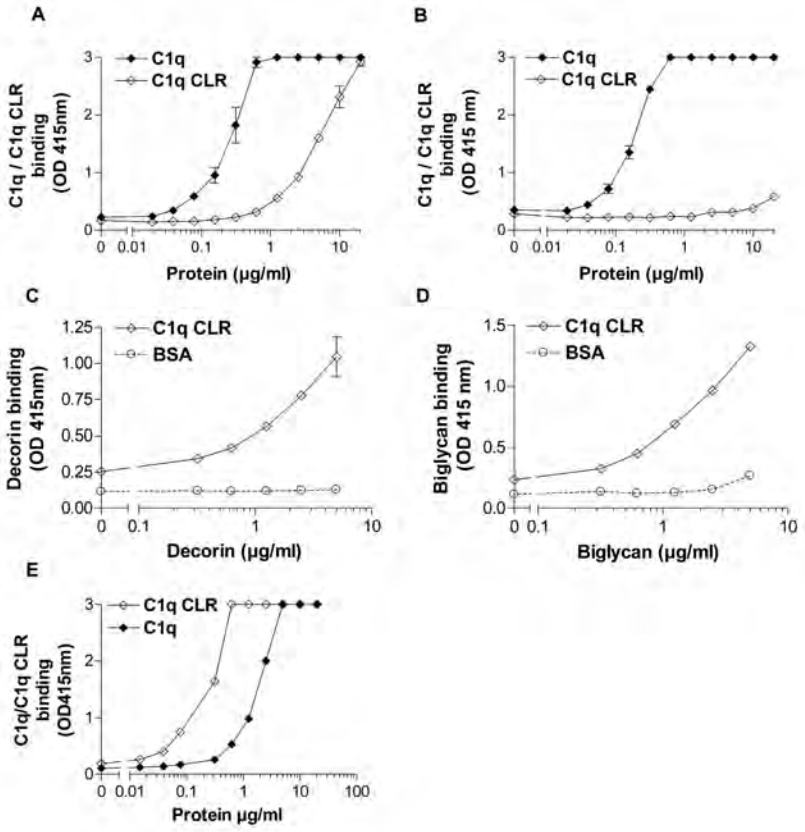

Figure 5. Decorin and biglycan bind the CLR domain of human C1q. Various concentrations of C1q or its CLR preparation were allowed to bind to immobilized decorin (*A*) or IgM (*B*). C1q and CLR binding was probed using mAb 2214. Different concentrations of decorin (*C*) or biglycan (*D*) were allowed to bind immobilized CLR or BSA. Binding of decorin and biglycan was probed using a rabbit anti-serum raised against decorin or biglycan, and then detected using goat anti-rabbit HRP conjugate. *E*, Microtiter wells were coated with different concentrations of C1q or CLR, and binding was detected using mAb 2214.

Decorin and biglycan bind to C1q and members of the collectin family

In view of the binding of decorin and biglycan to C1q, we further investigated their interaction with MBL and other collectins, considering similar overall structure and presence of triple-helical collagen regions. In a direct binding ELISA using a calcium-free buffer, both decorin and biglycan bound to immobilized C1q, native SP-D and rhSP-D, and the bovine conglutinin and CL-43 in a dose-dependent manner (Fig. 6, A and B). Decorin and biglycan showed the strongest binding to conglutinin, whereas only biglycan could detectably bind to immobilized MBL. This binding could not be improved by incubation in the presence of calcium (not shown). These data suggest that decorin and biglycan recognize a broad range of collagen-containing innate immune molecules without requiring calcium.

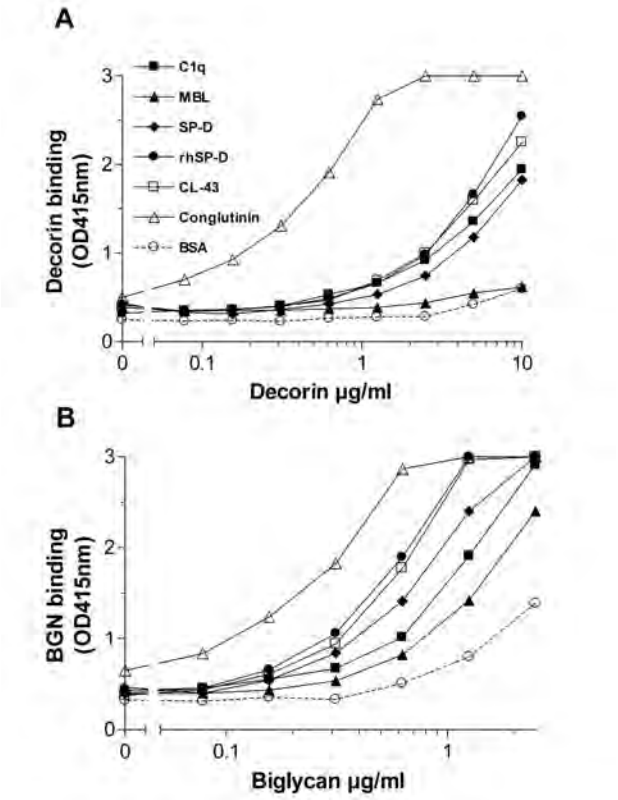

Figure 6. Decorin and biglycan interact with the members of the collectin family, including MBL, SP-D, conglutinin, and CL-43. Microtiter wells were coated with 2 µg/ml C1q, MBL, native SP-D, rhSP-D, bovine conglutinin, CL-43, or BSA. Wells were incubated with decorin (*A*) or biglycan (*B*), both in the presence of 2 mM EDTA. Proteoglycan binding was detected using specific rabbit antisera.

MBL binds to decorin in a Ca^{2+}-dependent way but does not inhibit the lectin pathway

The observation that biglycan can interact with MBL in Ca^{2+}-free conditions, prompted us to investigate 1) whether biglycan and decorin can interact with native MBL via its Ca^{2+}-dependent C-type lectin domain, 2) whether, by binding MBL, decorin and biglycan can modulate the lectin pathway, and 3) whether this modulation involves interference in the ligand binding of the C-type lectin domain. Purified MBL was incubated with immobilized decorin or biglycan in the absence or presence of Ca^{2+}, and binding was assessed using a mAb against MBL. Decorin and biglycan clearly bound MBL, but only in the presence of calcium (Fig. 7A), suggesting the involvement of the calcium-dependent C-type lectin domains of MBL in this interaction.

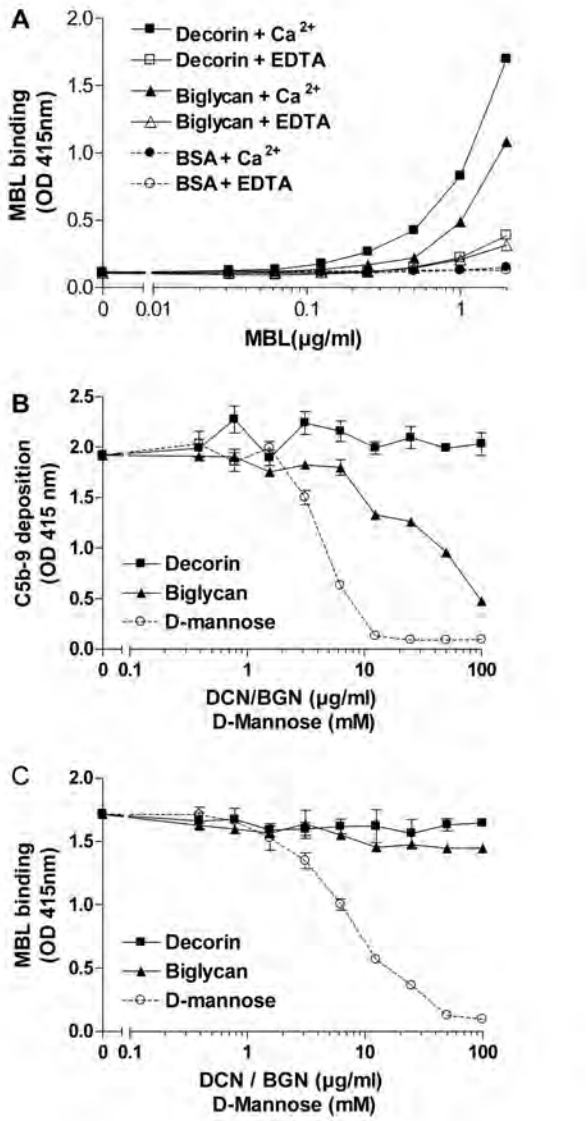

Figure 7. Decorin and biglycan bind MBL, but only biglycan inhibits the lectin pathway. *A*, Microtiter wells were coated with 5 µg/ml decorin, biglycan, or BSA and incubated with different concentrations of purified MBL in the presence or absence of Ca^{2+}. MBL binding was detected using an MBL-specific mAb. *B*, C1q-depleted serum was preincubated with different concentrations of decorin, biglycan, or D-mannose followed by incubation on a mannan-coated ELISA plate for 1 h at 37°C. C5b-9 deposition was detected using a specific mAb. *C*, MBL was preincubated in the presence of different concentrations of decorin, biglycan, or D-mannose and then allowed to bind to mannan-coated microtiter wells. Binding of MBL was detected using an MBL-specific mAb.

To assess the effect of the MBL-proteoglycan interactions on the activation of the lectin pathway, mannan-coated plates were incubated with C1q-depleted plasma as a complement source, followed by assessment of complement activation and generation of the C5b-9 complex as assessed with a mAb directed against C5b-9. Decorin was not able to inhibit the lectin pathway-mediated formation of C5b-9 (Fig. 7B). In contrast, biglycan nearly completely inhibited activation of complement via the MBL pathway with increasing concentration (IC_{50} 40 µg/ml). Also D-mannose, a known inhibitor of the lectin pathway, clearly inhibited complement activation (IC_{50} 5 µM).

To determine whether decorin and biglycan could modulate lectin pathway activation by inhibiting the binding of MBL to its ligand, MBL in the presence or absence of different concentrations decorin, biglycan, or D-mannose was incubated on a mannan-coated plate. Subsequently, binding of MBL was detected. Neither biglycan nor decorin could prevent the binding of MBL to its ligand, suggesting that inhibition of the lectin pathway by biglycan was not at the level of ligand binding (Fig. 7C). In contrast, D-mannose as a ligand for the C-type lectin domain could inhibit the binding of MBL to mannan completely.

