Complement aberrations and autoantibodies to complement proteins in relation to disease mechanisms.

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Abstract

The complement system, a part of the innate immune system with several links to the adaptive immune system, plays an important role in the pathogenesis of many diseases. The purpose of this thesis was to document and clarify some of these mechanisms. The thesis is based on four papers (I-IV).

(I and II) Autoantibodies to the C3 cleaving enzyme complex of the alternative pathway, C3 nephritic factors (C3 NeF), cause partial C3 deficiency and are associated with increased susceptibility to bacterial infections. By analysis of samples from 20 patients with C3 NeF, it was confirmed that C3 NeF are of at least 2 types; one with fluid phase (C3 NeF type I) and one with solid phase (C3 NeF type II) activity. Different types of C3 NeF were associated with different serum complement profiles and symptoms. Only C3 NeF type II were found to be associated with circulating autoantibodies to the collagenous region of C1q (aC1qCLR). Defense against Neisseria meningitidis in 26 patients with low C3 concentrations due to C3 NeF was investigated. In patients and control children, homozygosity for the IgG1 and IgG3 IGHG alleles G1M*f and G3M*b was found to be associated with higher serum bactericidal activity (SBA) than was heterozygosity. IGHG alleles correlated to IgG subclass binding to live meningococci, while IgG subclass binding did not correlate to SBA. Thus, the mechanism of influence from IGHG genes on SBA is unclear. IGHG variants are important for immune defense against meningococci in states of deficient complement function. This relationship should be examined further.

(III) Serum levels of mannan-binding lectin (MBL) and antibodies to proteins from a potentially nephritogenic Streptococcus pyogenes strain (serotype M1 strain AP1) were investigated in 73 patients with acute poststreptococcal glomerulonephritis (AGN). Antibody responses to the serotype M1-related antigens M1, protein H and streptococcal inhibitor of complement were increased in patients compared to controls. The presence of MBL deficient individuals (serum concentration <0.1 mg/L) among AGN patients showed that the lectin pathway is not required in the pathogenesis of AGN.

(IV) Autoimmunity has been implied to participate in the pathogenesis of idiopathic sudden hearing loss (ISHL). Autoantibodies were analysed in sera from 92 patients with ISHL. The most frequently occurring antibody was aC1qCLR, detected in 12 patients (13 %), all with normal serum concentrations of C1q. In ISHL, aC1qCLR probably represents cross-reactivity between C1q and inner ear protein(s).

Key words: Acute poststreptococcal glomerulonephritis, C1q autoantibodies, C3 nephritic factors, complement, idiopathic sudden hearing loss, GM allotypes, Neisseria meningitidis

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CONTENTS

LIST OF PAPERS

This thesis is based on the following original manuscripts, which are referred to in the text by their Roman numerals (I-IV).

I Skattum L, Mårtensson U, Sjöholm AG. Hypocomplementaemia caused by C3 nephritic factors (C3 NeF): clinical findings and the coincidence of C3 NeF type II with anti-C1q autoantibodies. J Intern Med, 242:455-64, 1997.

II Skattum L, Gullstrand B, Holmström E, Oxelius V-A, Truedsson L. Serum bactericidal activity against *Neisseria meningitidis* in patients with C3 nephritic factors is dependent on IgG allotypes. *Submitted*.

III Skattum L, Åkesson P, Truedsson L, Sjöholm AG. Antibodies against four proteins from a *Streptococcus pyogenes* serotype M1 strain and levels of circulating mannan-binding lectin in acute poststreptococcal glomerulonephritis. Int Arch Allergy Immunol, 140:9-19, 2006.

IV Skattum L, Axelsson S, Stjernquist-Desatnik A, Mårtensson U, Mongiat M, Marastoni S, Lindberg S, Truedsson L. Autoantibodies to C1q in idiopathic sudden hearing loss. *Manuscript*.

ABBREVIATIONS

Acute poststreptococcal glomerulonephritis	AGN
Acquired angioedema	AAE
Alternative pathway of complement activation	AP
Antibodies to DNaseB	aDNaseB
Antibodies to streptolysin	ASO
Anti-neutrophil cytoplasmic antibodies	ANCA
Atypical hemolytic uremic syndrome	aHUS
Autoantibodies to the collagen-like region of C1q	aC1qCLR
Autoimmune inner ear disease	AIED
C1 inhibitor	C1 INH
C3 nephritic factors	C3 NeF
C4b-binding protein	C4BP
Classical pathway of complement activation	CP
Dense deposit disease, membranoproliferative glomerulonephritis type II	DDD
Dendritic cells	DC
Distantly related to SIC	DRS
Elastin microfibril interface located protein-2	EMILIN-2
Enzyme-linked immunosorbent assay	ELISA
Heat shock protein-70	HSP-70
Hereditary angioedema	HAE
Hypocomplementemic urticarial vasculitis	HUVS
Idiopathic sudden hearing loss	ISHL
IgG heavy chain genes	IGHG
Immune complex	IC
Immunoglobulin	Ig
Isoelectric focussing	IEF
Lectin pathway of complement activation	LP
Lipooligosaccharide	LOS
Lipopolysaccharide	LPS
Mannan-binding lectin	MBL
MBL-associated serine protease 2	MASP-2
Mean hearing threshold	MHT
Membrane attack complex	MAC
Membrane cofactor protein	MCP
Membranoproliferative glomerulonephritis	MPGN
Neisseria meningitidis	*N. meningitidis*
Partial lipodystrophy	PLD
Polymerase chain reaction	PCR
Serum bactericidal activity	SBA
Sheep erythrocyte	ShE
Single nucleotide polymorphism	SNP
Streptococcal cysteine proteinase	SCP
Streptococcal inhibitor of complement	SIC
Streptococcus pyogenes, group A streptococcus	GAS
Systemic lupus erythematosus	SLE
Terminal complement complexes	TCC

INTRODUCTION

The complement system

The complement system is one of the phylogenetically oldest parts of the innate immune system [1]. It comprises more than 35 circulating and cell-bound proteins, participating in serial enzymatic reactions producing several intermediate effector mechanisms (figure 1). In humans, complement plays an important role in early childhood when innate immunity is most needed, before the development of the antibody repertoire. Further on in life, the complement system both mediates effects of immunoglobulins (Ig), and in itself contributes to B cell responses [2, 3]. Complement activation occurs through at least three different activation pathways, each with its own recognition molecules that initiate activation. Biophysical data of most complement proteins are shown in table 1.

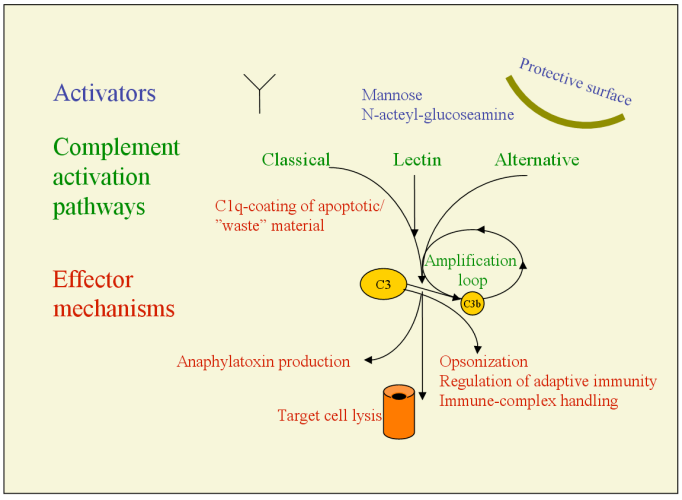

Figure 1. Schematic diagram of the complement system.

Overview of the complement system

Activation pathways

The classical pathway (CP, figure 2) is mainly activated by immune complexes (IC) containing bound IgG or IgM. The globular head region of C1q, the recognition molecule of the CP, binds to the activator. C-reactive protein [4] and several other non-immune molecules can also serve as ligands for C1q [5]. Binding of a ligand causes a conformational change in C1q, which leads to activation of C1r. C1r in its turn activates C1s. C1r and C1s are serine proteases which circulate in the inactive state, bound to C1q in a 2:1:2 (C1r:C1q:C1s) macromulecular, Ca^{2+}-dependent complex. Activated C1s cleaves C4, with formation of two fragments, C4a and C4b. The larger C4b may attach covalently to the activator by hydrolysis of an internal thioester while the smaller C4a diffuses away freely. C1s also cleaves C2, with

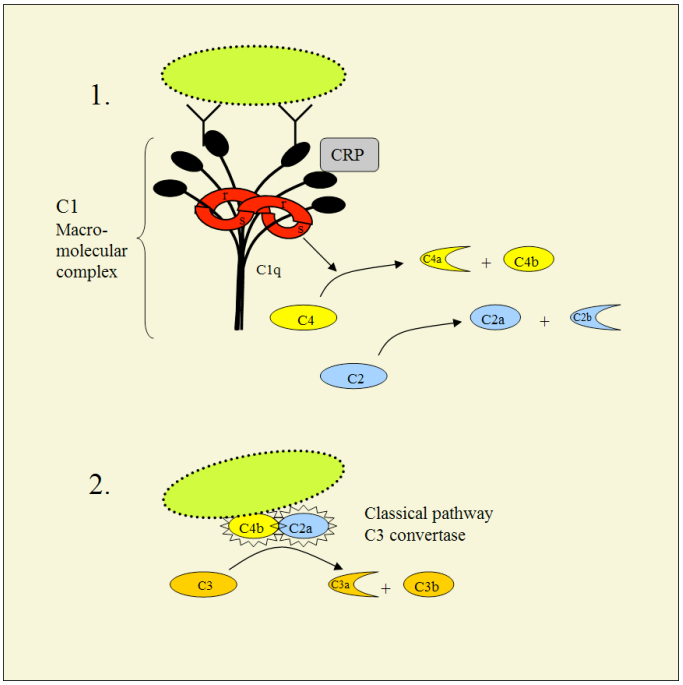

Figure 2. Activation of the classical pathway.

formation of C2a and C2b. The larger C2a binds to C4b and together C4b and C2a form the C3 convertase of the CP, a protein complex with enzymatic C3 cleaving activity.

The lectin pathway (LP, figure 3) is initiated when the oligomeric mannan-binding lectin (MBL) binds to specific carbohydrate residues not present on mammalian cells, for example mannose, N-acetylglucoseamine and L-fucose [6]. Circulating MBL is bound to Ca^{2+}-dependent homodimers of either of the MBL-associated serine proteases 1, 2 and 3 (MASP-1, MASP-2 and MASP-3) or a smaller enzyme; MBL-associated protein 19 (Map 19) [7]. Of the MASPs, MASP-2 is regarded to be the physiologically most important one. Another group of lectins, called ficolins, also circulate bound to MASPs and can activate the LP by binding to fibrinogen-like domains [8]. Upon bindning of activators, the

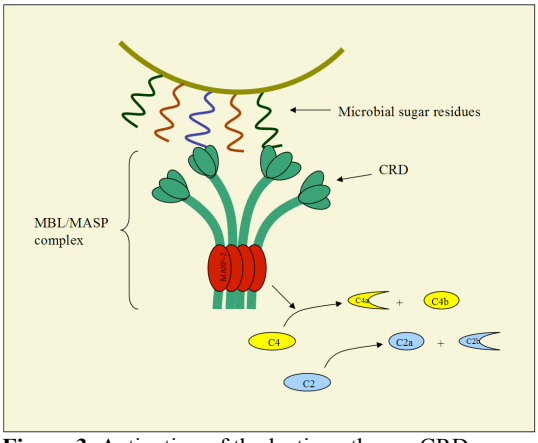

Figure 3. Activation of the lectin pathway. CRD; carbohydrate recognition domain.

Table 1. Biophysical data of fluid-phase complement components.

Component	MW[1] (kDa)	Plasma concentration (mg/L)	Main production site(s) (cell type)
C1q	460	80	Mø[2], other leukocytes
C1r	83	34	Hepatocyte
C1s	83	50	Hepatocyte, endothelial cell
C2	102	20	Hepatocyte
C4	202	150-400	Hepatocyte
MBL[3]	192-576[4]	0-7	Hepatocyte
MASP-2[5]	76	0.1-1.2	Hepatocyte
C3	185	800-1300	Hepatocyte, leukocytes, endothelial cell, epithelial cell
Factor B	93	200-400	Hepatocyte, Mø, lymphocyte, endothelial cell, epithelial cell
Factor D (adipsin)	24	1.4-2.2	Adipocyte, hepatocyte, Mø, astrocyte
Properdin	170-225[6]	20	Neutrophil, monocyte, T lymphocyte, hepatocyte
C5	192	70	Hepatocyte, Mø, monocyte, endothelial cell
C6	108	64	Hepatocyte, Mø, monocyte, endothelial cell
C7	97	56	Hepatocyte, Mø, monocyte, endothelial cell, neutrophil
C8	152	55	Hepatocyte, Mø, monocyte, endothelial cell
C9	71	59	Hepatocyte, Mø, monocyte, endothelial cell
Fluid phase C inhibitors	**(kDa)**	**(mg/L)**	**(cell type)**
C1 inhibitor	105	200	Hepatocyte, endothelial cell
C4b-binding protein	570	250	Hepatocyte
Factor H	155	200-600	Hepatocyte, monocyte, lymphocyte, endothelial cell, GMC[7]
Factor I	88	30-50	Hepatocyte, monocyte, lymphocyte, endothelial cell
Clusterin	80	50-100	Epithelial cells in many organs, some endocrine cells
Vitronectin	75	340	Hepatocyte

[1]Molecular weight, [2]Macrophage, [3]Mannan-binding lectin, [4]MBL is a multimer composed of two to six identical 96 kDa subunits, [5]MBL-associated serin protease-2, [6]Properdin is a multimer, predominantly composed of three or four identical 56 kDa subunits, [7]Glomerular mesangial cell.

MASPs are activated due to conformational changes, and subsequently MASP-2 cleaves C4 and C2. From this activation step onwards, the LP is analogous to the CP, forming identical C3 convertases (C4b2a).

Activation of the alternative pathway (AP, figure 4) is constantly ongoing in the fluid phase, due to spontaneous hydrolysis of the most abundant complement protein, C3. The internal thioester in C3 is broken by water, whereby C3(H_2O), also termed C3i, is formed. C3(H_2O) resembles C3b, a fragment formed upon cleavage of C3. C3b/C3(H_2O) can, in the presence of Mg^{2+}, bind factor B from the circulation. Factor B then becomes available for cleavage by factor D, giving rise to two fragments; Bb and Ba. The Bb fragment stays attached to C3b/C3(H_2O) and together the molecules form C3bBb, the C3 convertase of the AP [9]. C3 convertases cleave C3 into C3a (an anaphylatoxin) and C3b. C3b formed by C3 cleavage can bind to available surfaces and continue AP activation, creating an amplification loop. Binding of C3b and formation/stabilization of a C3 convertase is dependent on the nature of the surface and is promoted by, for example, a low content of sialic acid.

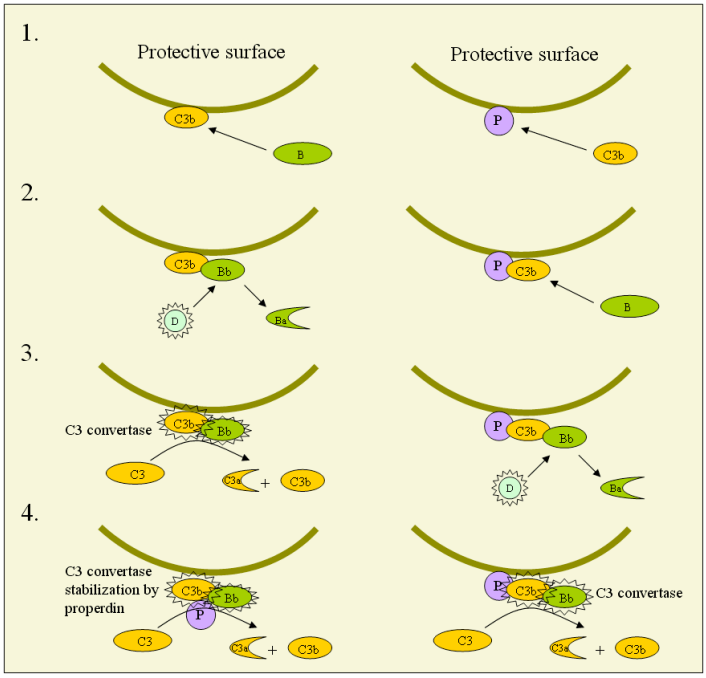

Figure 4. Activation of the alternative pathway, in sequential order. The right and left sequences show different scenarios. Properdin may serve as a stabilizer (left) and initiator (right) of the alternative pathway.

C3b molecules formed by activation of the CP or LP also initiate AP C3 convertase formation, whereby the AP increases complement activation initiated by the CP and LP. The AP also includes properdin, which binds to and stabilizes the C3 convertase, thereby prolonging its half-life. In addition, properdin has recently been ascribed a role as initiator or enhancer of the AP. In this function, properdin directs AP activation and functions as an AP specific recognition moleule [10, 11].

C3b molecules may also bind to C3 convertases formed by any complement activation pathway, whereby the convertases acquire C5 cleaving activity; that is, become C5 convertases. C5 is cleaved by C5 convertases into C5a and C5b. The former is an anaphylatoxin and has a chemoattractant effect for neutrophils and other leukocytes, while C5b is incorporated into the cell membrane. Alternatively, C5 activation has been reported to occur directly through proteolytic cleavage by non-complement

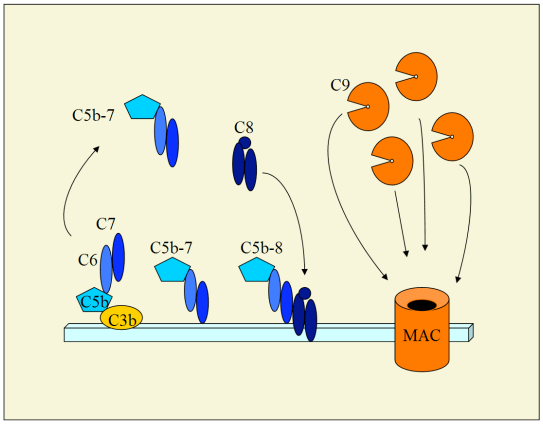

Figure 5. Assembly of the MAC of the terminal complement activation pathway. The light blue bar represents a cell membrane.

proteases [12, 13]. C5 cleavage marks the initiation of the terminal pathway (figure 5). By spontaneous sequential reactions, C5b, C6, C7, and C8 assemble to form the membrane attack complex (MAC). The terminal pathway culminates in the incorporation of multiple C9 molecules into target cell membranes. The C9 molecules polymerize to form a pore-shaped structure spanning the cell membrane, although lysis may occur, at a slower pace, in the absence of C9.

