Structure and function of complement component C3 and its fragments

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Introduction

Introduction

During their life, organisms are continually endangered by foreign pathogens and altered self structures that threaten the existence of the host. An effective apparatus had to evolve for preserving the consistency of the genetic code and this way making the survival of the species possible. The machinery which distinguishes self and non-self or altered self and fights against the causative agent is the immune system. During evolution, primitive pathogens, obeying the selection pressure, kept developing novel apparatus for the infection. The immune system also had to suit to these newer demands in protection of the host and this fight resulted the very complex and effective defence system of the mammals. The ancient arm of the immune system, innate immunity, did not disappear during evolution; on the contrary, it still tightly cooperates with the later formed adaptive immunity in protection of the vertebrates.

Pathogens, by breaking the physical barrier that separates the host form the environment, make contact with the components of the innate immune system. This ancient part of the immune system remained the first, instantly activated line of protection against invading pathogens. Cellular (macrophages, dendritic cells, granulocytes) and humoral (antibacterial peptides, cytokines, complement system) components of innate immunity destroy most of the pathogens in a short time. A certain proportion of invading agents survives the encounter with the innate immunity and challenge adaptive immunity, which provides an effective response in about two weeks following activation. It has to be emphasised that the cellular (B cells, T cells) and the humoral (antibodies, lymphokines) components of adaptive immunity are unable to work separately from the innate system. Components of the latter modulate adaptive responses and also take a crucial part in the effector phase. Some researches concentrate only on adaptive immunity, however without knowing the exact mechanisms of innate immunity some basic phenomena of the immune system can not be understood.

In this study, we focused on the complement system, the major humoral component of innate immunity. We have analyzed in great detail the third component (C3) of this system and its activation fragments which interact with several other molecules yielding a diverse set of biological responses.

1.1.1. Activation of the complement system

The complement system is an ancient component of the innate immune system; some of its major proteins appeared in echinoderms and exist in each deuterostome (Nonaka, 2001;Smith *et al.*, 1999). The complement system contains more than 30 proteins in various tissue fluids and on the surface of cells, including proteins of the enzymatic cascade, regulators and receptors. The cascade can be initiated by any of the three activation routes: the classical, lectin or alternative pathway (Figure 1.1). These pathways are analogous to the coagulation, fibrinolysis and kinin serine protease systems. The three routes converge at the point of the cleavage of the third component, C3, which is followed by the common terminal pathway. Proteins of the classical pathway are designated C1 through C9, while the components of the alternative pathway are called factors, followed by a letter (e.g. factor B, factor H). Several complement components are cleaved during activation of the system and normally the larger fragments are designated with 'b' while the smaller with 'a' suffixes (in the case of C2, the large fragment is designated C2a and the small C2b for historical reasons) (reviewed by (Walport, 2001)).

The classical pathway is initiated by the interaction of C1q, the subunit of the C1-complex with antigen-bound antibodies. The C1 complex consists of C1q and the two serine proteases, C1r and C1s, held together by Ca^{2+}. Conformational changes in the complex activate C1r, then C1s which first cleaves C4 and the larger C4b fragment binds covalently to the activating surface (bacteria, cell, etc.). C2 attaches to bound C4b, then it is cleaved by C1s leading to the formation of the C4b2a enzyme complex which is the C3 convertase of the classical pathway.

The lectin pathway is initiated by binding of the complex of mannose-binding lectin (MBL) or ficolins (Matsushita *et al.*, 2000) to arrays of sugar groups on the surface of bacterial cells. The mannose-binding lectin-associated serine protease 2 (MASP2) acts in a fashion similar to C1s, leading the formation of the C3 convertase enzyme, C4b2a (Matsushita and Fujita, 1992).

The alternative pathway is initiated by surface bound C3b fragments or by spontaneously hydrolysed C3 ($C3H_2O$) (Pangburn *et al.*, 1981), which in the presence of Mg^{2+} binds factor B, a protein homologous to C2. Factor D cleaves C3b-bound factor B and this way C3bBb, the C3 convertase of the alternative pathway is formed (Lesavre and Muller-Eberhard, 1978). The labile convertase complex has short half-life and is stabilised over 10-fold by the binding of properdin (Fearon and Austen,

1975;Hourcade, 2006). The C3 convertase enzymes cleave many C3 molecules and some of the generated C3b fragments bind covalently to the activating surface. These bound fragments are able to activate again the alternative pathway, making a positive feedback loop in the system, which increases dramatically the amount of activated C3b fragments. C3b is not able to distinguish between self and potentially harmful non-self structures, but other factors make this discrimination. The carbohydrate environment of the surface on which C3b fragments are deposited, determines the relative affinity of C3b for factor B or for a complement control protein, factor H. On the host cell surface that is abundant in polyanions such as sialic acid, factor H binds to C3b with higher affinity than does factor B and halts the formation of C3 convertases. On microbial surfaces that lack a polyanionic coating, factor B binds to C3b with a higher affinity than does factor H, and this leads amplified C3 cleavage (Meri and Pangburn, 1990).

In the terminal pathway, the C3b fragment binds to the C4b or C3b containing C3 convertases and forms the C5 convertase. This newly bound C3b acts as an acceptor site for C5 which will be cleaved to anaphylatoxin C5a and a metastabile C5b fragment (Takata *et al.*, 1987). C6 binds to C5b and forms a bimolecular complex, which remains loosely bound to C3b on the target cell surface until it reacts with C7. The C5b-7 complex then undergoes hydrophilic-amphiphilic transition and anchors itself firmly in the lipid bilayer (Preissner *et al.*, 1985). Membrane inserted C5b-7 functions as a receptor for C8. The binding of C8 changes its conformation and allows the α chain to penetrate into the lipid bilayer and finally the whole C5-8 complex drops deeper into the hydrophobic phase (Esser *et al.*, 1979), and forms a small functional channel of ~ 30 Å in diameter. The C5b-8 complex can mediate the binding of multiple C9 molecules (1-18), which incorporate into the membrane and form a channel of ~100 Å diameter (Tranum-Jensen *et al.*, 1978). This terminal complex (C5b-9), called membrane attack complex (MAC), causes cell lysis by disrupting the cell membrane.

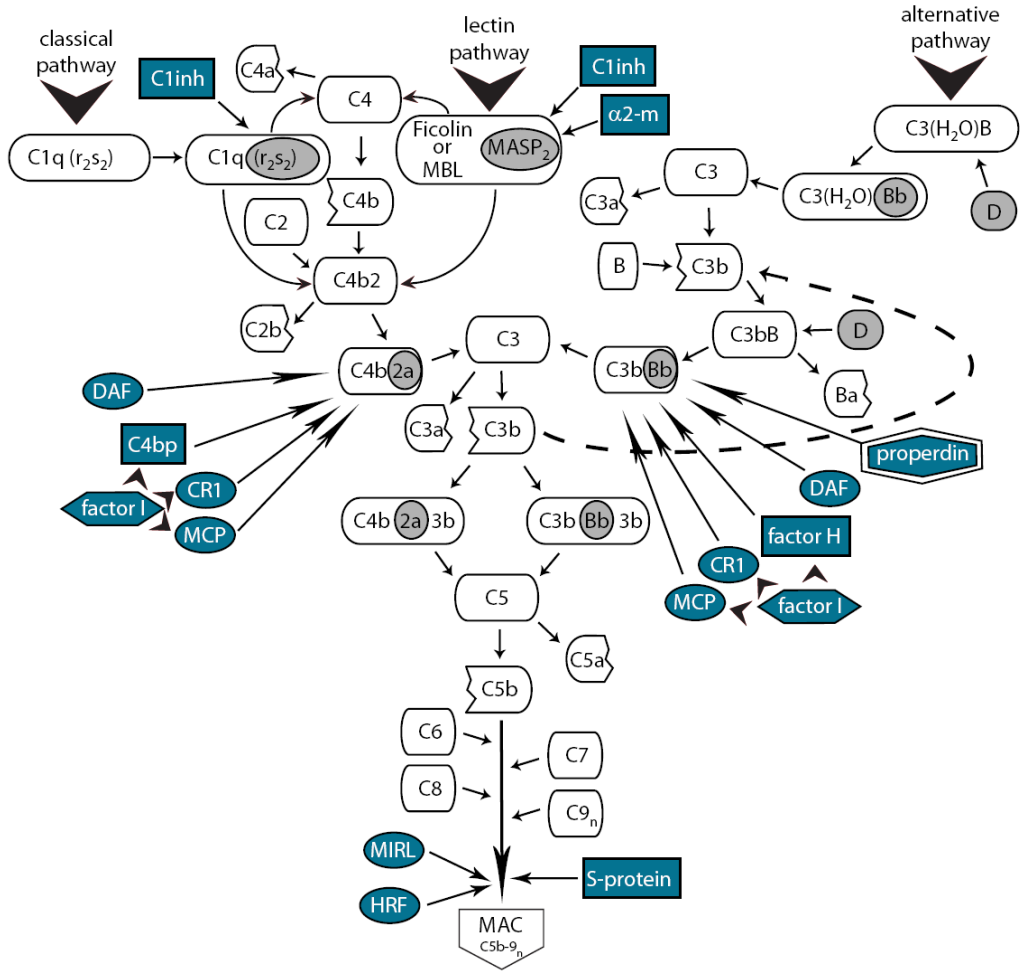


Figure 1.1. The activation and regulation of the human complement system. The initiation of the complement cascade can take place in three different ways (classical, lectin or alternative pathways), but following the convergence of these pathways at the level of the C3 molecule, a single route proceeds until the formation of the membrane attack complex. The positive feedback loop of the alternative pathway is illustrated by dashed line. The enzymatically active fragments are depicted in grey while the complement control proteins in blue background.

1.1.2. Regulation of the complement cascade

The complement system is a very effective apparatus against invading pathogens, but because of its effectiveness, it has to be well orchestrated or otherwise it can also destroy the host. The regulation starts at the level of the activators of the three pathways. The molecules or cells which initiate the cascade are listed in table 1.1.

Pathway	Initiator
Classical	Immune complex, apoptotic cell, certain viruses, Gram negative bacterial lipopolysaccharide, C-reactive protein bound to ligand
Lectin	Certain antibody glycoforms; carbohydrate structure on yeast, bacteria, viruses and parasites
Alternative	Many bacteria, fungi, viruses, tumor cells, aggregates of immunoglobulins

Table 1.1. Initiators of the three complement pathways

The complement activating properties of the antibodies are influenced by their isotype (Table 1.1) (Erdei, 2008;Janeway, 2001;Roitt, 1997), avidity and glycosylation (Arnold *et al.*, 2006).

The action of these activators should not result inevitable completion of the cascade, because this would cause unnecessary harm in the host, as well. There are many soluble and membrane bound complement control proteins (CCP) which are responsible for the proper functioning of this system. Three major targets in the complement cascade exist, where CCPs can exert their inhibitory effect; these are the serine proteases of the recognition complex, the C3 convertases and finally the MAC.

Isotype (human)	Isotype (mouse)	Complement activation
IgM	IgM	+++++
IgG1	IgG2a,c	+++
IgG2	IgG3	++
	IgG2b	+++
IgG3		+++
IgG4	IgG1	+
IgA1, IgA2	IgA	+ ?
IgE	IgE	-

Table 1.2. Complement activating properties of antibodies with different isotypes

The first control point is at the level of the initiator serine proteases (C1r, C1s, MASP1, MASP2). The C1 inhibitor (C1inh) irreversibly inactivates these enzymes (Munkvad *et al.*, 1990), so it blocks the classical and lectin pathway, as well. The other inhibitor at this point is α2-macroglobulin that can inactivate only the MASP enzymes (Terai *et al.*, 1995).

The major amplification of the cascade is carried out by the C3 convertases, where two mechanisms are involved in the regulation. The first mechanism prevents formation or accelerates dissociation of the C3 convertase by a process known as decay-accelerating activity. The second mechanism consists of the cleavage of the C3b or C4b molecule into iC3b or iC4b that can no longer participate in the formation of the C3 convertase enzymes (Molina, 2002). A serine protease, factor I, in the presence of cofactors, is responsible for the cleavage of the haemolytically active C3b and C4b fragments. The further listed CCPs, except for the membrane cofactor protein (MCP) (only cofactor) and decay-accelerating factor (DAF) (only decay-accelerating activity) are able to control the complement system by both mechanisms. Among the soluble cofactors of factor I, the C4 binding protein (C4bp) takes part in C4b, while factor H in C3b inactivation, while the membrane bound complement receptor 1 (CR1) and MCP cofactors inhibit the formation of both C4b2a and C3bBb. CR1 facilitates the complete degradation of C3, while the MCP helps only in the first cleavage of factor I (Muller-Eberhard, 1988). CR2 exhibits cofactor activity only on iC3b and helps its cleavage into C3dg and C3c fragments (Mitomo *et al.*, 1987). The decay-accelerating factor (DAF, CD55) prevents the assembly of the C3 convertase and dissociates the formed enzyme (Medof *et al.*, 1987). Interestingly, MCP has limited expression in mice, compared with the widespread expression in human tissues; here an additional CCP, called complement receptor 1 related protein (Crry), blocks the unnecessary formation of C3 convertases on self cells (Molina, 2002).

The formation of the MAC is the final event of the cascade and, in the same time, the last point where it can be stopped. The S-protein competes with the membrane binding site of MAC and this is the primary soluble MAC inhibitor of the serum (Podack and Muller-Eberhard, 1979); it allows binding of C8 and C9, but prevents C9 polymerization (Muller-Eberhard, 1988). The homologous restriction factor (HRF) is highly effective in inhibiting complement mediated channel formation, including the C5b-8 and MAC (Zalman *et al.*, 1986). The membrane

inhibitor of reactive lysis protein (MIRL, CD59) inhibits the incorporation of C9 into C5b-9 complexes formed in the membrane and reduces C9 polymerization (Rollins and Sims, 1990).

1.1.3. Biological effects of the complement system

Activation of the complement system initiates various physiological activities that help the host to protect its integrity against invading pathogens or altered self structures (Figure 1.2). The importance of the complement system is not restricted to innate immunity, but extends to adaptive responses as well, by forming a bridge between these two systems (Erdei *et al.*, 1991).

A remarkable feature of the complement system – which was actually the basis of its discovery – is its capability of lysing microbes. During the first contact with a pathogen, the alternative or lectin dependent pathway (or sometimes even the classical route – e.g. HIV) can be activated without the presence of antibodies, so the complement system can destroy the invading pathogens promptly as they appear in the body. If the host had already encountered a specific pathogen, the previously produced pathogen specific antibodies may also initiate the classical pathway, which is strongly augmented by the feed-back loop. The generated MAC disrupts the integrity of the pathogen cell membrane by pore forming, and thus eliminates the invading agent. Of course some pathogens try to avoid this protecting line, so during evolution several escape mechanisms have been developed by the pathogens, which hinder their clearance.

Every complement activating route results in C3 activation and a certain portion of the newly generated C3b fragments bind covalently to the activating surface. Following opsonisation, the covalently bound C3b and iC3b fragments serve as ‘tags’ on the pathogen. CR1 and CR3 receptors on the surface of macrophages bind to pathogen bound C3b and iC3b fragments increasing their phagocytosis and in this way facilitating presentation of antigens to T cells. On Kuppfer cells another receptor, the newly discovered complement receptor of the immunoglobulin superfamily (CRIg), is required for the efficient binding of C3-opsonized particles (Helmy *et al.*, 2006).

Complement activation also generates small peptides, called anaphylatoxins (C3a, C4a, C5a), that induce inflammation, promoting the effectivity of the immune process. The presence of C3a- and C5a receptors was confirmed on several cell types

(Gergely and Erdei, 2000;Johnson and Chenoweth, 1985). Anaphylatoxins induce the degranulation of the serosal type mast cells and basophils and the released histamine increases the permeability of blood vessels. Both C3a and C5a anaphylatoxins have chemotactic effect on eosinophils, while C5a attracts directly neutrophils to the site of inflammation, where these cells effectively eliminate microbes (Daffern *et al.*, 1995).

Formation of immune-complexes is a physiological phenomenon leading to the clearance of the antigen. In various pathological conditions however, excessive amounts of immune-complexes may be formed and can deposit in blood vessels, causing harmful inflammation. In the clearance of the immune complexes the complement system plays crucial role in two ways. On one hand C3 fragments can force apart the complexes by binding to them, and this way the large immune-complexes will be solubilised. On the other hand, the C3b fragments bound to the antigen-antibody complexes bind to human red blood cells via CR1. These cells then transfer the complexes to liver Kuppfer cells that phagocytose them, so they disappear from the bloodstream (Helmy *et al.*, 2006). In mice this clearance is not functioning, since mouse erythrocytes do not carry CR1 receptor on their surface (Kinoshita *et al.*, 1988).