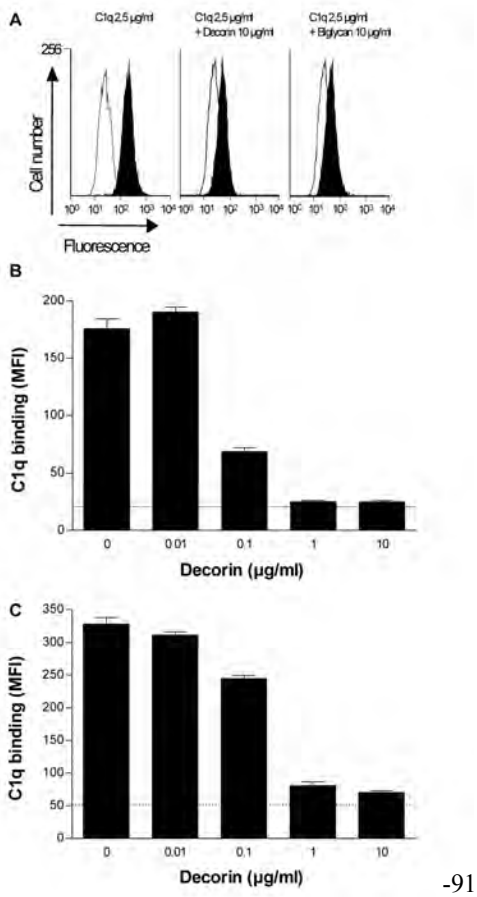

Figure 8. Decorin and biglycan inhibit the interaction of C1q with HUVEC and U937 cells. *A,* Purified C1q was preincubated with decorin or biglycan and then incubated with HUVEC. C1q binding was detected by flow cytometry using an polyclonal anti-C1q polyclonal Ab and followed by goat anti-rabbit-PE. Data are expressed as mean fluorescence intensity. *B* and *C,* C1q was preincubated with different concentrations of decorin in half isotonic buffer and then incubated with either HUVEC or U937 cells. C1q binding was detected using a C1q-specific polyclonal Ab followed by goat anti-rabbit-PE.

Decorin and biglycan inhibit the binding of C1q to HUVEC and U937 cells

Because C1q is known to modulate various immune cells through its interaction with C1q receptors (3), we sought to establish whether decorin or biglycan would interfere with C1q-cell interactions. Flow cytometry revealed that C1q was able to bind to both HUVEC and U937 cells. Decorin and biglycan strongly inhibited the binding of C1q to HUVEC (Fig. 8A). Decorin prevented the C1q binding to HUVEC in a dose-dependent manner with an IC_{50} of 0.1 µg/ml (Fig. 8B). Decorin was also able to inhibit the binding of C1q to U937 cells with an IC_{50} between 0.1 and 1 µg/ml (Fig. 8C).

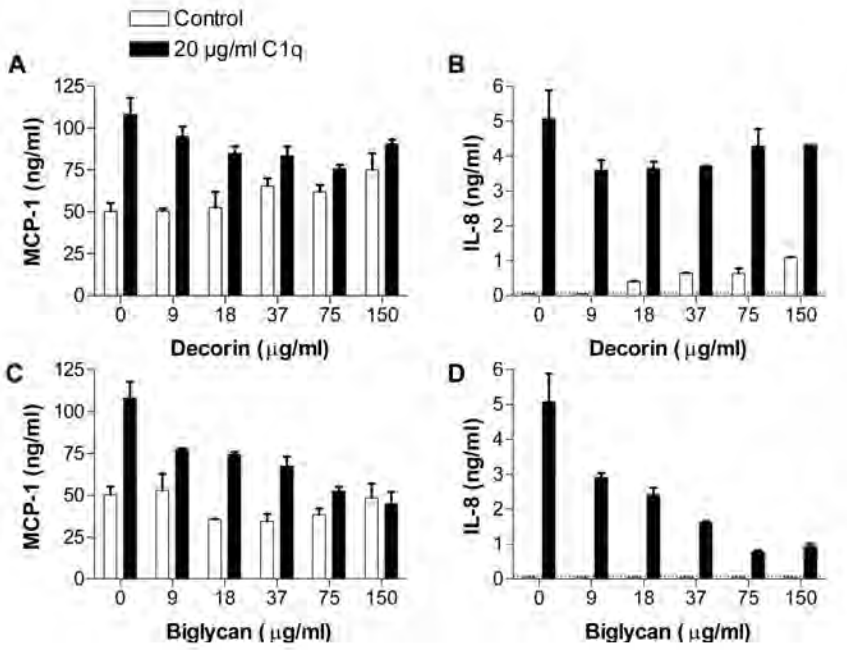

Figure 9. Biglycan inhibits the C1q-induced production of IL-8 and MCP-1 by HUVEC. HUVEC were cultured with (black bars) or without (white bars) 20 µg/ml C1q in the presence or absence of different concentrations of either decorin (*A* and *B*) or biglycan (*C* and *D*). After 48 h, the concentration of MCP-1 and IL-8 in the supernatant was determined by ELISA.

Biglycan inhibits C1q-induced MCP-1 and IL-8 production

Interaction of C1q with endothelial cells has been shown to result in production of inflammatory cytokines and chemokines, such as MCP-1 and IL-8. Therefore, we examined the effect of decorin and biglycan on C1q-induced MCP-1 and IL-8 production by HUVEC. Stimulation for 48 h with C1q alone resulted in strongly increased MCP-1 (~110 ng/ml) and IL-8 (~5 ng/ml) production (Fig. 9) compared with cells cultured in the presence of medium alone (~50 ng/ml and <0.1 ng/ml, respectively). The presence of decorin together with C1q

resulted in slight inhibition of MCP-1 production (Fig. 9A) and had no effect on the C1q-induced IL-8 production (Fig. 9B). It was noted that decorin by itself slightly increased MCP-1 and IL-8 production by HUVEC, which effect almost nullified the inhibitory effects of decorin on C1q-induced MCP-1 and IL-8 production. In contrast, addition of biglycan led to a dose-dependent decrease of C1q-induced MCP-1 production and completely abrogated the effect of C1q on IL-8 production (Fig. 9, C and D). Biglycan by itself had no effect on MCP-1 and IL-8 production.

Discussion

Components of the ECM are considered important for structural integrity, cell signaling, and survival within tissue organization. The ECM proteins can also play an active role in the innate immune response, as recently described for mindin (also called spondin 2), which binds bacteria and functions as opsonin for murine macrophages (22). Other ECM proteins have also been shown to regulate the complement system. Earlier, decorin, an ECM proteoglycan, was shown to bind C1q and inhibit the classical pathway of complement (8).

The ECM proteoglycans decorin and biglycan possess 55% similarity on the amino acid level. However, the secondary structure of both proteins as well as the spatial and temporal expression is different (6). Considering this, we wanted to investigate whether the recombinant forms of both proteins have similar effects on the classical pathway.

Human decorin and biglycan possess a higher affinity for immobilized C1q than for their well-known ligands, including collagens I, II, III, V, and VI (10), which suggests a possibly physiologically important interaction. In addition, we now show that the binding of C1q to immobilized decorin and biglycan is similar to the binding of C1q to its natural ligand IgM. Furthermore, human decorin, as described for bovine decorin, is capable of completely inhibiting the C1q-dependent lysis of Ab-opsonized erythrocytes. Interestingly, a similar effect was observed for biglycan.

Immobilized human decorin can bind intact C1, whereas biglycan is not able to bind C1. Furthermore, in contrast to other ligands that bind C1 such as IgM, IgG, and pentraxins, decorin completely fails to activate the classical pathway, although the protein was presented in a multimeric fashion by immobilization on plastic, a condition that is likely to facilitate complement activation. The ability of decorin to bind C1 is also reflected by its ability to inhibit the complement-mediated lysis of Ab-opsonized erythrocytes in the presence of whole serum as a complement source, where C1q is present in a calcium-rich environment and, therefore, predominantly present in the C1 form. Biglycan can also inhibit complement-

mediated erythrocyte lysis via the classical pathway, but at a much higher IC_{50} than decorin, which is consistent with its undetectable binding to C1.

Because the level of decorin in the circulation is low (0.9 ng/ml) (23), it seems unlikely that decorin plays a major role as inhibitor of the classical pathway in the circulation. However, at the cellular level, decorin is estimated to be present in the ECM in concentrations between 5 and 12.5 μg/ml (24). Thus, under conditions of tissue damage or remodeling of the ECM, decorin may play a role in inhibiting the classical pathway. However, biglycan is not able to bind C1 and has a much lower inhibitory effect on complement-mediated lysis of opsonized erythrocytes, which appears to suggest that under normal physiological conditions, biglycan may not be as relevant as decorin in the regulation of the classical pathway. However, infiltrating cells like macrophages have been shown to secrete biglycan in a model of renal inflammation, upon their stimulation with inflammatory cytokines (25). Hence, in inflammatory conditions, the additional biglycan could have an effect on the classical pathway of complement.

The mechanism of binding of C1q to decorin is a complicated issue. The ability of decorin to bind to several different collagens would favor binding via the CLR domain of C1q. However, C1q binding to bovine decorin has been described to be mediated via both the gC1q as well as the CLR of C1q (8), hence bovine decorin was proposed to bind at the hinge region between the gC1q and CLR domains. Consistent with this, Abs directed against the gC1q domain are able to completely inhibit the binding of C1q to immobilized decorin. Furthermore, the mouse IgG-Fc tails, as a natural ligand for the gC1q domain, appear to prevent C1q from binding to immobilized decorin. Curiously, individually expressed modules of the gC1q domain (ghA, ghB, and ghC) failed to interact with decorin, although they do bind various C1q ligands differentially, indicating their functional activity and proper folding (17). However, the native gC1q domain, prepared after collagenase digestion of native C1q, is able to strongly interact with decorin and biglycan, indicating that the C1q-proteoglycan interaction might require a combined heterotrimeric structure of the gC1q domain and the individual chains may contribute to proteoglycan binding.

We also noticed a direct interaction of C1q CLR domain with decorin and biglycan, consistent with the collagen-binding properties of these proteoglycans. However, relative to the binding to intact C1q, the interaction of decorin and biglycan was far less than as compared with the binding of C1q and the gC1q domain to both proteoglycans. Furthermore, recent data indicate that interactions with isolated C1q CLR could be a result of the preparation, altering the physical-chemical properties of the molecule, and the binding

characteristics of isolated CLR are different from those of intact C1q (26). Moreover, our experiments with inhibitory Abs against the gC1q domain clearly indicate that the primary interaction of proteoglycans with intact C1q involves the gC1q domain.