Effector functions of the complement system

The complement system takes part in the central action of the immune system: defense against external (microbial and physical) and internal (altered self) threats. It has both effectuating and regulating functions, which are to a great extent achieved through interaction with other parts of the immune system.

Many blood cells express complement receptors on their surfaces. Several functions of the complement system are exerted through binding of complement derived fragments to these receptors. Complement receptors and their complement derived ligands are listed in table 2, along with consequences of ligand binding.

Upon complement activation, the initiating particle is covered with C3 and/or C4 fragments, a process called opsonisation. These fragments are bound by complement receptors on phagocytes (the above paragraph), which facilitates phagocytosis.

The complement activation cascade produces three small fragments with proinflammatory effects; the anaphylatoxins C3a, C4a and C5a. These stimulate the release of mediators that increase vascular permeability, which contributes to local edema formation. C5a is also a chemoattractant for neutrophils and other cells that bear the C5aR.

The MAC formed by the terminal pathway of activation causes cell death by lysis. Both altered cells of the body (for example cancer cells) as well as foreign cells (bacteria) may be attacked.

An important task for the complement system is contribution to waste disposal. IC are constantly formed in the body and must be eliminated. Erythrocytes participate in this process via their C3b receptor complement receptor 1 (CR1, CD35). Deficient handling of IC leads to organ damage by excessive depostion of IC in tissues, which causes inflammation. Vessels of the kidney glomerulus, joints and skin are especially prone to IC deposition, probably because of blood flow conditions. IC deposition in these sites may cause glomerulonephritis, arthritis and vasculitis, respectively [14, 15]. Intracellular antigens from cells undergoing apoptosis are another type of material which may cause damage if not properly taken care of. Tolerance of the immune system to intracellular antigens may be broken upon prolonged or otherwise abnormal exposure in conjunction with apoptosis. In this way, autoreactivity may be provoked. C1q and MBL bind to apoptotic material, and its uptake by phagocytosis is mediated through receptors on macrophages and DC [16-19].

Table 2. Cell bound complement receptors.

Receptor (CD[1] designation)	Complement derived ligand(s)	Cellular distribution	Consequence(s) of complement ligand binding
CR[2]1 (CD35)	C4b, C3b, iC3b, iC4b, C1q	E[3], B[4], G[5], M[6], DC[7], T[8] subsets	IC[9] binding (E), inhibitory receptor (B), C3b breakdown (factor I cofactor), phagocytosis
CR2 (CD21)	C3d,C3dg, iC3b	B, FDC[10]	co-stimulation of B responses, EBV[11] receptor
CR3 (CD11b/CD18)	iC3b	G, M, Mø[12], NK[13], T, B, DC	phagocytosis, monocyte migration, adhesion
CR4 (CD11c/CD18)	iC3b	G, M, Mø, platelets, B, T	phagocytosis
CRIT[14]	C2	M, T, B, DC, platelets	inhibition of CP activation
C3aR	C3a	N[15], Ma[16], T, DC, EC[17], EpC[18], PC[19]	release of proinflammatory mediators, regulation of adaptive responses
C5aR (CD88)	C5a, C5adesArg	Mø, N, Eo[20], Ma, T, DC, EC, EpC, PC	like C3aR, and in addition chemoattraction, regulation of tissue regeneration and tolerance
C5L2	C5a, C5adesArg, possibly C3adesArg	Mø, N, Ma, DC, PC	probably inhibition of C5aR-mediated responses
cC1qR/CR	C1q, MBL[21]	Mø, DC, EC, platelets, B, T	Mø/DC; phagocytosis, EC/P; increased cell adhesion, B/T; activation and proliferation
gC1qR/p33	C1q globular head region, vitronectin	Mø, DC, EC, platelets, B, T	Mø/DC; phagocytosis, EC/P; increased cell adhesion, B/T; activation and proliferation
C1qRp (CD93)	C1q	M, N, EC, SC[22]	phagocytosis, clearance of apoptotic material, cell adhesion
CRIg	C3b, iC3b	Mø	in the liver: phagocytosis, inhibition of AP C3 convertase

[1]CD; cluster of differentiation, [2]CR; Complement receptor, [3]E; erythrocytes, [4]B; B lymphocytes, [5]G; granulocytes, [6]M; monocytes, [7]DC; dendritic cells, [8]T; T lymphocytes, [9]IC; immune complex, [10]FDC; Follicular dendritic cells, [11]EBV; Epstein-Barr virus, [12]Mø; macrophages, [13]NK; natural killer cells, [14]CRIT; complement C2 receptor inhibitor trispanning, [15]N; neutrophils, [16]Ma; Mast cells, [17]EC; endothelial cells, [18]EpC; epithelial cells, [19]PC; parenchymal cells, [20]Eo; eosinophils,[21]MBL; mannan-binding lectin, [22]SC; stem cells.

The complement system is involved in the stimulation and regulation of the adaptive immune system. This is exemplified by patients with deficiencies of the CP, who exhibit low Ig levels and impaired specific immune responses [20-25]. There are several possible mechanisms which explain the link. B cell responses are stimulated by binding of C3dg to CR2 [2, 3]. Recently, such cross-linking was even reported to overcome B cell anergy [26]. B cells also carry CR1, which is an inhibitory receptor on those cells, and partial differences in ligands for CR1 and CR2 enable regulation of B cell responses depending on the type of C3 fragments [27]. Regulation of immune responses by complement proteins may occur indirectly, through several

mechanisms. DC maturation is increased by C1q binding [28], and interactions between T cells and DC are influenced by C3 fragments [29, 30]. Coligation of membrane cofactor protein (MCP, CD46) and the T cell receptor induces development of T cells into regulatory T cells [31], and such CD46-induced regulatory T cells suppress CD4+ T cells but not the maturation of DC [32].

Inhibition of the complement system

Because of the powerful effects of complement, its activation must be tightly regulated to avoid tissue damage. To ensure protection, a number of fluid-phase and cell-bound inhibitors are present in the organism. Besides being protected by these, host cells are also protected by the nature of their surface, which, partly because of its high content of sialic acid, does not protect the AP C3 convertase from degradation [33].

The inhibitor of the C1 macromolecular complex is called C1 inhibitor (C1 INH). This is a large serpin (serine protease inhibitor) which is the sole inactivator of C1r and C1s of the CP as well as of MASP-2 of the LP. In addition, C1 INH inactivates serine proteases of other cascade systems in the circulation, for example factor XII and kallikrein [34].

Factor H is the fluid-phase inhibitor of the AP, which acts to dissociate the AP C3 convertase by binding C3b. In addition, factor H is a cofactor for factor I, another fluid phase regulatory protein. Factor I cleaves C3b, whereby C3b becomes iC3b, which is unable to bind factor B. With the help of factor H or other cofactors, factor I degrades iC3b further into C3c, producing the small fragment C3dg. Both C3dg and the intermediate forms iC3b and C3b remain attached and may bind to their respective receptors (table 2). The counterpart of factor H in the CP is C4b-binding protein (C4BP), which dissociates the CP C3 convertase and acts as a cofactor of factor I in C3b degradation analogous to factor H activity in the AP. Vitronectin is a fluid-phase protein, which inhibits C9 polymerization [35] and attachment of the MAC complex to cell membranes [36]. Clusterin is another circulating MAC-inhibitor [37]. Host cells are protected from complement deposition and lysis by several membrane-bound complement inhibitors. Both CR1 and MCP act as cofactors for factor I in the degradation of C3b and C4b. In addition, MCP and CR1 share the ability of decay-accelerating factor (DAF, CD55), to accelerate the intrinsic decay of CP and AP C3 convertases. CD59 is a membrane-bound inhibitor of MAC-mediated cell lysis [38]. The genes encoding MCP, DAF, CR1, factor H and C4BP all belong to the regulators of complement activation (RCA) gene family, on the long arm of chromosome 1 [39].

Complement deficiencies

The most common inheritable complement deficiency state is low concentrations of MBL due to low-expressing gene variants. MBL deficiency is commonly defined as serum levels <0.1 mg/L, occurring in about 10 % of a Caucasian population [7]. In combination with other types of immunodeficiency, the subtotal MBL deficiency is

associated with slightly to moderately increased susceptibility to infection [40, 41]. For example, during immunosuppressive treatment, or in the immunologic "window" between 6 and 18 months of age, MBL deficiency increases the risk of contracting bacterial infections. The impact of MBL deficiency on disease risks is a matter of discussion. Homozygous MASP-2 deficiency was originally described in a patient with multiple autoimmune disease manifestations as well as recurrent pneumococcal infections [42]. The allele frequency of the A→G substitution at position 120 in exon 3 of the MASP-2 gene, detected in the first discovered MASP-2 deficient patient, was reported to be 1.3 % in 200 healthy individuals [43].

C2 deficiency is the most common of the deficiencies of CP components, with a frequency of ~1:20,000 in Caucasian populations [44]. Around 90 % of C2 deficiency cases (referred to as type 1) are caused by a 28bp deletion in the C2 gene. This deletion is mostly found in a specific MHC haplotype [45, 46]. All deficiencies of the CP are associated with an increased risk of systemic lupus erythematosus (SLE) or SLE-like IC disease. There is a hierarchy within the CP with regard to susceptibility for SLE, with the risk sizes C1q>C4>C2 of the respective complement factor deficiencies. These differences are not completely understood, but may be caused by differing impacts on waste disposal or IC handling of the various components in the CP. In addition to SLE, CP deficiencies are associated with proneness to bacterial infections; in particular those caused by encapsulated organisms like *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitidis* (*N. meningitidis*; meningococcus). Cardiovascular disease is overrepresented in C2 deficiency, irrespective of SLE [47], while the frequency of such associations is difficult to assess for the less common deficiencies of the CP.

Deficiencies of the AP are uncommon [44], and are mainly associated with bacterial infections. In particular, properdin deficiency markedly increases the risk of meningococcal infection. Properdin deficiency is X-linked, and 3 different forms have been described: complete deficiency (type I deficiency), incomplete deficiency, with circulating properdin concentrations of ~10 % of normal (type II deficiency), and a form caused by a dysfunctional protein (type III) [48]. Heterozygous female carriers of a properdin deficiency gene are generally not clinically affected.

The sole consequence of deficiencies of the terminal pathway is an increased risk of neisserial infections (*Neisseria gonorrhoeae* and meningococci). In these patients, as opposed to meningococcal infections in properdin deficiency, infections are often recurrent, but rarely fatal [49]. In the Japanese population, deficiency of C9 is common, with a frequency of almost 1:1000 [50]. Patients with deficiency of C9 are much less prone to neisserial infections than other terminal pathway-deficient individuals, which suggests that suboptimal lytic capacity is often sufficient for neisserial killing [51].

Inherited deficiency of C1 INH, termed hereditary angioedema (HAE), affects not only the complement system, as it is involved in the control of other biological systems like the contact activation system and the coagulation cascade [52]. The

clinical symptoms are recurrent localized edemas, which result from deficient inhibition of kallikrein, and the resultant excessive formation of bradykinin [34]. The edemas locate to soft tissues and mucous membranes, and may be life-threatening if occurring in the throat. Such cases need immediate intensive care and injections of purified human or recombinant C1 INH concentrate; this should therefore be accessible at all emergency care hospitals. HAE is caused by heterozygous deficiency of C1 INH, with prevalence around 1:50,000 [53], and a substantial proportion of cases result from novel mutations [54]. HAE may be caused by lack of circulating C1 INH (HAE type I) or by mutations leading to dysfunctional variants of the protein (HAE type II). Homozygous complete deficiency as well as some single nucleotide polymorphisms (SNPs) of the fluid-phase AP regulator factor H are associated with membranoproliferative glomerulonephritis (MPGN) [55, 56], while a subgroup of cases of atypical hemolytic uremic syndrome (aHUS) are associated with heterozygous factor H gene polymorphisms [57], particularly mutations located in gene segments coding for the C terminal region of factor H [58]. Some factor H gene SNPs are also associated with increased risk of developing age-related macular degeneration, which is the leading cause of blindness in the elderly [59, 60]. Complete deficiencies of both factor I and factor H lead to increased susceptibility for bacterial infections due to secondary C3 deficiency [50], while this is not the case with most gene variants associated with aHUS. Genetic variants in the genes for factor I, factor H and MCP are all associated with aHUS, but disease penetrance is variable and often requires a combination of SNPs in two or more genes [61].

Acquired complement deficiency may result from decreased production, increased protein loss, or more commonly, from increased consumption of components through excessive complement activation. For obvious reasons, as opposed to inherited deficiencies, acquired deficiencies rarely are complete, and their clinical significance depends on the magnitude of the decrease in native protein concentration.

Acquired C3 deficiency without pronounced decrease of concentrations of other complement components develops in conditions with increased activation of the AP, like acute post-streptococcal glomerulonephritis (AGN) [62], and circulating autoantibodies to the AP C3 convertase, C3 nephritic factors (C3NeF) [9]. Like in inherited C3 deficiency, the acquired form is associated with increased risk of bacterial infections [63].

Several diseases are associated with increased CP consumption. The CP activating agents in such conditions as SLE and cryoglobulinemia are IC. Excessive CP activation leads to secondary low serum concentrations of C4 in particular, and less often of C1q. Total C2 levels as detected by conventional methods are affected only in conjunction with pronounced CP activation, as the large C2a fragments are determined along with native C2 by immunochemical analysis [64, 65].

Acquired deficiency of C1 INH is termed acquired angioedema (AAE). Patients with this condition show low concentrations of all CP components including C1q, which helps to differentiate it from HAE, where only C4 and C1 INH are low [66]. AAE is a rare condition which occurs as a paramalignant phenomenon, or in association with autoimmune diseases [66]. It may also present as an isolated entity, and may in any setting be associated with or caused by autoantibodies to C1 INH. The clinical symptoms of AAE are the same as those of HAE, but are often unresponsive to symptomatic treatment unless the associated disease is successfully overcome.

Paroxysmal nocturnal hemoglobinuria refers to an acquired condition of recurrent acute attacks of intravascular hemolysis, most often occurring during the night. The hemolysis causes hemoglobinuria, observable as macrohematuria. The hemolysis results from loss of membrane-bound inhibitors of the MAC; CD59 and/or DAF on blood cells due to a somatic mutation in a gene involved in assembly of glycosylphosphatidylinositol (GPI)-anchors. Paroxysmal nocturnal hemoglobinuria is an acquired hematopoetic stem cell disorder [67].

Autoantibodies to complement proteins

Like all autoantibodies, antibodies specific for complement proteins are present in low amounts in healthy individuals as well as in patients, and increase in concentration with increasing age. Some complement autoantibodies with strong disease associations are presented below.

C3 NeF [68] are often of IgG1 or IgG3 subclass [69, 70]. C3 NeF are associated with MPGN, mainly type II (dense deposit disease; DDD), acquired partial lipodystrophy (PLD) and increased susceptibility to infection by encapsulated bacteria due to acquired C3 deficiency. Acquired PLD is a very rare disease [71] characterized by gradual loss of subcutaneous fat of the face and upper half of the body. The cause of acquired PLD is unknown, although factor D is produced by adipocytes and C3 NeF can lyse adipocytes experimentally [72]. There is no specific therapy for acquired PLD. After some years, about 50 % of patients also develop MPGN [71]. There are two or more types of C3 NeF, recognizing different or partly different antigens. Some are only active in the fluid phase and probably bind to C3 convertases. Another type, which only can be detected by solid-phase assays, may be directed to C5 convertases [73, 74]. All C3 NeF stabilize C3 convertases both against intrinsic and extrinsic decay, whereby convertase half-life is prolonged and C3 is continuously activated. The resulting persistently low serum C3 concentrations induces increased susceptibility to infection [75].

Autoantibodies that stabilise the CP C3 convertase (C4b2a) are termed C4 nephritic factors. Like C3 NeF, C4 nephritic factors induce low C3 concentrations in serum due to complement consumption. C4 nephritic factors are rarely found in patients with MPGN, AGN and SLE [76-78].

C1q, the recognition protein of the CP, is a macromolecular heterotrimer composed of 18 polypeptide chains of three types; the A, B and C chains. The C-terminal ends of one A, one B and one C chain together form one of six globular "head" regions. At the C-terminal ends, 6 heterotrimers form a partly triple-helical structure, the collagen-like part of C1q. This arrangement gives C1q the shape of a bunch of tulips (figure 6). Autoantibodies to the collagen-like part of C1q (aC1qCLR) [79, 80] are found in about 30-50 % of unselected SLE patients, especially in conjunction with nephritis [81], and are disproportionately often of IgG2 subclass [82, 83]. The autoantibodies recognize only bound C1q, but not C1q in solution [79]. In SLE, aC1qCLR are associated with low C1q concentrations and titres often follow disease activity [84]. More recently, autoantibodies directed to the globular head region have been described in SLE [85]. At least 95 % of patients with hypocomplementemic urticarial vasculitis (HUVS) have aC1qCLR, regularly accompanied by low C1q levels [86]. Lower frequencies of aC1qCLR-positive individuals are found among patients with rheumatoid vasculitis and MPGN, as well as in human immunodeficiency virus (HIV) and hepatitis C virus infected patients [87-90]. The majority of aC1qCLR-containing HUVS sera show IgG reactivity with the separated B and C chains of C1q in Western blot analysis. Most aC1qCLR-containing SLE sera show no such reactivity, and this difference in reaction pattern may be used for differential diagnosis [91, 92].

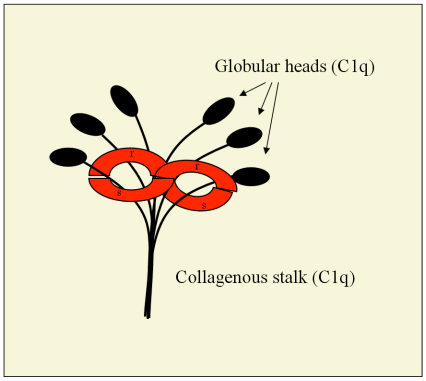

Figure 6. Schematic picture of the C1 macromolecular complex. C1q is in black, C1r and C1s in red.