Activation of the complement system also has an effect on acquired immunity. Antigen bound C3d fragments function as molecular adjuvant: the CD19/CR2/TAPA-1 complex augments signalling through the B cell antigen receptor. The blockade of CR2 receptor suppresses the primary antibody response (Dempsey *et al.*, 1996;Fearon and Carter, 1995).

CR1 and CR2 on the surface of follicular dendritic cells (FDCs) are critical for the generation of normal humoral immunity, especially during the later stages of the primary immune response and the generation of B cell memory. The FDC plays a crucial role in germinal center reactions, like somatic hypermutation of Ig genes, Ig isotype switching and the generation of memory cells, by trapping immune complexes via CR1 and CR2 receptors (Fang *et al.*, 1998).

C3 fragments fixed to the antigen presenting cells enhance the proliferation of antigen-specific T cells by interacting with the C3 receptors expressed on activated T lymphocytes. C3 fragments form a bridge between the antigen presenting cell (APC) and T cells, enhancing cell-to-cell contact and intracellular signalling (Kerekes *et al.*, 1998).

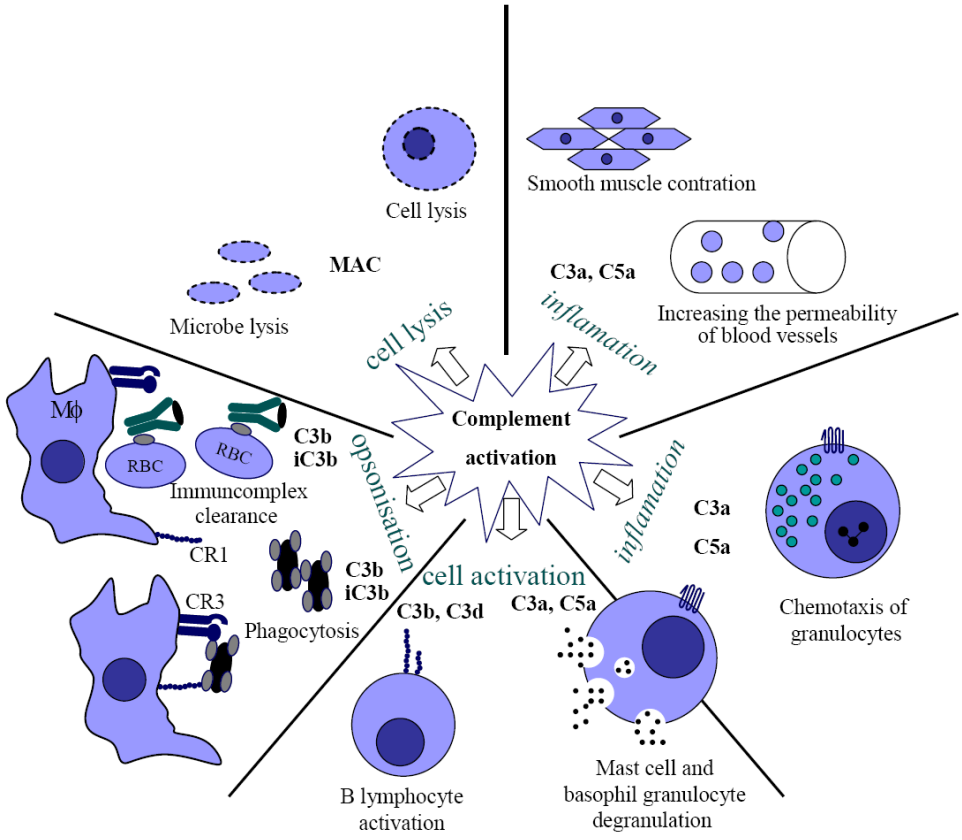


Figure 1.2. The multiple effects of the complement system (Gergely and Erdei, 2000)

The role of the complement system is not restricted only to the protection against invading pathogen, but it also modulates several other biological processes (Mastellos and Lambris, 2002). It is suggested that C3 and C3-binding proteins facilitate gamete membrane fusion and thereby promote sperm penetration and oocyte fertilization (Anderson *et al.*, 1993). The involvement of C3 molecules was suggested also in the dedifferentiation process and the muscle differentiation in the limb regeneration of urodele species (Del Rio-Tsonis *et al.*, 1998). The role of C5 was proved in liver regeneration following toxic injury (Mastellos *et al.*, 2001). There is evidence, which suggests that C3a also has a role in the maturation and lineage commitment of hematopoetic progenitors (Mastellos and Lambris, 2002).

Because of the multiple roles of the complement system, it is reasonable to make any effort to understand every detail of this ancient part of humoral immunity.

1.2.1 Structure of C3

Component C3 is the most abundant complement protein in the serum ($c_{human\ C3}$ ~1.2 mg/ml, $c_{mouse\ C3}$ ~ 0.5 mg/ml). Its main source is the hepatocyte (Alper *et al.*, 1969), but macrophages (Zimmer *et al.*, 1982) and, with less efficiency, endothelial cells (Warren *et al.*, 1987) can also secrete this crucial complement component. The C3 molecule belongs to the α2-macroglobulin family, whose members, like C4 and the proteinase inhibitor α2-macroglobulin contain an internal thioesher group and have important roles in the immune response. C5 is a homologue of C3 and C4, but lacks the thioesther group. Human and mouse C3 have 77% amino acid identity (de Bruijn and Fey, 1985;Fey *et al.*, 1984) and their genes are located on chromosome 19 (Whitehead *et al.*, 1982) and 17, respectively. The molecule consists of α and β chains which are linked by a disulphide bond and non-covalent forces (Figure 1.3).

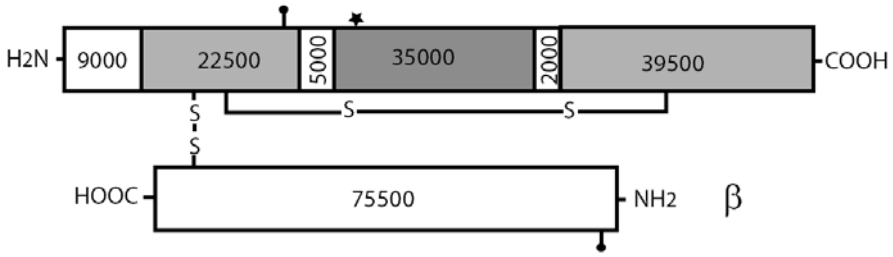


Figure 1.3. Schematic representation of the mature human C3.
The molecular massses of the fragments (in Da) have been calculated from amino acid sequences and do not include carbohydrate content. The location of the thioester group (asterix) and N-linked carbohydrate (closed balloon) sites are also indicated in the figure.

The human C3 molecule is translated into a precursor molecule, the prepro-C3 of 1663 amino acid residues. At the NH_2 terminal end there is a 22-residue signal sequence which is followed by the β and the α chain sequence separated by tetra-arginine residues (Table 1.3). During maturation a furin-like enzyme cleaves out the four arginine residues between the β and α chain (Misumi *et al.*, 1991), so the molecule can attain its final structure. In the Golgi, N-linked carbohydrate moieties also attach at asparagine residue 917 of the α chain [$Man_8(GlcNAc)_2$ +

$Man_9(GlcNAc)_2$] and 63 of the β chain [$Man_5(GlcNAc)_2$ + $Man_6(GlcNAc)_2$] and together account for 1.5% of the molecular weight of human C3 (Hirani *et al.*, 1986;Sahu and Lambris, 2001).

	Human	Mouse
Molecular weight	187148 Da	186484Da
NCBI Protein Database	NP_000055	P01027
Precursor molecule	1663aa	1663aa
Signal sequence	22	24
β chain	645	642
α chain	992	993

Table 1.3. Major properties of human and mouse C3

The crystal structure of the C3 is already solved (Janssen *et al.*, 2005), the structure of its 13 domains are shown in figure 1.4.

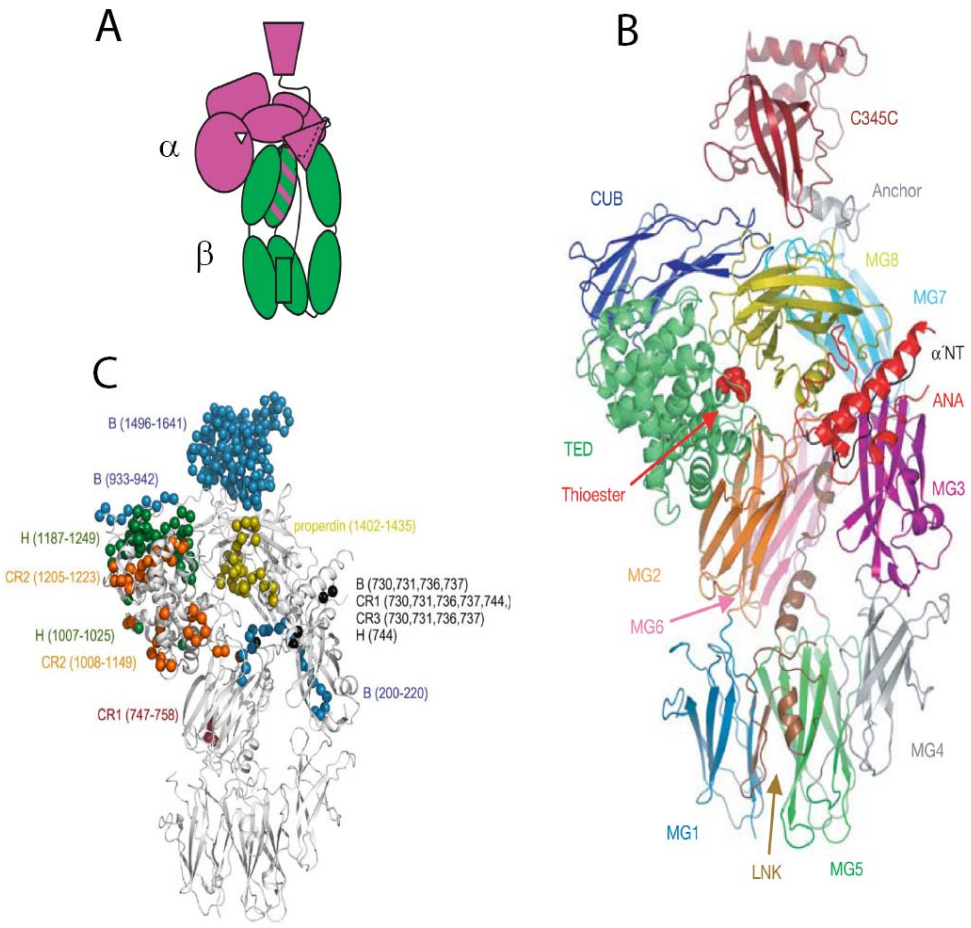


Figure 1.4. The structure of the human C3 molecule.

A) *The molecule consists of 13 domains, 6-6 domains are formed by α and β chains, while surprisingly, one domain is formed by parts of both chains.*

B) *The structure study revealed that C3 contains 8 macroglobulin domains (MG); six of these domains along with the linker domain (LNK) form the β chain. MG1-6 shape a structurally stable platform, that is called the β ring, onto which the flexible domains of the α chain are crafted. The α chain starts with the anaphylatoxin domain (ANA), which when cleaved off, forms the C3a fragment. Most of the C3g and C3f form the CUB domain while the remaining part of C3g and C3d together shape the thioester-containing domain (TED). Finally, residues 1496-1641 make up a carboxyl-terminal C345C domain with a netrin-like fold.*

C) *Putative binding sites mapped on the structure of C3 (Janssen and Gros, 2007)*

1.2.2. The function of the internal thioester in the C3 molecule

One of the most arresting feature of the C3 molecule is that following activation the C3b fragment can bind covalently to the activating surface (Law and Levine, 1977). For this attribution, a thioester bound is responsible which is formed during post-translational modification, as a result of intramolecular transacylation between the thiol group of cysteine and the γ-amide group of the glutamine within the sequence Cys^{1009}-Gly-Glu-Gln^{1012} (Sahu and Lambris, 2001;Tack *et al.*, 1980).

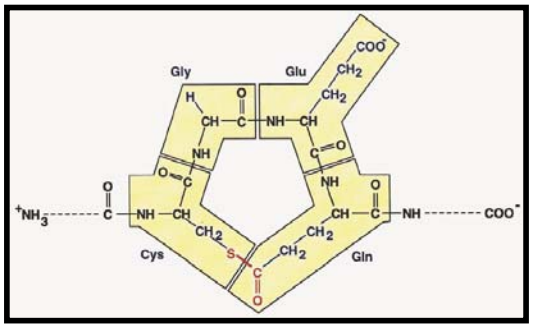


Figure 1.5. Composition of the C3 thiolactone ring (Law and Dodds, 1997)

The process of covalent binding goes through short half-life intermediers. First a histidine at position 1126 attacks the thioester to form an acyl-imidasole intermedier that is possibly stabilized by Glu^{1128}. The released thiol then acts as a base to catalyse the transfer of the acyl group to hydroxyl-nucleophiles, including water (Figure 1.6) (Dodds *et al.*, 1996).

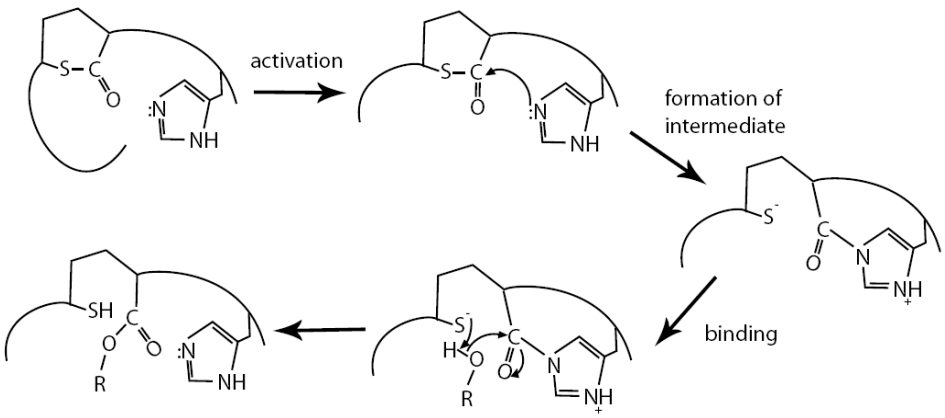


Figure 1.6. Covalent binding reaction of C3 (Law and Dodds, 1997).

The circulating native C3 is an inert molecule, the highly reactive thioester is shielded in a hydrophobic/aromatic pocket, between the MG8 and TED domains, 85Å away from the solvent, and the His^{1126} and Glu^{1128} that are necessary for the formation of the reactive intermedier are also far from the thioester group. The ANA domain has critical role in protecting the thioester, it may serve to keep MG8 in a correct position for interaction with TED and possibly, to induce a conformation of MG8 that enhances interaction with TED. The removal of ANA, yielding C3b, weakens the interactions between MG8 and TED, thereby allowing TED to swing out of its nested position (Figure 1.7) (Janssen *et al.*, 2005;Janssen *et al.*, 2006). The thioester completely exposes to the surface of the C3b molecule and it can bind preferentially to hydroxyl and at much more less extent to amide groups, forming an ester or amide linkage to the targeted surface on which complement activation is occurring. The half-life of C3b is only ~60μs (Sim *et al.*, 1981), the binding reaction is not efficient, typically only about 10% of generated C3b binds to target while the remaining 90% remains in fluid phase, where it is inactivated rapidly (Law and Dodds, 1997). The metastable C3b does not react randomly or non-specifically, rather it prefers specific OH groups and specific residues on proteins. Yet it needs to be emphasized that the nascent C3b fragment does not have the ability to discriminate between self and non-self structures.

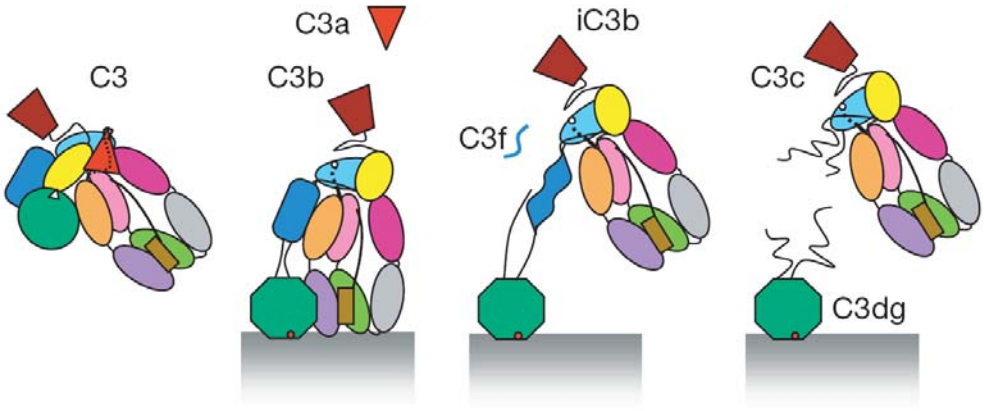


Figure 1.7. Proposed model for the conformational pathway of C3.
The native C3 is an inactive molecule, but following activation, the formerly buried thioester bond appears on the exterior of the molecule and binds covalently to hydroxyl groups of the activator surface. In the next steps factor I and cofactors cleave off the small C3f then the large C3c fragment, so finally only C3dg stays attached to the surface (Janssen et al., 2006).