C1q has a number of characteristics in common with members of the collectin family. These molecules are all characterized by a multimeric structure consisting of trimeric subunits, as well as by similar collagenous domains containing Gly-X-Y repeats. Ligand recognition takes place via gC1q domain for C1q and via C-type lectin domain for collectins (27). Recently, SP-D has been shown to bind decorin (28), and this interaction involves C-type lectin domain binding to decorin-attached glycosaminoglycan chain, whereas the decorin core protein can bind via SP-D collagen region (28). In the present study, we validated the interaction of decorin and biglycan with native SP-D and rhSP-D, which represents the trimeric C-type lectin domains. We observed that immobilized CL-43, conglutinin, rhSP-D, and SP-D can bind decorin and biglycan in a dose-dependent and calcium-independent manner, suggesting a protein-protein interaction. Furthermore, in the absence of calcium, only biglycan showed an interaction with immobilized MBL, indicating that biglycan may bind to the CLR domain of MBL. However in the presence of Ca^{2+}, purified MBL binds efficiently to immobilized decorin and biglycan, suggesting that the C-type lectin domains of MBL could also bind to carbohydrates present on decorin and biglycan, as is the case for SP-D (28).

The ability of decorin to bind to MBL is not reflected in its ability to inhibit the lectin pathway of complement. Most likely, the interaction of decorin with the C-type lectin domain of MBL in the fluid phase is insufficient to inhibit complement activation significantly because addition of decorin has no effect on the binding of MBL to a immobilized ligand. In contrast, the interaction of decorin with C1 does result in the inhibition of complement. As opposed to decorin, biglycan is capable of inhibiting activation of the lectin pathway, although it does not seem to inhibit the MBL-ligand interaction. Therefore, it is likely that the inhibitory effect of biglycan on the lectin pathway activation involves the interaction of biglycan with the CLR domain of MBL, possibly interfering in binding and/or activation of MASP-2.

The C1q molecule is known to bind to immune cells via cell membrane receptors and to induce the production of inflammatory cytokines and chemokines, such as MCP-1 and IL-8, by endothelial cells (3). We found that decorin and biglycan strongly inhibit the binding of C1q to cells, presumably interfering in receptor-mediated interactions. In addition, biglycan clearly had an inhibitory effect on the C1q-induced MCP-1 and IL-8 production by

endothelial cells. Therefore, decorin and biglycan not only function as inhibitors of the classical pathway of complement activation, but may have an additional role in down-regulating the proinflammatory effects of C1q on cells, by inhibition of the production of cytokines. In contrast, it has been observed that biglycan, via interaction with TLR-2 and -4 on macrophages, can induce the expression of inflammatory mediators like TNF- and MIP-2 (25). Furthermore, in biglycan-deficient mice, the absence of biglycan has been associated with a survival benefit in a mouse model of sepsis (25). Together, these results indicate that biglycan is able to modulate inflammation in several ways, and its final effect may be strongly dependent on the context, the site, and the phase of the inflammatory process.

It has been described previously that infusion of decorin can ameliorate fibrosis in an experimental model of kidney disease, induced by anti-Thy1 Abs, which was explained by an inhibitory effect on TGF- function (29). However, because this is a complement-dependent model, it is possible that the ameliorating effects of decorin might be partially explained by its ability to prevent the activation of the classical complement pathway. Accordingly, complement inhibition may contribute to a reduction of the degree of damage inflicted to tissues, ultimately resulting in less fibrosis. Moreover, in an experimental model of unilateral uretal obstruction, mice deficient in decorin showed exaggerated apoptosis, mononuclear cell infiltration, tubular atrophy, and matrix deposition, as compared with wild-type mice (30). Whether increased tissue damage in decorin-deficient mice is associated with activation of the classical complement pathway, is worth investigating.

In summary, we have shown that the human proteoglycans decorin and biglycan interfere differentially with various aspects of complement-mediated inflammatory responses, including activation of the complement cascade via classical and lectin pathways, and endothelial cell activation. Furthermore, interaction of these proteoglycans with collectins suggests a potential role in the modulation of collectin function. Our results indicate that decorin might function at the tissue level as a modulator of complement-dependent inflammation. It appears that local expression of decorin and biglycan may limit tissue damage after injury.

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Footnotes

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Abbreviations used in this paper: MBL, mannose binding lectin; ECM, extracellular matrix; SP-D, surfactant protein D; CL-43, collectin 43; CLR, collagen-like region; gC1q, C1q globular head; C1qHA, C1q-dependent hemolytic assay; NHS, normal human serum; EA, Ab-opsonized erythrocyte; MASP, mannose-binding lectin-associated serine protease; rhSP-D, recombinant human SP-D; HSA, human serum albumin.

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Human neutrophil peptide-1 inhibits both the classical and the lectin pathway of complement activation

5

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Abstract

Human neutrophil peptide-1 (HNP-1) is a member of the α-defensin family. Defensins are cationic antimicrobial peptides, which play an important role in the antimicrobial response to microorganisms. In addition, recent studies have revealed the involvement of defensins in inflammation, immunity and wound repair. Defensins are present in the azurophilic granules of neutrophils and are released upon neutrophil stimulation. Previous studies showed that HNP-1 binds to C1q and inhibits the classical complement pathway. In view of the structural and functional similarity between C1q and MBL, we have now examined the interactions between HNP-1 and MBL.

We observed a dose-dependent binding of HNP-1 to MBL in calcium-free buffer, indicating that HNP-1 binds to MBL most likely via the collagenous domains. To identify the binding sites in HNP-1 involved in the binding to C1q and MBL, we used a series of overlapping synthetic linear peptides that spanned the entire HNP-1 sequence. Both MBL and C1q showed a dose-dependent binding to the same set of peptides, suggesting a similar binding site in HNP-1 for both MBL and C1q. Strongest binding was observed to peptides containing the C- or N-terminal part of the HNP-1 molecule. Using an ELISA based system, we demonstrated that HNP-1 inhibits activation of both the classical pathway and lectin pathway of complement. Furthermore, we demonstrated that C1q and MBL can form complexes with HNP-1 in solution.

Together, the data indicate that HNP-1 interacts with both C1q and MBL efficiently resulting in inhibition of both the classical and the lectin pathway of complement. We conclude that HNP-1 may play a role in protection against tissue injury during inflammatory conditions by inhibiting the early phase of complement activation.

1.Introduction

Human neutrophil peptide-1 (HNP-1) is a member of the α-defensin family. Defensins are small cationic antimicrobial peptides present in the azurophilic granules of neutrophils. The human α-defensins are comprised of human neutrophil peptide 1–4, which constitute about 50% of the protein content of the azurophilic granules of neutrophils, and HD-5 and HD-6 which are localized in the intestinal Paneth cells (Ganz, 2003). HNP-1 is a 3.4 kDa peptide that consists of 30 amino acids containing three intra-chain disulfide bridges. It is synthesized as preproHNP-1 which is then processed to proHNP-1, and subsequently converted to mature and active HNP-1 which is stored in the azurophilic granules.

Defensins play a role in the defense against microorganisms ranging from Gram-negative to Gram-positive bacteria, fungi and enveloped viruses (Faurschou and Borregaard, 2003). Because the defensins are cationic, electrostatic interactions allow efficient binding to the anionic microbial cell wall (Hill et al., 1991). Binding of defensins to microorganisms disrupts the integrity of the cell membrane by the formation of pores that result in target cell injury and cell death (Lehrer et al., 1989).

Human serum contains several proteins that can bind to defensins. HNP-1 binds to serine protease inhibitors (serpins) such as α1-proteinase inhibitor and α1-antichymotrypsin suggesting a role for these protease inhibitors in controlling defensin activity and vice versa (Panyutich et al., 1995). Moreover, it is known that the first component of the classical pathway of complement activation, C1q, binds to HNP-1. Our group has previously shown that binding of HNP-1 to the collagenous tail of C1q results in inhibition of classical pathway activation (van den Berg et al., 1998).

The complement system is part of the innate immune system and is involved in the defense against invading pathogens and in the acquired immune response. The complement system consists of three different pathways; the classical pathway, the alternative pathway and the lectin pathway. Activation of complement results in formation of the membrane attack complex (MAC complex) and cell lysis. The complement system can be activated by binding of the recognition molecules of the different pathways to surfaces of microbial pathogens and via binding to adaptor molecules such as antibodies and acute phase proteins.

The lectin pathway of complement activation can be triggered by binding of mannose-binding lectin (MBL) to carbohydrates which are present on the surfaces of microorganisms. However, complement activation can also be involved in tissue injury and inflammation.

More recently it has become clear that the lectin pathway of complement activation also plays a key role in ischemia-reperfusion injury (Walsh et al., 2005 and Hart et al., 2005). MBL is a collagenous lectin with calcium-dependent lectin domains (CRD domains) for target recognition and collagen-like domains for effector functions. Collagenous tails of C1q and MBL are homologous and have been shown to share binding to several molecules (Bohlson et al., 2007).

Because of the homology between MBL and C1q with respect to structure and function we envisaged a possible role for HNP-1 in lectin pathway regulation. Therefore, we investigated whether HNP-1 could bind to MBL and inhibit lectin pathway activation. Furthermore we studied the binding site in HNP-1 for MBL and C1q using a set of peptides spanning different parts of the HNP-1 molecule. The results of this study indicate a similar binding site for C1q and MBL on HNP-1. Furthermore, we show that HNP-1 inhibits both classical and lectin pathway activation.

2. Materials and methods

2.1. Isolation of HNP-1

HNP-1 was isolated as described earlier (van Wetering et al., 1997). In short, neutrophils were isolated from human buffy coats. Granules were obtained from the supernatant after disruption by nitrogen cavitation and centrifugation. Granules were extracted with 5% acetic acid, fractionated by gel filtration using Sephacryl S-200 HR columns (GE, Roosendaal, The Netherlands) and reverse phase high-performance liquid chromatography (HPLC) on C18 (Vydac, The Separation Group, Hesperia, CA) and followed by analysis for contaminating proteins by tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), acid urea PAGE and laser desorption mass spectrometry (Lasermat; Finnigan MAT, Hemel Hempstead, UK). An aliquot of the highly purified HNP-1 was conjugated to digoxigenin (Dig) using Dig-3-O-methylcarbonyl--aminocaproicacid-N-hydroxysuccinimide ester (Boehringer Mannheim, Mannheim, Germany) according to instructions provided by the manufacturer.