A proportion of patients with AAE produce autoantibodies to C1 INH. Analysis of autoantibodies to C1 INH is helpful in the differentiation between hereditary and acquired C1 INH deficiency [66, 93]. Both IgG and IgM autoantibodies have been described in AAE, and increased levels of IgM autoantibodies to C1 INH have also been reported in HAE [94].

Autoantibodies to MBL have been found in SLE [91, 95] and rheumatoid arthritis [96]. MBL activity showed inverse correlation with autoantibodies to MBL in both diseases while autoantibodies to MBL did not correlate with disease activity in SLE.

Autoantibodies to factor H have been found in aHUS [97], where they correlate with deficiency of complement factor H related proteins 1 and 3 [98]. Factor H antigenic levels remain normal in the presence of autoantibodies to factor H despite interference with the complement regulating function of factor H.

Disease associations of the complement system

Complement and infections

As described under "Complement deficiencies", bacterial infections are overrepresented in complement deficient individuals. This applies to deficiencies in all complement activation pathways. Functions of complement proteins and effects of complement activation explain this association. A functional complement system is required for efficient development of adaptive immune responses [99], direct killing of microorganisms and opsonization for promotion of phagocytosis.

In the presence of a functional complement system, infectious organisms have evolved many strategies to overcome these obstacles. The impact of encapsulation is diverse, as some encapsulated bacteria, like *Escherichia coli*, are readily killed by the normal complement system, while others, like *Streptococcus pyogenes* (*S. pyogenes*; group A streptococci; GAS) exhibit resistance to complement mediated defense in the absence of serotype-specific antibodies [100]. In some species, i.e. *Haemophilus influenzae*, sialylation of polysaccharide residues plays an important role in the protection against complement mediated attack, and several organisms even capture sialic acid from the environment [101]. Gram-positive bacteria in general are resistant to complement attack. The protection is mediated by the thick peptidoglycan of the cell wall and the capsule, as well as by other means such as acquisition of complement regulating proteins from the host. A vast number of micro-organisms utilize host complement regulators for their own protection in this way [102]. The complement regulating proteins often retain their function after having been bound by the bacterium [103]. Some intracellular microorganisms utilize membrane-bound complement receptors for entrance into host cells [104]. In sepsis caused by the Gram-negative bacteria, the AP is strongly activated by the lipopolysaccharide (LPS), which is a typical feature of Gram-negative bacteria such as *Escherichia coli*. CP activation by lipid A also contributes [105]. The resulting excessive complement activation is often detrimental to the host, as it contributes to the shock and disseminated intravascular coagulation that is often induced by these species.

Complement and autoimmune disease

Complement is involved in autoimmune disease in two principally different ways. On the one hand, deficiencies of CP complement components confer an increased risk of autoimmune disease, mainly SLE. On the other hand, complement activation contributes to inflammation and organ damage in several autoimmune diseases.

The mechanisms by which complement protects against autoimmunity are incompletely understood. There are different explanatory theories. One is that complement contributes to the organism´s scavenger system, which handles apoptotic and other "waste" material. During cell death by apoptosis, intracellular antigens are transiently expressed at the surface of cell membrane-bound vesicles called apoptotic blebs. Apoptotic blebs bind C1q which activates complement and thereby facilitates uptake by phagocytes and dendritic cells (DC) [17, 18, 106]. In

the absence of complement, exposure to the adaptive immune system of intracellular antigens will be prolonged. This increases the risk of an autoimmune response [107]. Another hypothesis implies a role for complement in the autoimmune response as a function of its importance for stimulation and regulation of adaptive immunity, including development and maintenance of tolerance [2, 108, 109].

Complement as a harmful pro-inflammatory agent in autoimmune disease is exemplified by its role in SLE. In active SLE, most evidently in conjunction with kidney engagement, systemic complement activation occurs. Complement is believed to be activated by IC formed by autoantibodies and their respective antigens, for example double-stranded DNA. The activation leads to consumption of complement factors in excess of the synthesis rate. These circumstances lead to an imbalance in serum complement levels, with low levels of CP factors like C4 and C1q. Since the complement activation in SLE proceeds mainly via the CP, concentrations of AP complement components are rarely affected. Serum concentrations of complement activation products such as C3dg and soluble terminal complement complexes (TCC) increase. Systemic complement activation correlates to disease activity in about one third of SLE patients, while the rest have normal complement concentrations also during flares [81]. The reason for this diversity in SLE is not clear. SLE patients with systemic complement activation and low C1q serum levels often show increased concentrations of aC1qCLR. The reason for this is not fully understood, but aC1qCLR may be pathogenic [110].

The CP is systemically activated also in HUVS, an SLE-like autoimmune disease of unknown etiology [86]. Patients with HUVS exhibit low serum concentrations of C1q in almost 100 % of cases, along with aC1qCLR in the circulation. C3 and C4 levels are usually moderately low.

Activation of the CP occurs locally in other autoimmune diseases, contributing to pathogenesis. For example, local complement activation occurs in the joints of patients with arthritis [111].

Recent evidence indicates a role for the AP in the development of glomerular damage in vasculitis associated with anti-neutrophil cytoplasmic antibodies (ANCA) [112, 113]. In a mouse model, animals deficient in factor B and/or C5 were protected from organ damage caused by injection of ANCA directed against myeloperoxidase. The mechanism of AP complement activation in ANCA-associated vasculitis is still unclear, but several AP complement factors including not only properdin but also the targets for ANCA (most commonly myeloperoxidase and proteinase 3) are produced by the neutrophil itself [114]. Neutrophil activation by ANCA [115] might lead to increased release or *de novo* production of AP complement factors, which might initiate complement activation locally. The recent finding that properdin may in fact act as an initiator of the AP [10] provides a possible key to the understanding of the AP dependency of ANCA-associated glomerular injury. An alternative explanation is provided by additional molecules, able to directly activate C5 (e.g. elastase), which are also produced by neutrophils [12].

Complement and renal disease

Functions of the complement system are central to many renal diseases. One group of conditions arise due to uncontrolled AP activity, while CP activation causes inflammation and organ damage in IC-related disorders. In addition, recent evidence suggests that the LP is involved in the pathogenesis of some types of kidney disease. Some glomerular renal diseases and their relation to the complement system are listed in table 3.

Table 3. Involvement of complement in different types of glomerulonephritis.

GN[1] with major complement involvement	Type of complement involvement
GN associated with mixed cryoglobulinemia	Systemic classical pathway activation
SLE nephritis	Systemic classical pathway activation
MPGN I (primary or secondary; eg in SLE)	Systemic classical pathway activation
MPGN II	Systemic alternative pathway activation
AGN	Systemic alternative pathway activation
GN with minor complement involvement	**Type of complement involvement**
IgA nephropathy	Low serum C3 concentration in a minority of cases
GN associated with ANCA	C5b and factor B required for GN development in mouse model
Henoch-Schönlein nephritis	Low serum C3 concentration in ~5 % of cases

[1]Glomerulonephritides

MPGN type II (DDD), is a rare chronic disease which leads to renal failure in 50 % of patients after 10 years. Renal biopsies show deposits of electron-dense material of unknown nature along the glomerular basement membrane, as well as deposited C3, but little or no Ig [58]. In fact, it has been proposed that DDD is in fact not an MPGN, as only a proportion of cases show the membranoproliferative histopathological picture [116]. DDD is associated with deficient control of the AP. Such deficient control occurs in inherited total factor H deficiency, due to certain factor H and factor H-related protein mutations, but also in acquired states in conjunction with C3 NeF [55, 117]. Recently, an explanation was provided for the clinical observation that complete factor I deficiency does not cause increased susceptibility to DDD, although it is associated with deficient AP C3-convertase control. It seems that the C3-breakdown products of factor I-mediated C3 cleavage are required in the pathogenesis [118]. Included among the thrombotic microangiopathies, aHUS is characterized by hemolysis, thromocytopenia and acute renal failure [57]. A subgroup of aHUS cases are caused by deficient function of factors controlling the AP C3-convertase, but in contrast to DDD, only the activity on the endothelial cell surface seems to be important in this disease, and heterozygous mutations are often sufficient to cause disease[119, 120]. Certain polymorphisms of genes encoding proteins involved in the AP are associated with development of aHUS. In addition to factor H variants, this association has been shown for mutations and polymorphisms in the genes for factor I, MCP and factor B

[61, 121, 122]. Recently, mutations in the C3 gene were also demonstrated in aHUS patients [123]. Incomplete penetrance of the disease-associated genes indicates that other factors, such as infections, are necessary as trigger factors. The AP is activated in AGN, but the reason for and the possible role of activation in pathogenesis is largely unknown (see "AGN" further on).

The CP is activated in several diseases of wich nephritis is a common manifestation, including SLE, HUVS, and mixed cryoglobulinemia. In these conditions, Ig and complement components are deposited in the glomeruli, and serum levels of CP complement components are often low. However, the AP has been shown to be also of importance in a model of cryoglobulin-induced IC glomerulonephritis [124].

LP involvement has been suggested for the disease processes of IgA nephropathy and AGN [125-127].

Complement and inflammatory diseases

Due to the production of anaphylatoxins, the complement system is involved in the development of organ damage in many inflammatory diseases. Some examples are ischemia-reperfusion damage [128], allergy/asthma [129], cardiovascular disease [130], and sepsis [131]. In several of these conditions, C5a and the C5a receptor seem to play a crucial role. In these conditions, C5a is often formed via activation of the AP, and inhibition of C5a and other complement effector molecules has been tried as a means of treatment.

Complement analysis

Analysis of the complement system in patient samples is warranted in investigation of possible complement deficiency, and in cases of suspected excessive complement activation. Complement deficiency is mainly suspected in conjunction with proneness to bacterial infection not explained by other immunodeficiency, in certain renal conditions, and in cases of non-allergic angioedema. Inherited complement deficiency should also be excluded in SLE patients. Assessment of increased complement activation is of interest in IC disorders.

Functional analysis

Functional activity of the three complement activation pathways is measured as a screening procedure on suspicion of complement deficiency. If functional assays indicate a deficiency, investigations proceed with further analysis of proteins involved in the affected pathway/s, also depending on the clinical symptoms [132].

The original gold standard is hemolytic assays employing sensitized sheep erythrocytes for the CP (CH50) and rabbit or guinea-pig erythrocytes for the AP (AP50). Less time-consuming hemolytic tests for the CP and AP have been developed to provide alternatives, both quantitative [133] and qualitative [134]. An enzyme-linked immunosorbent assay (ELISA) for quantitative analysis of all three activation pathways was developed and commercialised more recently [135]. This assay utilizes a monoclonal antibody to a neo-epitope expressed in C9 upon

activation [136]. Functional assays of single complement components may again employ the lytic activity of complement (hemolytic assays). A functional hemolytic analysis for factor H has been described, which can be used as a screening method in aHUS to indicate which patients carry functionally significant factor H mutations/ SNPs [137].

Complement protein analysis

Serum or plasma concentrations of complement proteins are measured as part of the investigations of suspected complement deficiency (hereditary or acquired), or to indirectly assess increased complement activation, which in many instances induces decreased complement concentrations due to consumption. Immunochemical techniques such as turbidimetry, nephelometry, electroimmunoassay, single radial immunodiffusion and ELISA are commonly used for this purpose. Recently, protein microarray technique has been applied to analyze complement proteins on a research basis [138], and comparison of results showed good agreement with conventional methods.

Complement activation products

In vivo complement activation occurs in many diseases and through different activation pathways. For example in SLE, complement activation mainly via the CP correlates with disease activity in a subset of patients and may even predict flares [81]. In other IC mediated diseases and in hereditary angioedema, CP activation also occurs. Assessment of complement activation that has occurred by any one or more of the three activation pathways is sufficient in most cases, and is achieved by measurement of concentrations of C3dg and/or TCC in the circulation. If it is considered necessary to determine which activation pathway is involved, activation products specific for the respective activation pathways may be analyzed. No activation product specific for LP activation has been described. TCC is usually measured by sandwich ELISA, employing the monoclonal antibody against a neo-epitope in C9 for detection of TCC bound to the plate by an antibody to some other part of the complex [136]. C3d or C3dg may be analyzed by immunoelectrophoresis, provided that all C3 fragments containing C3c are first separated from the sample [139]. Sampling and transport conditions for the specimen must be taken into account when evaluating results, as spontaneous *in vitro* complement activation occurs with prolonged storage at temperatures above -80°C. *In vitro* activation leads to formation of complement activation products and thus may produce falsely pathological results [140, 141].

Complement autoantibody analysis

As already mentioned (under “Autoantibodies to complement proteins”), clinically significant autoantibodies to C1q in SLE are directed against the collagenous region. ELISA techniques employing the collagenous part as antigen would therefore be preferable to analysis of reactivity with the whole molecule. However, the risk of obtaining differing results when using the whole C1q molecule as antigen is likely to

be negligible (unpublished observations), and commercial kits employ the whole C1q molecule. Western blot analysis of IgG reactivity to the separated polypeptide chains of C1q (A, B and C) is useful to differentiate between SLE and the similar HUVS. IgG from the majority of patients with patients with SLE shows no Western blot reactivity, while most HUVS sera show IgG Western blot reactivity with the C1q B and C chains [91, 92].

C3 NeF are usually analyzed by methods that detect their convertase stabilizing effect. The classical test is a hemolytic test in which the convertase is built stepwise on sheep erythrocytes with purified complement proteins. After addition of patient IgG, decay of the convertase is quantified [68]. This analysis does not take the effect of factor H into account as whole serum is not used. Later, a simpler hemolytic test using whole serum was described [142]. Another functional test measures *in vitro* C3 breakdown after mixing patient and normal serum [143]. ELISA tests using purified complement proteins have been described. These types of tests show the IgG nature, but not the C3 convertase stabilising effect of C3 NeF [144]. Some methods for C3 NeF analysis will be thoroughly described later in this thesis.

Autoantibodies to other complement factors are measured by conventional techniques, such as ELISA.

Genetic analysis

Most complement deficiencies have an extensively heterogenous genetic background, and are not caused by one or a few genetic variants. In these cases, gene sequencing is required to determine the genetic cause of deficiency. A few exceptions are present, including C2 deficiency and MBL polymorphism.

A genetically based diagnosis of C2 deficiency, may be made by polymerase chain reaction (PCR) assay using primers specific for a fragment containing the 28 bp-deletion in the C2 gene, which causes >90 % of all cases. The MBL structural and promotor polymorphic variants causing low circulating levels may be determined in a similar fashion. In HAE, gene sequencing is necessary to determine the genetic basis for low circulating concentrations (HAE type I) or dysfunction (HAE type II) of C1 INH. In HAE, novel mutations of the long C1 INH gene are not uncommon.

Immunoglobulins

An Ig, or antibody, is the soluble form of the B lymphocyte antigen receptor, and represents the humoral effector arm of acquired immunity. In short, Ig:s are composed of four polypeptide chains; two of which are 24 kDa in size (light chains), and two of the size of 55-70 kDa (heavy chains). Antigen binding sites are formed by each pair of polypeptide chains composed of one light and one heavy chain. Structurally, Igs are Y-like in shape, with the variable antigen binding (Fab) parts, forming two identical arms, and the constant part (Fc), responsible for interaction with C1q and phagocyte receptors, forming the base (figure 7). Parts of the Ig chains fold into globular domains, two on the light chains (designated VL and CL; V for

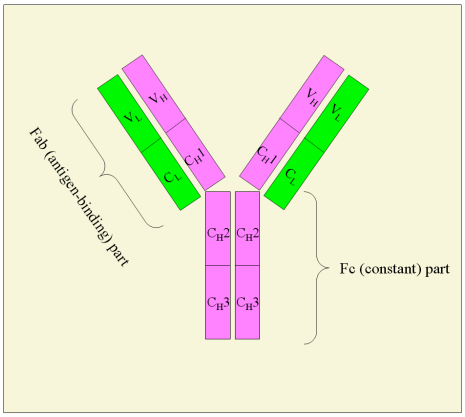

Figure 7. Principal structure of an Ig molecule. Heavy chains are in purple and light chains in green. Amino acid substitutions responsible for differences between allotypic variants of GM are located in CH1/CH3 (G1M), CH1/CH2/CH3 (G3M) and CH2 (G2M).

variable, C for constant and L for light chain), and three or four on the heavy chains (VH, CH1, CH2, CH3 and CH4). There are five main classes, called isotypes, of Igs; IgM, D, G, A and E, which all have partly different functions in the immune system. IgD is mainly present on immature B cells and is found only in trace amounts in the circulation. IgM is a pentamer and the main component of the primary immune response. With maturation of antigen responses, IgA and IgG are formed. There are two IgA subclasses: IgA1 and IgA2, and four IgG subclasses: IgG1, IgG2, IgG3 and IgG4. IgE is formed mainly in allergic immune responses and against parasite antigens. Ig isotypes differ in their capacity to activate complement, with IgG and IgM being the most efficient. Properties of different IgG subclasses are summarized in table 4. Some important functions of Igs are a) direct neutralization of harmful exogenous antigens, b) activation of complement, c) transport of antigens to the reticulo-endothelial system by erythrocytes, after opsonization with C3b, d) binding to receptors on phagocytes, and thereby elimination of antigen and, e) uptake of antigen on B cell-bound Igs, an event which is necessary for antigen-processing and affinity maturation of specific antibodies. Development of antibody responses depends on a functional complement system, indicated by low IgG4 and IgG2 levels [20, 24, 25] and impaired production of specific antibodies in complement deficient individuals [22]. B cells express both the stimulatory CR2 [2, 3] and the inhibitory CR1 [27].

Table 4. Properties of IgG subclasses.

Subclass	Serum concentration (g/L)	C[1] activation	FcγR[2] specificity
IgG1	7.5	++	all FcγRs
IgG2	3.5	+	mainly FcγRIIa
IgG3	1.5	+++	all FcγRs
IgG4	0.5	-	mainly FcγRIIb

[1]Complement, [2]Fcγ receptor.