1.2.3. Limited proteolysis of the C3 molecule

The three pathways of complement activation converge at the C3 glycoprotein, when fragments are cleaved off from the molecule as a result of limited proteolysis. The formation of classical (C4b2a) or alternative (C3bBb) C3 convertases leads to the cleavage of native C3 between residues 726 and 727 (Arg-Ser), thus the anaphylatoxin C3a and the major fragment C3b are generated (Figure 1.8). A carboxypeptidase is responsible for the inactivation of the C3a anaphylatoxin by the removal of the C terminal arginine and formation of the inactive $C3a_{desArg}$ molecule (Bokisch and Muller-Eberhard, 1970;Zwirner *et al.*, 1998).

The formation of C3b results conformational changes (Janssen *et al.*, 2006;Wiesmann *et al.*, 2006) and binding sites appear on the molecule for the C5, properdin (P), factors H, B and I, complement receptor1 (CR1) and the membrane co-factor protein (MCP). Inactivation of C3b by factor I proceeds in three steps with the help of co-factor molecules (MCP, CR1, or factor H). The cleavage between residues 1281 and 1282 (Arg-Ser) results in the formation of $iC3b_1$ and the cleavage between residues 1298 and 1299 (Arg-Ser) liberates the small C3f fragment and yields $iC3b_2$. The third factor I cleavage site, cut with the help of CR1 or factor H, is at residues 932-933 (Arg-Glu) of the α chain. This cleavage generates the C3c and C3dg fragments.

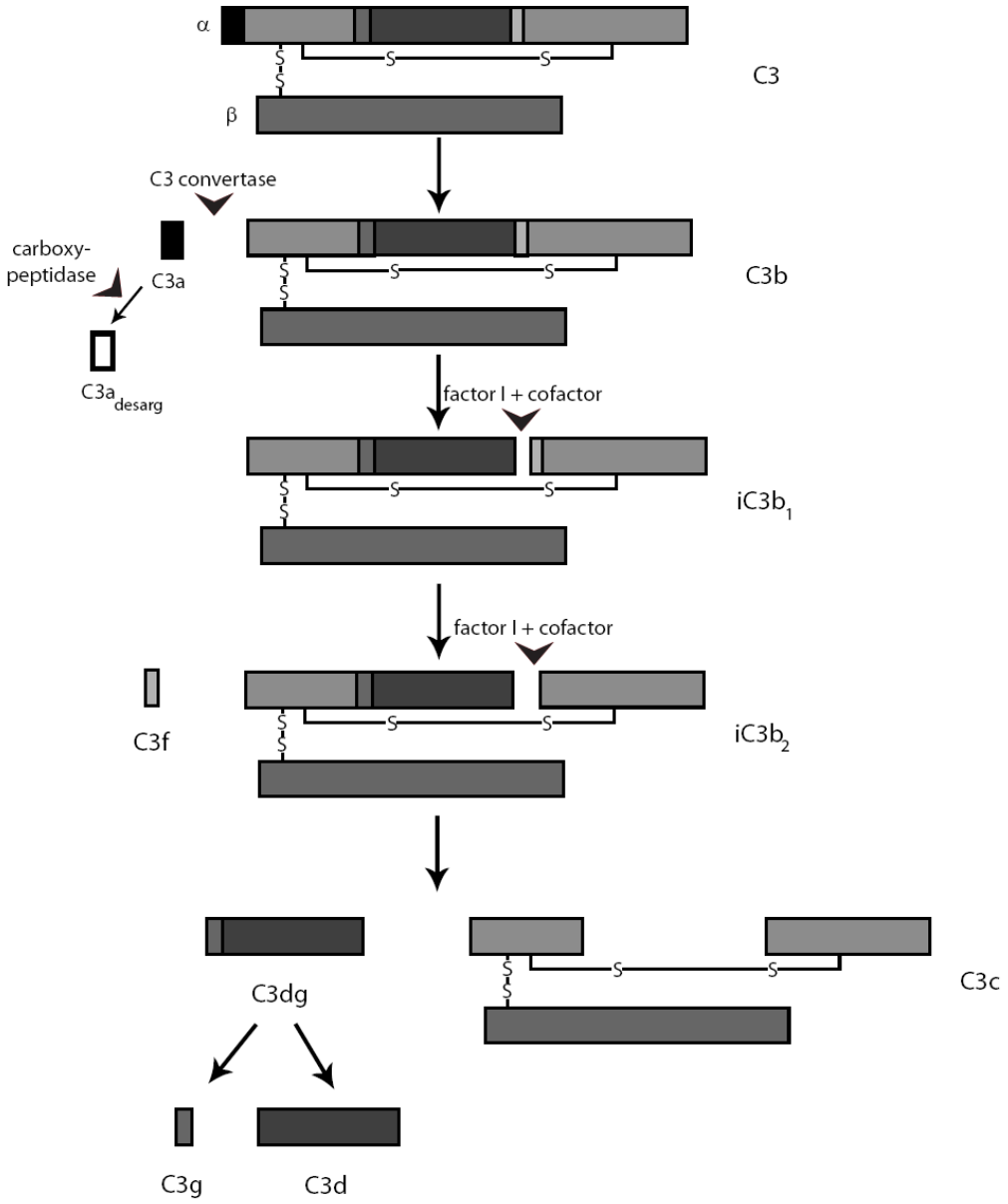
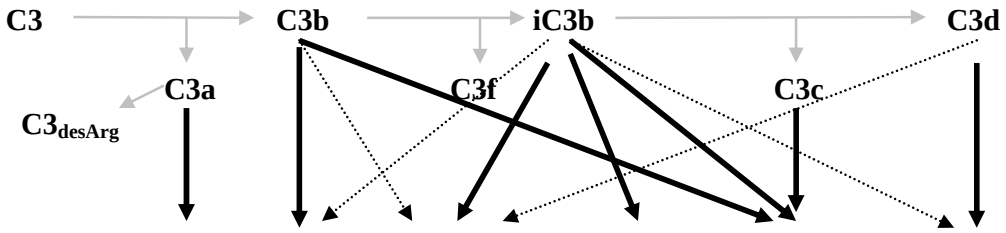


Figure 1.8. The limited proteolysis of the C3 molecule

Only disulphide bridges relevant for interchain bonds are shown.

1.2.4. Receptors for various C3 fragments

Native C3 does not bind to any receptors, thus does not exhibit biological activity, while the C3 degradation products interact with various proteins, yielding a diverse set of biological responses. The following six cell surface receptors responsible for the various effects of the C3 fragments have been identified: C3aR, CR1, CR2, CR3, CR4 (Gergely and Erdei, 2000) and the newly discovered CRIg (Gros *et al.*, 2008;Helmy *et al.*, 2006;Wiesmann *et al.*, 2006). The major properties of these receptors are listed in table 1.4.



	C3aR	CR1 CD35	CR3 CD11b/CD18	CR4 CD11c/CD18	CRIg	CR2 CD21
granulocyte	+	+	+	+	-	-
monocyte	+	+	+	+	-	-
macrophage	+	+	+	+	+ (K)	-
erythrocyte	-	+ (-)	-		-	-
T cell	-	+ (-)	-		-	+ act.
B cell	-	+	-		-	+
FDC	-	+++	+		-	+++
DC	?	?	+/-	+	-	?
Mast cell	+	+				

Table 1.4. Tissue distribution of the C3 fragment specific receptors
K: Kupffer cells; brackets refer to murine expression
(Helmy et al., 2006)

1.3. Diagnostic methods for the detection of complement activation

In the last several decades routine complement analysis in the clinic was usually associated only with the quantification of C3 and C4, measurement of C1-inhibitor and screening for complement activity. In recent years, the field of complement analysis has expanded considerably and new methods were developed makeing the survey of this enzyme cascade easier, faster and more precise (Mollnes *et al.*, 2007).

The functional assays of the complement system assess the integrity of the individual activation pathways. These assays give global view about this system and with a subsequent analysis of various components the cause of the complement deficiency can be assigned. The haemolytic assay is a commonly used technique for the measurement of the classical pathway. The result of the CH50 test is expressed as a reciprocal dilution of the sample needed to achieve 50% lysis of fixed amount of antibody-coated sheep erythrocytes. In a similar setup, where rabbit or guinea pig erythrocytes are used in the presence of Mg^{2+} but not Ca^{2+}, the activity of the alternative pathway and the value of AH50 can be determined. Several variants of these assays are known, which are easier to perform and are able to handle large numbers of various samples (Nilsson and Nilsson, 1984;Truedsson *et al.*, 1981). The haemolytic assays are frequently used, but one has to keep in mind some of its disadvantages, e.g. it does not give information about the lectin pathway and properdin deficiency; furthermore, the use of erythrocytes can be problematic. A new commercially available ELISA based method (Wielisa®, Wieslab, Lund, Sweden) for the functional assay of the complement system can provide a solution for the limitations of the haemolytic assays. This method is based on the observation that following initiation of the complement pathway, complement components (even the terminal complex) bind to the plastic surface of the ELISA plate. For the activation of the classical, lectin and alternative pathways, IgM, mannan and LPS coat is used, respectively. During the separate detection of a pathway, the other two have to be blocked. Using the suitable ions (Ca^{2+} or Mg^{2+}) or selective blocking of a pathway (anti-C1q antibody for the blockade of classical beside lectin route measurement) guarantees the selective measurement of a pathway. The readout of these measurements is the detection of the incorporated C9 by a suitable antibody (Roos *et al.*, 2003;Seelen *et al.*, 2005). These assays are suitable for measuring only the overall condition of the complement system. In the future it will be necessary to develop a suitable technique for the detection of complement activation when it reacts with a specific pathogen derived antigen or with an autoimmune disease related autoantigen (Atkinson and Frank, 2006).

When a pathway is defective, measurement of the concentration of complement components can assign the missing molecule. Various tests like Western blot, ELISA, nephelometry, turbidometry, radial immundiffusion, are available for the quantitative or semi-quantitative measurement of the complement proteins. When

complement deficiency is present but the levels of complement proteins are in the physiological range, the presence of a non-functional or dysfunctional variant of the complement component is suspected. The best way to detect the functional activity of a single component is to test the capacity of the sample to reconstitute the total complement activity of a serum that is deficient for a known component. This can be done either by haemolytic or ELISA assays (Seelen *et al.*, 2005). The measurement of the regulatory proteins has clinical relevance, as well. For the diagnosis of haemolytic uraemic syndrome, the functional detection of C1-inhibitor is suggested by an assay which is based on the action of C1inh as an enzyme inhibitor and the use of chromogenic substrate (Munkvad *et al.*, 1990). Another important complement regulator protein is factor H. For the detection of factor H deficiency, unsensitised sheep erythrocytes are incubated with the serum in question. In normal serum, factor H binds to the erythrocytes and protects them from lysis, whereas sera lacking functional factor H cause erythrocyte lysis (Sanchez-Corral *et al.*, 2004).

The haemolytic activity and individual component measurements are useful as a first level screening technique but are not suitable for the measurement of the pathologically increased complement activation *in vivo*. For assessing in vivo complement activation the measurement of the various complement activation products is the better solution. One always has to consider the half-life of the measured active component in these assays. The half-life of C5a, SC5b-9 and C3 activation products are 1min, 50-60 min and a few hours, respectively. (Mollnes *et al.*, 2007). ELISA and flow cytometry microbeads are suitable techniques for the quantification. Both techniques need monoclonal antibodies which recognize only those neoepitopes which are exclusively exposed on active split products, thus inactive proteins do not disturb the quantification.

Genetic analysis of complement components is also available, but these techniques are more expensive and harder to use for screening purpose. The determination of point mutations and polymorphic variants in genes of complement component is carried out usually for research and not diagnostic purpose.

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Chapter 2

Objectives

The complement system is a fundamental element of humoral innate immunity but it has an important effect on adaptive response, as well. Complement proteins form a bridge between these two arms of the immune system. The most versatile molecule of this cascade is its third component, C3. This complement protein is situated in a key position of the complement cascade and it exerts several effects through different receptors specific to various activation fragments.

Studying the structural and functional properties of the C3 molecule we set the following four goals:

1. **Generation and characterization of monoclonal antibodies recognizing various mouse C3-fragments.**

   For the better understanding of the human complement system, animal models are necessary. Our aim was to produce and characterize a series of monoclonal antibodies specific to various C3 fragments; these antibodies can help in the study of the mouse complement system.

2. **Defining conformational changes of the C3-C3b conversion in fluid phase.**

   As a consequence of complement activation the C3a fragment is deliberated from C3 by the convertase enzymes. Following this cleavage, approximately one-tenth of nascent C3b binds covalently to the activator surface and induces multiple effects through binding to its receptors. Our goal was to study the conformational changes of the C3-C3b conversion in fluid phase; these results could complete the already known static picture which was derived from x-ray crystallographic experiments.

3. **Following the fate of cell surface bound C3-fragments.**

   C3 fragments can bind not only to the surface of the pathogens, but onto the membrane of various cells, as well. C3 fragments deposited on the cell membrane of antigen presenting cells facilitate their antigen presenting capability to T cells in the presence of suboptimal antigen dose. Our aim was to follow the fate of these cell membrane-bound C3 fragments.

4. Developing a new technique for the simultaneous detection of antibody binding and complement activation by various antigens.

The complement system is known to have a role in the pathogenesis of several diseases, so the measurement of its activation has an important diagnostic value. Our aim was to develop a new method which makes easier the parallel detection of the complement activating properties of antigens and antibodies.

Chapter 3

Summary of the new scientific results

1. Generation and characterization of monoclonal antibodies recognizing various mouse C3-fragments.

We generated 8 rat monoclonal antibodies which are specific to various fragments of mouse C3. Clone 3/11 detects C3a, while the others recognize the larger fragments. Usability of these antibodies was determined in ELISA, Western-blot, cytofluorimetry and immunhistochemistry. Two of the eight antibodies are capable of modulating the complement system; they can inhibit the lysis of red blood cells in hemolytic assay. Antibodies produced by clone 3/26 were found to increase C3 deposition following incubation of B cells in autolougos serum (chapter 4.).

2. Defining conformational changes of the C3-C3b conversion in fluid phase.

H/D exchange method coupled with mass spectrometry was carried out for the comparison of the conformational changes of C3-C3b conversion. By combining two forms of mass spectrometry (MALDI, ESI) we could reach 61% coverage on the C3b sequence. Based on our result most part of the molecule has the same conformation both in C3 and C3b. Eight out of the 82 detected peptides showed higher solvent accessibility in C3b, in good agreement with the crystallographic result. These peptides concentrated on TED, CUB, C345C and MG8 domains which are the binding sites for factor H, factor B and properdin, i.e. those components that bind only to C3b. In contrast, seven peptides were found that are more exposed in C3 than in C3b, what does not fit to the known 3d structure of these molecules. Surprisingly, these peptides cover the binding site for the CRIg receptor (chapter 5.).

3. Following the fate of cell surface bound C3-fragments.

Our results revealed that in mice the CR1/2 receptor is not the major acceptor-site for the nascent C3 fragments – in contrast to its human counterparts. Following the fate of cell surface deposited C3-fragments we found that they are concentrated in small patches and their amount decreases in time. We demonstrated that the cells remove these fragments by forming exosomes, which are able to increase the antigen presenting properties of these small vesicles (chapter 6.).

4. **Developing a new technique for the simultaneous detection of antibody binding and complement activation by various antigens.**

We developed a protein chip based method for the measurement of complement activation which allows the parallel measurement of the complement activating properties of many antigens and antibodies easily. We proved that following serum treatment on a protein array, bound antibodies and their complement activating capacity is simultaneously measurable. Drawing data of IgG and C3 measurements on the same 2D plot has immunological representation; in this way the Th1 or Th2 dominance following immunization is easily distinguishable. These results show the possible applicability of this technique in diagnostic (chapter 7-8.).