2.2. Isolation of C1q

C1q was isolated as described earlier (Nauta et al., 2002). In short, 2 L of re-calcified human plasma was precipitated by treatment with polyethylene glycol 6000 (PEG-6000, Sigma–Aldrich, St. Louis, MO) to a final concentration of 3% and incubated on ice for 1 h. After centrifugation the pellet was resuspended in 150 ml of Veronal-Buffered saline (VBS; 1.8 mM Na-5,5-diethyl-barbital, 0.2 mM 5,5-diethylbarbituric acid, 145 mM NaCl) containing 10

mM EDTA. C1q was isolated using a rabbit IgG-Sepharose column (human IgG-Sepharose incubated with an excess of rabbit IgG anti-human IgG and washed with PBS). C1q was eluted from the column using PBS containing 10 mM EDTA and 1M NaCl. Fractions were tested for C1q by single radial immunodiffusion, C1q-containing fractions were pooled, dialysed against PBS containing 4.5 mM EDTA and applied to a Biorex 70 column (Bio-Rad Laboratories, Hercules, CA). C1q was eluted from the column using a salt gradient; fractions were tested for C1q by a hemolytic assay, pooled and concentrated. The C1q-containig pool was then applied to a Superdex 200 gel filtration column (Pharmacia Biotech, Uppsala, Sweden). Peak fractions containing C1q were pooled, checked for impurities with SDS-PAGE electrophoresis and stored at −80 °C.

2.3. Isolation of MBL

MBL was isolated as described earlier (Groeneveld et al., 2005). Human plasma was precipitated using polyethylene glycol 3350 (Sigma, St. Louis, MO) at a final concentration of 7%. The precipitate was dissolved in TBS-T/Ca^{2+} (50 mM Tris, 0.15 M NaCl, 0.05% Tween 20, 20 mM $CaCl_2$, pH 7.8) and incubated overnight at 4 °C with mannan-coupled sepharose beads (Sigma, St. Louis). The beads were then washed with TBS-T/$Ca^{2+/}$1 M NaCl and bound proteins were eluted with TBS-T containing 10 mM EDTA. Presence of MBL was determined in ELISA and fractions were pooled and concentrated. To remove contaminating immunoglobulins in the MBL preparation the pool was absorbed on 4E8 (mAb anti-IgA, produced in Laboratory of Nephrology, Leiden, the Netherlands) coupled to Biogel A5 (Bio-Rad, Hercules, CA), HB57 (mAb anti-IgM, hybridoma obtained from the American Type Culture Collection, Manassas, VA) coupled to Biogel A5, and protein G coupled to Sepharose (Pharmacia, Uppsala, Sweden). The purified MBL was free of IgA, IgG, IgM and C1q as assessed by ELISA. The MBL concentration was determined by ELISA, aliquotted and stored at −80 °C.

2.4. ELISA protocol

For all ELISAs, Nunc Maxisorp plates (Nunc, Roskilde, Denmark) were used. Plates were coated using coating buffer (100 mM Na_2CO_3/$NaHCO_3$, pH 9.6) containing the appropiate concentration of antibody or protein overnight at room temperature or for 2 h at 37 °C. Plates were washed three times after each step with PBS containing 0.05% Tween 20 and blocked with PBS containing 1% BSA for 1 h at 37 °C. All following steps were performed in PBS containing 0.05% Tween 20 and 1% BSA and incubated for 1 h at 37 °C, unless mentioned

otherwise. Antibodies used for detection were conjugated to digoxigenin (Dig) using Dig-3-O-methylcarbonyl--aminocaproic acid-N-hydroxysuccinimide ester (Boehringer Mannheim, Mannheim, Germany) according to instructions provided by the manufacturer. Detection of Dig-conjugated antibodies was performed with sheep anti-Dig antibodies conjugated to HRP (Fab fragments, Boehringer Mannheim). Enzyme activity of HRP was detected with 2,2'azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma). The OD 415 nm was measured using a microplate biokinetic reader (EL312e; Biotek Instruments, Winooski, VT).

2.5. ELISA for detection of binding of HNP-1 to C1q and MBL

To determine binding of HNP-1 to C1q and MBL, plates were coated with either 5 μg/ml C1q, 1 μg/ml MBL or BSA as a negative control. After blocking, Dig-conjugated HNP-1 (prepared as described above) was incubated on the plate in different doses. Binding of HNP-1-Dig was detected with sheep anti-Dig antibodies as mentioned above.

2.6. Binding of C1q and MBL to different HNP-1 derived peptides

On the basis of the HNP-1 amino acid sequence, a set of seven different overlapping peptides was synthesized. Synthetic peptides were made by solid phase technology on TentagelS resin (Rapp, Tübingen, Germany) using N-Fmoc-t-butyl-protected amino acids, piperidine deprotection, and PyBOP/N-methylmorpholine activation. All produced peptides were analyzed by analytical reversed phase HPLC and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFF MS), dissolved in dimethyl-sulfoxide (DMSO, Merck, Schuchardt, Germany) at a concentration of 10 mM and stored at −80 °C. Peptide stock solutions were diluted in coating buffer and coated on microwells in different concentrations starting at 10 μM. Next, C1q or MBL (1 μg/ml) were incubated on the plate for 1 h at 37 °C. Binding of C1q and MBL was detected using F(ab')2 from rabbit IgG anti-human C1q and 3E7 Dig (mAb anti-MBL mouse IgG1, kindly provided by T. Fujita, Fukushima Medical University School of Medicine, Fukushima, Japan), respectively, both conjugated to Dig.

2.7. Detection of HNP-1 mediated complement inhibition

The effect of HNP-1 on complement activation via the classical pathway or the lectin pathway was assessed as follows. Plates were coated with IgM (3.25 μg/ml) for the classical pathway or mannan (100 μg/ml) for the lectin pathway and blocked with PBS containing 1% BSA. HNP-1 or equal amounts of solvent as control were preincubated together with normal human serum diluted 1/1000 as a complement source for classical pathway activation, or C1q-

depleted plasma diluted 1/50 as a complement source for lectin pathway activation for 30 min on ice in gelatin/Veronal buffer (GVB^{++}; Veronal buffered saline (VBS), 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1% gelatin, 0.05% Tween 20, pH 7.5). C1q was depleted from normal human serum as described previously (Roos et al., 2001). After incubation of the mixture on the plate for 1 h at 37 °C, C4 deposition was determined using anti-C4 mAb conjugated to Dig (kindly provided by D. Wouters, Sanquin Blood Supply Foundation, Amsterdam).

2.8. Detection of direct complement activation by HNP-1

To establish whether HNP-1 is able to directly activate complement by itself, plates were coated with HNP-1 (5 μg/ml), IgM (3.25 μg/ml), mannan (100 μg/ml) or BSA (5 μg/ml) as control. This was followed by incubation with different concentrations of normal human serum diluted in GVB^{++} for 1 h at 37 °C. As a measure for complement activation C3 deposition was determined with a Dig-conjugated mAb anti-human C3 (clone RFK22, prepared by lab. of Nephrology, Leiden).

2.9. Detection of complex formation in the fluid phase

To determine whether HNP-1 forms complexes with C1q and MBL in solution, different concentrations of HNP-1 were preincubated with purified C1q (2 μg/ml; in the presence of 1M NaCl) or MBL (2 μg/ml; in buffer only) for 1 h at 37 °C and subsequently assessed for complexes using a sandwich ELISA. For this purpose, plates were coated with a monoclonal antibody against HNP-1 (Aarbiou et al., 2002) followed by incubation with the protein mixtures for 1 h at 37 °C. C1q or MBL complexed with HNP-1 were detected with a specific antibody against C1q and MBL respectively, as described above.

3. Results

3.1. HNP-1 binds to C1q and MBL in a dose-dependent manner

Earlier studies have shown that HNP-1 binds specifically to the collagenous tail of C1q. In the present study, we examined whether HNP-1 also binds to MBL. ELISA plates were coated with C1q, MBL or BSA as a negative control and subsequently incubated with increasing concentrations of Dig-conjugated HNP-1. A dose-dependent binding of HNP-1 to C1q and MBL was detected (Fig. 1A and B) as compared to the negative control using immobilized BSA. In these experiments, HNP-1 bound MBL in the absence of calcium, which suggests that HNP-1 binds to the collagenous tail part of MBL.

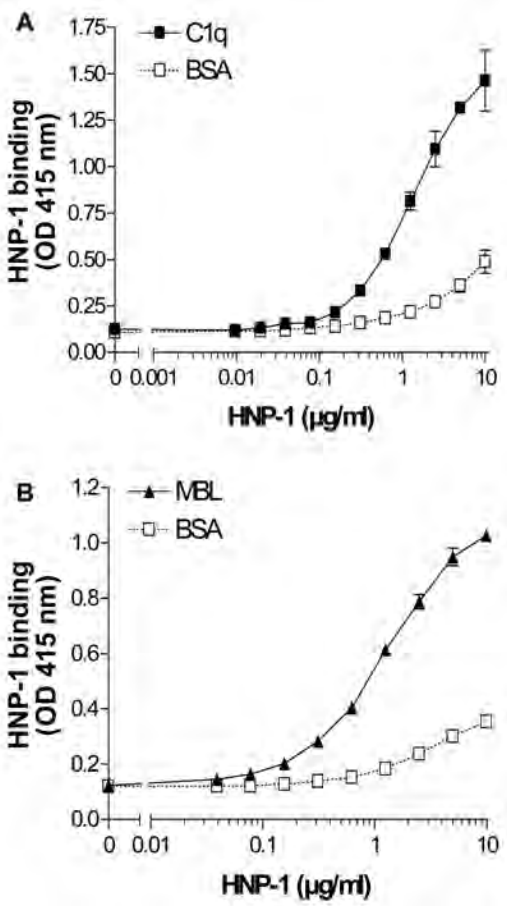

Figure. 1. HNP-1 binds to C1q and MBL. C1q (5 µg/ml); (A) MBL (1 µg/ml); (B) or BSA were coated to a plate. After blocking, different concentrations of HNP-1 conjugated to Dig were added for 1 h at 37 °C followed by $F(ab')_2$ sheep anti-Dig coupled to HRP (n = 2).

3.2. MBL and C1q bind to the same domain of HNP-1

To study the binding site for C1q and MBL on HNP-1, a number of overlapping peptides spanning the HNP-1 molecule were synthesized (Table 1). These peptides were coated to ELISA plates in increasing doses. As a negative control BSA was used. C1q or MBL were then incubated in a fixed concentration on the plate and binding of C1q or MBL was assessed with specific antibodies. Results of five independent experiments were summarized in Table 1. Peptides A1, B1 and D4 bound C1q very well, while peptide A4 showed intermediate binding of C1q. MBL bound well to the peptides A1, A4, B1 and D4. C1q and MBL did not bind to BSA. Taken together, these experiments indicate that both C1q and MBL bind preferentially to peptides that contain sequences located in the N- and C-terminal part of the HNP-1 molecule.