Variation of the IgG heavy chain genes

The IgG heavy chain gene locus (IGHG) on chromosome 14q32 is polymorphic. Several variants of IgG1, IgG2 and IgG3 (GM allotypes) have been described, and are uneqally distributed between different ethnic groups [145]. The official nomenclature system for description of IGHG genes is presented on the IMGT

database [146], http://imgt.cines.fr/textes/IMGTrepertoire/Proteins/allotypes/human/IGH/IGHC/. In this thesis, a simplified form of the alphabetical nomenclature is used. In Caucasian populations, there are two main allelic variants of IGHG genes for IgG1; *G1M*a* and *G1M*f* . The alternative IGHG genes for IgG2 are *G2M*n* and *G2M*n-*, while the IgG3 heavy chain gene is encoded by *G3M*g* or *G3M*b*. All IGHG genes are inherited together in fixed haplotypes.Within IGHG, there is a linkage disequilibrium, with *G1M*a* being linked to *G3M*g* and *G1M*f* to *G3M*b* in IGHG haplotypes. Three IGHG haplotypes are common in Caucasian populations. These are *G3M*g;G1M*a;G2M*n-* (31 %), *G3M*b;G1M*f;G2M*n-* (23 %) and *G3M*b;G1M*f;G2M*n* (45 %) [147]. No allelic variants of IgG4 genes have been described. The amino acid substitutions responsible for differences between *G1M*a* and *G1M*f* are located in the CH1 and CH3 Ig domains [148], while differences between G3M alleles, which are much more complex, involve both CH1, CH2 and CH3 [149] (figure 7). The mutation leading to the amino acid substitution in IgG2 that gives rise to the different G2M variants is located in CH2 [150]. The CH2 region is directly involved in interactions between Ig on the one hand, and complement/Fc-receptors on the other [148].

Functional and clinical importance of IGHG gene variants

IGHG genes influence the concentrations of IgG subclasses [145], and both individual genes and the IGHG haplotype are of importance [147]. *G1M*f* homozygosity is associated with lower levels of IgG1 than *G1M*f/*a* or *G1M*a/*a* genotype [151, 152], IgG2 concentrations in sera of adults increase with increasing number of *G2M*n* alleles, and *G3M*b* homozygous individuals have the highest IgG3 levels [153]. The effect of GM genotypes is strongest on IgG3 levels, intermediate for IgG2 and weakest for IgG1 [151].

Not much is known about possible functional differences between products of IGHG allelic variants. No studies on Fc-receptor binding capacities have been reported. One paper dealing with differences in complement activation has been published. No difference in ability to initiate complement mediated cell lysis could be shown for recombinant human IgG1 of different GM allotypes, although some differences in binding of complement components were noted. GM allotypes of IgG2 or IgG3 were not studied [154].

IGHG genes influence production of specific antibodies, in particular to polysaccharide antigens [155, 156]. Susceptibility to several groups of diseases correlates to IGHG gene alleles. These include both infections [25, 157-160], immunological disorders like allergy/asthma [161] and autoimmune diseases [162], and lymphoproliferative states [163]. In addition, IGHG genes may be of particular importance in complement deficiency states [25, 164].

Complement and meningococcal infections

Neisseria meningitidis

N. meningitidis is a Gram-negative, facultatively aerobic diplococcus, whose sole habitat is the human nasopharyngeal mucosa. Meningococci belong to the bacterial family of *Neisseriaceae*, which also includes gonococci and commensal *Neisseria*. The bacterium is surrounded by a polysaccharide capsule, the antigen properties of which form the basis for division of strains into serogroups. Serotyping is based on expression of class 2/3 outer membrane protein (Por B) and serosubtyping on expression of class 1 outer membrane protein (Por A). Meningococci can also be classified on the basis of lipooligosaccharide (LOS) content in the outer membrane (immunotype) [165]. The meningococcus is a commensal of the nasopharyngeal microbial flora which rarely causes disease and the prevalence of carriage has been reported to be around 10 % in industrialized countries.

Meningococci possess several types of virulence factors. The capsule protects the bacteria from phagocytosis and desiccation. Except in serogroup A, the capsule is composed mainly of sialic acid. This protects the bacterium against AP-mediated complement attack. Clinical isolates causing disease are usually encapsulated. Meningococci are capable of switching capsule [166] and may also turn off capsule synthesis and become uncapsulated. Pili, LOS and other adhesins are necessary for attachment to epithelial cells. LOS, or endotoxin, is also the cause of extensive inflammation causing shock in meningococcemia. Complement regulators are recruited by meningococci by specific complement binding proteins. Por A binds C4BP [167] and a lipoprotein termed GNA 1870 binds factor H [168]. Binding of these regulators has been shown to increase serum resistance. Meningococci express several proteins that enable acquisition of iron from the host. Iron is a prerequisite of bacterial metabolism. Bleb formation, i.e. budding of outer membrane vesicles, is a means of diverting host immune response from the bacterium itself. The blebs have a high LOS content, and are also composed of outer membrane proteins and lipids [169-171].

The relative frequencies of different serogroups vary between countries and continents, both in terms of carriage and disease. Serogroup A dominates in sub-Saharan Africa, known as the meningitis belt, where meningococcal epidemic disease occurs every 5-10 years. Serogroups B and C are the most common serogroups in Europe and the USA, although during the 1990s there were local outbreaks of disease caused by serogroup Y [165, 170]. In the year 2000, a serogroup W-135 strain was spread in Africa, Asia, Europe and the USA by Muslim pilgrims returning from Mecca [172].

Meningococcal disease

Meningococcal infection is relatively uncommon, affecting approximately 0.5-7 individuals per 100,000 per year in industrialized countries [173]. However, in spite of access to effective antibiotics and modern intensive care, meningococcal infection

is still associated with an unusually high risk of fatal outcome. Only 6 out of the 13 meningococcal serogroups cause infection, namely serogroups A, B, C, Y, W-135 and X [170]. Incidence rates are highest among infants and adolescents. In endemic regions, most cases are sporadic, but large outbreaks may also occur, regularly so in Africa, affecting up to 1 % of the population.

The main clinical manifestations of *N. meningitidis* are meningitis and sepsis, with fatality rates of about 10 % in spite of treatment (highest for sepsis) [170]. The disease course is often rapid, especially in sepsis, with symptoms evolving within hours. Meningococcal sepsis causes death by inducing shock and/or disseminated intravascular coagulopathy. LOS is central to the pathogenesis of these manifestations. For example, endotoxin levels in the circulation correlate with the severity of shock [169]. Endotoxin activates both the complement and contact activation systems, with resulting massive release of pro- and anti-inflammatory cytokines, attraction and activation of neutrophils, vasodilation, capillary leakage, disseminated intravascular microthrombus formation, and vasculitis. However, a contradictory finding is that complement activation was shown to be independent of endotoxin in a whole blood model [174]. Meningitis can become fatal due to raised intracerebral pressure. Disabling *sequelae* are not uncommon after survival of invasive disease, examples being hearing loss and loss of limbs due to thrombosis. Rare cases present with chronic meningococcemia, which denotes a state of relapsing fever and arthralgia, but without the dramatic signs of shock and coagulopathy ususally associated with meningococcal invasive disease.

Meningococci may also cause local disease like otitis media, pneumonia, pericarditis, septic arthritis and, rarely, urethritis. Serogroup Y and W-135 more frequently cause pneumonia than other serogroups [165]. IC-mediated carditis or arthritis may also arise after some days to weeks.

The hallmark of meningococcal sepsis is a petechial rash, representing hemorrhagic lesions due to vasculitis. Other symptoms are uncharacteristic and indistinguishable from sepsis in general. Traditionally, diagnosis is based on bacterial culture of blood or cerebrospinal fluid. A more rapid, but unspecific way of establishing the etiologic cause of meningitis is direct Gram-staining of cerebrospinal fluid with detection of Gram-negative diplococci. Anticapsular antisera may be used for rapid detection in coagglutination tests. More recently, PCR-based techniques have come in to use. Penicillin resistance is still rare [170].

Defense against meningococcal infection

Natural defense

The defense against *N. meningitidis* is based in the first line on local mucosal immunity, illustrated by the fact that smoking is a risk factor for disease. Other causes of damage to the nasopharyngeal mucosa, such as dry environment and viral respiratory infection like influenza, also increase disease susceptibility. Another important circumstance influencing the risk of contracting disease is age, with the

highest incidence rates found among infants and adolescents. Further risk factors have been described. The strongest association is with complement deficiencies, which will be outlined below. Certain genetic variants of lung surfactant protein A increase susceptibility to infection [175]. Lung surfactant protein A is a recognition protein belonging to the collectin family of proteins and is involved in innate mucosal immune defense. Polymorphisms in the genes of other innate immunity molecules, e.g. toll-like receptor 4 and carcino-embryonic antigen cell adhesion molecule [176, 177], as well as Fcγ-receptor II variants [178], are further examples of host immune defense variables that influence disease susceptibility.

Clearance of meningococci from the bloodstream depends on serum bactericidal lysis and opsonophagocytosis. Both mechanisms involve specific antibodies. Serum bactericidal lysis is thought to be mainly mediated by antibodies which bind to the capsule or outer membrane proteins and subsequently activate complement via the CP, although some evidence indicates additional activation of the AP by meningococcal antibodies [179]. The recently described role of properdin as an initiator of AP activation may also be important [10]. Serum bactericidal lysis of meningococci may to an uncertain extent occur through direct activation of the AP by LOS without involvement of specific antibodies [11]. Like serum bactericidal activity (SBA), opsonophagocytosis is also mediated by anti-meningococcal antibodies. The antibodies may induce phagocytosis either directly by binding to Fc-receptors on phagocytes, or indirectly by activation of complement and subsequent uptake via complement receptors. Mechanisms for clearance of invading

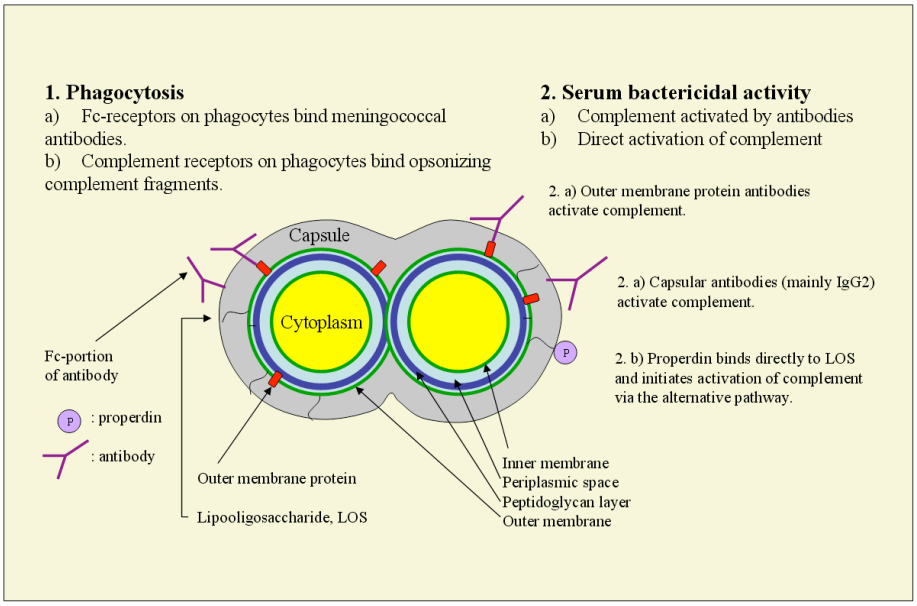

Figure 8. Principal structure of *N. meningitidis* diplococci with important cell wall and other antigenic features indicated. Mechanisms for clearance of meningococci from the blood are presented.

meningococci are shown in figure 8. Judging from the striking susceptibility for meningococcal disease among complement-deficient individuals, serum bactericidal lysis, which is always complement-dependent, is usually regarded as the most important defense mechanism, but experimental evidence indicates that opsonophagocytosis plays an important role in the defense against serogroup B meningococci [180]. The "gold standard" of evaluation of an individual´s immunity to meningococci is the serum bactericidal assay. It employs rabbit or human serum as an external source of complement, and provides a functional estimate of protective anti-meningococcal antibody level [181]. Alternatively, immunity may be assessed by investigation of opsonophagocytic activity [180].

Specific antibodies are thought to be acquired during asymptomatic carriage and through colonization by apathogenic *Neisseria lactamica*. Cross-protective antibodies are probably also induced by enteric bacteria with capsular antigens similar to meningococci, like *Bacillus pumilus* and *Escherichia coli* K1 [169]. The majority of children between 6 months and 5 years of age lack protective antibodies against meningococci from serogroups A, B and C, and this is the likely reason for the higher disease rates in young children [181]. The second incidence peak in adolescence is less well understood, but coincides with the increased social interaction occurring in that age-group.

Vaccines

Development of vaccines has gained much interest as infection is associated with high risks of death or severe morbidity. To minimize the susceptibility of populations to meningococcal disease, effective vaccines are much wanted, especially so in Africa. Since their introduction in the 70´s and 80´s, vaccines against serogroups A, C W-135 and Y based on polysaccharide capsular antigen have been available. These give rise to a non-T cell-dependent immune response without immune memory, and therefore must be repeated every 3 to 5 years. In addition, children under 2 years of age are unable to respond. In addition, hyporesponsiveness to the C polysaccharide has been reported to occur after booster doses. In 1999 vaccination with conjugate vaccines made up of capsular antigens from serogroup C conjugated with diphtheria or tetanus toxins were included in the general vaccination programme in the UK. These vaccines had been shown to elicit sufficient immune responses in small children, and to induce immunological memory. In the UK, this measure led to a decline in serogroup C-associated infections [182] and induction of herd immunity [183]. More recently, a conjugate vaccine against serogroups A, C, Y and W-135, has become available in the USA and Canada. The tetravalent conjugate vaccines have not yet been sufficiently evaluated for infants, but they are safe and efficient from 2 years and up [173].

The capsule of serogroup B cross-reacts with an adhesin expressed on developing neural tissue, and exhibits low immunogenicity. For this reason, vaccines based on serogroup B capsular antigen are inefficient in stimulating protective antibodies, and are potentially harmful [184]. Instead, several attempts have been made to find

alternative antigens suitable for a serogroup B vaccine. Vaccines based on outer membrane proteins/vesicles have been efficient during local outbreaks, but confer only serosubtype-specific protection due to extensive inter-strain variation of the main antigenic outer membrane protein Por A. At present, there is an ongoing search for outer membrane proteins more conserved between different serogroup B strains [185]. For example, five relatively conserved outer membrane antigens identified by reverse vaccinology, have been combined in a vaccine shown to elicit bacteridal antibodies in mice [186]. Recently discovered antigens of the serogroup B capsule have also been proposed as putative vaccine components [187].

Complement deficiency and meningococcal disease

Most inherited complement deficiences cause an increased risk of meningococcal infection [171, 188]. The importance of the complement system in the defense against *N. meningitidis* is also emphasized by the association of some acquired complement deficiences and meningococcal disease [50, 63, 189]. Deficiencies of the terminal pathway show the strongest association with meningococcal disease. Infection is often recurrent, but fatality rates are lower than in meningococcal disease in complement-sufficient individuals [49]. The relatively mild disease may be due to less endotoxin release in the absence of MAC formation and hence no cell lysis taking place. In properdin deficiency, fatal outcome is common, while recurrent disease is rare, indicating a protective effect of specific antibodies in these patients [44, 190]. Against the background that CP activation by specific antibodies has been regarded as the principal defense mechanism, the strong clinical association between properdin deficiency and meningococcal disease has been difficult to explain. However, a recent report showed that complement activation by LOS and LPS is properdin-dependent, providing an explanation for this association [11]. This finding is also consistent with the reported association between meningococcal disease and a factor H SNP resulting in higher factor H serum concentrations, again showing the importance of the AP in the defense [191]. Patients with deficiencies of other AP components are also among the groups with increased susceptibility [192]. Patients deficient in components of the CP show a general increase in susceptibility to bacterial infections, among them meningococcal infections [44, 47]. Several studies have shown that complement-deficient patients may benefit from vaccination against meningococci [179, 193]. This has been shown even in the case of terminal complement component deficiency, explained by increased opsonophagocytic capacity after vaccination [194]. Vaccination studies in complement deficiency have so far evaluated the effect of polysaccharide vaccines, and not of the novel conjugated vaccines. In cases with recurrent disease continuous prophylactic or on-demand antibiotic treatment is necessary.

Group A streptococcus and acute poststreptococcal glomerulonephritis

Streptococcus pyogenes

S. pyogenes is a strictly human pathogenic Gram-positive coccus, which causes disease in all parts of the world. In most GAS strains the coccus is surrounded by a hyaluronic acid capsule, and as a Gram-positive bacterium its cell wall has a thick peptidoglycan layer. The capsule is not immunogenic. The different serotypes are identified by type-specific cell wall proteins called M proteins [195]. The M proteins belong to a family of proteins with similar structure. Typing is also based on presence or absence of the opacity factor and on analysis of T antigens, which is another group of cell wall protein antigens exhibiting inter-strain diversification. GAS are cultured on blood agar and typing can be performed serologically (which has become less common), or by PCR.

To enable infection, GAS possesses a number of virulence factors. One example is the antiphagocytic M proteins, which are surface-attached. Each strain expresses its own serotype-specific M protein. Antibodies to M proteins confer serotype-specific immunity. Several secreted exotoxins and the hyaluronic acid capsule also contribute to escape from host defense. As mentioned under "Complement and infections", GAS can also bind the complement regulators factor H and C4BP, which helps it to resist the immune system of the host [100, 196]. In addition, the M5 protein may induce local immunosuppression through binding of CD46 [197].

Group A streptococcal antigens

A vast number of *S. pyogenes* antigens are described in the literature, some surface-bound and some secreted [198]. Four streptococcal antigens are of special interest to this thesis, as antibodies to them were analysed in paper III. These will therefore be outlined here. One of the streptococcal exotoxins is called streptococcal cysteine proteinase (SCP; *S. pyogenes* pyrogenic exotoxin B, SpeB). It is produced by the vast majority of GAS strains [199]. SCP is a positively charged enzyme, with a wide spectrum of substrates, including streptococcal surface proteins. These can be released by SCP cleavage, and thereby disseminate further into tissues [200]. Other substrates for SCP include complement proteins properdin and C3 [201, 202]. SCP has several pro-inflammatory effects, for example it increases cytokine production from leukocytes [203], and it has been implicated in the pathogenesis of AGN [204, 205]. M1 is the serotype-specific M protein of the M1 GAS serotype, which constituted a large proportion of invasive GAS isolates during the 1990s [206]. It binds the Fc-part of IgG and has antiphagocytic properties [207]. M1 has recently been shown to be a superantigen, a quality likely to contribute strongly to the ability to cause invasive disease [208]. Protein H belongs to the M protein family, is expressed only by M1 strains, and like M1 it binds IgG [209]. As protein H can be released from the streptococcal surface by SCP, it may form soluble complexes with IgG [200]. Such complexes activate complement via the CP, while protein H inhibits

IgG-mediated complement activation on surfaces by blocking C1q binding to immobilized IgG [210]. Streptococcal inhibitor of complement (SIC) [211], is a secreted streptococcal protein expressed by streptococcal strains M1 and M57. SIC takes it name from its ability to inhibit complement-mediated cell lysis, and apart from this it inhibits antibacterial peptides [212, 213]. SIC genes show an unusually high sequence variation among clinical M1 isolates [214, 215]. Streptococcal strains M12 and M55 possess genes with some homology with the SIC gene (*sic*). These genes are called distantly related to SIC, *drs* [216]. The products of these genes (DRS), bind the MAC and inhibit antibacterial mucosal peptides in a similar fashion to SIC, but in contrast to SIC they do not inhibit complement-mediated cell lysis [217, 218].