Chapter 4

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Novel monoclonal antibodies against mouse C3 interfering with complement activation: description of fine specificity and application to various immunoassays

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Novel monoclonal antibodies against mouse C3 interfering with complement activation: description of fine specificity and applications to various immunoassays

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Abstract

The role of complement proteins in various pathophysiological settings has been studied primarily using mouse models of disease. However, the specific contribution of C3-derived fragments to these biologic processes has not been addressed in a rigorous manner because of a lack of antibodies that can selectively recognize mouse C3 or any of its degradation fragments. Here we report the generation and characterization of a panel of rat monoclonal antibodies reacting with mouse C3 and its degradation products. We describe their performance in various immunological assays such as ELISA, Western blotting, flow cytometry and immunohistochemistry. Of all the antibodies generated, one selectively recognized the C3a anaphylatoxin, and all other reacted with C3c. Furthermore, two monoclonal antibodies preferentially reacted with the cleaved C3 fragments C3b/iC3b/C3c but not native C3. Except for the one recognizing C3a, all antibodies were suitable for detecting C3 deposited on cells and tissues, two effectively inhibited the hemolytic activity of mouse complement and one enhanced C3-deposition to the cell membrane. These novel monoclonal antibodies may serve as useful reagents for elucidating functions mediated by C3-derived fragments in various pathophysiological conditions.

Keywords: Mouse; C3; Monoclonal antibody; ELISA

1. Introduction

C3, the most abundant complement protein in serum, plays a central role in the complement activation cascade. Its cleavage product, C3b, forms an integral part of the C3 and C5 convertases (Rawal and Pangburn, 2001), promoting complement activation and the subsequent formation of the membrane attack complex. Covalent attachment of C3b to the activating surface results in opsonization of foreign antigens and provides vital co-stimulatory signals to elements of the acquired immune response via specific interactions with complement receptor-bearing cells (Nielsen and Leslie, 2002; Barrington et al., 2001; Sahu and Lambris, 2001; Volanakis, 2002). C3a, a peptide released from the N-terminus of the α-chain of C3, possesses anaphylatoxic as well as various immunoregulatory properties (Hugli, 1990).

Apart from serving as an essential link between innate and adaptive immunity and acting as an inflammatory mediator, C3 has recently been implicated in developmental and non-inflammatory processes such as hematopoiesis, skeletal and vascular development, and reproduction (Mastellos and Lambris, 2002). Complement activation also occurs in numerous pathological and clinical conditions (e.g., infection, autoimmunity, and transplantation) and is thereby associated with detrimental effects such as sustained tissue injury and excessive inflammation.

C3 deficiency results in impaired immune responses against a variety of antigens and pathogens (Singer et al., 1994). A critical role for C3 and its receptors in regulating acquired immunity is also underscored by the inability of mice deficient in CR1/2 (Haas et al., 2002) or CR3

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(Rosenkranz et al., 1998) to mount a proper immune response to pathogens.

The use of appropriate transgenic mouse models has facilitated the study of C3-mediated functions in vivo. However, a lack of antibodies that can selectively recognize murine C3 or any of its degradation fragments has considerably impeded the in-depth characterization of C3-mediated functions. Thus, developing tools for the detection of C3 and its split products in mice has become a necessity for complement researchers, to better understanding the functions of the complement system both in physiological and pathological conditions. Moreover, antibodies inhibiting complement activation in mice may provide useful therapeutic tools once successfully tested in appropriate mouse models. Here we describe the fine specificity and possible applications of a set of novel monoclonal antibodies (mAbs) generated against mouse C3.

2. Materials and methods

2.1. Animals

Eight to 12-week-old female LOU/M/WSL rats and Balb/c mice obtained from the National Institute of Oncology, Budapest were used in all experiments performed according to EC regulations.

2.2. Generation of hybridomas

Rats were immunized by intraperitoneal injection of mouse C3-coated Sepharose beads (Tosic et al., 1989) and 100 μg purified mouse C3 emulsified in complete Freund's adjuvant for priming or in incomplete Freund's adjuvant for boosting. Mouse C3—purified according to Van Berg et al. (1989)—was dissolved in phosphate-buffered saline and administered intravenously three days before the fusion of rat spleen cells and Sp2/0-Ag14 mouse myeloma cells in the presence of PEG 6000 (Sigma–Aldrich, Hungary) using standard techniques Tosic et al. (1989). Clones producing specific anti-mouse C3 antibodies were selected based on antibody reactivity with the target antigen, assessed by solid-phase enzyme assay and Western blotting.

2.3. ELISA to measure the binding of rat mAbs to mouse C3 fragments

Ninety-six-well microtiter plates (Propilén Kft., Pécs, Hungary) coated with purified mouse C3 (5 μg/ml) were used for screening hybridoma supernatants for the presence of monoclonal antibodies. Serial dilutions of rat hybridoma supernatanats were added, and biotinylated mouse or goat anti-rat IgG and streptavidin-peroxidase (Sigma–Aldrich, Hungary) were used to detect bound mAb. For the selection of hybridomas reacting with mouse C3c, microtiter plates were coated with purified mouse C3c (5 μg/ml), and bound mAbs were detected by the addition of HRP-conjugated goat anti-rat IgG. Similarly, for selecting mAbs reacting with synthetic mouse C3a (Spruce et al., unpublished observations), plates were coated with mouse C3a (1 μg/ml), and bound mAbs were detected with HRP-conjugated goat anti-rat IgG. Plates coated with rat isotype-specific, purified mouse mAbs (Serotec) were used to determine the IgG subclass of the selected mAbs.

2.4. ELISA to measure native and activated C3 fragments in mouse plasma

Microtiter wells coated with 50 μl of 2.3 μg/ml anti-rat IgG Fc (ICN Cappel) in PBS pH 7.4 for 2 h at 25 °C or overnight at 4 °C were then saturated with 200 μl of 10 mg/ml BSA (Sigma) in PBS (blocking buffer) The rat anti-mouse C3 mAb supernatants of clone 2/11 and 2/16, were diluted in blocking buffer and added at their optimal concentration based on previous titration experiments.

Mouse plasma was collected by intracardiac puncture of isoflurane anesthetized mice using 50 μg/ml Lepirudin (Refludan®, Aventis) (Mollnes et al., 2002). A portion of the plasma was activated with 2 mg/ml zymosan for 30 min at 37 °C. Samples of activated, non-activated, and 20 mM EDTA-treated plasma were serially diluted in blocking buffer (starting dilution 1:1000) and added to wells. Bound mouse C3 was detected with 3.2 μg/ml of HRP-conjugated goat anti-mouse C3 in blocking buffer (ICN Pharmaceuticals, Inc., OH). The reaction was developed by adding ABTS (Roche), and 0.033% H_2O_2 in 0.1 M CH_2COONa pH 4.2. The optical density was measured in an ELISA reader at 405 nm. All incubations following the blocking step were performed at 25 °C for 1 h, and unbound proteins were removed by washing with PBS, pH 7.4, containing 0.05% Tween 20.

To assess which antibodies recognize specifically the C3b fragment of mouse C3, degradation of C3b to iC3b in zymosan treated serum was prevented using K76COOH as previously described (Hong et al., 1981). Briefly, one volume of mouse serum was mixed with three volumes of 4 mg/ml K76COOH (kindly provided by Dr. T. Kinoshita, Osaka, Japan) dissolved in GVB^{++} or PBS as control and incubated with rotation for 30 min at 25 °C. The generated C3 fragments were detected as described above.

2.5. Western blotting

Electrophoresis of purified mouse C3 (1 μg; a kind gift of Barbara Uzonyi, ELTE, Budapest, Hungary) was performed under reducing or non-reducing conditions with 7.5% polyacrylamide gels. Proteins were transferred onto a nitrocellulose membrane (Bio-Rad), which was then blocked with 5% milk and incubated with supernatants of anti C3 mAbs diluted 1:10 for 1 h at room temperature. Biotin-conjugated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA)

diluted 1:1000 was used as a secondary antibody and the detection was carried out using chemiluminescence.

2.6. Flow cytometric detection of C3 fragments fixed to the cell membrane

Mouse splenocytes (3×10^5) were incubated in 100 μl of 10×-diluted autologous, freshly drawn mouse serum at 37 °C for 1 h, and washed three times with PBS. Cells were then incubated with 80 μl of supernatants of anti-C3 mAbs for 20 min at 4 °C, then washed again three times. FITC-conjugated goat anti-rat IgG (Organon Teknika Cappel, Durham, NC) was used to detect bound primary antibody. After washing, cells were incubated with Cy3-conjugated goat anti-mouse kappa light chain (Southern Biotechnology, Birmingham, USA) for 20 min at 4 °C. FACS analysis was carried out on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) and data were analyzed by WinMDI software.

2.7. Immunohistochemistry

Frozen kidney sections (5 μm) from mice treated with anti-glomerular basement membrane antibodies (generous gift of Dr. Wen-Chao Song) (Sogabe et al., 2001) were fixed in acetone for 5 min at room temperature. Endogenous peroxidases were quenched by incubation at room temperature in freshly prepared 3% hydrogen peroxide in methanol. Avidin and biotin blocking steps were performed using a Vectastain avidin–biotin blocking kit (Vector Laboratories, Burlingame, CA), followed by incubation with 4% rabbit serum in Tris Base Saline (TBS). Culture supernatants containing anti-C3 antibodies, diluted 1:5–1:25 in 1% bovine serum albumin in TBS, were applied to sections and incubated overnight at 4 °C. Biotinylated rabbit anti-rat IgG (Vector Laboratories), diluted 1:200 in 1% bovine serum albumin in TBS, was added to tissue sections. Standard peroxidase ABC reagent and 3,3′-diaminobenzidine substrate incubations were performed according to manufacturer's instructions (Vector Laboratories). Slides were counterstained with hematoxylin and assessed by light microscopy (Olympus BX 60).

2.8. Hemolytic assay to assess the ability of mAbs to inhibit mouse complement activation

The procedure described by Tanaka et al. (1986) was used with minor modifications. Antibody against rabbit erythrocytes (RE) was produced in guinea pigs. 300 μl of a 1% suspension of RE mixed with the same amount of complete Freund's adjuvant was injected subcutaneously, followed by seven sequential injections with incomplete adjuvant at weekly intervals. Seven days after the last injection, animals were exsanguinated under ketamin anesthesia by cardiac puncture. Serum was inactivated for 1 h at 56 °C and stored at −20 °C until use. RE were sensitized by adding antiserum diluted four-fold to 3×10^8 ml^{-1} RE in 10 mM EDTA*GVB solution at a 1:1 ratio. After incubation for 30 min at 37 °C, the RE were washed three times with PBS, and their concentration was adjusted to 1.5×10^8 cells/ml.

Hemolytic assays were carried out in 96-well U-bottom plates using 8.3 μl mouse serum diluted four-fold in GVB and 33 μl of 1.5×10^8 cells/ml sensitized RE. Hybridoma supernatants were added at the indicated dilutions, and samples were incubated for 1 h at 35 °C. Reactions were stopped by placing the plates on ice for 10 min. After centrifugation, 10 μl of the supernatants were mixed with 100 μl tetra-methyl-benzidine solution. The reaction was stopped with 100 μl 2 N H_2SO_4, and the OD of the samples was measured at 450 nm.

2.9. Modulation of C3 deposition on B cells by mAb 3/26

Mouse splenocytes (3×10^5) were incubated in a mixture of 10 μl freshly drawn mouse serum and 80 or 20 μl mAb supernatant, made up to 100 μl with complete RPMI medium. After 1 h of incubation at 37 °C, cells were washed three times with PBS. FITC conjugated goat anti-mouse C3 $F(ab')_2$ fragment (Cappel) was used to detect cell-bound C3. After washing, cells were incubated with Cy3-conjugated goat anti-mouse kappa light chain (Southern Biotechnology, Birmingham, USA) for 20 min at 4 °C. FACS analysis was carried out on FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) and data were analyzed by WinMDI software.

3. Results

3.1. Reactivity of mAbs with mouse C3 and its degradation fragments

The clones producing anti-mouse C3 antibodies were selected based on reactivity with ELISA plate-bound purified C3. However, when we used purified C3c fragments or zymosan-activated mouse serum instead of purified C3 in the detection assays, we saw noteworthy differences among the hybridomas. All mAbs except clone 3/11 recognized C3c (Fig. 1A and B). This finding prompted us to test the reactivity of clone 3/11 with the C3a anaphylatoxin, and, as demonstrated in Fig. 1C, clone 3/11 was indeed positive for binding to C3a (Table 1). Adsorption of C3 to a plastic surface can result in conformational modifications that expose the mAb site(s) of the molecule (Andersson et al., 2002). As a result, we were unable to distinguish between reactivity with the native and fragments of C3. Two separate approaches were employed in order to overcome this problem. First, a sandwich ELISA was designed with the mAbs captured by anti-rat IgG-Fc antibodies. The mAbs bound various C3 forms present in non-activated or zymosan-activated serum. Subsequently, a polyclonal

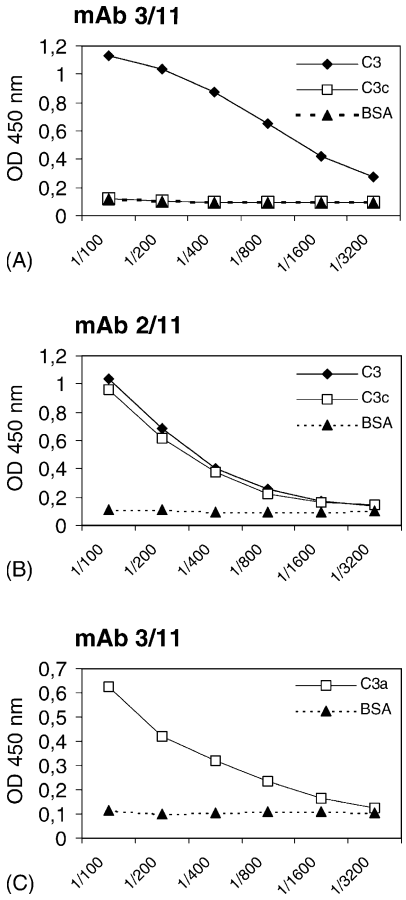

Fig. 1. mAb 3/11 specifically recognizes mouse C3a. Plates were coated with C3, C3c, and BSA (A and B) or C3a and BSA (C), and plate-bound proteins were detected with mAbs 3/11 (A and C) or 3/26 (B). Note that 2/11 is representative of all other mAbs except 3/11.

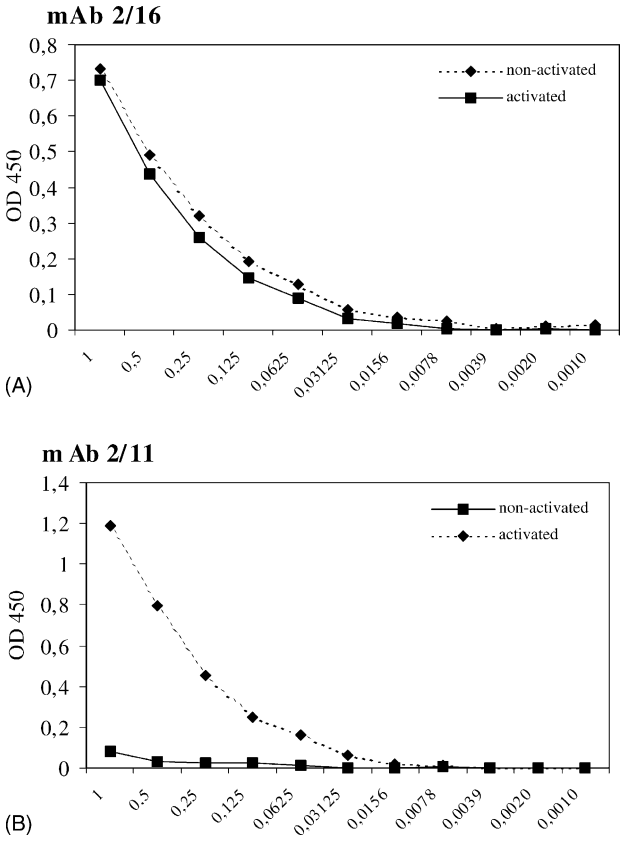

Fig. 2. mAb 2/11 preferentially recognizes activated C3 fragments. mAb 2/16 (A) or 2/11 (B) was bound to the plate by anti-rat IgG Fc antibody, then zymosan-activated or non-activated plasma was added. Captured C3 and C3 fragments were detected with HRP-conjugated goat anti-mouse C3 IgG.

antibody raised against mouse C3 was applied to detect all forms of C3 generated in the serum bound by the mAbs. Antibody 2/11 preferentially reacted with activated C3 fragments as shown in Fig. 2A. In the second approach, serum was pretreated with the monocarboxylic acid K76COOH to prevent cleavage of C3b to iC3b during zymosan-mediated activation of serum. Two of the antibodies, 2/11 and 3/26, showed significant reactivity with K76COOH-treated sera (Fig. 3).