Table 1 C1q and MBL bind to the C- and N-terminal domain of HNP-1

Peptide	HNP-1 Sequence (N→C) ACYCRIPACIAGERRYGTCIYQGRLWAFCC	C1q binding	MBL binding
A1	IAGERRYGTCIYQGRLWAFCC	+	+
B1	ACYCRIPACIAGERRYGTCI	+	+
C1	RIPACIAGERRYGTCIYQGR	-	-
A4	ACYCRIPACIAGERR	+/-	+
B4	IPACIAGERRYGTCI	-	-
C4	AGERRYGTCIYQGRL	-	-
D4	YGTCIYQGRLWAFCC	+	+

Synthetic truncated HNP-1 peptides were coated to a plate. After blocking, C1q (1 μg/ml) or MBL (1 μg/ml) were added to the plate for 1 h at 37 °C, followed by detection of C1q and MBL with $F(ab')_2$ from rabbit anti-human C1q and a monoclonal anti-MBL antibody (3E7), respectively, both coupled to Dig. Strong binding to C1q or MBL is indicated by (+), no binding is indicated by (−). Intermediate binding to C1q or MBL is indicated by (+/−) (n = 5).

3.3. HNP-1 inhibits both the classical pathway and the lectin pathway and does not activate complement by itself

To study the effect of HNP-1 on the classical and lectin pathway of complement activation, we assessed whether HNP-1 could prevent C4 activation induced by classical or lectin pathway activation. Increasing concentrations of HNP-1 were preincubated with diluted serum as a complement source for classical pathway activation, or diluted C1q-depleted plasma as a complement source for lectin pathway activation, followed by activation of the classical pathway or the lectin pathway on plates coated with IgM or mannan, respectively. With increasing concentrations of HNP-1, C4 activation by the classical pathway was completely inhibited with an IC_{50} of approximately 30 μg/ml HNP-1 (Fig. 2A). Furthermore HNP-1 dose-dependently inhibited lectin pathway induced C4 activation with an IC_{50} of approximately 100 μg/ml (Fig. 2B). No inhibitory effect was observed for solvent only.

We further tested whether HNP-1 is able to activate complement by itself by determining whether immobilized HNP-1 could activate complement and lead to deposition of C3. HNP-1 was coated on a plate, followed by incubation with normal human serum and detection of deposited C3 with a monoclonal antibody. HNP-1 was not able to support activation of C3 (Fig. 3), whereas both IgM and mannan strongly induced complement activation and C3 deposition. Immobilized BSA was used as a negative control, and did not induce complement activation.

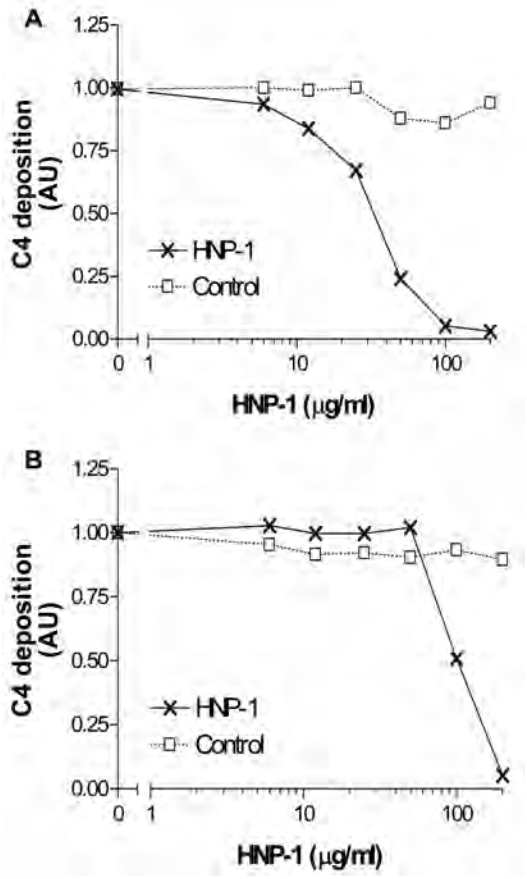

Figure. 2. HNP-1 inhibits both the lectin and the classical pathway of complement. Plates were coated with 3.25 μg/ml IgM (A) or 100 μg/ml mannan (B), followed by incubation with either normal human serum (A) or C1q depleted plasma (B) in the absence or presence of different concentrations of HNP-1 or buffer as control. Complement activation was determined by detection of C4 deposition with a specific antibody directed against C4 (n = 3)

3.4 Detection of complex formation between HNP-1 and C1q or MBL

Studies presented above indicate that both immobilized MBL and C1q bound HNP-1 (Fig. 1). Next, we studied whether these molecules also interacted with HNP-1 in the fluid phase. Complexes were allowed to form during an incubation at room temperature, followed by detection in a sandwich ELISA using immobilized anti-HNP mAb and F(ab')2 anti-C1q or mAb anti-MBL as detecting antibodies. In order to prevent the interaction of the Fc region of the coated antibody with free C1q in the HNP-C1q mixture, the incubation was performed in a high salt buffer. With increasing concentrations of HNP-1, fluid phase complex formation was clearly detectable, both between C1q and HNP-1 (Fig. 4A) and between MBL and HNP-1 (Fig. 4B). No signal was obtained upon incubation with HNP-1 alone.

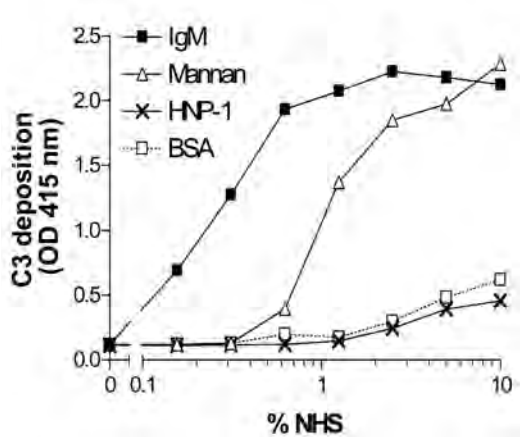

Figure. 3. HNP-1 does not activate complement. HNP-1 (5 μg/ml) was coated to a plate and incubated with different concentrations of normal human serum for 1 h at 37 °C and subsequently assessed for complement activation. Complement activation was determined by detection of C3 deposition with a specific antibody directed against C3. IgM (3.25 μg/ml), Mannan (100 μg/ml) and BSA were included as control (n = 2).

4.Discussion

In the present study, we investigated the interaction of human neutrophil peptide-1 with the recognition molecules of the classical and lectin complement pathway, C1q and MBL. Here, we demonstrate that HNP-1 most probably binds the collagen-like region of MBL and that it inhibits the activation of both the classical and lectin pathway of complement. We show with truncated HNP peptides that predominantly the C-terminal and N-terminal domains are involved in the HNP-1 binding to C1q and MBL. Furthermore, we show that HNP-1 can form complexes with C1q and MBL in solution.

It is thought that the majority of HNPs that are produced have their effect on microbes within the neutrophil, after fusion of the azurophilic granule with the phagolysosome containing the microbe (Ganz, 2003). However HNPs have been shown to be released by neutrophils under conditions of inflammation like sepsis and meningitis. In plasma from sepsis patients, the levels of circulating HNPs are high, up to 170 μg/ml (Panyutich et al., 1993), while in patients suffering from cystic fibrosis, defensin levels have been measured of 300–1600 μg/ml in sputum (Soong et al., 1997). Furthermore, recently it has been discovered that both monocytes (Mackewicz et al., 2003) and NK Cells (Chalifour et al., 2004) express HNPs which could enhance the amount of HNP locally. These findings indicate that high concentrations of extracellular HNP can be present at sites of inflammation, and thus could affect complement activity.

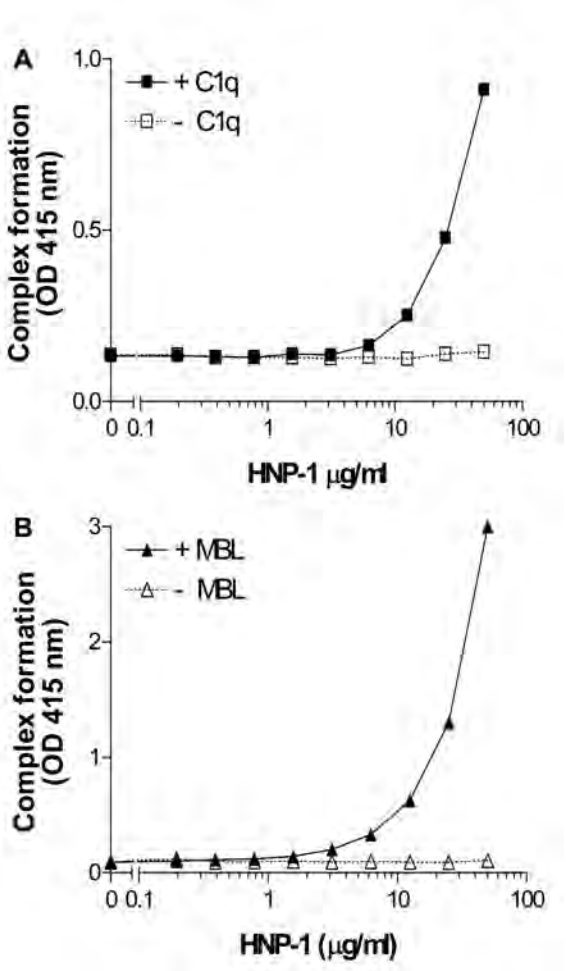

Figure 4. HNP-1 forms complexes with C1q and MBL in solution. Complexes with C1q or MBL were generated using increasing concentrations of HNP-1 and assessed for complex formation in a sandwich ELISA. A monoclonal antibody against HNP-1 was coated on a plate. Different concentrations of HNP-1 were preincubated with 2 µg/ml C1q in the presence of 1M NaCl or with 2 µg/ml MBL in normal buffer for 30 min at 37 °C, and then added to the plate for 1 h at 37 °C. The presence of MBL or C1q was detected using specific antibodies (n = 2).