Diseases caused by Group A streptococcus

GAS are strictly human pathogens which cause both relatively mild diseases, like acute pharyngitis and impetigo, and severe diseases as erysipelas, necrotizing fasciitis, sepsis and the streptococcal toxic shock syndrome [198]. After a GAS infection, so called non-suppurative *sequelae* may follow. These are erythema nodosum, rheumatic fever and AGN. The complications are caused by the immunologic response to the preceding infection. The 1990s saw a rise of incidences of severe GAS infections in industrialized countries. The background for this sustained high prevalence of invasive disease is not understood [219].

AGN

The symptoms of AGN start 1-3 weeks after a GAS infection. Incidences are low in the Western world, while in developing countries it is still the leading cause of acute renal failure in children, with more than 470,000 yearly cases worldwide [220]. Nephritis symptoms are often accompanied by hypertension and edema, but renal failure occurs only in a minority of cases. AGN is mostly self-healing, and is treated symptomatically, as there is no specific treatment. Recurrence is extremely rare, but has been reported [221, 222]. It is generally considered that only GAS strains of certain M types are potentially nephritogenic, as these M types are overrepresented among clinical isolates. The pathogenesis of AGN is incompletely understood, although it has been regarded as an IC-mediated disease, since Ig and complement proteins are deposited in glomeruli of AGN patients [223]. The concept of AGN as an IC disease is consistent with the time aspect of disease onset in relation to the preceding infection, as an immune response to the streptococcus would take around 10 days to develop. According to the IC model, the streptococcal antigen would reach glomeruli via the circulation, but local inflammation and symptoms of nephritis would develop only after mounting of a specific immune response to the "planted antigen". To become trapped in glomeruli, such an antigen might require special properties, e.g. to be positively charged in order to bind the negatively charged glomerular basement membrane [224]. An alternative hypothesis for glomerular trapping is the idea that an IgG-binding streptococcal antigen would form IC-like non-immune complexes that would deposit in glomeruli [210, 225].

Table 5. Proposed nephritogenic streptococcal antigens described in the literature.

Antigen	M serotype origin	Suggested mechanism of nephritogenicity	Reference(s)
M protein	All	Molecular mimicry with glomerular antigens	[265]
NAPlr[1]	Nearly all	Plasminogen binding → C[2] activation and local damage	[266]
Neuraminidase	Mainly 1, 4 and 12	Alteration of IgG, rendering it autoimmune	[267]
Ig-binding proteins (eg protein H)	Several M types	IgG-binding; formation of IC-like complexes	[210], [268]
SCP[3]	All	Combination of direct inflammatory tissue damage and IC-formation (IC formed *in situ* or deposited)	[205]
Streptokinase	All	Plasminogen activation → C activation and local damage	[269]

[1]Nephritis-associated plasmin receptor, [2]Complement, [3]Streptococcal cysteine proteinase/Streptococcal erythrogenic toxin B

Since only some GAS strains and a minority of infection cases develop AGN, there has been an extensive search for a "nephritogenic antigen". Such proposed antigens are listed in table 5.

The AP is consistently activated in AGN, as shown by low serum levels of C3 and properdin [62]. Serum concentrations of CP components like C1q and C4 are regularly normal, although increased breakdown of C2 is seen in some patients [65]. The LP has also been implicated in the etiology of AGN. MBL has been shown in glomerular biopsies from AGN patients [127, 226], and binds to streptococci [227]. A circumstance that is often overlooked in discussions of AGN pathogenesis is that the typical serum complement profile for AGN is not typical of IC disease, which usually shows signs of CP activation [9]. Possibly, the disease process in AGN consists of two separate entities: one that produces systemic AP complement activation and another that produces local glomerular inflammation. As complement activation is always present in AGN, both of these processes would have to be initiated by the same infecting organism.

Host factors predisposing for disease undoubtedly play an important role in the etiology of AGN, as very few patients infected with potentially nephritogenic GAS develop the disease. For example, certain HLA genes have been shown to be associated with susceptibility to AGN [228, 229], while few other host immunogenetic factors have been investigated.

Idiopathic sudden hearing loss and autoimmune inner ear disease

ISHL and AIED – an overview

Idiopathic sudden hearing loss, (ISHL), or sudden sensorineural hearing loss is a syndrome of acute onset (24 hours to 3 days) of usually unilateral hearing loss. The incidence is 10-20:100,000 per year, and the syndrome is most common among middle-aged persons [230]. Although the cause is usually unknown, viral infection, vascular events and autoimmune processes are believed to be responsible for some cases [231]. The prognosis in ISHL is favourable, with spontaneous full or partial

recovery in about two thirds of patients. A short corticosteroid course is the only treatment that is widely used and accepted [230]. Other treatment options like hyperbaric oxygen, hemorrheological regimes and antiviral therapy have been proposed, but there are conflicting data on their effect. The existence of an autoimmune subgroup in ISHL was reported in 1979 [232], in a cohort of 18 patients with good response to steroid treatment. Since then more knowledge on autoimmune inner ear disease (AIED) has gradually accumulated. AIED is usually bilateral, may be sudden to rapidly progressive, and responds well to corticosteroids. It would be preferable to be able to distinguish AIED from ISHL, as AIED is an entity with potentially greater benefit from immunosuppressive treatment. As AIED is initially often unilateral, the clinical distinction is difficult to make [233]. To improve diagnosis of AIED, routine immunological laboratory tests have been recommended in sudden and progressive hearing loss [234]. Immunologically caused inner ear pathology can also be part of a systemic autoimmune illness like SLE or systemic vasculitis, e.g. Wegener´s granulomatosis [233].

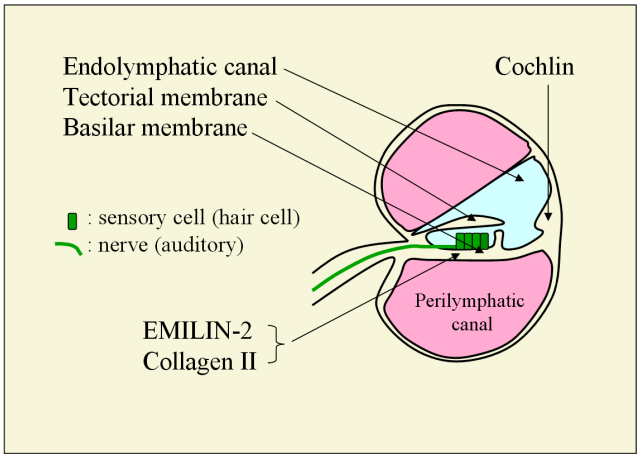

Figure 9. Cross-section through the cochlea showing the localization of EMILIN-2 in the cochlear basilar membrane. The localization of two other putative inner ear autoantigens, collagen type II and cochlin, are also indicated.

Etiology and pathogenesis of AIED

The inner ear has been regarded as an immunoprivileged site, separated from the immune system by the blood-labyrinthine barrier. However, the barrier is only relative, as Ig and lymphocytes are present in the inner ear, which in animal studies has shown capacity to mount an immune response after challenge [235]. For example, acoustic trauma initiates cochlear inflammation in mice [236]. Mainly as the result of animal studies, both cell-mediated and humoral adaptive immunity, as well as innate immunity, have been implicated in the pathogenesis [237-239]. Several disease-specific and non-specific autoantibodies have been associated with AIED, reviewed in [234]. Proposed inner ear autoantigens include a 68kDa bovine temporal bone protein, to which 35 % of ISHL patients were originally reported to show IgG reactivity [240]. Such antibodies cross-react with bovine heat shock protein-70 (HSP-70), which is the antigen used in a commercially available test for ISHL/AIED. Another example is cochlin, a major inner ear protein [241].

Inner ear antigens with collagenous structure

Several inner ear antigens are of collagenous or partly collagen-like structure. Such antigens have the theoretical potential to cross-react immunologically with the collagenous region of C1q. Collagenous/partly collageous inner ear proteins include type II and IX collagens, which are suggested ISHL/AIED autoantigens [242, 243]. However, cross-reactivity between the collagenous region of C1q and collagen type II could not be demonstrated in a previous study [244]. Two ear proteins purified from fish species: saccular collagen [245] and otolin-1 [246], the latter with a putative human gene homologue [247], have not been investigated for possible relation to hearing loss disorders. A cochlear antigen of particular interest to this thesis is elastin microfibril interface located protein-2 (EMILIN-2) [248]. Together with other EMILINs, EMILIN-2 forms a family of matrix glycoproteins, present in blood vessel walls and other elastic tissues [249]. EMILINs contain a C-terminal C1q globular domain, a collagenous sequence, a long coiled-coil region and a specific N-terminal EMI domain [250]. Recently, EMILIN-2 was shown to induce cell death via the extrinsic apoptotic pathway [250]. EMILIN-2 is a major constituent of the cochlear basilar membrane and is also present in the heart, lung and brain [251]. Cochlear basilar membrane movements stimulate the sensory hair cells of the cochlea, which initiate nerve signalling in response to the auditory stimulus. Figure 9 shows a schematic cross-section of the cochlea with localization of EMILIN-2, collagen II, and cochlin.

AIMS OF THE PRESENT INVESTIGATIONS

I
- To make an inventory of the causes of and clinical conditions associated with persistently low serum C3 concentrations in consecutive clinical samples.
- To evaluate available diagnostic laboratory analyses and improve the diagnostics of C3 NeF and associated diseases.
- To establish an ELISA for investigation of differences between C3 NeF types.
- To analyze aC1qCLR in association with C3 NeF.

II
- To assess the bactericidal activity against *N. meningitidis* in serum from patients with low C3 concentrations due to C3 NeF.
- To study Ig and complement parameters with a possible influence on SBA in patients with C3 NeF.
- To study IgG allotype distribution in patients with C3 NeF.

III
- To analyze presence of antibodies to four streptococcal proteins in patients with AGN.
- To investigate MBL serum concentrations in patients with AGN.

IV
- To investigate aC1qCLR and other common autoantibodies in unselected consecutive patients with ISHL.
- To analyze potential cross-reactivity between C1q and a recently described protein, EMILIN-2, in patients with ISHL, SLE or HUVS.
- To investigate the relation between clinical data and autoantibodies in patients with ISHL.

PRESENT INVESTIGATIONS

I and II

Subjects and methods

Patients and controls

In paper I, serum samples from 25 persons with prolonged or unexplained low serum concentration of C3 were examined in order to document clinical associations and occurrence of C3 NeF. Follow-up samples were available in 22 patients. All included individuals had C3 concentrations below 0.43 g/L (33 % of mean concentration of a normal serum pool of 100 blood donor sera). Patients with other causes of low C3 such as SLE or AGN were excluded, except for one SLE case with selective and prolonged C3 deficiency difficult to explain by the SLE disease alone. In all but 3 cases, clinical records were reviewed in collaboration with, or clinical information was forwarded by, the responsible physician.

In paper II, serum samples from 30 individuals with C3 NeF were included. Presence of C3 NeF had been determined by a combination of a sheep erythrocyte (ShE) hemolytic assay [142], and a fluid phase C3 cleavage assay [143], described under Methods; autoantibody analysis. Samples were decoded after registration of age. All but one serum showed C3 concentrations below the reference interval (0.77-1.38 g/L, based on measurements in 100 healthy blood donors). The serum with normal C3 concentration was not used for serum bactericidal assays.

Serum samples from 51 controls, selected in order that their GM allotypes would approximately match the GM allotype distribution of the patients, were used in paper II. Of these, 22 were from healthy adult volunteers and 29 were from children whose samples had been sent to the Clinical Immunology Laboratory as part of diagnostic investigations. Samples of control children showed normal values of IgG, IgG subclasses, C3, C4, C1q, properdin and complement function, and were decoded after registration of age.

All samples used in these studies had been stored at -80°C. Patient samples had been sent to the Clinical Immunology Laboratory as part of diagnostic investigations.

Methods

Complement analyses (I and II)

Performed analyses of serum concentrations of complement proteins are presented in table 6. All complement concentrations were measured by electroimmunoassay except for MBL, which was measured by a sandwich ELISA [23], and C3 and C4, which were measured by electroimmunoassay or turbidimetry. Complement function (paper II) in patient samples was determined quantitatively by a commercial ELISA (CP function) [135] or a rabbit erythrocyte hemolytic assay (AP function) [133], and in control samples by a qualitative hemolytic assay (CP and AP functions) [134].

Table 6. Analyses of complement protein serum concentrations performed in papers I and II.

I

Subjects	Complement proteins
All patients:	C3, C4, C1q, factor B, C5, properdin
Five patients without C3NeF:	factor H, factor I, C4BP

II

Subjects	Complement proteins
Patients and controls:	C3, C4
Patients and control children:	MBL
Patients only:	factor B, properdin, C5, factor H, factor I

Autoantibody analyses (I)

Presence of C3 NeF was analyzed by previously described methods, with partial modifications. In a ShE hemolytic assay using a mixture of patient and normal whole serum in buffer supporting only AP activation, the AP C3 convertase was formed and stabilized if C3 NeF was present. The upper reference limit was defined as 15 % hemolysis, based on results of 20 healthy individuals. In a fluid phase assay detecting C3 cleavage, patient and normal serum were mixed and incubated at 37°C. C3 cleavage was assessed by crossed immunoelectrophoresis of the mixtures. Based on measurements in 20 healthy persons, the normal reference limit of C3 cleavage was </= 10 %. The third, qualitative method was a combination of isoelectric focussing (IEF) of samples in an agarose gel followed by overlay of a second agarose gel with properdin-deficient serum as complement source, and guinea-pig erythrocytes in buffer supporting only AP activation. Hemolytic bands were produced if C3 NeF was present.

An ELISA using purified complement proteins to build AP C3 convertase-like complexes in a microtiter plate [144] was employed to investigate binding specificities of C3 NeF.

An in-house ELISA was used to analyze aC1qCLR of IgG isotype. The ELISA was performed with the collagenous part of C1q as antigen and using high salt buffer (1 M NaCl), as described previously [91]. Western blot analysis of IgG reactivity with the separated polypeptide chains of C1q was performed essentially as described earlier [91].

Ig analyses (II)

Serum concentrations of IgG subclasses were analyzed in all subjects by turbidimetry or radial immunodiffusion. IgG concentrations were measured in adult controls only, by turbidimetry. In other subjects, IgG concentration was calculated as the sum of IgG subclass values.

Antigenic markers corresponding to allelic variants of IGHG (GM allotypes) were determined serologically in all subjects by ELISA [252] and double immunodiffusion assay [253]. Markers of the following alleles were analyzed: *G1M*a*, *G1M*f*, *G2M*n*, *G2M*n-* and *G3M*b*. Numbers refer to IgG subclass identity, and the gene name is separated from the allele designation by an asterisk.

Presence of *G3M*g* was deduced by comparing the content of G3M(b) allotype determined by ELISA with the total IgG3 content of the sample.

Serum defense against meningococci (II)

The following three meningococcal strains were used: C:2b:P1.5, Y:NT:P1.2 and W-135:2a:P1.2 [179].

Antibodies against purified meningococcal capsular polysaccharides from strains C (98/730), W-135 (01/428) and Y (01/426) (all from the National Institute for Biological Standards and Control, UK), were measured in all subjects by ELISA [190]. Results were calibrated against standard reference serum CDC1992 (Centers for Disease Control, Atlanta, GA, USA).

SBA against meningococci in sera from 26 patients (10 children and 16 adults) and 51 controls (29 children and 22 adults) was investigated. Results were expressed as percent surviving meningococci, with the number of surviving meningococci after incubation in heat inactivated pooled normal human serum representing 100 %.

Patient´s serum binding of IgG1, IgG2 or IgG3 to live meningococci (serogroup C) was measured by flow cytometry. Serum from a healthy individual vaccinated with tetravalent polysaccharide meningococcal vaccine one year before sampling was used as positive control, and IgG free serum (affinity purified with protein G) from a healthy individual was used as negative control. Results were expressed as the proportion of IgG subclass binding to meningococci with the positive control representing 100 % binding.

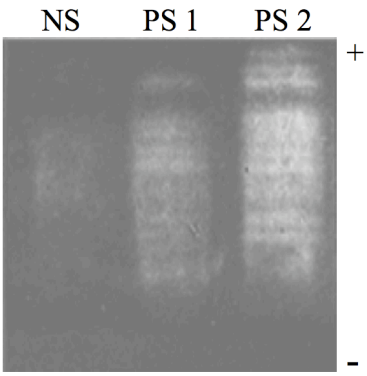

Figure 10. Isoelectric focussing of serum samples combined with overlay of a hemolytic agarose gel containing guinea-pig erythrocytes and properdin deficient human serum as complement source. C3NeF-containing patient sera produce multiple hemolytic bands in the region where immunoglobulins migrate. PS=patient serum, NS=normal human serum. The anode (-) and cathode (+) positions are indicated.

Ethics

The investigations presented in paper I were conducted as part of extended clinical studies, in most patients performed in collaboration with the responsible physician. In paper II, patient samples were decoded before extension of studies. Control samples were either from healthy volunteers (adults) or were used after decoding (children).

Statistical methods

In paper II, non-parametric methods were used throughout. For comparison of medians, the Mann-Whitney U test was used. The Spearman rank correlation test was used to calculate coefficients of correlation. Fisher's exact test was used for analysis of contingency tables. To compensate for multiple comparisons, the

Bonferroni test was employed. *P*-values <0.05 were considered significant.

Results and discussion

Autoantibody findings and their relation to complement profiles and clinical associations (I)

C3 NeF was present in the serum of 20 out of 25 investigated patients with unexplained subnormal C3 levels. In addition to MPGN and acquired PLD, which are the clinical conditions commonly associated with C3 NeF, other diagnoses were present among the patients. One patient had anaphylactoid (Henoch-Schönlein) purpura without overt nephritis, which is a novel association. The spectrum of infectious diseases was broader than expected, including both pneumonia and streptococcal septicemia in addition to the commonly associated neisserial infections.