Table 1
Summary of mAb reactivities

Clone (isotype)	Fragment specificity[a]	K76 COOH test[b]	N/A C3[c]	SB-C3b/iC3b[d]	Inhibition of hemolysis[e]	WB-NR	WB-R
2/1 (IgG1)	C3/iC3b/C3c	−	N + A	+	n.d.	+	+
2/11 (IgG1)	C3b/iC3b/C3c	+	A	+	Yes	+	−
2/16 (IgG1)	C3/iC3b/C3c	−	N + A	+	No	+	+
2/19 (IgG1)	C3/iC3b/C3c	−	N + A	+	No	+	+
2/20 (IgG1)	C3/iC3b/C3c	−	N + A	+	n.d.	+	+
2/26 (IgG1)	C3/iC3b/C3c	−	N + A	+	No	+	+
3/11 (IgG2a)	C3a	−	−	−	n.d.	+	−
3/26 (IgG2a)	C3b/iC3b/C3c	+	−	+	Yes	+	−

[a] Summary of all tests by ELISA using purified C3c and synthetic C3a.
[b] By ELISA after pretreatment of zymosan-activated serum with K76COOH inhibitor.
[c] Antibody against rat Fc was used to capture anti-C3 mAbs. After adding non-treated or zymosan-activated mouse serum, the C3-fragments were detected with an HRP-conjugated goat anti-mouse C3. N, native; A, activated.
[d] Tested by indirect cytofluorimetry using B cells incubated with fresh mouse serum (1:10 dilution). Binding of various mAbs to cell-bound C3 was visualized using FITC-labeled anti-rat Ig.
[e] Hemolytic assay using rabbit erythrocytes sensitized with antibodies generated in guinea pig.

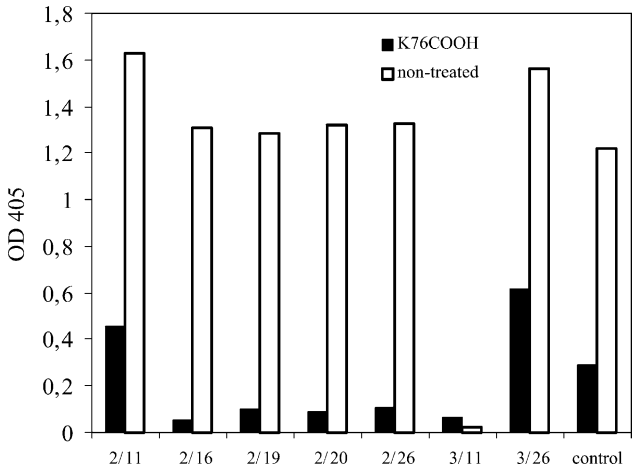

Fig. 3. Reactivity of mAbs with C3 fragments in K76COOH treated and non-treated sera. The goat anti-mouse C3 polyclonal antibody was used as positive control. Two mAbs—2/11 and 3/26—showed stronger reactivity with K76COOH-treated sera than the others.

3.2. Mapping of mAb recognized epitopes to mouse C3 chain

All eight mAbs that were found to recognize C3 by ELISA were also able to detect the entire C3 molecule under non-reducing conditions (Fig. 4). Some of the mAbs also recognized the β-chain of purified C3 under reducing conditions in Western blotting experiments (Fig. 4 and Table 1).

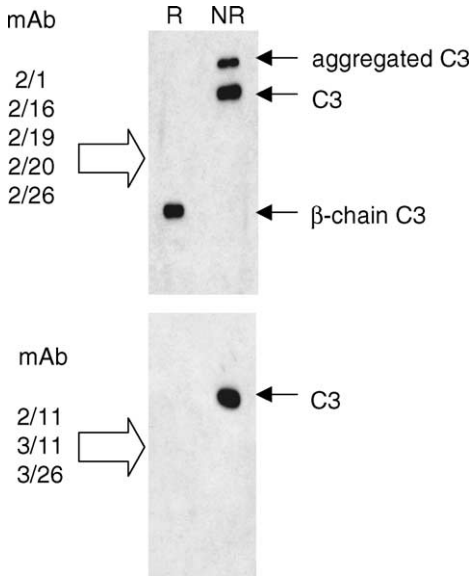

Fig. 4. Reactivity of monoclonal mAbs in Western blotting with purified C3 run under non-reducing (NR) or reducing (R) conditions.

3.3. Reactivity of mAbs with C3 fragments deposited on cells

The deposition of C3 fragments on the surface of B cells is the consequence of incubation of those cells with autologous serum and subsequent activation of the complement system via the alternative pathway (Kerekes et al.,

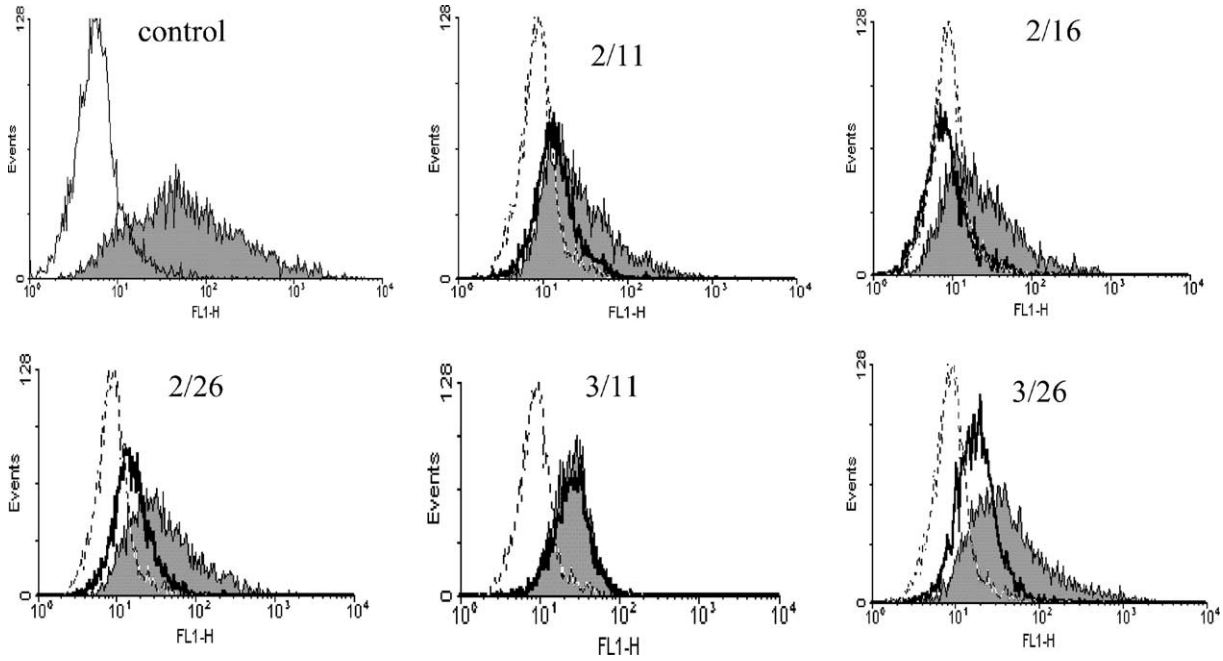

Fig. 5. Flow cytometric analysis of selected mAb binding to C3 fragments fixed on B cells. Splenocytes were incubated in autologous fresh mouse serum (shaded histogram) or EDTA-complemented serum (thick lines) to allow deposition of C3 on the cells, then the indicated monoclonal antibodies and FITC-conjugated goat anti-rat IgG were used to detect bound C3 fragments. Thin or dotted lines indicate fluorescence of unstained cells. Live B cells were gated on the basis of light scatter characteristics and Ig kappa chain positivity. Fluorescein conjugated polyclonal goat anti-mouse C3 antibody was used as a control.

1998). This model was applied in our experimental setting. Mouse splenocytes preincubated with serum from the same animal were stained with mAbs for the presence of C3 fragments. Kappa light chain of immunoglobulin positive cells (B cell lineage) were gated and analyzed for mAb binding. All mAbs except 3/11 showed positive reactivity in flow cytometry analysis. The most prominent increase in the fluorescence level was observed with the antibody 3/26 (Fig. 5). As a positive control for the detection of cell surface-bound C3, a polyclonal antibody (fluorescein

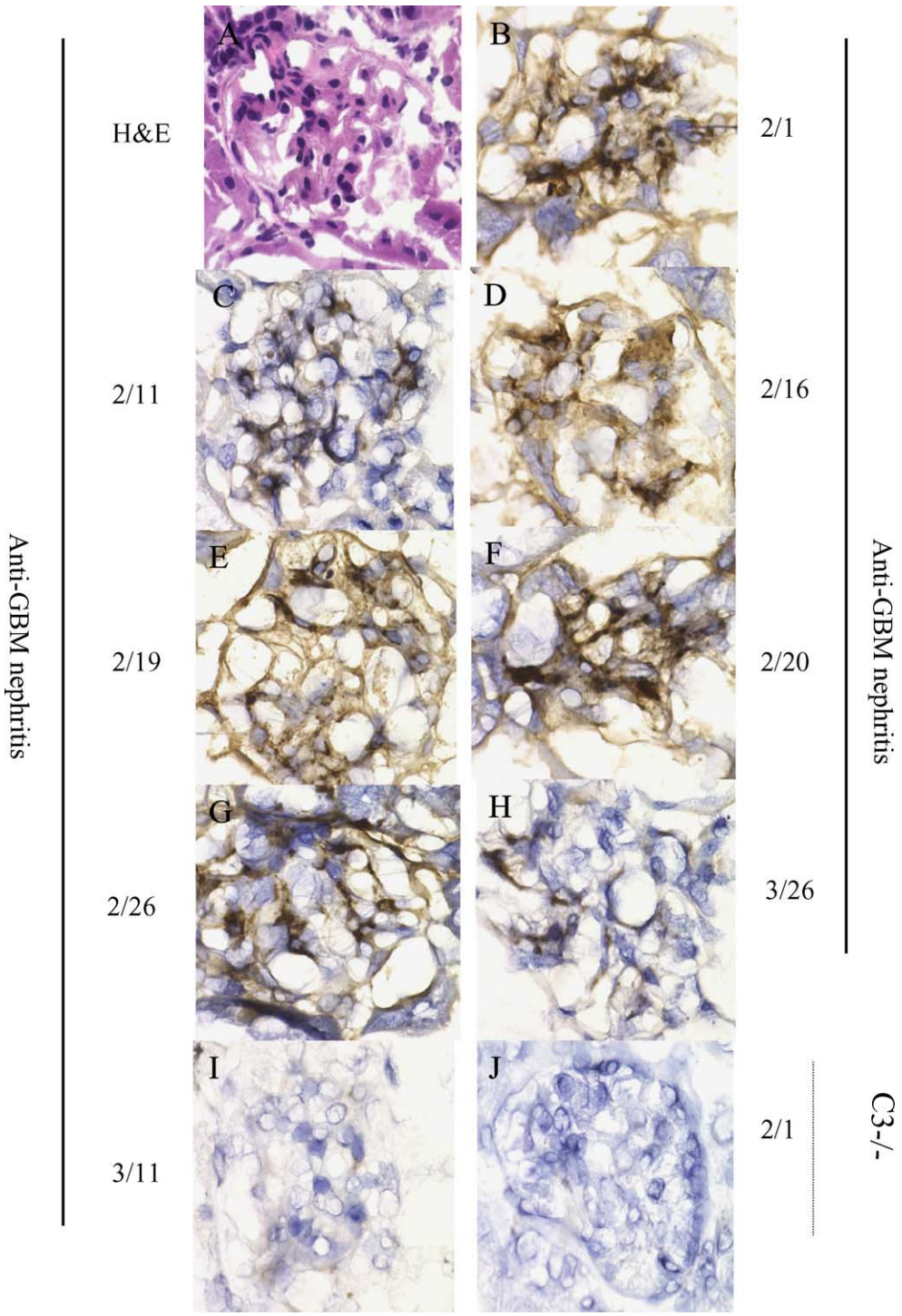

Fig. 6. Reactivity of anti-C3 mAbs with C3 deposited on glomerular basement membrane: (A) H and E staining and (B–I) immunohistochemistry of kidney sections presenting morphological features of GBM nephritis. Images B–I show immunohistochemical staining of the kidney glomeruli with mAbs 2/1, 2/11, 2/16, 2/19, 2/20, 2/26, 3/26, 3/11, respectively. (J) Histological section from a C3-deficient mouse stained with mAb 2/1 (magnification of all shown images, 600×).

conjugated goat F(ab′)$_2$ anti-mouse C3, Cappel, PA) was used.

3.4. Use of the mAbs for immunohistochemical detection of mouse C3

Histological sections from kidneys of mice treated with anti-glomerular basement antibody revealed morphological features of experimental anti-glomerular basement (anti-GBM) nephritis: segmental capillary lumen obliteration, an increase in segmental glomerular matrix and diffuse glomerular capillary wall thickening, which reflects the deposition of immune complexes and complement (Fig. 6A). All antibodies, except 3/11 (Fig. 6I), showed positive immunoreactivity, indicating C3b/iC3b deposition on the glomerular capillary walls (Fig. 6B–H). The diffuse linear pattern of C3 staining closely resembled that of immunofluorescence typical for anti-GBM nephritis. None of the mAbs reacted with kidney sections from C3-deficient mice. A representative example of this negative control is shown in Fig. 6J.

3.5. Effect of the mAbs on the hemolytic activity of mouse serum

The studies described above indicated that the mAbs could react with both native and degraded mouse C3. It is reasonable to hypothesize that they also might inhibit the formation of the C3 and C5 convertases. To verify this hypothesis, we assessed the ability of the mAbs to block complement activation and subsequent cell lysis. For this purpose we developed a sensitive hemolytic assay that allowed the detection of red blood cell lysis in the presence of culture medium. The amount of mouse serum required to achieve 70–100% hemolysis was initially determined in preliminary experiments. Culture supernatants containing the mAbs were mixed with freshly drawn mouse serum, and then added to the sensitized erythrocytes. Two of the mAbs, 2/11 and 3/26,

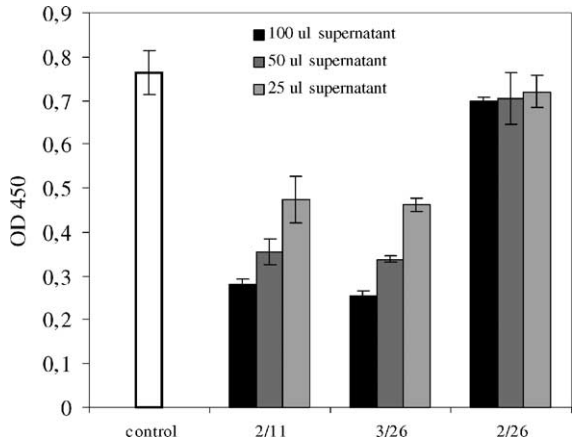

Fig. 7. Interference of mAbs 2/11, 3/26 and 2/26 with hemolytic activity of mouse serum. Fresh mouse serum was mixed with supernatants of the indicated hybridomas or with buffer as control before addition to sensitized rabbit erythrocytes. Hemolysis was measured on the basis of the peroxidase activity of the released hemoglobin using tetra-methyl-benzidine as substrate. Results are representative of at least three experiments, columns indicate mean optical densities, and error bars indicate S.D.

consistently inhibited hemolysis in a dose-dependent manner, while clone 2/26 had no effect in this system (Fig. 7).

3.6. mAb 3/26 enhances the deposition of C3 on B cells

We hypothesized that binding of the antibodies to C3 or its fragments could modify the extent of complement deposition on B cells. To test such an effect we incubated splenocytes with fresh mouse serum in the presence or absence of mAbs, and deposited C3 was detected by flow cytometry. As demonstrated in Fig. 8, mAb 3/26 facilitated C3 deposition onto B cells resulting in high C3 levels on the total B-cell population. The isotype-matched control mAb, 3/11 had no such effect. It has to be emphasized that in all experiments where mouse serum was the source of C3, sera drawn immediately before the experiment proved to be considerable more efficient than stored serum.