HNP-1 binds to the recognition molecule of the classical complement pathway C1q and inhibits the activation of the classical pathway (van den Berg et al., 1998). Here, we show that HNP-1 has a similar effect on MBL and the lectin pathway. Furthermore, HNP-1 binds to MBL in the absence of Ca^{2+} which suggests that HNP binds to MBL via the collagen-like tail, which is similar to the observed interaction of C1q and HNP (van den Berg et al., 1998). Supporting our results Hartshorn et al. (2006) have recently found weak but statistically significant binding of recombinant MBL to HNP-2. In this study they reported that surfactant

protein-D (SP-D), another member of the collectin family containing a collagen-like region and a CRD domain, has a strong binding to HNP-2 which was also calcium-independent. These findings suggest that the interaction between HNP-2 and SP-D also involves the collagenous domains. The binding of HNP to the collagen-like region of both C1q and MBL molecules suggests that the observed inhibitory effects of HNP-1 on the activation of the classical and lectin pathway could be caused by interference of HNP-1 with the binding or function of the subsequent enzymes in the complement activation cascade, e.g. C1r/C1s and the MASPs.

While others have reported a direct activation of complement by immobilized HNP-1 (Prohaszka et al., 1997), using normal human serum as a complement source, we were not able to detect significant activation of complement by immobilized HNP-1. This difference could be due to the fact that in our experiments we did not allow the HNP peptides to dry in the wells before we added the complement source. Drying could induce changes in the conformation of the peptides and cause aggregation which is known to induce complement activation. Our results also indicate that HNP-1 is not able to activate the lectin pathway or alternative pathway even though the conditions used (Fig. 3.) would enable activation of these pathways.

Using truncated HNP peptides we demonstrated that both C1q and MBL bind preferentially to the N- and C-terminal sites within the HNP-1 molecule. This further confirms the similarity between the interaction of HNP-1 with C1q and HNP-1 with MBL respectively. However we do observe a difference in the binding of MBL and C1q to peptide A4 (Table 1), suggesting that some of the 5 aminoacids that are present in peptide B1 but not in A4 could play a minor role in the interaction of HNP-1 with C1q, as we do not see any binding of C1q to peptides that contain this region but not the N- and C-terminus (peptides B4, C4; Table 1).

Models of three dimensional structure of the HNP molecules (Yang et al., 2004) indicate that the N- and C-terminus are lying opposite each other at the same end of the molecule, further supporting the involvement of both domains in MBL and C1q binding. However, our truncated peptides lack the spatial alignment of the C- and N-terminus. They do not possess the three intrachain S-S bridges that are present in native HNP-1 and are probably more linear shaped.

In our experiments, we used purified HNP-1 only. Even though there have been reports that there are differences in the candicidal (Lehrer et al., 1988) and chemotactic effects

(Chertov et al., 1996) of the individual HNP peptides, it might be possible to extrapolate these results to the HNP-2 and -3 peptides. The sequences of HNP-1, 2 and 3 differ only in the first amino acid at the N-terminal domain (Selsted et al., 1985), and our results show that even with 7 of the N-terminal aminoacids missing (peptide A1; Table 1) MBL and C1q bind very well. This suggests that small differences at the N-terminus of the individual HNPs could be compensated by the C-terminal domain.

We further show that we can detect formation of complexes between HNP and C1q/MBL in vitro even under high salt conditions. In accordance, Panyutich et al. (1995) have shown that the interactions of α1-proteinase inhibitor and α1-antichymotrypsin with HNP are not influenced by a high salt buffer. The presence of high HNP levels as well as C1q in conditions of inflammation would suggest that these complexes might also occur in vivo. We have tested the sera of a group of approximately 100 sepsis patients (results not shown) for the presence of elevated HNP levels and the presence of HNP-C1q complexes. These patients showed elevated levels of HNP in the circulation (up to 1000 ng/ml). However, experiments aimed to detect complexes of HNP and C1q in these sera failed due to interference of free C1q even in the presence of high salt concentrations in the buffer.

The observed binding and complex formation with HNP could be part of the overall debris-clearing functions of C1q and MBL. However, the tails of both molecules have been shown to be predominantly involved in signaling and activating functions, while the globular head domains of C1q and the CRD domains of MBL are specialized in recognizing and binding targets (Bohlson et al., 2007). The involvement of the collagen-like region in the binding to HNP therefore suggests that HNP regulates the function of C1q and MBL rather than the other way around. The formation of soluble complexes between HNP-1 and MBL/C1q could be a way to prevent both recognition molecules to take part in activating complement at the site of HNP-1 release.

HNP-1 is a multifunctional molecule and has also been shown to stimulate wound healing in vitro (Aarbiou et al., 2004) and cell growth (Aarbiou et al., 2002) which involves this molecule also in the aftermath of the inflammatory process, the repair phase. This phase could benefit from down regulation of complement activation by HNP.

In summary, we have shown that HNP-1 binds and forms complexes with C1q and MBL and inhibits the activation of both the lectin and the classical pathway of complement activation. Hence, HNP-1 might play next to its anti-microbial role also a complement inhibitory and therefore anti-inflammatory role at sites of inflammation.

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Summary & General discussion

6

Chapter 6: General Discussion

In the present thesis the contribution of complement was assessed in different models of renal disease in mice. More specifically, two models were addressed. In the first model, the contribution of anti-C1q antibodies in renal inflammation was assessed. In the second model, an anti-GBM nephritis was induced in a controlled fashion using fixed amounts of rabbit anti-GBM and a mouse mAb against rabbit IgG. In both of these models, a clear contribution of complement was observed. In the anti-C1q-model, a clear preference for classical pathway activation was demonstrated. Interestingly, in the anti-GBM model, it was observed that both the classical and the alternative pathway were involved in wildtype mice, whereas under conditions of classical pathway deficiency, the alternative pathway was sufficient to cause inflammation and renal injury.

Initially it was the purpose of the studies to use these models for assessment of the potential of novel complement inhibitors. In this regard, it was demonstrated that endogenous proteins such as defensins and proteoglycans are suitable candidates for further investigation.

6.1 Summary.

Chapter 2 presents a mouse model in which it is demonstrated that C1q and anti-C1q antibodies can lead to glomerulonephritis if there is a preceding deposition of an antibody in the glomerulus. Immunizing C1q-deficient mice with purified mouse C1q generated anti-C1q monoclonal antibody-producing cells. This resulted in three monoclonal antibodies directed against mouse C1q (mAb JL-1, 2, 3). When mAb JL-1 was administrated to wild type mice, C1q and anti-C1q were deposited in the glomerulus, but no significant renal damage was observed. However, combination of this mAb with a preceding injection of a sub-nephritogenic dose of C1q-fixing anti-GBM antibodies resulted in synergistic effects as indicated by overt renal damage, characterized by albuminuria, granulocyte influx and histological changes. The dependency of this synergistic effect on C1q that is already present in the glomeruli was established using different combinations of antibodies in wildtype and C1q-deficient mice.

Furthermore, using both antibodies and complement-knockout mice we were able to show that this glomerulonephritis is also dependent on the presence of C4, C3 and Fc-receptors. This model provides insight into how anti-C1q antibodies could be involved in the development of lupus nephritis in patients suffering from SLE.

Chapter 3 describes a novel complement-dependent anti-GBM nephritis model. Nephritis is induced by injecting mice with rabbit anti-GBM antibodies followed 6 days later by an injection of a monoclonal antibody directed against rabbit IgG. Albuminuria and strong complement deposition is only induced in mice that receive both antibodies, not in mice that receive only the first antibody. After the initial onset phase of acute glomerulonephritis this develops into a chronic situation, with persistent albuminuria, and complement deposition observed after 3 weeks.

Using C1q, C4, C3 and Fcγ-receptor knockout mice, we show that glomerulonephritis is partially dependent on the presence of C3 while Fc-receptors are crucial for the development of disease. However, although deposition of C3 was shown to be partially dependent on C1q and C4, indicating a contribution of the classical pathway in complement activation, the classical pathway was not required for induction of renal injury. Therefore, in this model of antibody-mediated renal disease, the alternative pathway of complement is responsible for disease induction.

In **Chapter 4** the interactions between C1q, collectins and two matrix proteoglycans decorin and biglycan are presented. Both proteoglycans are able to inhibit complement activation by binding the C1q molecule. Furthermore, this interaction has also inhibitory effects on the pro-inflammatory actions of C1q on cells: the cellular C1q binding and subsequent induction of IL-8 and MCP-1 release by endothelial cells. Further experiments show that proteoglycans can bind to other members of the structurally similar family of collectins. In conclusion, decorin and biglycan can serve an anti-inflammatory role by inhibiting complement activation.

Chapter 5 describes the inhibitory action of human neutrophil peptide-1 (HNP-1) on the complement pathway. HNP-1 is present in high amounts in the circulation during inflammatory disease like sepsis, a situation that also involves complement activation. In vitro studies show that HNP-1 is able to inhibit both the activation of the classical and the lectin pathway. We show that HNP-1 binds to C1q and MBL via both the N- and C-terminal domains of the peptide by making use of truncated peptides. Furthermore we show that HNP-1 can form complexes with C1q and with MBL, respectively that can be detected in solution.

6.2 General Discussion & Future

6.2.1 Complement activation in disease

Complement has dual role in systemic lupus erythematosus. On the one hand, C1q and other complement components are shown to be deposited in the kidneys of SLE patients, suggesting an active participation of complement to inflammation and damage in this disease. On the other hand, deficiencies of C1q, C1s and C4, respectively, are know to predispose to the development of SLE, suggesting that these molecules plays a protective role [1]. The latter point fits well with one of the current hypotheses concerning the development of SLE: a disturbed clearance of apoptotic and necrotic cells leading to circulating cell debris which may be involved in a loss of self tolerance and consequently the development of the auto-antibodies that are characteristic for SLE [2]. Auto-antibodies against C1q are frequently observed in SLE patients and higher titers in circulation are associated with a renal flare [3] [4].

In studies presented in *chapter 2* the role of the classical pathway molecule C1q and anti-C1q antibodies was investigated in a mouse model of glomerulonephritis. We observed that renal disease only develops in this model when the glomerular basement membrane is first primed with C1q attached to immune complexes whereas injection of anti-C1q antibodies alone is not sufficient to induce disease. This observation is supported by the finding that some patient groups (e.g. hypocomplementemic urticarial vasculitis patients (HUVS) [5]) and healthy persons have circulating anti-C1q antibodies but do not develop renal disease [6]. Hence, findings presented in this chapter suggest that anti-C1q antibodies alone are not necessarily pathogenic. However, anti-C1q antibodies can enhance complement activation and Fc-receptor-mediated injury when they recognize C1q that is bound by immune complexes already present in the GBM. These immune complexes might also contain anti-dsDNA or anti-nucleosome antibodies [7]. Therefore, these mechanisms could directly play a role in the development of lupus nephritis in SLE patients.