Based on the combination of results from three assays, C3 NeF could be confirmed to dissociate into two partly different entities [73, 254]. One type was associated with increased fluid phase C3 cleavage and bands of hemolysis in the combined IEF and guinea-pig erythrocyte gel overlay assay (figure 10). To varying degree these sera were also positive in the ShE hemolytic assay. This type of C3 NeF, which was referred to as type I, was also associated with normal serum levels of C5 (9 out of 10 patients with C3 NeF type I, one with C5 level at the lower limit of the reference interval), and absence of aC1qCLR (all 10 patients). The other type of C3 NeF activity could not be detected by IEF. C3 cleavage in the fluid phase was negative or in the low range in all but one patient. This type of C3 NeF, which was referred to as C3 NeF type II, was detected by ShE hemolytic assay and was associated with low serum levels of C5 (8 out of 10 patients). The results are summarized in figure 11. In sera from 6 out of 10 patients with C3 NeF type II autoantibodies to C1q were detected (5 with aC1qCLR detected by ELISA, and 1 with Western blot reactivity to the C1q C chain). Of the 6 patients with autoantibodies to C1q, one had a slightly low serum C1q level, and 5 had normal levels. C1q concentrations were not correlated

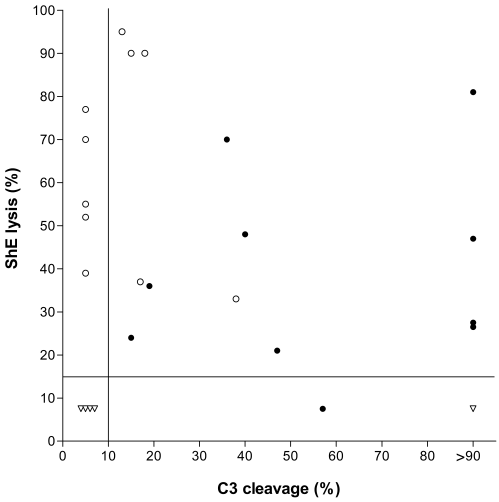

Figure 11. Results from ShE hemolysis assay (ShE lysis) and C3 cleavage assay in samples from 25 patients with low C3 concentrations. ●= patients with C3 NeF, who showed positive results in the assay combining IEF and guinea-pig erythrocyte gel hemolysis. ○= patients with C3 NeF, who showed negative results in IEF assay. Triangles symbolize patients without C3 NeF.

to concentrations of aC1qCLR.

Sera from 8 patients with C3 NeF type I and 5 patients with C3 NeF type II were investigated by C3NeF-ELISA (5 additional patients investigated compared to published data in paper I). Seven out of 8 samples containing C3 NeF type I bound IgG to C3bBb complexes. Only one C3 NeF type II-containing serum bound IgG in the C3 NeF-ELISA, when C3, factor B, factor D and properdin had been added. This serum was from a patient with MPGN and previous meningococcal infection, and showed a normal serum properdin level. The other 4 sera containing C3 NeF type II did not promote IgG-binding (figure 12).

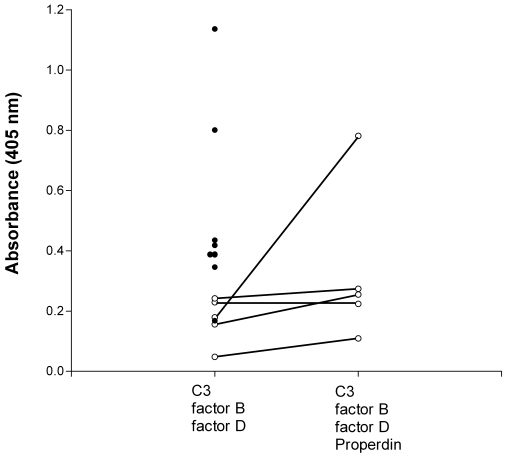

Figure 12. Results from an ELISA assay using purified complement components to investigate IgG reactivity with C3 convertase-like protein complexes. Absorbance values (405 nm) of sera containing C3 NeF type I (●, n=8) and type II (○, n=5). Purified proteins used are indicated on the x-axis.

Among 10 patients with C3 NeF type II, 7 had MPGN and 3 had other types of glomerulonephritis, while 3 out of 10 patients considered to have C3 NeF type I had MPGN, (glomerulonephritis vs not: p=0.02, Fisher´s exact test). Thus, C3 NeF type II is more strongly associated with glomerulonephritis than is C3 NeF type I. The 5 patients without detected C3 NeF also had glomerulonephritis. C3 NeF type II, but not C3 NeF type I, was associated with aC1qCLR (p=0.01, Fisher´s exact test). The observed association between C3 NeF type II and glomerulonephritis might explain the occurrence of aC1qCLR in these patients, as aC1qCLR are known to be associated with glomerulonephritides of varying causes. Alternatively, the coexistence of C3 NeF type II and aC1qCLR might be due to a common autoantigen, composed of an AP convertase containing or associated with C1q molecules. The observed differences between C3 NeF type I and type II may also result from different autoantigens. The target for C3 NeF type I is clearly an AP C3 convertase, judging from the associated serum complement profile with low C3 but normal C5 levels, and from ELISA results. The target for C3 NeF type II, on the other hand, might be a C3/C5-convertase, which would explain the low C5 levels in patients with C3 NeF type II. As IgG from a C3 NeF type II-containing sample investigated by ELISA was shown to bind a C3bBbP complex, reactivity with a C3-convertase containing properdin may be present in other patients with C3 NeF type II, in accordance with another report [254].

As to the nature of the C3 activating factors investigated in paper I, the IgG identity of C3 NeF type I has been shown by 2 methods; IEF combined with hemolytic gel overlay [255], and ELISA. The first of these methods detects hemolytic bands present in the region where Igs are expected to migrate, and the ELISA directly detects IgG-binding to the coated antigens. The nature of C3 NeF type II, on the other hand, remains to be solved. C3 NeF type II is mainly detected by hemolytic assay [142], and could theoretically be any factor or even combination of factors that increase the function and/or decrease the inhibition of the AP, including dysfunctional complement inhibitors.

This paragraph refers to results of previously unpublished experiments. In ELISA experiments, 9 samples containing C3 NeF type II were analyzed for possible IgG-reactivity with factor H, on the assumption that such antibodies could cause C3 NeF-like activity in the ShE hemolysis assay by causing dysregulation of the AP. Although no anti-factor H antibodies were detected, antibodies to factor H might still influence results of the ShE hemolysis assay if present. Samples from 20 patients with AGN were also analyzed by the hemolytic assay, and 6 (30 %) were positive, all but one in the low to moderate range (20-30 %, unpublished results). This finding points to the unspecific character of the hemolytic assay [142], and indicates a functional similarity between the C3 converting factor(s) in AGN and C3 NeF. Indeed, C3 NeF has been described in some AGN patients [256]. Possible cross-reactivity of described ShE hemolytic C3 NeF assay with a hemolytic assay for assessment of factor H function [137] was investigated. Results of the factor H functional assay are often pathological in patients with mutations in factor H leading to aHUS. Samples from 10 patients (most of whom have MPGN) showing positive results in the C3 NeF ShE hemolytic assay, were also analyzed by the factor H functional assay and were found to be negative.

Table 7. Complement function and serum concentrations of complement components in 30 patients with C3 NeF in paper II.

Complement activation pathway	**Median**	**Range**	**Reference interval**
The classical pathway (ELISA, %)	1.5	0-105	69-129
The alternative pathway (RaE[1], %)	7.5	0-164	70-130
Complement component			
Factor B (%)	91.5	7-198	59-154
Properdin (%)	90	<25-165	54-157
C5 (%)	73.5	<2.5-162	72-171
Factor H (%)	91	<2.5-144	69-154
Factor I (%)	108	72-180	60-152
C3 (mg/L)	150	30-1580	770-1380
MBL (mg/L)	0.50	0.01-6.55	>0.1
C4 (mg/L)	195	68-420	120-330

[1]RaE=quantitative rabbit erythrocyte hemolytic assay

Serum mediated defense against meningococci in patients with C3 NeF (II)

Complement findings in the C3 NeF patients included in paper II are summarized in table 7. The frequency of MBL serum levels consistent with MBL deficiency (MBL serum level <0.1 mg/L) among patients and controls did not significantly differ from the frequency among previously published controls [43]. Several C3 NeF patients showed low levels of IgG1 (7; 23%), IgG2 (7; 23 %) and IgG4 (2; 7 %). As severe kidney disease may cause increased loss of Ig through protein leakage, the association between MPGN and C3 NeF may have caused acquired IgG deficiency in some patients. The extent of protein leakage could not be investigated due to the study design, but nephritic patients showed lower median concentrations of IgG (p=0.006), IgG1 (p=0.01) and IgG2 (p=0.02) than patients with other diagnoses. Ig and complement values were within normal ranges in samples from the controls, with 2 exceptions: the serum C3 level in one subject was 760 mg/L and the IgG4 level in another was 0.04 g/L.

The IGHG genotype *GM*b;f;n/GM*g;a;n* was overrepresented among C3 NeF patients compared to previously published controls [147, 257], (10 % vs 2 %, p=0.04, Fisher´s exact test). In C3 NeF patients, G1M genotypes were reversely associated with IgG and IgG1 concentrations compared to controls and compared to previous reports [145, 151]. In the patients, homozygosity for *G1M*f* was associated with higher IgG (6.9 g/L) and IgG1 (6.1 g/L) than was heterozygosity (*G1M*a/*f*, IgG: 11.1 g/L, p=0.03 and IgG1: 5.3 g/L, n.s.). The opposite was true for the controls, with heterozygotes showing higher IgG (ns) and IgG1 (p=0.007) than homozygotes. As only 2 patients were homozygous for *G1M*a*, data from these patients were not used for comparisons. The group of G1M heterozygous patients contained a non-significantly larger proportion of glomerulonephritis cases, and the presence of overt glomerular disease influenced concentrations of IgG and IgG1 (above) in the same direction as did G1M/G3M heterozygosity (figure 13).

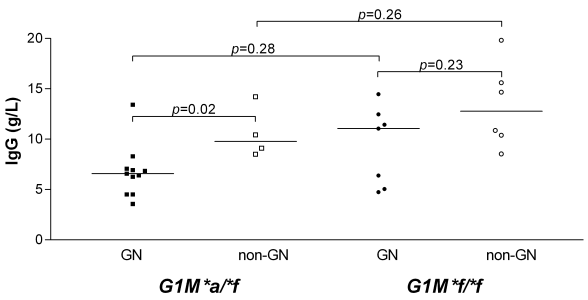

Figure 13. IgG serum concentrations in 28 C3 NeF patients in paper II. Horizontal bars show medians. Patients are stratified according to G1M genotype and glomerulonephritis (GN) or no glomerulonephritis (non-GN). *G1M*a/*f* with GN: n=11, *G1M*a/*f* without GN: n=4, *G1M*f/*f* with GN: n=7, *G1M*f/*f* without GN: n=6.

C3 NeF showed less efficient SBA against serogroup Y and W-135 meningococci than controls (figure 14). However, after stratification according to G1M genotype, differences in SBA between patients and controls were found only in heterozygous individuals (figure 15). The less efficient killing

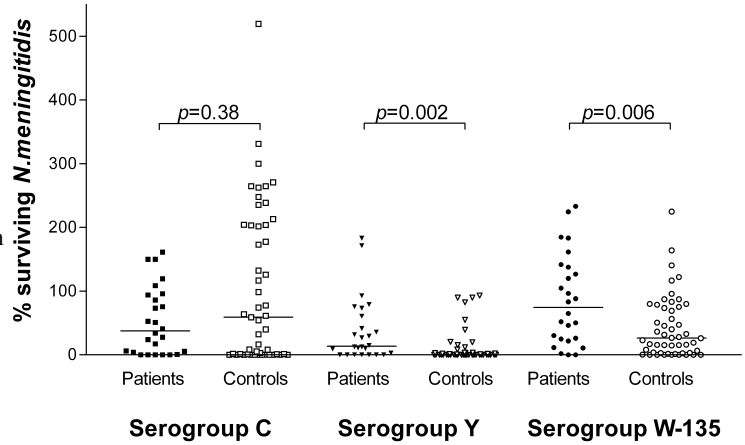

Figure 14. Serum bactericidal activity against serogroup C, Y and W-135 meningococci in sera from patients with C3 NeF (n=26) and controls (n=51). Horizontal bars represent medians.

of serogroups Y and W-135 in sera from patients compared to controls may help explain the clinical observation that these serogroups are overrepresented in meningococcal infections in complement-deficient individuals. Among patients, serum from individuals homozygous for *G1M*f* or *G3M*b* showed higher SBA against meningococci of all investigated serogroups than heterozygous individuals (*G1M*a/G1M*f* and *G3M*g/G3M*b*). This was also true for control children (serogroup C only), but not for adults (data not shown). The similarity in results between C3 NeF patients and control children were unexpected. Childhood might represent a state of relative complement deficiency, as concentrations of some complement factors are lower in children [258, 259]. After compensation for multiple comparisons, SBA was not correlated to complement serum levels or concentrations of IgG or IgG subclasses in patients or controls. Antibodies to meningococcal capsular polysaccharides were detected in few subjects and capsular antibodies were not related to SBA.

In an attempt to elucidate the mechanism behind the relation between SBA and G1M genotypes, the capacity of patient sera to promote IgG subclass binding to live meningococci was investigated by flow cytometry. In accordance with SBA results, sera from patients homozygous for *G1M*f* or *G3M*b* showed more binding of both IgG1 and IgG3 to bacteria after incubation. However, as SBA was not correlated to the amount of IgG1 or IgG3 binding, the influence of IGHG genes on meningococcal killing is still unexplained. It may be due to functional differences between products of different IGHG alleles, for example in complement activating capacity, although such differences have not been shown for IgG1 molecules [154]. Some amino acid substitutions associated with the G3M alleles are located in the complement/Fc-receptor interacting CH2 region of the Ig, or involve both CH2 and CH3 [149]. Alterations induced by the *G1M*a* and *G1M*f* alleles are located in the CH1 and CH3 regions, these not being directly involved in complement – Ig binding [148], so that they are perhaps less likely to influence complement activation.

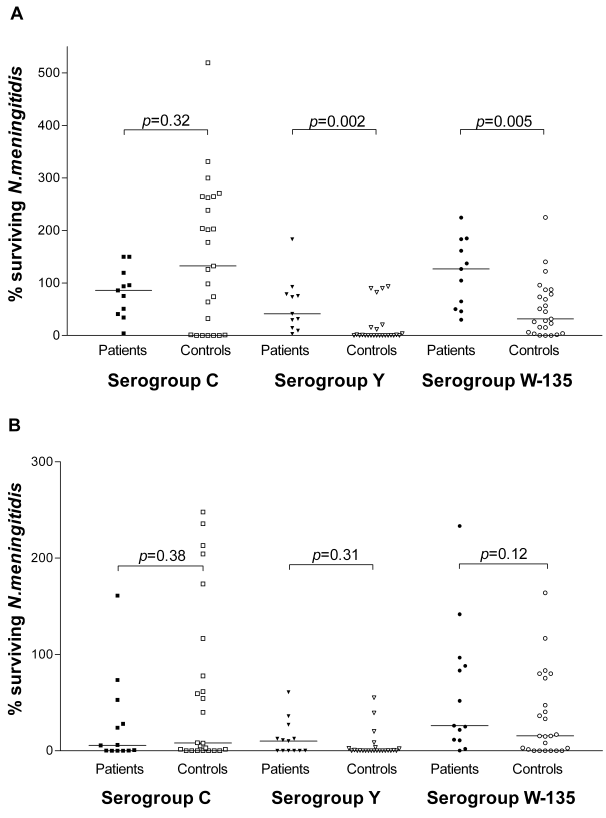

Figure 15. Serum bactericidal activity against serogroup C, Y and W-135 meningococci in sera from patients with C3 NeF and controls. Horizontal bars represent medians. A. Subjects with heterozygous G1M genotype (*G1M*a/*f*). Patients: n=11, controls: n=25. B. Subjects with homozygous G1M genotype (*G1M*f/*f*). Patients: n=13, controls: n=24.

Alternatively, the differences between patients with different IGHG genes may depend on linked genes in the extended haplotype.

In controls, serum C3 and C4 levels did not correlate to IgG or IgG subclass levels. Glomerulonephritis was related to Ig levels in the patients, and G1M genotypes were unevenly distributed among patients with or without nephritis. Therefore, patients were stratified according to G1M genotype and presence of nephritis, before investigation of the relation between serum complement concentrations and Ig levels. Only samples from nephritic G1M heterozygous patients (n=11) showed significant correlations between concentrations of IgG or IgG1 and C3 (IgG: r=0.83, p=0.003; IgG1: r=0.95, p<0.0001), C4 (IgG: r=0.62, p=0.048; IgG1: r=0.79, p=0.006) and C5 (IgG: r=0.76, p=0.01; IgG1: r=0.77, p=0.007), respectively, while samples from nephritic G1M homozygous patients did not.

In summary, G1M and/or G3M allotypes influence serum killing of meningococci in patients with acquired C3 deficiency. A mechanism explaining this finding remains to be elucidated. GM allotypic variation has previously been shown to influence susceptibility to infection in inherited complement deficiency states. As GM

allotypes have turned out to be important for defense against meningococci in acquired C3 deficiency, IGHG gene variants may play a role in the defense against bacteria in other conditions with acquired complement deficiency, such as SLE.

In addition, G1M allotypes are related to Ig concentrations in a reverse way in patients compared to controls, and complement concentrations are related to IgG concentration only in sera from G1M heterozygous patients. These findings might suggest a greater dependency on complement for regulation of Ig concentrations in G1M heterozygous compared to homozygous patients.

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Subjects and methods

Patients and controls

Samples from 73 patients with AGN (17 adults and 56 children) were collected consecutively during 1969-1977 and 1992-2001, among samples that had been sent to the Clinical Immunology Laboratory for diagnostic reasons. Inclusion was based on the following criteria: clinical symptoms of acute nephritis, positive culture of group A streptococci/elevated concentrations of ASO or aDNaseB, and serum C3 concentration below the reference interval (0.77-1.38 g/L, based on measurements in 100 healthy blood donors). Normalization of C3 concentration within 10 weeks was observed in all but 5 patients, in whom follow-up samples were not available. These 5 patients were included because of typical clinical symptoms of AGN.

Decoded control samples consisted of 61 sera collected in 1989 from healthy blood donors, and sera from 34 children whose samples had been sent to the laboratory in 1997 for other diagnostic purposes.