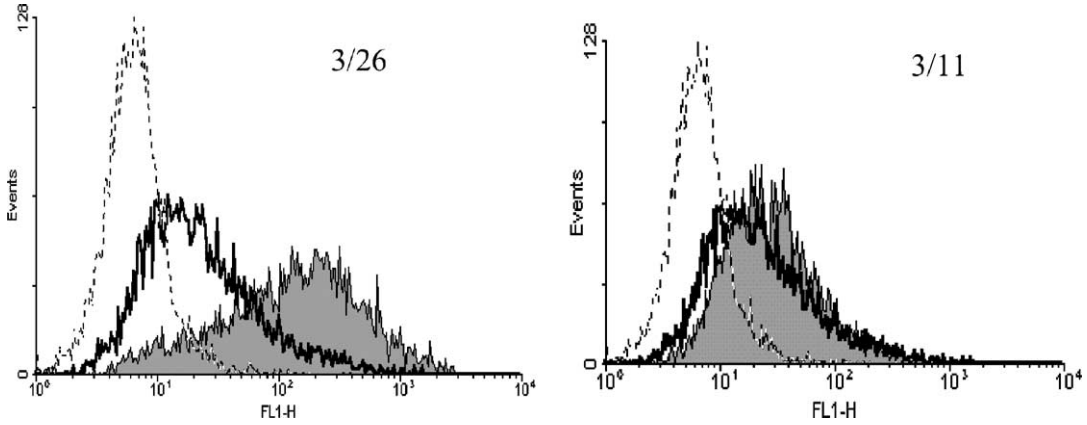

Fig. 8. Enhancement of C3 deposition on autologous B cells by mAb 3/26. Splenocytes were incubated in medium (thin line) or in fresh mouse serum without (thick line) or with 3/26 mAb (shaded histogram) for 1 h at 37 °C. As control, an isotype-matched mAb (clone 3/11) was used. Deposited C3 was stained with FITC conjugated goat anti-mouse C3. Live B cells were gated on the basis of light scatter and Ig kappa chain positivity.

4. Discussion

C3 and its activation products have been implicated in the pathophysiology of several diseases. In this respect, the advent of transgenic technology has enabled the investigation of C3-mediated functions in various pathophysiological settings, through the establishment of appropriate mouse models of disease. However, this field of complement research has long suffered from the lack of systematic reagents that can target mouse complement components and, in particular, from the lack of selective antibodies that can recognize C3 and its various bioactive degradation fragments. Despite the fact that previous studies have begun to address this problem by reporting the generation of rat monoclonal antibodies against mouse components C3 and C4 (Kremmer et al., 1990), this past effort has not yielded any antibodies that could differentially recognize fragments of C3 generated upon complement activation, or any antibodies that could effectively inhibit complement activation at various proteolytic steps of the cascade.

In the present study we report the generation and characterization of a panel of novel monoclonal antibodies that specifically recognize murine C3 as well as several of its degradation products. We have also demonstrated that these antibodies can be employed in various immunological assays to detect mouse C3 in both the fluid (serum) and solid phase (e.g., deposited on cells and tissues). Having developed a sensitive ELISA method for the detection of native (non-activated) versus activated C3, we were able to select those mAbs that preferentially reacted with the activation products of C3. Using a selective inhibitor of iC3b formation, we then showed that two of these mAbs, clones 2/11 and 3/26, selectively bind to C3b fragment of C3. The use of these mAbs will allow for the quantitation of complement activation levels in mouse fluids (e.g., serum) and provide a unique tool for the detection of C3 activation in mouse models of disease.

Our studies have also led to the identification of a single mAb (clone 3/11) that selectively binds the C3a anaphylatoxin. This antibody may prove to be a unique resource for developing sensitive ELISA schemes to quantitate complement activation in mice. It is generally accepted that ELISA assays detecting C3a generation constitute a particularly reliable and accurate means of measuring fluid-phase complement activation in various pathological conditions in humans. The identification of clone 3/11 may now provide a similar tool for monitoring acute complement activation (C3a generation) in experimental mouse models.

Studies using flow cytometric analysis and immunohistochemical staining revealed that all the mAbs, except clone 3/11, could detect C3b/iC3b fragments deposited on cells and tissues. These antibodies can serve as useful reagents for monitoring both acute and chronic complement activation as well as for detecting C3 deposition in disease mouse models.

In addition, our in vitro studies using a sensitive hemolytic assay demonstrated that mAbs 2/11 and 3/26 can also inhibit complement-mediated lysis. Interestingly clone 3/26 effectively enhanced C3 deposition on murine B cells, as well. This finding can be explained by the following main differences between the two experiments: on one hand hemolysis is initiated by the classical pathway, whereas deposition on B cells in this setting is alternative pathway mediated, on the other hand cells of different species are used in the assays. Since complement regulatory proteins function in a species specific manner, the quality and outcome of complement activation are different in the two experiments. Still, the apparently contradictory results can be resolved theoretically even for an identical system as follows. A possible mechanism of enhancement is that after recognizing cell-bound C3 (Fig. 5) 3/26 itself activates complement. This is in agreement with the finding that C3 deposition on T cells was not affected by 3/26 (K. Papp, unpublished observations). Enhancement of complement C3b(i) deposition by a monoclonal antibody against human C3b(i) has already been reported (Kennedy et al., 2003). Lysis of cells by complement requires the cleavage of C5 in order to generate the membrane attack complex. Since C3b is a constituent of the C5 convertase complex, 3/26 may bind to and inactivate these complexes, explaining its inhibitory effect in hemolysis.

The efficacy of these antibodies in interfering with complement activation in vitro will allow for the rational design of inhibition experiments targeting C3 and C3-derived fragments (C3a, C3b/iC3b) in vivo. Such studies will help clarify the mechanisms by which C3 and its bioactive fragments mediate their functions in various pathophysiological conditions. Experiments assessing the ability of these mAbs to interfere with complement activation in vivo are currently in progress in our laboratory.

Acknowledgements

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Chapter 5

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Dynamic structural changes during complement C3 activation analysed by hydrogen/deuterium exchange mass spectrometry

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Dynamic Structural Changes During Complement C3 Activation Analyzed by Hydrogen/Deuterium Exchange Mass Spectrometry

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Abstract

Proteolytic cleavage of component C3 to C3b is a central step in the activation of complement. Whereas C3 is largely biologically inactive, C3b is directly involved in various complement activities. While the recently described crystal structures of C3 and C3b provide a molecular basis of complement activation, they do not reflect the dynamic changes that occur in solution. In addition, the available C3b structures diverge in some important aspects. Here we have utilized hydrogen/deuterium exchange coupled with mass spectrometry (HDX-MS) to investigate relative changes in the solution-phase structures of C3 and C3b. By combining two forms of mass spectrometry we could maximize the primary sequence coverage of C3b and demonstrate the feasibility of this method for large plasma proteins. While the majority of the 82 peptides that could be followed over time showed only minor alterations in HDX, we observed clear changes in solvent accessibility for 16 peptides, primarily in the α-chain (α'NT, MG6-8, CUB, TED, C345C domains). Most of these peptides could be directly linked to the structural transitions visible in the crystal structures and revealed additional information about the probability of the structural variants of C3b. In addition, a discontinuous cluster of seven peptides in the MG3, MG6, LNK and α'NT domains showed a decreased accessibility after activation to C3b. Although no gross conformational changes are detected in the crystal structure, this area may reflect a structurally flexible region in solution that contributes to C3 activation and function.

1. Introduction

As a central component of innate immunity, the human complement system plays a major role in the recognition and elimination of microbial intruders and other pathogenic cells.

Abbreviations: ANA, anaphylatoxin domain; C3(H_2O), hydrolyzed C3; C3(N), nucleophilically activated C3; CRIg, complement receptor of the immunoglobulin superfamily; CUB, C1r/C1s, Uegf, Bmp1 domain; ESI, electrospray ionization; HDX, hydrogen/deuterium exchange; LNK, linker domain; MALDI, matrix-assisted laser desorption/ionization; MG, macroglobulin domain; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PDB, protein data bank; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TED, thioester domain; TFA, trifluoroacetic acid.

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Recent research revealed even more important functions for this tuned cascade of soluble and membrane proteins, such as bridging to adaptive immune responses and additional cascades (e.g. coagulation system). Activation of complement component C3 is the point of convergence in the initiation of the complement cascade by the lectin, alternative and classical pathways. This proteolytic conversion leads to the production of the biologically active effector protein C3b and the anaphylatoxin C3a from the biologically inactive protein C3. Covalent attachment of C3b on the surface of foreign cells (i.e. opsonization) induces a variety of terminal complement actions from cell lysis and phagocytosis to the stimulation of downstream immune responses (Markiewski and Lambris, 2007). Since complement activation on host cells has devastating effects and may lead to a number of severe diseases, the cascade activity has to be carefully controlled. The ability to enable and restrict molecular interaction within a single protein template is a central aspect of the control strategy.

The biological activities of C3b are directly related to the dynamic exposure of binding sites that are necessary for protein-protein interactions (Gros et al., 2007). While native C3 has a very limited amount of physiological binding partners, C3b gains the ability to bind a variety of essential proteins, including C5; properdin; factors B, H and I; membrane co-factor protein; decay accelerating factor; and complement receptor 1 (Sahu and Lambris, 2001). It is through these interactions that C3b and its breakdown fragments are capable of propagating the innate immune response and influencing adaptive immunity. It is not surprising, therefore, that increased activation of C3 or impaired regulation of C3b have been attributed to an increasing number of diseases (Ricklin and Lambris, 2007; Thurman, 2006; Volanakis and Frank, 1998), rendering these proteins as potential targets of therapeutic intervention (Ricklin and Lambris, 2007). In addition, the C3-to-C3b transition is also critical for complement evasion of human pathogens (Lambris et al., 2007). For example, the extracellular fibrinogen-binding protein from *Staphylococcus aureus* has been shown to preferentially bind the native over the activated form of C3, which may be pivotal for its inhibitory activity (Hammel et al., 2007b). Numerous biological and biophysical techniques have been utilized to investigate the structural changes accompanying the increase in function upon activation of C3. Earlier evidence accumulated through neoepitope mapping with antibodies (Alsenz et al., 1990), chemical modification strategies (Isenman et al., 1981), solution scattering (Perkins and Sim, 1986), and electron microscopy (Smith et al., 1984) indicated significant structural rearrangements during this process. These observations have recently been confirmed by the publication of crystal structures for C3 (Janssen et al., 2005) and C3b (Abdul Ajees et al., 2006; Janssen et al., 2006; Wiesmann et al., 2006) as well as detailed electron microscopy studies (Nishida et al., 2006). While these studies offered a first insight into the mechanism by which C3 activation is propagated, they all were taken under static, non-solute conditions. Even more, the available crystal structures for C3b diverge in some important points and are currently matters of scientific debate (Janssen et al., 2007). Given these structural uncertainties, the versatile biological functions of C3b, as well as the dynamic process in which C3 activity is regulated, more detailed information about how this rearrangement takes place in solution is highly sought after.

Hydrogen deuterium exchange (HDX[1]) coupled with mass spectrometry (MS) has evolved into an indispensable tool for characterizing such dynamic structural changes in solution. HDX takes advantage of the ability of amide backbone hydrogen atoms to exchange with water hydrogens in solution (Busenlehner and Armstrong, 2005; Hoofnagle et al., 2003; Wales and Engen, 2006). When D_2O is substituted for H_2O in the buffer in which the protein is dissolved, solvent deuterium atoms exchange with backbone hydrogen atoms at a rate influenced by the local structure of the protein. Amide hydrogens that are buried within the protein or are involved in hydrogen bonds exchange more slowly with solvent deuterium atoms than do more accessible hydrogen atoms at the protein surface (Englander and Kallenbach, 1983). By analyzing the rate and/or magnitude of the deuterium incorporation into the protein backbone, one can make inferences about the relative structure of the protein. To monitor the amount of deuterium incorporation, a physical technique such as nuclear magnetic resonance (NMR) or MS is employed (Englander, 2006). The choice of analytical technique depends upon the size of the protein; NMR is best suited for smaller proteins (less than ~30 kDa) while MS allows for the investigation of proteins in excess of 30 kDa. However, low sequence coverage and other experimental parameters render the analysis of larger proteins increasingly difficult for MS as well (Cravello et al., 2003). Characterization of multidomain plasma proteins such as C3 (184 kDa; Fig. 1A) therefore demands an especially high level of instrumental and experimental precision. Although HDX-MS does not provide a three-dimensional structure of the protein, it is capable of reporting structural changes when the protein is studied under varying conditions or in different states (Eyles and Kaltashov, 2004; Hamuro et al., 2003; Schuster et al., 2007). Furthermore, the technique has also been utilized to identify interacting surfaces in protein-protein (Melnyk et al., 2006) and protein-ligand interactions (Hamuro et al., 2006). HDX-MS reflects changes in protein structure that occur in solution but might not be evident from crystallographic structures. As a consequence, the combination of HDX-MS with high-resolution crystal structures can provide an unequaled insight into the solution phase dynamics of proteins.

In a previous study, we have successfully used HDX-MS to investigate the structural changes in C3 that take place during its hydrolysis to C3(H_2O) (Schuster et al., 2007; Winters et al., 2005). Here we have utilized this approach to investigate the relative solution structures of C3 and C3b. Two MS techniques were employed, matrix-assisted laser desorption/ionization time of flight (MALDI) and electrospray ionization with ion trap (ESI), to maximize the sequence coverage for these large proteins. When analyzed in the context of the available crystal structures for C3 and C3b, our data reveal that there is a cluster of four discontinuous peptides within the MG3, MG6, LNK, and α'NT domains that exhibit increased deuterium exchange in C3 when compared to C3b. The same regions only showed minimal differences in the two crystallographic structures, suggesting that this area may exhibit conformational flexibility in solution. These data not only indicate that this region plays an important role in the activation process but also demonstrate the applicability of the technology to large plasma proteins.

2. Materials and Methods

2.1. Protein Preparation

C3 was purified from human plasma (University of Pennsylvania Blood Bank) using established methods (Hammer et al., 1981; Katragadda et al., 2006; Sahu et al., 2000), and was precipitated by dialysis against 5 mM MES buffer (pH 6.0) at 4 °C. C3b was obtained from C3 by limited digestion with trypsin as previously described (Janssen et al., 2006) and was purified by gel filtration over a Superdex 200 size-exclusion column, followed by anion exchange chromatography over a Mono-Q column. Isolated C3b was treated with iodoacetamide to prevent dimerization, purified over a Mono-S column and then concentrated using an Ultrafree-MC centrifugal filter device. Protein purity was carefully controlled by SDS-PAGE to exclude contaminations, and aliquots of the proteins were stored at -70 °C. At the time of use, protein samples was thawed on ice, centrifuged at 3000 rpm in a Sorvall Biofuge Fresco (Thermo Scientific, Waltham, MA) at 4 °C and reconstituted in the appropriate buffer.

2.2. HDX-MS using MALDI

Aliquots of C3 or C3b were deuterated by the addition of 6 µl protein solution (4 mg/ml in PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) to 120 µl D_2O (99.9%, Cambridge Isotope Laboratories, Andover, MA). After timed intervals at room temperature (10 s, 30 s, 1 min, 2.5 min, 5 min, 10 min, 15 min), 10-µl aliquots of the protein/D_2O solution were removed, added to 10 µL of 0.2% trifluoroacetic acid (TFA) and immediately frozen in liquid nitrogen to quench the amide HDX process (final pH=2.5). To control for HDX that occurred after the quenching process, a reference exchange sample was prepared by mixing 2 µl of protein solution with 40 µl 0.2% TFA; 10 µl of this solution was then mixed with 10 µl D_2O and immediately frozen in liquid nitrogen.

Prepared samples were analyzed by MALDI MS on the same day. A single frozen aliquot was thawed at room temperature and the solution was mixed with 20 µl of immobilized pepsin (Pierce, Rockford, IL) at pH 2.6 and 0 °C for 5 min. The pepsin was quickly removed by centrifuging the sample for 30 s at 12,000 × g at 4 °C. Immediately following centrifugation, 1 µl of the peptide mixture was mixed with a 1-µl aliquot of matrix solution (20 mg/ml α-cyano-4-hydroxycinnamic acid in 1:1 acetonitrile: 0.1% TFA). 1 µl of this mixture was quickly spotted onto an ice-chilled MALDI target plate, dried under moderate vacuum for 1 min, and then analyzed on a MALDI time-of-flight mass spectrometer (MALDI micro MX; Waters, Milford, MA) at an acceleration voltage of 20 kV. Typically, 40 single-shot mass spectra were summed to give a composite spectrum. Care was taken to ensure a consistent timing between all steps for each aliquot.

Sequence assignment of the pepsin-generated peptides was performed using an MS/MS-derived C3 peptide map of 354 peptides spanning 80% of the native C3 primary structure. This peptide map had been generated in a previous study (Winters et al., 2005) using an ESI-Ion Trap LCQ-Duo mass spectrometer (Thermo Scientific).

All MALDI data are reprocessed using MassLynx software (Waters) and centroid values of the isotopic envelopes were calculated using MagTran (version 1.02, Dr. Zhongqi Zhang, Amgen Inc. (Zhang and Marshall, 1998)) as described previously (Winters et al., 2005). MALDI-based exchange reactions were performed in triplicate. For a given peptide, the average difference in deuteration was calculated by comparing the percentage of deuterium incorporation (measured deuterium incorporation divided by theoretical maximum deuterium incorporation, multiplied by 100) at each time point from the C3 sample with the corresponding time point in C3b. The mean of the resulting percentages across all time points was used.