Our model also suggests that inhibition of C1q binding to deposited immune complexes in the GBM might provide a way to prevent the development of LN in SLE patients. However, continuous inhibition of C1q in the light of the above mentioned hypothesis may lead to the development of auto-antibodies as C1q plays a role in clearing debris. It might however be possible to apply potential inhibitors at the moment as anti-C1q levels in the circulation start to rise.

6.2.2. Complement-dependent disease models

Over the years several rat and mouse models for glomerulonephritis have been developed. However, the general consensus of these models is that most of the effects are either complement-independent, mainly involving cellular interactions, or complement-dependent and mediated by C3 and C5, via the alternative activation pathway.

In our model (*chapter 3*) we make use of an attenuated model. We first infuse a limited amount of rabbit anti-mouse GBM antibodies that deposit in the kidney without causing disease, followed by a mouse monoclonal antibody against rabbit IgG that induces glomerulonephritis within 24 hours. This model is more close to a situation where immune complexes are deposited in the kidney. We also made use of autologous (mouse) antibodies to induce disease. In fact, these are IgG2a antibodies that are known to induce complement very well.

In agreement with the well-known interaction of antibodies with C1q, clear evidence was obtained that the classical pathway was at least partially involved in complement activation. However, disease induction was shown to be independent form C1q and C4 but required C3. Therefore, it is assumed that the alternative pathway of complement is responsible for renal injury in this model of antibody-mediated renal injury. Furthermore, cellular interactions play an essential role in the effector phase, as indicated by experiments with Fc-receptor deficient mice. Fc receptors presumably play a key role in activating and sequestering polymorphonuclear leukocytes and macrophages at the globular basement membrane in the kidney, leading to production of cytokines and oxygen radicals, associated with glomerular injury.

This model could be used to further assess the contribution of various molecules of the alternative and the classical complement pathways to the disease process. Furthermore, the development of chronic glomerulonephritis in this model facilitates interference studies with potential therapeutic inhibitors at different time points, during development of disease but also when the disease is established.

6.2.3. Regulation of Complement

Chapters 4 and 5 of the thesis describe the complement-inhibitory functions of host-derived molecules that do not classically belong to the family of complement-regulatory molecules. *Chapter 4* describes how the extracellular matrix molecules decorin and biglycan inhibit the classical pathway and prevent cell-mediated inflammatory effects. Furthermore, we show that

the antimicrobial peptide HNP-1 also functions as an inhibitor of the classical pathway and the lectin pathway (see *chapter 5*).

With respect to decorin and biglycan, it is demonstrated that these molecules interact with C1q and MBL but do not serve as a regular complement-activating ligand. Recently, Sjöberg et al [8] have confirmed our results of decorin on the classical pathway and showed at the same time that other family members of the Small Leucine-rich proteoglycans are not inhibiting, but instead are activating complement, such as fibromodulin. This emphasizes that complement inhibition by decorin and biglycan is a specific effect of these matrix molecules. Furthermore, these authors have shown by electron microscopy, using gold-labeled anti-decorin antibodies, that decorin binds C1q on the interface of the head and the tail domain (*personal communication*), as was also suggested in *chapter 4* and by Krumdieck et al. [9]. This indicates that the interaction of decorin with C1q is different from that with complement-activating ligands of C1q.

Further experiments indicate that decorin is able to bind the C1 complex, but subsequently can not activate the C4 molecule. Combining our data and those of Sjöberg suggests that decorin binds very close to the site were the $C1r_2$-$C1s_2$ quartet is supposed to bind on C1q[10]. The observed inhibitory action of decorin might be mediated by the decorin molecule directly dislocating C1r and C1s from C1q or by preventing activation of these proteases.

Among the regular functions of the matrix proteins decorin and biglycan is the arrangement of collagens in tissue. Possibly, these molecules may interact with collagen and with C1q and/or MBL simultaneously. In support for this hypothesis, it has been reported that decorin might have more that one binding place for collagens [11]. Furthermore, decorin has been shown to be able to bind TGF-β and collagens at the same time [12].

Therefore, these molecules, being a part of the extracellular matrix, may sequester C1q and MBL, thereby preventing activation of the complement system. In conditions were the extracellular matrix is damaged, or subject to rearrangement, such as during or after an inflammatory process, decorin and biglycan may be further exposed. They can subsequently interact with C1q and MBL, thereby downregulating undesired complement activation as a consequence of tissue injury.

Purified decorin has already been used in a rat model to inhibit anti-Thy1.1 induced nephritis, but the ameliorating effects were then attributed to the inhibitory effects that decorin has on TGF-β [13]. Our research suggests however, that it is possible that inhibition of complement activation by decorin might play a role in this situation as the anti-Thy1.1 experimental model is dependent on complement activation [14] [15]. Hence these matrix molecules are potential candidates to be applied as inhibitors of the classical and lectin pathway. However, as these molecules have been shown to have other effects, e.g. on growth and the alignment and composition of the matrix, designing synthetic inhibitors of C1q on the basis of the structure of these two molecules would be preferred.

HNP-1 is produced and secreted by neutrophils during inflammatory conditions. As we (*results not shown*) and others [16] [17] have observed, patients suffering from sepsis have significantly increased amounts of HNP-1 in the circulation (20-4000 fold [16]). The local concentration of HNP-1 at the site of inflammation and neutrophil degranulation, is assumed to be even higher. In *chapter 5* it is presented that HNP-1 binds MBL and C1q and inhibits the activation of the classical and lectin pathway in vitro. Such a complement-inhibitory action of a molecule directly involved in host defense could be rather unexpected.

HNP molecules are very effective in lysing bacteria. Furthermore, products that are co-secreted with HNP, like myeloperoxidase (MPO) and lactoferrin, also have pathogen-killing properties. The contents of the neutrophil granules, the presence of activated complement components and the opsonizing capacity of the neutrophil, all cooperate with each other to achieve rapid pathogen clearance. The results presented in chapter 5 suggest that, during such a response, HNP-1 may prevent unnecessary and excessive complement activation, thereby protecting against tissue injury.

HNP-1 might be useful as a complement inhibitor. However, the purification of HNP from neutrophils is a time-consuming process and dependent on available blood donors. Since HNP-1 is only a small peptide composed of 30 amino acids, synthetic production could be feasible and has been attempted for anti-microbial purposes. However, this synthesis is hampered by the presence of three disulfide bridges involved in the folding of the molecule [18]. To circumvent this, Lundy et al. [19] have recently looked at the anti-microbial activities of truncated analogues without the disulfide bridges of the HNP-1 molecule. One of the

truncated forms containing the C-terminal portion displayed similar anti-microbial activity as the complete HNP-1 molecule.

Next to complement inhibition and anti-microbial functions, HNP-1 has other effects, for example on expression of IL-8 [20], cell growth [21], and cell survival etc.. C1q and MBL were shown to bind to the C and N-terminal portion of the HNP-1 molecule. Therefore, its application as a therapeutic complement inhibitor requires a complex design of such a synthetic molecule, containing its complement-inhibitory domains but excluding other, undesired functions.

6.3 General conclusion

Appropriate complement activation is an act of balance between activating and inhibitory systems. The interplay between these two in a normal situation results in a level of complement activation that is sufficient for host protection and does not induce significant host injury. However, complement is inappropriately activated in circumstances where the inhibitory system is overwhelmed. This situation occurs in several diseases when excessive local activation of complement is taking place (e.g. in sepsis), when the trigger of complement activation is inappropriate (such as deposition of autoantibodies), or when, due to genetic or acquired deficiencies, there is insufficient complement control (e.g. a-typical HUS). Such conditions lead to undesired and/or uncontrolled complement activation leading to inflammation and tissue injury. In these situations, interfering in complement activation using synthetic or host-derived complement inhibitors is a potential therapeutic option. The mouse glomerulonephritis models presented in the thesis could serve as test platforms for the use of complement inhibitors in vivo. Furthermore, research presented in this thesis points to novel inhibitors outside the complement system that might play a role in the delicate balance of suppression and activation and that could be potential candidates for use as inhibitor or for the design of novel inhibitors.

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Nederlandse Samenvatting

Het complementsysteem is één van de belangrijkste verdedigingslinies van het lichaam. Door middel van een opeenvolgende activatie van eiwitten worden o.a. ziekteverwekkers en celresten verwijderd. Er zijn vier activatie-routes bekend van het complementsysteem; de klassieke route, de alternatieve route, de lectine route en de C2-bypassroute met elk hun eigen specifieke activatoren (zie figuur 1, **hoofdstuk 1**). Het complementsysteem is erg reactief en ongecontroleerde activatie kan leiden tot ontsteking en uiteindelijk schade aan eigen cellen en weefsels. Daarom staat het onder strenge controle van complementremmers die onderdeel uitmaken van het complement systeem.

In bepaalde auto-immuunziekten zoals het syndroom van Goodpasture (anti-GBM ziekte) is complement aantoonbaar geactiveerd, echter het debat is nog steeds aan de gang in welke mate complement een rol speelt in het ontwikkelingsproces van deze en andere ziekten. Ook is het nog niet duidelijk welke van de complement routes voor de activatie van belang is. Met behulp van diermodellen worden deze ziekteprocessen nagebootst en de bijdrage van complement onderzocht. Het is aannemelijk dat complementactivatie die plaatsvindt in een aantal ziekten de reeds aanwezige orgaan en weefselschade versterkt. Het is dus van medisch belang dat we inzicht krijgen in hoe complement geremd kan worden in zulke situaties. Er wordt actief gezocht naar natuurlijke en synthetische remmers van het complementsysteem die straks gebruikt kunnen worden bij de behandeling van ziekten waarin is aangetoond dat de activatie van het complementsysteem een nadelig effect heeft.

In dit proefschrift wordt het complementsysteem vanuit verschillende gezichtspunten onderzocht: de rol die complement activatie speelt in een muismodel voor de auto-immuunziekte Systemic Lupus Erythematosus (SLE) (**hoofdstuk 2**), de rol die complement heeft in een muismodel voor anti-GBM gemedieerde nierontsteking (**hoofdstuk 3**) en in **hoofdstuk 4 en 5** wordt bestudeert welke moleculen buiten de bekende complementremmers complementactivatie kunnen beïnvloeden.