Methods

Complement analysis

Serum C3 concentrations were measured by electroimmunoassay or turbidimetry. MBL levels were measured by sandwich ELISA.

Streptococcal proteins

The following streptococcal proteins were purified from the serotype M1 strain AP1: SCP, M1, SIC and protein H [200, 207, 209, 211]. DRS12 and DRS55 were purified from serotype M12 and M55 *S. pyogenes* strains, respectively [213].

Antibodies against streptococcal proteins

ASO levels were measured by turbidimetry and aDNaseB levels were measured by a commercial kit (REF OUMP13, Dade Behring, Marburg, Germany).

IgG (or in the case of protein H, IgA) and IgM antibodies against SCP, M1, SIC and protein H were measured by ELISA. Except for IgM antibodies against SCP,

antibody concentrations were expressed in mg/L, by means of a heterologous ELISA procedure [23]. IgM-antibodies to DRS12 and DRS55 were also investigated by ELISA, and results were expressed as absorbance values.

Ethics

The study was approved by the Ethical Research Committee of Lund University.

Statistical methods

Non-parametric methods were used. The Mann-Whitney U test was used for comparison of medians. To compensate for multiple comparisons, only differences of medians with a p-value ≤ 0.008 were considered significant. The Spearman rank correlation test was used for calculation of correlations, and Fisher´s exact test for analysis of contingency tables.

Results and discussion

MBL serum levels in AGN

The frequency of MBL deficiency (MBL serum level <0.1 mg/L) among the AGN patients (8 out of 73) did not significantly differ from the frequency among previously published controls (31 out of 200, p=0.44, [43]). The presence of MBL deficiency in AGN argues strongly against an important role for LP activation in pathogenesis of the disease. This does not exclude that involvement and activation of the LP may occur in the disease course, as has been indicated previously [226].

Median MBL serum levels were increased compared to the levels in the previously published controls (figure 16). We interpret this increase as part of the acute phase reaction, which occurs early in AGN [62], as MBL has been reported to be a weak acute phase reactant [8].

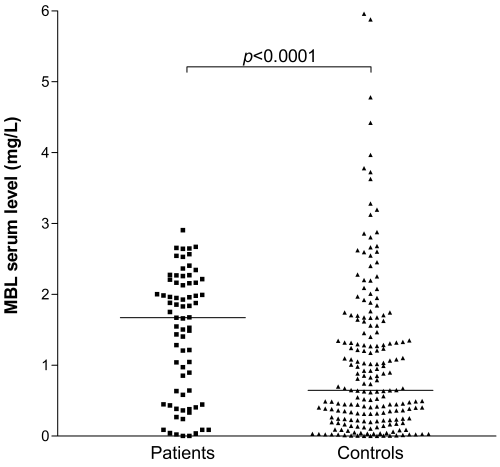

Figure 16. Serum concentrations of MBL in patients with AGN (n=73) and published controls [43]. Horizontal bars represent medians.

Antibodies to antigens from S. pyogenes serotype M1 in AGN

Adult patients showed significantly increased antibody levels only to SCP (IgG, p=0.004), which is expressed by all group A streptococci [199], while antibody responses in children with AGN were directed

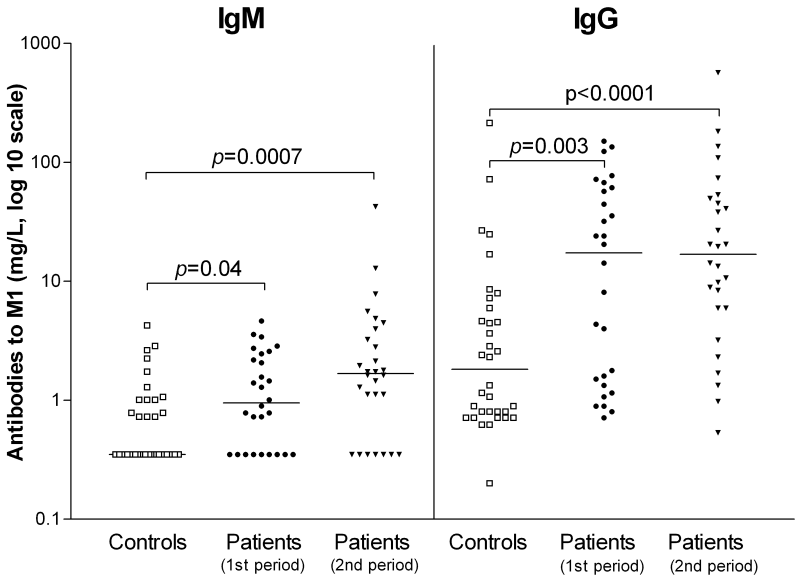

Figure 17. IgM and IgG antibodies to M1, the serotype-specific M protein of *S.pyogenes* strain AP1, in sera from children with AGN and children controls. First period: 1969-1977, second period: 1992-2001. Horizontal bars represent medians.

against all investigated antigens, more or less extensively. Child patient samples collected during both time periods showed increased antibody responses to the M protein of the AP1 strain; M1 (figure 17). In children samples from the early period, IgA responses to protein H were increased compared to controls (p=0.001). Child patients from the later period did not show increased antibody responses to protein H. Instead, we detected increased serum levels of IgM antibodies to SIC (figure 18) in this patient group as well as in samples from children from the first period.

Cross-reactivity with conserved parts of other M family proteins might influence the results for antibodies to M1 and protein H [195] and cross-reactions may also affect levels of antibodies to SIC. We found that IgM-reactivity to SIC was correlated to IgM-reactivity against DRS12 (r=0.56, p=0.04) and DRS55 (r=0.62, p=0.02), but cross-reactivity was not investigated. Indeed, in a previous study, AGN patients showed increased antibody responses to DRS, but not to SIC. Although serotype M1 belongs to the more common serotypes among clinical isolates [206], the results nevertheless indicate recent encounter with serotype M1 streptococci in an unexpectedly high number of patients, suggesting an overrepresentation of SIC and/ protein H-expressing M1 strains in AGN. The reason for this may be that antigens which elicit these antibody responses contribute to the pathogenesis of AGN, or that strains expressing such antigens are nephritogenic for other, unknown, reasons. None of the investigated antibodies proved to be more reliable markers of AGN than the markers traditionally used, like ASO and aDNaseB.

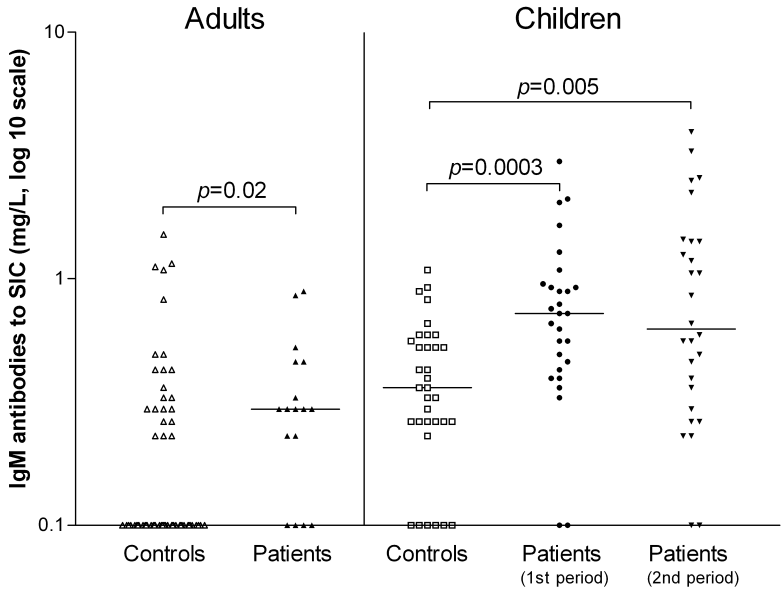

Figure 18. IgM antibodies to SIC from *S.pyogenes* strain AP1, in sera from patients with AGN and controls. First period: 1969-1977, second period: 1992-2001. Horizontal bars represent medians.

IV

Subjects and methods

Patients and controls

Serum samples from 92 consecutive patients with ISHL were collected between January 2004 and July 2006. An additional 8 patients with elevated concentrations of aC1qCLR were collected during a different time period. Diagnosis of ISHL was made at the Department of Otorhinolaryngology, Lund University Hospital, using the following diagnostic criteria: rapid onset of hearing loss (within 24 h), and mean hearing threshold (MHT) >30 dB. MHT is the mean value of bone conduction at frequencies 500, 1000, 2000, 4000 Hz. Among ISHL patients, 20 individuals negative for aC1qCLR were age-matched to aC1qCLR positive patients (total 20).

Sera from 10 ISHL patients (from the 92 consecutive patients described above), 5 patients with SLE and 5 patients with HUVS, all positive for aC1qCLR, together with 10 sera from healthy blood donors without aC1qCLR, were used for analysis of IgG reactivity with EMILIN-2.

All samples were stored at -80°C.

Methods

Collection of clinical data

Clinical records of the ISHL patients were reviewed by specialists in otorhinolaryngology at the Department of Otorhinolaryngology, Lund University Hospital, and data concerning disease development, treatment, and other simultaneous diagnoses were collected. Audiometric data of hearing thresholds (MHT and at 4000 Hz) at diagnosis and 3 months after diagnosis were recorded and compared in 20 patients positive for aC1qCLR and in 20 age-matched aC1qCLR-negative patients.

Autoantibody and C1q analyses

Analysis of aC1qCLR was performed as previously described [91].

Anti-nuclear antibodies, antibodies to cardiolipin, rheumatoid factors, and anti-nuclear cytoplasmic antibodies (ANCA) were analyzed as previously described [260]. Antibodies to HSP-70 were determined by a commercial Western blot (OTOblot®, IMMCO Diagnostics, Buffalo, NY, U.S.A.) at Wieslab AB, Lund, Sweden.

In 12 aC1qCLR positive ISHL patient samples (from the January 2004 to July 2006 period), C1q was measured by electroimmunoassay, and Western blot analysis of IgG reactivity with separated C1q chains was performed as described for paper I [91].

Reactivity with EMILIN-2

IgG reactivity with EMILIN-2 was investigated in 40 serum samples, by ELISA: 10 sera from ISHL patients with aC1qCLR, 10 sera from ISHL patients without aC1qCLR, 10 sera from healthy blood donors without aC1qCLR, 5 sera from HUVS patients and 5 sera from SLE patients with aC1qCLR.

Ethics

Studies of the ISHL patients were part of an extended clinical investigation. Informed consent had previously been obtained from the SLE and HUVS patients.

Statistical methods

Non-parametric methods were used. The Mann-Whitney U test was used for comparison of medians. For calculation of correlations, the Spearman rank correlation test was applied, and Fisher´s exact test was used for analysis of contingency tables.

Results and discussion

Autoantibody findings in ISHL

The most common autoantibody among the 92 ISHL patients in this study was aC1qCLR. It was detected in 12 patients (13 %). All detected autoantibodies are shown in table 8. Reactivity with the separated C1q C chain, as detected by Western blot, was seen in 2 patients. This is an unusual finding of uncertain significance. C1q serum levels were normal in all patients with aC1qCLR, and C1q levels were not correlated to concentrations of aC1qCLR. In active SLE, especially with nephritis, where the aC1qCLR are associated with disease activity, C1q and aC1qCLR are often inversely correlated. Several factors suggest that aC1qCLR in ISHL represents a cross-reaction: the lack of correlation between concentrations of C1q and aC1qCLR, the absence of other diagnoses previously associated with aC1qCLR, and the existence of at least 3 proteins in the inner ear with partly collagen-like structure. We therefore went on to investigate possible IgG reactivity of patient sera with EMILIN-2, the murine counterpart of which is present in the cochlear basilar membrane [251]. Analysis of aC1qCLR should be included in clinical examination of ISHL patients.

Table 8. Autoantibodies detected in 92 patients with idiopathic sudden hearing loss.

Autoantibody	Number of patients positive (%)
Autoantibodies to the collagen-like region of C1q	12 (13)
Anti-nuclear antibodies	10 (11)
Antibodies to cardiolipin	8 (9)
Antibodies to HSP-70	6 (7)
Rheumatoid factors	6 (7)
Anti-neutrophil cytoplasmic antibodies	1 (1)

IgG reactivity with EMILIN-2 in ISHL, SLE and HUVS

Sera from HUVS and SLE patients showed increased IgG reactivity with EMILIN-2 compared to blood donor samples, as shown by ELISA. The IgG reactivity with EMILIN-2 of ISHL sera with or without a-C1qCLR was not different from the IgG reactivity of blood donor sera (figure 19). IgG reactivity with EMILIN-2 did not correlate to aC1qCLR concentrations in any investigated group. The increased IgG reactivity with EMILIN-2 in SLE and HUVS suggests increased or altered exposure of this antigen in these diseases. This is of particular interest against the background of a described role for EMILIN-2 in apoptosis [250], as defective handling of apoptotic material is believed to contribute to the pathogenetic process in SLE. On the other hand, the autoantibody repertoire in SLE is vast [261], and not all of the produced antibodies can be expected to be central to disease mechanisms or prognosis.

IgG reactivity with EMILIN-2 and aC1qCLR; relation to clinical findings in ISHL patients

Median levels of MHT and 4000 Hz at diagnosis did not differ significantly between 20 patients with aC1qCLR and 20 age-matched patients without aC1qCLR. Nor was there any significant difference in median values for improvement of MHT or 4000

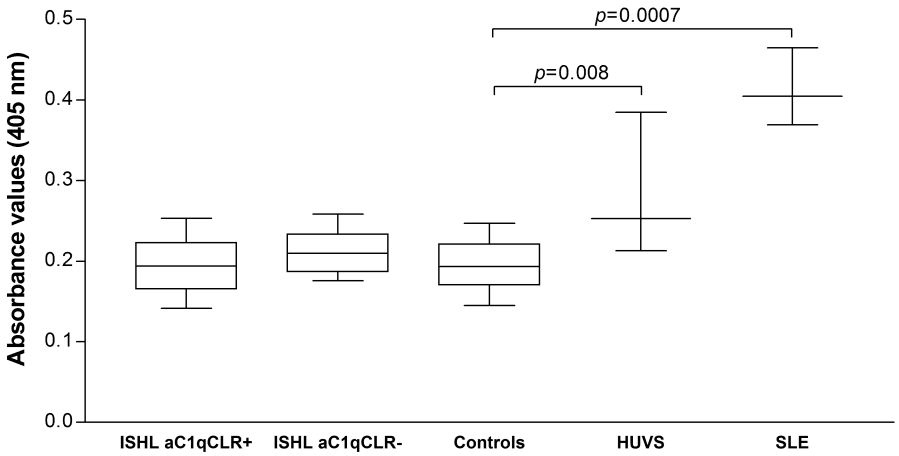

Figure 19. IgG reactivity with EMILIN-2 in sera from SLE patients (n=5), HUVS patients (n=5), ISHL patients with aC1qCLR (ISHL aC1qCLR+, n=10), ISHL patients without aC1qCLR (ISHL aC1qCLR-, n=10) and blood donor controls (Controls, n=10). *P*-values of statistically significant different medians compared to the median value of blood donors are shown. Boxes represent interquartile ranges. Minimum and maximum values are indicated. Horizontal bars show medians.

Hz after 3 months between aC1qCLR positive and negative patients, although there was a tendency to greater improvement at 4000 Hz in the aC1qCLR negative group (data not shown). There was also a non-significant tendency to negative correlation between aC1qCLR values and hearing threshold values (MHT: r= -0.41, p=0.07; 4000 Hz: r= -0.32, p=0.16) at diagnosis, but no correlation to improvement or to audiometric results at follow-up. Taken together, these results might suggest a protective effect of aC1qCLR in ISHL.

The frequency of patients treated with corticosteroids was similar among aC1qCLR positive (15 out of 20) and aC1qCLR negative patients (17 out of 20; Fisher's exact test, p=0.69) and improvement of audiometric parameters was not significantly different between treated and non-treated patients.

In the 10 aC1qCLR positive ISHL patients investigated for IgG reactivity with EMILIN-2, there was no correlation between EMILIN-2 IgG reactivity and MHT or 4000 Hz values at diagnosis or improvement in MHT or 4000 Hz (data not shown).

CONCLUSIONS AND FUTURE PERSPECTIVES

Conclusions

I Presence of C3 NeF in the circulation is the most common explanation for continuously low C3 levels.

The existence of (at least) 2 different forms of C3 NeF, one associated with normal (type I) and another associated with low (type II) serum concentrations of C5, was confirmed. C3 NeF type II are more often associated with glomerulonephritis than C3 NeF type I. Only C3 NeF type II are associated with aC1qCLR.

Only C3 NeF type I, and not C3 NeF type II, could be proved to be an IgG autoantibody. Hence, the nature of C3 NeF type II activity is uncertain.

Better screening tests for C3 NeF are needed, as the assays commonly used are not entirely specific for C3 NeF.

The association between C3 NeF and meningococcal disease was confirmed.

II SBA against meningococci is dependent on IgG allotypes in sera from patients with low C3 due to C3 NeF, and from children with complement function normal for their age. Homozygosity for *G3M*b* and *G1M*f* is associated with higher SBA than the heterozygous genotypes *G3M*g/*b* and *G1M*a/*f*.

In patients with low C3, only individuals heterozygous for IgG1/IgG3 allotypes show less SBA against meningococci than controls.

The distribution of IgG genotypes in patients with C3 NeF differs from that in the normal population.

III MBL is not required in the pathogenesis of AGN.

AGN patients show increased antibody responses to antigens from *S. pyogenes* serotype M1, implying that a) these antigens are involved in the pathogenesis, or b) *S. pyogenes* strains expressing these antigens are nephritogenic for other reason(s).

IV In sera from patients with ISHL, aC1qCLR was the most commonly found autoantibody out of 6 investigated, including antibodies to HSP-70.

In ISHL, aC1qCLR are associated with normal C1q concentrations, and ISHL patients with aC1qCLR have no additional diagnoses associated with aC1qCLR. These findings suggest that aC1qCLR in ISHL may represent a cross-reaction.

There was no evidence for cross-reaction between aC1qCLR and antibodies to EMILIN-2 in patients with ISHL.

Future perspectives

The observations made in the present investigations have implications for understanding and diagnosis of the investigated conditions and raise new questions to be addressed in the future.