2.3. Generation of C3 Peptide Map for ESI Experiments

In order to obtain peptide fragments of suitable size for ESI analysis, digestion conditions were optimized using non-deuterated C3. A pepsin column was prepared by immobilizing porcine pepsin on Poros 20 AL medium (Applied Biosystems, Foster City, CA) at 30 mg/ml according to the manufacturer's instructions (Hamuro et al., 2003). In general, 20 µl of 30 µM C3 was diluted with 30 µl of cold acidic buffer containing urea and tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Pierce) at varying concentrations. 45 µl of this solution was digested over a refrigerated pepsin column and separated by reversed-phase HPLC prior to analysis by ESI MS. The composition of the acidic buffer solution, the proteolysis, and the HPLC gradient were all optimized for analysis of C3. Several aliquots of non-deuterated C3 were processed using optimized conditions, and the resulting peptides were analyzed by ESI in the data-dependent MS/MS mode with dynamic exclusion. SEQUEST software (Thermo Scientific) was used to identify the sequence-selected parent peptide ions dynamically. The tentatively identified peptides were verified by visual confirmation of the parent ion charge state assigned by SEQUEST. These results provided a map of C3 peptides correlated by m/z, peak retention time, and sequence identity.

2.4. HDX-MS using ESI

Samples containing C3 (15.3 mg/ml in PBS, pH 7.4) or C3b (16.7 mg/mL in PBS, pH 7.4) were analyzed independently under the same conditions. A single time-point deuteration reaction was accomplished by mixing 10 µl of protein solution with 10 µl D_2O and incubating

the mixture at 4 °C for a predetermined time of 15 s, 50 s, 2.5 min, or 8.5 min, at 4 °C. To quench the exchange reaction, 30 µl of a solution containing 8 M urea and 1 M TCEP at pH 3.0 and 4 °C was added to the mixture. 45 µl of the quenched solution was immediately injected to a pepsin column (104-µl bed volume) in 0.05% TFA (200 µl/min) for 3 min, with subsequent collection of proteolytic products by a trap column (Magic C4, Michrom BioResources, Inc.; 4-µL bed volume). The peptide fragments were eluted from the trap column and separated on a C18 column (Magic C18, Michrom BioResources, Inc.) with a linear gradient of 13-40% solvent B over 23 min (solvent A, 0.05% TFA in water; solvent B, 95% acetonitrile, 5% solvent A). Mass spectrometric analyses were carried out with a Thermo Finnigan LCQ mass spectrometer (Thermo Scientific, Waltham, MA) with capillary temperature at 215 °C. These partially deuterated samples were then subjected to the analysis, along with control samples of non-deuterated (run without deuterated buffers) and fully deuterated C3 (incubated in 50 mM TCEP in 50% D_2O for 5 h at 60 °C).

The centroids of probe peptide isotopic envelopes were measured using a program developed in-house in collaboration with Sierra Analytics. The deuterium incorporation was calculated and corrected for back-exchange by using previously described methods (Hamuro et al., 2006; Zhang and Smith, 1993). The percent deuterium incorporation at each time point was compared for the corresponding C3- and C3b-derived peptides in order to calculate the average difference in deuteration across all time points (Tables 1 and 2).

2.5. Mapping of identified HDX peptides on the C3/C3b crystal structures

The crystal structure data for C3 (PDB accession code: 2A73 (Janssen et al., 2005)), C3b (PDB: 2HR0 (Abdul Ajees et al., 2006) and 2I07 (Janssen et al., 2006)), and a C3b:CRIg complex (PDB: 2ICF (Wiesmann et al., 2006)) were visualized and aligned in PyMol (version 0.99, DeLano Scientific LLC). The C3b structure 2I07 has been used for all mapping studies of C3b unless indicated otherwise. In accordance with similar studies (Brudler et al., 2006; Hamuro et al., 2006; Horn et al., 2006), changes in deuterium incorporation of more than ±10% were considered to be significant. Peptides that showed an increased (≥10%), decreased (≤-10%), or not significantly altered hydrogen/deuterium exchange were highlighted in red, blue, and yellow, respectively.

3. Results

3.1. MALDI Experiment – Hydrogen/Deuterium Exchange

A set of 35 peptides that are common to C3 and C3b and cover 27% of the primary sequence of C3b have been selected based on their peak quality and intensity (Tables 1 and 2; Fig. 1C and D). The differential deuterium incorporation for these 35 peptides was calculated across all time points. Peptides that exhibited a significant difference in average deuterium incorporation (>+10% or <-10%) between C3 and C3b included eight peptides from the α-chain (742-750, 830-839, 956-968, 1037-1043, 1189-1206, 1189-1209, 1237-1248, 1237-1251) and two peptides from the β-chain (235-248, 550-558). The remaining 25 peptides had an absolute average difference in deuteration of less than 10% (Tables 1 and 2).

The results of the MALDI HDX data were mapped onto the crystal structures of C3 and C3b (Janssen et al., 2006; Janssen et al., 2005) (Fig. 2). In the case of four peptides (1189-1206, 1189-1209, 1237-1248, and 1237-1251), the increased deuterium uptake in C3b could be accounted for by the large conformational changes that occurred during C3 activation. However, several other peptides showed a decrease in exchange upon conversion to C3b (peptides 235-248, 550-558, 742-750, 830-839, 1037-1043), which could not be entirely explained by the structural changes evident in the crystal structure models. As a consequence, we performed additional HDX experiments using ESI in order to improve upon sequence coverage and validate our results.

3.2. Generation of C3 Peptide Map for ESI Experiments

We optimized the digestion conditions to produce C3 fragments of suitable size and distribution for ESI analysis. The best results were obtained with acidic buffer containing 8 M urea and 1 M TCEP at pH 3.0, a protein flow of 200 µl/min over the immobilized pepsin column and a linear gradient of 13-40% solvent B over 23 min on the C18 HPLC column. Under these conditions, 155 peptides spanning 68% of the amino acid sequence of C3 were identified. None of those peptides spanned one of the two known glycosylation sites of C3/C3b (Asn-63, Asn-917 (Hirani et al., 1986)).

3.3. ESI Experiment – Hydrogen/Deuterium Exchange

The deuterium incorporation for 47 well-suited peptides from C3 and C3b was followed across all time points (Tables 1 and 2; Fig. 1C and D). These peptides spanned 55% of the C3b primary sequence and included 26 peptides from the α-chain and 21 peptides from the β-chain. For each peptide, the percent deuterium incorporation was determined at each time point, and the

C3 Sequence Number	Chain	Domain	Average Difference in Deuteration [a]	
			MALDI	ESI
1-14	β	MG1	n/a	0.2%
5–15	β	MG1	-5.5%	n/a
21–47	β	MG1	2.1%	-0.5%
23–47	β	MG1	-1.1%	n/a
101-108	β	MG1-2	n/a	-3.7%
109-124	β	MG2	n/a	-6.7%
125–142	β	MG2	-2.4%	-2.5%
143-158	β	MG2	n/a	2.4%
165-176	β	MG2	n/a	9.4%
177–186	β	MG2	-4.0%	n/a
187-199	β	MG2	n/a	-1.7%
221–230	β	MG3	-3.1%	n/a
235–248	***β***	***MG3***	***-18.9%***	***n/a***
249-277	β	MG3	n/a	-5.4%
259–277	β	MG3	-7.1%	n/a
307-335	β	MG3-4	n/a	-4.9%
336–352	β	MG4	-4.2%	n/a
336-350	β	MG4	n/a	0.6%
353–373	β	MG4	-2.7%	n/a
353-387	β	MG4	n/a	-2.3%
388–411	β	MG4	-1.5%	2.6%
412–436	β	MG4-5	-3.4%	1.8%
437-453	β	MG5	n/a	-2.4%
440–449	β	MG5	-3.7%	n/a
454-470	β	MG5	n/a	1.9%
471–491	β	MG5	1.2%	n/a
472-491	β	MG5	n/a	1.9%
492-499	β	MG5	n/a	3.0%
513–526	β	MG5	-1.5%	n/a
542-560	***β***	***MG6β***	***n/a***	***-11.1%***
550–558	***β***	***MG6β***	***-24.1%***	***n/a***
574-599	***β***	***MG6β-LNK***	***n/a***	***-17.7%***
600-622	β	LNK	n/a	0.3%

Table 1. Differential deuteration in the β-chain of C3- and C3b-derived peptides.

[a] Average percentage change in amide backbone deuterium level after correction for side chain contributions (MALDI) or deuterium back-exchange (ESI). Positive and negative numbers indicate increased and decreased deuterium incorporation in C3b compared to C3, respectively. Changes greater than ±10% in at least one method were considered significant and are highlighted in bold italic type.

differential change between C3 and C3b was calculated as described above. Six peptides were observed to have an absolute average difference in deuteration of at least 10% (>+10% or <-10%) including four peptides from the α-chain (957-968, 1189-1215, 1386-1422, and 1553-1570; Table 2) and two peptides from the β-chain (542-560, 574-599; Table 1). The remaining peptides exhibited an absolute average difference in deuteration of less than 10%.

C3 Sequence Number	Chain	Domain	Average Difference in Deuteration [a]	
			MALDI	ESI
737-750	α	α'NT	n/a	-8.5%
742–750	***α***	***α'NT -MG6α***	***-10.9%***	***n/a***
751-770	α	MG6α	n/a	-1.9%
811–822	α	MG7	-1.7%	n/a
830–839	***α***	***MG7***	***-21.3%***	***n/a***
873-887	α	MG7	n/a	-6.1%
922–945	α	CUBg	0.1%	n/a
922-955	α	CUBg	n/a	4.6%
956–968	***α***	***CUBg-TED***	***11.0%***	***n/a***
957-968	***α***	***CUBg-TED***	***n/a***	***35.4%***
994-1026	α	TED	n/a	-2.3%
1012–1025	α	TED	-0.9%	n/a
1012–1026	α	TED	-0.4%	n/a
1027-1044	α	TED	n/a	1.8%
1037–1043	***α***	***TED***	***-10.4%***	***n/a***
1047–1058	α	TED	0.5%	n/a
1048–1056	α	TED	-3.2%	n/a
1061-1071	α	TED	n/a	-6.9%
1108–1121	α	TED	-6.9%	n/a
1108–1123	α	TED	-9.2%	-9.2%
1169-1188	α	TED	n/a	0.8%
1189–1206	***α***	***TED***	***12.9%***	***n/a***
1189–1209	***α***	***TED***	***12.5%***	***n/a***
1189-1215	***α***	***TED***	***n/a***	***14.6%***
1224-1236	α	TED	n/a	-2.1%
1237–1244	α	TED	2.1%	n/a
1237–1248	***α***	***TED***	***18.1%***	***n/a***
1237–1251	***α***	***TED***	***15.0%***	***n/a***
1252-1272	α	TED-CUBf	n/a	-0.6%
1273–1296	α	CUBf	-1.6%	1.3%
1342-1364	α	MG8	n/a	4.6%
1386-1422	***α***	***MG8***	***n/a***	***12.1%***
1416–1422	α	MG8	-6.2%	n/a
1440-1463	α	MG8	n/a	-0.4%
1508-1520	α	C345C	n/a	-5.8%
1521-1535	α	C345C	n/a	-0.2%
1536-1542	α	C345C	n/a	4.2%
1543-1561	α	C345C	n/a	3.6%
1553-1570	***α***	***C345C***	***n/a***	***12.6%***
1575-1589	α	C345C	n/a	-1.8%
1590-1605	α	C345C	n/a	6.4%
1606-1629	α	C345C	n/a	4.6%
1635-1641	α	C345C	n/a	2.0%

Table 2. Differential deuteration in the α-chain of C3- and C3b-derived peptides.

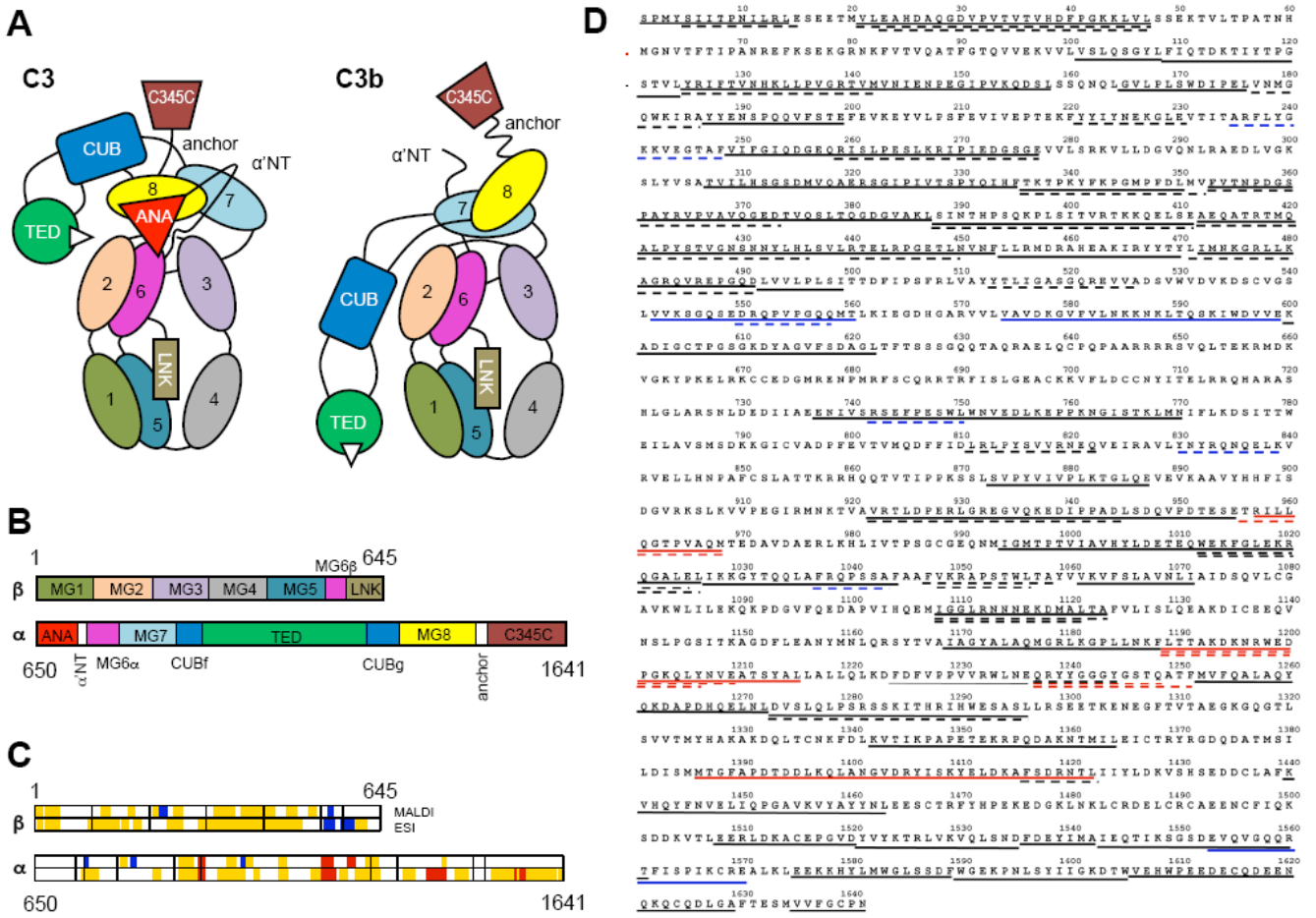

Fig. 1. Domain organization of C3/C3b, and sequence coverage by mass spectrometry. (A) Individual domains in C3 and C3b are represented by different colors and labeled with the domain name abbreviation. Numbered domains signify the corresponding macroglobulin domains (MG1-MG8). Panel (B) shows the domain locations on the primary sequence of the C3 α and β-chains. Upon activation, the ANA domain is removed from C3 and allows the α'NT domain to shift from one face of the protein to the other. As a consequence, the MG8, CUB, and TED domains undergo a large relocation in relation to the protein core (MG1-6) and expose the thioester moiety (white triangle) in C3b that is essential for opsonization of foreign surfaces. A set of 82 peptides spanning 61% of the C3 sequence have been utilized for the analysis and are plotted against the domain scheme (C) and the primary sequence of C3 (D). Areas with significantly increased HD exchange in C3b (≥10%; red) were mainly identified in the β-chain, while the α-chain contained more peptides with significant decrease in HD exchange (≤-10%; blue). Yellow and black areas signify peptides with no significant change (>-10% or <10%) in (C) and (D), respectively. Dashed and solid lines represent MALDI- and ESI-derived peptides, respectively, in the sequence plot (D).