In **hoofdstuk 2** wordt een model voor SLE beschreven waar muizen worden geïnjecteerd met een antilichaam gericht tegen C1q (anti-C1q), het herkenningsmolecuul van de klassieke complementroute. In de meeste gevallen is dit antilichaam duidelijk aanwezig in SLE patiënten die een nierontsteking ontwikkelen (lupus nefritis) maar het komt ook voor in de

circulatie bij verschillende andere ziekten en ook bij gezonde personen. Met behulp van muizen die genetisch deficiënt zijn voor verschillende complement moleculen, en door het combineren van verschillende antilichamen, hebben we kunnen aantonen dat het vóórkomen van anti-C1q antilichaam alleen niet genoeg is om voldoende complement te activeren om nierontsteking te ontwikkelen. Hiervoor moet er eerst een precedent zijn, antilichaam alleen of antilichamen in complex met hun ligand moeten reeds gebonden zijn in de nier. C1q uit de bloedcirculatie zal vervolgens hieraan binden aangezien gebonden antichamen het natuurlijke ligand zijn voor C1q. De gebonden C1q moleculen kunnen weer herkend worden door het in de circulatie aanwezige antilichaam tegen C1q. Deze opstapeling van moleculen veroorzaakt dat complement en infiltrerende cellen zodanig geactiveerd worden dan het leidt tot nierontsteking en nierschade.

In **hoofdstuk 3** werd onderzocht welke componenten en activatieroutes van complement van belang zijn in het ontwikkelen van nierontsteking en nierschade in dit anti-GBM muismodel. Daartoe werden muizen eerst geïnjecteerd met een antilichaam gericht tegen de glomerulaire basaalmembraan in de nier. gevolgd door een tweede antilichaam dat specifiek het eerste antilichaam herkent en bindt. Met behulp van genetisch deficiënte muizen werd aangetoond dat in dit model activatie van de alternatieve complement route via het C3 molecuul voornamelijk van belang is en dat ook de cellulaire Fc receptoren op infiltrerende cellen een cruciale rol spelen. Muizen deficiënt voor deze moleculen ontwikkelen geen of minder nierschade in tegenstelling tot wildtype muizen. Muizen deficiënt in bepaalde onderdelen van de klassieke- en lectine route (C1q, C4) ontwikkelen daarentegen wel eenzelfde nierschade als wildtype muizen. Dit geeft aan dat de rol van de klassieke route overgenomen kan worden door de alternatieve route.

In **hoofdstuk 4** werd onderzocht of de nauw verwante moleculen decorin en biglycan in staat zijn om activatie van de verschillende complement routes te remmen en hoe ze dat doen. Decorin en biglycan binden de complementherkenningsmoleculen C1q en MBL en voorkomen hiermee verdere activatie van de klassieke en lectine complementroutes. Verder kunnen ze andere complementeffecten blokkeren, zoals de binding aan endotheelcellen en de productie van ontstekingsbevorderende moleculen door deze cellen.

In **hoofdstuk 5** werd onderzocht of human neutrophil peptide-1, een antimicrobieel peptide dat uitgescheiden wordt door neutrofiele witte bloedcellen effect kan hebben op de activatie van complement. Het blijkt dat dit peptide in staat is om de klassieke route en lectine route van complement te remmen door te binden aan C1q en MBL. Met behulp van gesynthetiseerde delen van dit peptide is vastgesteld dat vooral de beide uiteinden van het peptide van cruciaal belang zijn voor de binding aan C1q en MBL.

In conclusie, dit proefschrift beschrijft een mogelijk mechanisme hoe anti-C1q antilichamen een rol kunnen spelen in de ontwikkeling van nierontsteking in SLE. In een nieuw muismodel voor anti-GBM gemedieerde nierontsteking wordt vastgesteld dat complement, met name via de alternatieve route, tesamen met Fc receptoren een cruciale rol spelen in de ontwikkeling van nierontsteking en schade. Tenslotte wordt aangetoond dat behalve de bekende complementremmers ook moleculen die in gezonde situaties andere functies vervullen, zoals extracellulaire matrixeiwitten en anti-microbiële peptiden, een rol spelen in de regulatie van complement.

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Het vervaardigen van een proefschrift is geen monografie maar in feite een compilatie van menselijke bijdragen in de verschillende aspecten van het leven. Voor al hun bijdragen wil ik alle collega's van het laboratorium nierziekten en de rest van de "D3 vloer", het secretariaat en de staf bedanken. Ik heb het geluk gehad om te mogen samenwerken met drie uitstekende analisten gedurende mijn AIO tijd, daarom een speciaal dankjewel aan Ria, Astrid en Sandra voor al hun harde werk en dat ze het met mij uitgehouden hebben. Ook mijn studenten Sabine, Patrick, Lili en Layla hebben stenen en steentjes bijgedragen aan de berg die het nu is geworden, dank jullie wel en veel succes verder! Ook bedank ik hier Professor Anna Blom voor de tijd die ze mij gegeven heeft om dit proefschrift af te maken en ook al mijn huidige collega's op het Wallenberg laboratorium. Verder wil ik Leendert, Rinze, Nicole en Katharina bedanken voor hun hulp en niet-werk gerelateerde ondersteuning gedurende de afgelopen tijd. Als laatste wil ik mijn familie bedanken en vooral mijn ouders voor de steun die ze me altijd gegeven hebben. Dank jullie allemaal!

Curriculum Vitae

De auteur van dit proefschrift werd op 5 augustus 1976 geboren te Geleen. Na het behalen van het HAVO diploma in 1993, gevolgd door het VWO diploma in 1995 aan de scholengemeenschap Sint Michiel te Geleen, werd in 1995 aangevangen met de studie biologie aan de Universiteit Utrecht. Onderzoeksstages werden gedaan bij de vakgroep Endocrinologie aan de Universiteit Utrecht onder leiding van Prof. H. Goos en Dr. D. Consten, evenals bij Pathologie aan het Universitair Medisch Centrum Utrecht onder leiding van Dr. R. Goldschmeding en Dr. I. Blom. Na het behalen van het doctoraal examen in november 2000 en een tijdelijke aanstelling als research medewerker bij de afdeling pathologie, vertrok de auteur naar Australië voor een jaar Work & Travel. In augustus 2002 werd het promotietraject aangevangen aan het LUMC, bij de afdeling nierziekten onder leiding van Prof. Dr. M.R. Daha en Dr. A. Roos. Op dit moment is de auteur werkzaam bij Prof. Dr. A.M. Blom, departement Laboratory Medicine, Universiteit Lund, Wallenberg laboratorium te Malmö, Zweden.

Curriculum Vitae

The author of this thesis was born on the 5th of August 1976 in Geleen, the Netherlands. After completing secondary education with HAVO in 1993 and VWO in 1995, the author continued his education in the same year with the study Biology at the University of Utrecht. During this study two research projects were completed: at the department of Endocrinology, Utrecht University under the supervision of Prof. Dr. H. Goos and Dr. D. Consten and at the department of Pathology, University Medical Center Utrecht under the supervision of Dr. R. Goldschmeding and Dr. I. Blom. He received his MSc in Biology in November 2000 and started with a temporary assignment as research assistant at the department of Pathology. Thereafter he left for a year of Work & Travel in Australia. In August 2002 the author started his PhD training under the supervision of Prof. Dr. M.R. Daha and Dr. A. Roos at the department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands. Currently he is employed at the laboratory of Prof. A.M. Blom, department Laboratory Medicine, Lund University, Wallenberg laboratory, Malmö, Sweden.

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Groeneveld, T.W.L., Flierman, R., Otten, M.A., Rastaldi, M.P., Trouw, L.A., Faber-Krol, M.C., Essers, M., Verbeek, J.S., Daha, M.R. and Roos, A.
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Color figures Chapter 2

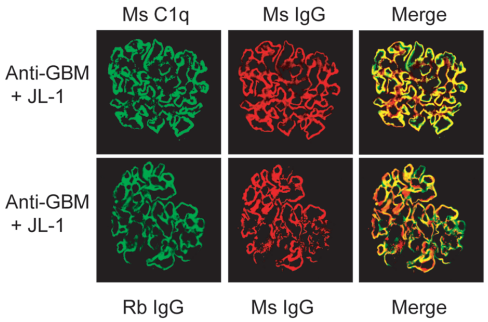

Figure 4C. (C) Confocal analysis of kidney sections of mice injected with rabbit anti-GBM and JL-1. Representative pictures are shown for the colocalization (yellow) of mouse C1q (green) and mouse IgG (red) and the colocalization (yellow) of rabbit IgG (green) and mouse IgG (red). They indicate that rabbit IgG, mouse C1q, and mouse IgG do colocalize in these mice, in a linear, GBM-like pattern.

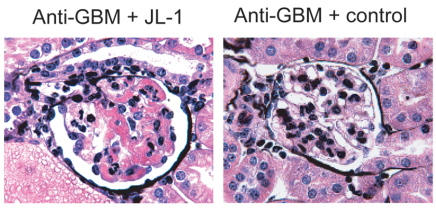

Figure 6A. Histological analysis of Silver-stained renal sections of mice injected with rabbit anti-mouse GBM and coinjected with either mAb JL-1 or lgG2b control mAb, obtained at 24 hours after injection. For JL-1-coinjected mice, images show pronounced inflammatory cell influx, focal capillary tuft occlusion by microthrombi, necrotizing lesions, nuclear debris, and wireloop-like lesions. Control-coinjected miceonly display marginal inflammatory cell influx. Original magnification, x400.

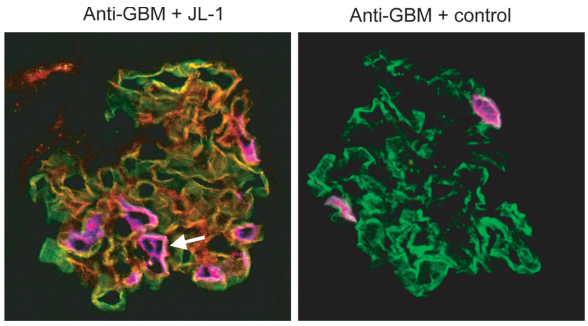

Figure 7B. Confocal analysis of sections stained for mouse C1q (green), mouse IgG (red), and mouse granulocytes (purple). The pictures are merged and show, in yellow, colocalization of green and red, and, in white, colocalization of green and purple. The anti-GBM antibodies induced linear fixation of C1q in both groups, but only in the JL-1-coinjected mice is there colocalization between C1q and IgG and a pronounced influx of granulocytes. Original magnification, x400. The white arrow indicates the white colocalization between C1q and granulocytes.