The renal condition DDD, which is associated with C3 NeF, has a heterogeneous background and C3 NeF assays are not altogether specific. In addition to C3 NeF analysis, patients with DDD should be investigated for other forms of dysregulation of the AP according to recent recommendations [262]. The nature and specificity of C3 NeF type II needs further investigation, and a specific test for C3 NeF type II should be established. Also, the stronger association of C3 NeF type II with glomerulonephritis in comparison to C3 NeF type I is interesting, and might have implications for pathogenesis and prognosis.

GM allotypes were found to be important for serum mediated defense against meningococci in patients with low C3 concentration. The mechanism of this relation to GM allotypes is not clear, as the level of IgG subclass binding to meningococci in the performed experiments did not correlate to bactericidal activity. Different allotypes may have different capacities for complement activation. Investigations of such possible relations have been initiated. IGHG genes are already known to influence antibody concentrations and repertoire. Alternative of a direct IGHG gene impact, linked genes in the extended haplotype may be responsible. The possible influence of IGHG genetic variants on defense against other species susceptible to serum killing like for example *Haemophilus influenzae* or *N. gonorrhoeae* should also be investigated. Although the matter has not been much studied, G2M allotypes are important for the immune defense in some states of inherited complement deficiency [25, 164]. As the present findings demonstrate the influence of GM allotypes in acquired complement deficiency, it would be of interest to further study the role of IGHG genes for bacterial defense in other conditions with acquired low complement function, e.g. SLE or AGN.

The role of complement in AGN still remains elusive. The LP pathway may be activated in AGN, but is not required for disease development, as MBL-deficient AGN patients were found. Signs of CP activation are rare in AGN. Thus, the AP is the pathway which is most prominently activated in this disease. Direct AP activation by plasmin through plasminogen-binding streptococcal antigens has been considered earlier, but the recent data which propose a role for properdin as an AP initiator open the way for a new possible mechanism of complement activation in AGN. Properdin might bind streptococci and/or IC and provide a platform for AP activation. Properdin binding to IC has previously been shown to occur in the presence of C1q and C3 [263]. Such a mechanism would be consistent with the previous observation that properdin concentrations may decrease before C3 concentrations in the course of AGN [264].

The presence of aC1qCLR in ISHL is clearly not related to complement activation. Instead, it probably represents a cross-reaction to one or more out of several partly

collagen-like inner ear proteins. The lack of IgG reactivity with one such antigen, EMILIN-2, in ISHL does not exclude this possibility. The potential cross-reactivity of aC1qCLR with cochlear antigens could be preferably investigated by immunohistochemical analysis.

An unexpected finding was the increased reactivity with EMILIN-2 observed in sera from patients with SLE and HUVS. The fact that EMILIN-2 has been reported to regulate apoptosis is particularly interesting concerning SLE, as deficient handling of apoptotic material predisposes to SLE development. Hypothetically, antibodies to EMILIN-2 might inhibit its function, thereby leading to increased apoptosis.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

(Popularized summary in Swedish)

Komplementsystemet är en grupp av proteiner (äggviteämnen) som kallas komplementfaktorer, vilka tillsammans utgör en del av det medfödda immunförsvaret. Av de drygt 30 olika kända faktorerna som ingår i komplementsystemet cirkulerar vissa fritt i blodet medan andra sitter fast, bundna till celler. I samband med sjukdom eller skada kan komplementsystemet aktiveras. Vid aktivering reagerar komplementfaktorerna med varandra i en kaskadreaktion med flera biologiska effekter som bidrar till immunförsvaret. Bland annat leder komplementaktivering till att ytan på mikroorganismer och andra skadliga ämnen täcks av komplementfaktorer. Detta underlättar för fagocyter (”ätarceller”) att ta upp (fagocytera) och förstöra/borttransportera dessa skadliga substanser. Andra effekter av komplementaktivering är t ex direkt avdödning av bakterier och andra främmande celler genom att deras väggar löses upp, ökning av inflammation och vätskeutträde i vävnad (ödembildning), hantering av rester av döda celler, och stimulering av det förvärvade immunförsvaret.

Aktivering av komplementsystemet kan ske via 3 olika aktiveringsvägar: klassiska vägen, lektinvägen och alternativa vägen. Varje väg har sina delvis egna ingående komplementfaktorer och aktiveras på sitt eget specifika sätt, skilt från de andra vägarna. Aktivering via vilken som helst av de 3 vägarna leder till aktivering av komplementfaktor 3 (C3). Sista delen av komplementsystemets kaskadreaktion (den terminala vägen) är gemensam för alla 3 aktiveringsvägar. Bristande komplementaktivering är skadlig, framför allt därför att den leder till sämre infektionsförsvar. Även överdriven komplementaktivering kan vara skadlig pga. att den ger ökad inflammation. Vid vissa sjukdomar förbrukas för mycket komplementfaktorer till följd av överdriven aktivering. Om detta inte kan kompenseras av nybildning, leder det till sänkta komplementhalter i blodet. Komplementsystemet är involverat i sjukdomsprocessen vid många sjukdomar, och komplementsystemets roll för några sådana mekanismer för sjukdomsutveckling har undersökts i denna avhandling.

En annan viktig del av immunförsvaret är antikroppar. Antikroppar eller immunglobuliner (Ig) är proteiner som bildas av en typ av blodceller (B-lymfocyter) efter att immunförsvaret har stött på ett främmande ämne. Ig är sammansatta av 2 lätta och 2 tunga peptidkedjor (beståndsdelar i proteiner). Varje antikropp binder specifikt till det ämne (antigen) som har startat dess produktion. Antikroppar finns av 5 huvudtyper (IgD, IgM, IgA, IgG och IgE). IgG förekommer i 4 olika undergrupper, eller subklasser (IgG1, IgG2, IgG3 och IgG4). IgM och IgG kan aktivera den klassiska vägen för komplementaktivering efter att de bildat ett s.k. immunkomplex (IC) genom att binda sitt antigen. Antikroppar bidrar också i immunförsvaret genom att underlätta fagocytos och borttransport av skadliga ämnen. Vid vissa sjukdomar, framför allt vid s.k. autoimmuna sjukdomar men också

övergående i samband med infektioner, bildas antikroppar mot kroppens egna ämnen och strukturer (autoantikroppar). Detta leder till skador på kroppen, t ex genom komplementaktivering. Ett specialfall av autoantikroppar är autoantikroppar mot komplementproteiner. Två sådana typer av autoantikroppar har studerats i två av delarbetena i denna avhandling.

I delarbete I undersöktes serumprover (koagulerade blodprover där blodkroppar har avskilts) från 25 patienter med låg halt av komplementfaktor C3 i blodet utan känd förklaring. Hos 20 av dessa patienter påvisades en autoantikropp riktad mot ett komplex av komplementfaktorer. Komplexet är ett s.k. ”C3-konvertas”, som bildas vid komplementaktivering. C3-konvertaset är ett enzym, med förmåga att klyva C3. Autoantikroppen kallas ”C3 nephritic factor” eller C3 NeF, och stabiliserar C3-konvertaset genom att binda det. Därmed förlängs konvertasets halveringstid och mer C3 klyvs än normalt. Detta leder till låg C3-halt i blodet. Med användande av flera testmetoder för C3 NeF kunde det bekräftas att C3 NeF är av (minst) 2 typer, typ I och typ II. C3 NeF typ II hittas företrädesvis med en metod som påvisar dess stabiliserande effekt på ett C3-konvertas bildat på celler, medan C3NeF typ I kan visas även med tester där C3-konvertaset bildas och stabiliseras i fri lösning. Vi kunde också bekräfta att typ I är associerad med normal serumhalt av komplementfaktor C5, medan typ II har låg halt av C5. Ett nytt fynd var att C3 NeF typ II oftare än typ I hittas hos patienter med njursjukdomen membranproliferativ glomerulonefrit (MPGN). Bland patienter med C3 NeF typ I förekom mer varierande sjukdomar, bland annat hade en patient anafylaktoid purpura (en kärlinflammation i huden), en sjukdom som tidigare inte har rapporterats hos någon patient med C3 NeF. En annan ny observation var att endast patienter med C3 NeF typ II (6 av 10) samtidigt hade en annan autoantikropp, C1q-antikroppar (aC1qCLR). aC1qCLR är antikroppar riktade mot den del av komplementfaktor C1q, som till sin struktur liknar kollagen (ett protein som är vanligt i t ex bindväv). Ingen patient med C3 NeF typ I hade aC1qCLR. En analysmetod sattes upp, i vilken C3-konvertas byggs upp med hjälp av renade komplementfaktorer. Därefter mäts binding av IgG till C3-konvertaset. Med denna test kunde bara antikroppar av typ I påvisas. Trots försök att tillsätta komplementfaktorer i olika kombinationer för att bygga kända former av C3-konvertas, kunde inte specificiteten för C3 NeF typ II fastställas. Detta innebär att det är osäkert om C3 NeF typ II verkligen är en autoantikropp, eller om det är t ex en kombination av autoantikropp och komplementfaktorer eller defekter hos komplementfaktorer som förklarar den ökade C3-klyvningen hos dessa patienter.

Immunförsvaret mot meningokocker är nedsatt hos patienter med låg C3-halt i blodet (t ex till följd av C3 NeF). Meningokocker är bakterier som kan ge hjärnhinneinflammation och blodförgiftning. I delarbete II undersöktes serumprover från 30 patienter med C3 NeF. Hos 26 av dessa undersöktes serums förmåga till avdödning av 3 meningokockstammar. Meningokocker indelas i serogrupper på basen av skillnader i deras yttre hölje (kapsel). De tre stammarna tillhörde olika serogrupper: grupp C, Y och W-135. Mot grupp Y och W-135 var förmågan till

avdödning nedsatt hos patienterna jämfört med hos 51 kontroller med normalt komplementsystem. Dessa 2 serogrupper förekommer ofta vid meningokockinfektioner hos komplementdefekta personer, men infekterar mera sällan personer med normalt komplementsystem. Försöken visade att serogrupp C-meningokocker överlevde även i serum från kontrollerna. Hos patienter med C3 NeF och i viss utsträckning hos barnkontroller (29 stycken) visade sig avdödningsförmågan vara kopplad till vissa varianter av gener för den tunga kedjan av IgG (GM). Sådana gener förekommer i flera vanliga varianter. Hos vuxna kontroller, som nästan samtliga hade god förmåga till meningokockavdödning, fanns ingen koppling mellan GM-varianter och avdödning. Eftersom den tidiga barndomsperioden även hos friska individer innebär en tid av relativ komplementbrist, kan detta förklara varför GM-gener har betydelse hos barn med normal komplementfunktion för åldern. Vilken uppsättning av GM-gener man har får betydelse när komplementsystemet inte fungerar optimalt. Andra faktorer för serumavdödning av meningokocker undersöktes: total halt av olika IgG-subklasser, halter av flera komplementfaktorer, och antikroppar specifika för de olika undersökta stammarna. Ingen av dessa parametrar visade någon statistiskt signifikant koppling till avdödning.

I delarbete III undersöktes serumprover från 73 patienter med akut poststreptokock-glomerulonefrit (AGN). AGN är en njurinflammation som kan uppstå 1-2 veckor efter en infektion orsakad av streptokockbakterier (t ex halsfluss). AGN yttrar sej som varierande grad av akut njursvikt, högt blodtryck och ödembildning. Samtidigt har patienten övergående låga halter av komplementfaktorerna C3 och properdin i blodet. Eftersom properdin ingår i den alternativa vägen för komplementaktivering, talar komplementprofilen vid AGN för att den alternativa vägen blir aktiverad. Det har även rapporterats att lektinvägen skulle vara inblandad i sjukdomsuppkomsten. Sjukdomsmekanismen är oklar, men den vanligaste hypotesen är att ett streptokockprotein och antikroppar riktade mot detta bildar immunkomplex som aktiverar komplementsystemet. I vävnadsbitar (biopsier) tagna från njuren hos patienter med AGN finns både komplementfaktorer och antikroppar. På något sätt måste streptokockproteinet fastna i de delar av njuren som inflammeras – glomeruli - för att kunna orsaka skada där. Under många år har flera forskargrupper letat bland streptokockproteiner och i njurbiopsier från patienter efter ett så kallat nefritogent (som orsakar nefrit) antigen, som skulle kunna förklara sjukdomsuppkomsten. Flera sådana antigen har föreslagits, men inget har visat sej övertygande. Vissa grupper av streptokockstammar har också ansetts vara mer nefritogena än andra. I delarbete III mättes serumhalter av mannan-bindande lektin (MBL) hos patienterna med AGN. MBL är startproteinet för lektinvägen, och brist på MBL förekommer hos ca 10 % av normalbefolkningen. Hos patienterna fanns så stor andel individer med MBL-brist som förväntat. Detta fynd talar mot att aktivering av lektinvägen är nödvändig för utveckling av AGN. I delarbetet mättes också serumhalter av antikroppar mot 4 streptokockproteiner. De 4 proteinerna är ”streptococcal cysteine proteinase” (SCP), M1, protein H och ”streptococcal inhibitor of complement” (SIC). Alla proteiner renades fram från en streptokockstam som tillhör en nefritogen grupp kallad M1.

Patienterna hade högre nivåer av antikroppar mot alla 4 streptokockproteinerna, särskilt mot SIC och M1. Detta talar för att dessa proteiner kan vara inblandade i sjukdomsuppkomsten vid AGN, men det är oklart hur.

I delarbete IV undersöktes serumprover från 92 patienter med oförklarad plötslig dövhet (SD). SD definieras som dövhet som utvecklas inom 24 timmar och inte har en känd orsak. I vissa fall har SD kunnat kopplas till infektioner, kärlsjukdom eller autoimmun sjukdom. I ca 2/3 av fallen kommer hörseln helt eller delvis tillbaka spontant, men alla patienter behandlas för säkerhets skull med kortison eftersom det är den enda behandlingen med någon effekt. Det finns idag inga säkra sätt att vid insjuknandet avgöra vem som kommer att ha nytta av behandling, eller att skilja ut olika undergrupper av patienter med olika sjukdomsorsak. Dessutom förblir orsaken okänd i majoriteten av fallen. Flera olika autoantikroppar har påvisats hos patienter med SD, och dessa analyseras rutinmässigt i blodprover vid nyinsjuknandet. Autoantikroppar ses dock bara hos en liten andel patienter, och det är oklart om och hur de är relaterade till sjukdomsmekanismen. Flera olika delar av immunsystemet har föreslagits vara inblandade i sjukdomsutvecklingen. Hos de 92 patienterna i delarbete IV analyserades halter av 6 olika autoantikroppar och patienternas journaler studerades. 12 av 92 patienter (13 %) hade förhöjda nivåer av aC1qCLR (se delarbete I). Detta är första gången aC1qCLR rapporteras vid SD, och aC1qCLR var den vanligast förekommande av de undersökta antikropparna. Alla patienter med aC1qCLR hade normal koncentration av C1q i serum, och ingen hade någon annan sjukdom som är associerad med aC1qCLR. Detta talar för att aC1qCLR egentligen kan vara riktade mot något annat antigen än C1q, så kallad korsreaktivitet. I innerörat finns flera proteiner med liknande struktur som den kollagen-lika delen av C1q. Ett immunsvar mot något eller flera av dessa skulle kunna tänkas ligga bakom förekomsten av aC1qCLR vid SD, och även kunna spela roll för sjukdomsutveckling. För att undersöka möjlig korsreaktivitet analyserades antikroppsbindning till ett sådant protein, kallat EMILIN-2, i prover från patienter med SD (med och utan aC1qCLR), systemisk lupus erythematosus (SLE), hypokomplementemisk urticariavaskulit (HUVS) och blodgivare. SLE och HUVS är autoimmuna sjukdomar där aC1qCLR är vanligt förekommande. Prover från patienter med SLE och HUVS visade ökad reaktivitet med EMILIN-2 jämfört med blodgivare, medan prover från patienter med SD inte gjorde det. Avsaknaden av antikroppsbindning till EMILIN-2 hos dessa patienter med SD utesluter dock inte möjligheten av att aC1qCLR vid SD representerar en korsreaktion mot något annat protein i innerörat. Halten av aC1qCLR var inte statistiskt signifikant relaterad till sjukdomsprognosen hos patienterna med SD, och inte korrelerad till nivån av antikroppsbinding till EMILIN-2

Sammanfattningsvis har betydelsen av komplementsystemet och/eller autoantikroppar mot komplementfaktorer vid flera sjukdomar belysts i avhandlingen.

I Autoantikroppar mot den alternativa komplementaktiveringsvägens C3-konvertas, C3 NeF, visades vara den vanligaste orsaken till oförklarad låg C3-halt i blodet, och

de två typerna av C3 NeF förekom vid delvis olika sjukdomar. Endast C3 NeF typ II var associerad med förekomst av autoantikroppar mot den kollagen-lika delen av komplementfaktor C1q (aC1qCLR). Mer specifika analysmetoder för C3 NeF behöver utarbetas, och fynden leder till frågan om typen av C3 NeF kan ha betydelse för sjukdomsutveckling och prognos.

II Hos patienter med låg C3-halt i blodet till följd av C3 NeF visades uppsättningen av vissa gener för IgG (GM) vara kopplad till serums förmåga att avdöda meningokockbakterier. En liknande koppling visades hos barn med normalt komplementsystem. IgG-genvarianter kan tänkas inverka på immunförsvaret mot meningokocker även hos andra patientgrupper med förvärvad komplementbrist. Betydelsen av GM för försvar mot andra bakterier har inte heller studerats hos patienter med förvärvad komplementbrist, och skulle kunna undersökas.

III Brist på mannan-bindande lektin påvisades hos patienter med akut post-streptokockglomerulonefrit (AGN), vilket talar emot en central roll för lektinvägen för komplementaktivering i utveckling av AGN. Alternativa vägen kvarstår som den viktigaste komplementaktiveringsvägen vid AGN, och nya rön antyder en roll för komplementfaktorn properdin i initieringen av komplementaktiveringen. Halterna av antikroppar mot streptokockproteinerna M1 och SIC var ökade hos patienter med AGN.

IV Hos patienter med oförklarad plötslig dövhet (SD) var aC1qCLR den vanligast förekommande autoantikroppen. Samtidig normal C1q-nivå och avsaknad av aC1qCLR-associerade sjukdomar talar för att aC1qCLR vid SD beror på korsreaktivitet, särskilt mot bakgrund av flera proteiner med delvis liknande struktur finns i innerörat. Analys av aC1qCLR bör kunna bli ett diagnostiskt hjälpmedel i utredningen av SD, och eventuell korsreaktivitet kan utredas vidare med andra metoder.

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