3.4. Combining MALDI and ESI HDX Data - Deuterium Incorporation Upon Conversion of C3 to C3b

The combined MALDI- and ESI-based HDX strategies provided for the assessment of 82 peptides common to C3 and C3b, accounting for 61% of the C3b primary sequence (Fig. 1C and D). Comparison of the deuteration profiles showed that nine peptides exhibited increased exchange in C3b when compared to their exchange in C3 (956-968, 957-968, 1189-1206, 1189-1209, 1189-1215, 1237-1248, 1237-1251, 1386-1422 and 1553-1570), and seven peptides exhibited decreased exchange in C3b when compared to their exchange in C3 (235-248, 542-560, 550-558, 574-599, 742-750, 830-839 and 1037-1043). The remaining 66 peptides exhibited a lower difference (<10%) in deuterium incorporation.

The exchange data for all peptides was overlaid onto the C3 and C3b crystal structures (Fig. 2). All peptides that showed increased deuterium exchange in C3b were located within the α-chain (Table 2, Fig. 3). These peptides were distributed among four crystallographic domains: TED, CUB, C345C, and MG8 (Fig. 1B and C). The overlapping peptides 956-968 and 957-968 reside

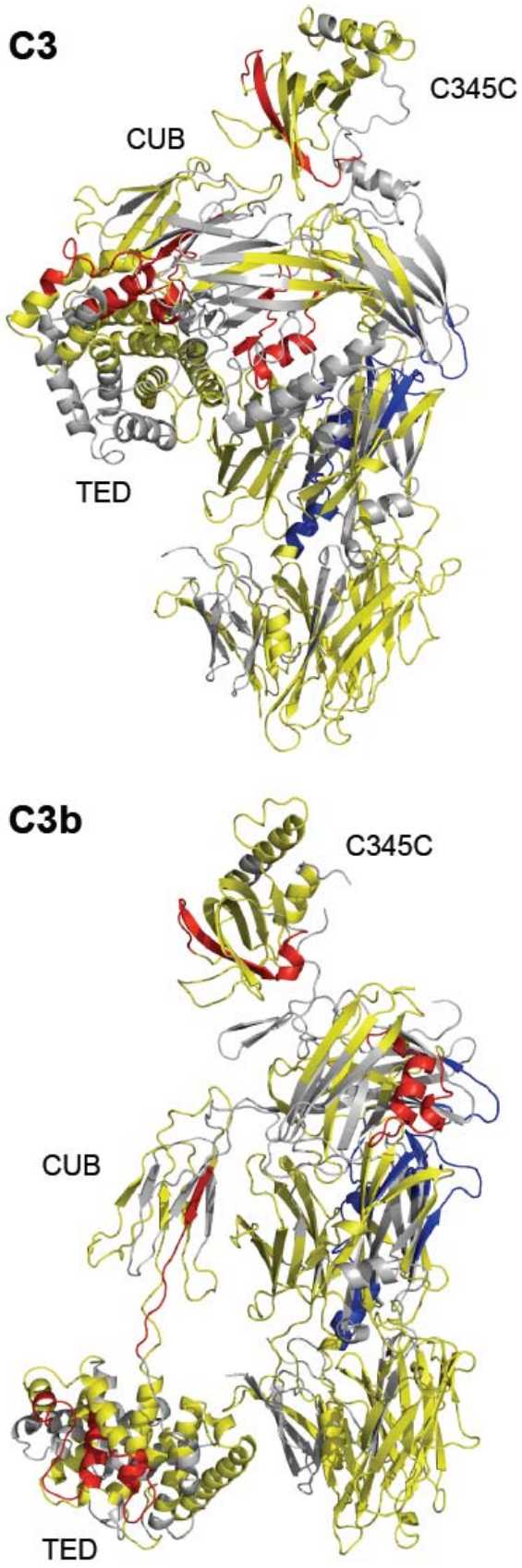

Fig. 2. Mapping of differential HDX data on the C3 and C3b crystal structures. Peptides with increased exchange in C3b as compared to C3 are highlighted in red (average difference in deuteration ≥10%); peptides with decreased exchange in C3b as compared to C3 are in blue (≤-10%); peptides with minimal difference in exchange are in yellow (>-10% or <10%). Peptides for which no HDX data are available are represented in gray.

within the TED-CUB interface, which is largely involved in the conformational changes accompanying C3 activation. Examination of the respective crystal structures revealed that this portion of the protein is clearly more solvent-exposed in C3b than in C3, a conclusion with which the HDX data were in full agreement. Two groups of overlapping peptides (1189-1206, 1189-1209, 1189-1215 and 1237-1248, 1237-1251) were in the TED domain, which undergoes a translation of >45 Å upon conversion of C3 to C3b. Also, the peptides in the first of these two groups included a putative binding site for factor H (Herbert et al., 2006; Lambris et al., 1988), a complement regulatory protein with affinity for C3b but not C3, suggesting that the binding site gains solvent accessibility upon structural change. Together, these observations support the increased deuterium exchange observed in the HDX experiments.

The peptide 1386-1422 is found within the MG8 domain, which undergoes rotation and a 24-Å translation upon conversion of C3 to C3b (Abdul Ajees et al., 2006; Janssen et al., 2006; Wiesmann et al., 2006). This transformation exposes the proposed properdin binding site at residues 1402-1435, which may be reflected by the increased deuterium exchange of peptide 1386-1422 in C3b (Daoudaki et al., 1988). Finally, within the C345C domain, peptide 1553-1570 also exhibited increased exchange in C3b when compared to C3. The C345C domain rotates approximately 30º upon conversion of C3 to C3b and possesses a binding site for factor B, a key protein in the formation of the alternative pathway convertase (Janssen and Gros, 2007). In summary, the observed increases in exchange for these peptides are consistent with the available crystal structures for C3 and C3b.

In contrast to the situation for peptides that exhibited increased deuterium exchange, the HDX data for the peptides that exhibited decreased deuterium exchange in C3b compared to C3 were not consistent with the changes observed in the crystal structures (Fig. 4). These peptides spanned the following domains: MG3 (peptide 235-248), MG6 (542-560, 550-558), the interfaces of MG6-LNK (574-599) and α'NT-MG6 (742-750), MG7 (830-839), and TED (1037-1043) (Fig. 1B and C). With the exception of the TED domain, these domains were found to undergo minimal structural changes upon

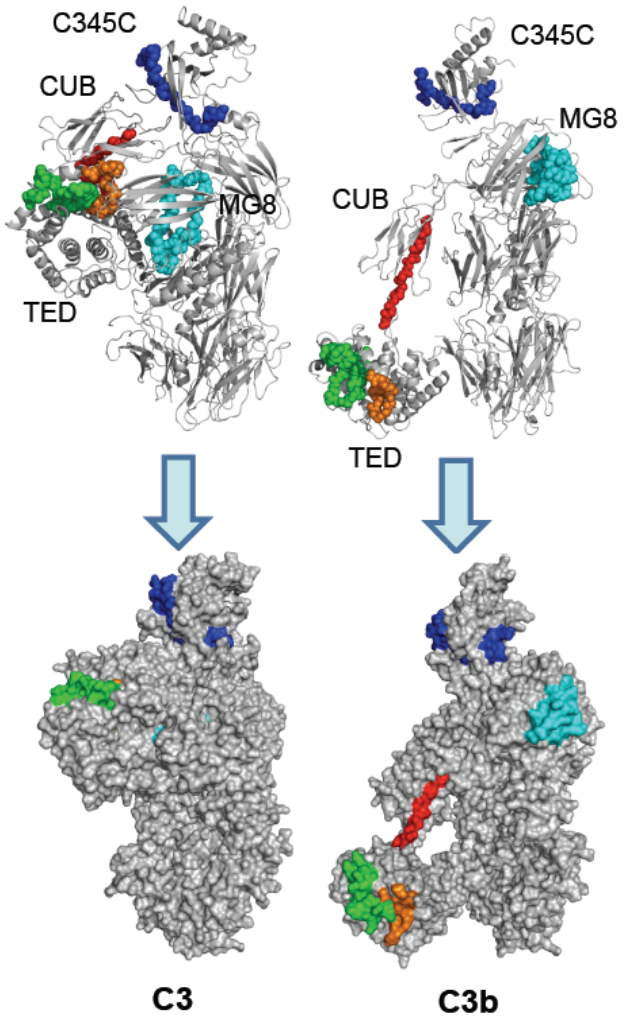

Fig. 3. Areas that exhibit increased deuterium exchange upon activation of C3. Peptides with significantly increased HDX in C3b (≥10%) are highlighted in the crystal structures of C3 (left) and C3b (right). The backbones of these peptides are represented as balls in a cartoon representation in the top panels. In the bottom panel, the structures are represented as the calculated solvent-exposed surface. Peptide sequence identification: red = 956-968; green = 1189-1215; orange = 1237-1251; light blue = 1386-1415; blue = 1553-1570. In general, HDX data for these peptides are in agreement with reported changes in protein structure upon conversion of C3 to C3b.

conversion of C3 to C3b (Janssen et al., 2006). Interestingly, mapping of the peptides that were identified in these domains (235-248, 542-560, 574-599, 742-750 and 830-839) on the crystal structures of C3 and C3b revealed that they form a cluster of loops in close proximity to one another (Fig. 4).

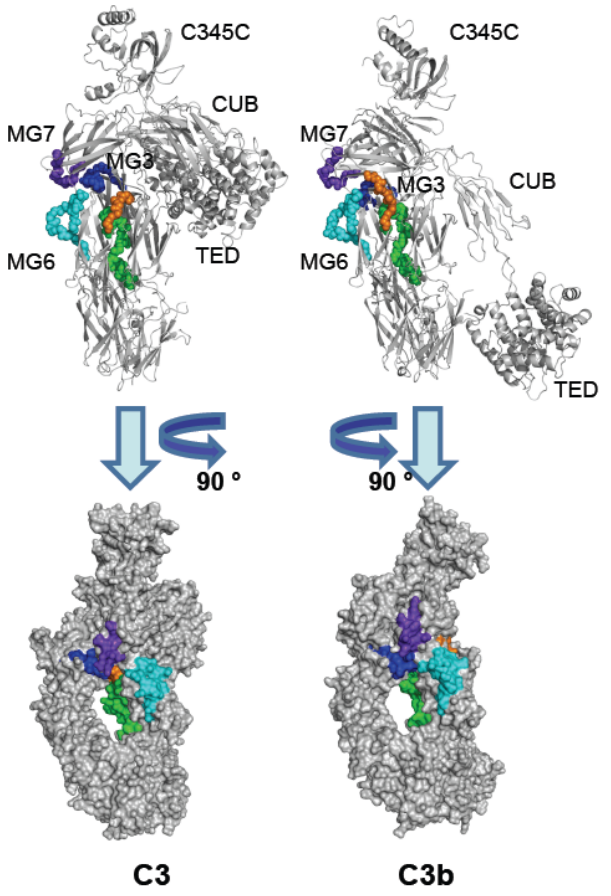

Fig. 4. Areas that exhibit decreased deuterium exchange upon activation of C3. Peptides showing a significantly lower HDX (≤-10%) in C3b as compared to C3 are highlighted in the crystal structures of C3 (left) and C3b (right). The backbones of these peptides are represented as balls in a cartoon representation in the top panels to improve clarity. In the bottom panel, the structures have been rotated and represented as the calculated solvent-accessible surface. Peptide sequence identification: blue = 235-248; light blue = 542-560; green = 574-599; orange 742-750; purple = 830-839. While no large structural changes have been observed in the corresponding area of the crystal structures, these peptides form a cluster at the interface of the MG3, MG6, MG7, LNK, and α'NT domains that may show a higher degree of structural flexibility in solution. Peptide 1037-1043, which also showed decreased HDX in C3b, is located on the TED domain and does not contribute to the cluster described above. Its exact location and differential exposition is shown separately in Fig. 6.

When comparing this region between C3 and C3b, only slight changes in the arrangement of these peptides but no frank conformational changes are evident. As a consequence, this area may exhibit local fluctuations in secondary or tertiary structure that are more apparent in solution than in the crystal. At first view, the significance of peptide 1037-1043 seems to be contradictory between

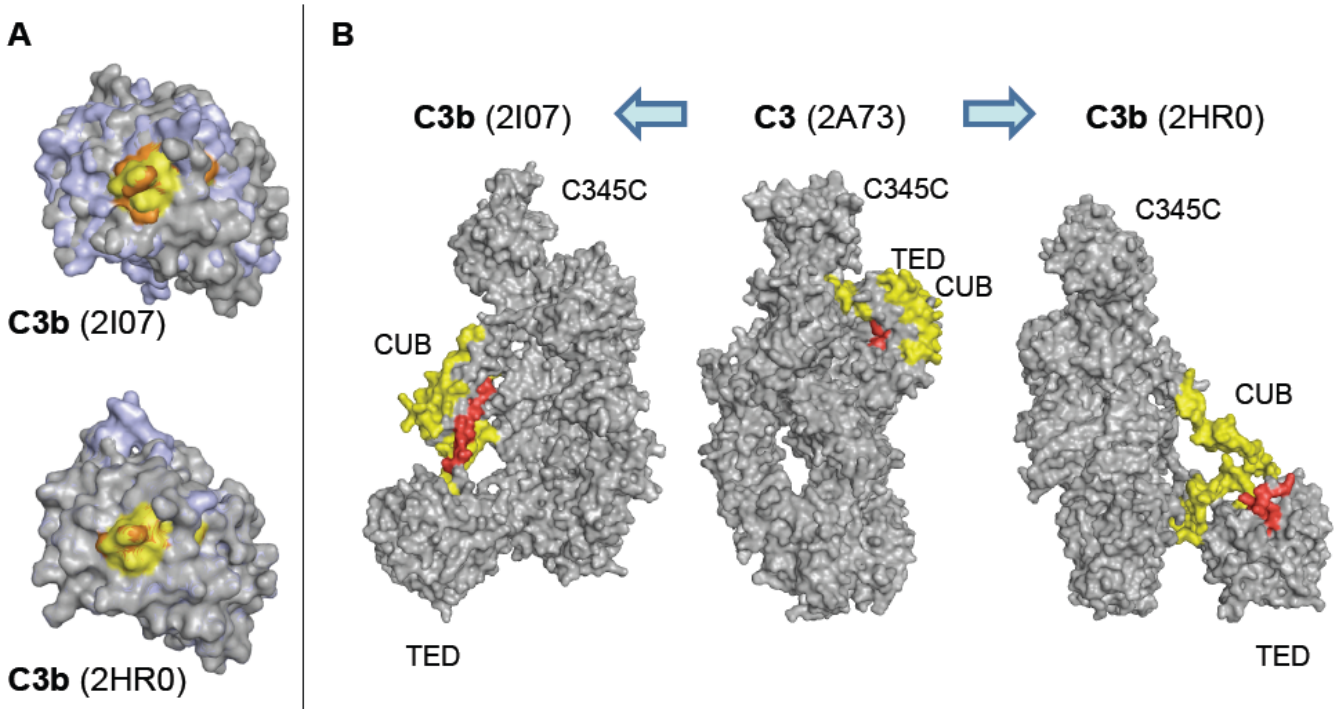

Fig. 5. Structural changes in the TED (a) and CUB (b) domains between C3 and the contradicting crystal structures of C3b. (A) Significant conformational changes between the TED domains of C3 (grey) and C3b (pale blue) are only observed for the C3b structure by Janssen et al. (2I07; left) but not for the alternative structure by Abdul Ajees et al. (2HR0, right). In the case of peptide 1037-1044 (yellow and orange in C3 and C3b, respectively), the observed changes in HDX are therefore in closer agreement with structure 2I07. (B) While the CUB domain remains in a compact state in 2I07, the same domain appears rather unfolded and elongated in 2HR0. In the HDX analysis, the majority of the CUB-derived peptides show no significant change in deuteration (yellow), which would again support the 2I07 structure. In agreement with HDX data, peptide 957-968 (red) gets more solvent-exposed in both C3b variants.

experiments. This peptide in the TED domain loses solvent exposure upon conversion from C3 to C3b in the MALDI experiment (-10.4%; Table 2). Structural differences in this area are only visible in the C3b structures by Janssen and Wiesman (Janssen et al., 2006; Wiesmann et al., 2006), whereas this TED area is largely unaltered between C3 and the C3b structure by Abdul Ajees (Abdul Ajees et al., 2006) (Fig. 5A). However, the larger, encompassing peptide 1027-1044 exhibited no significant change in deuterium uptake when analyzed by ESI MS (+1.8%). While this discrepancy may be based on experimental conditions, it may also indicate a statistical compensation due to higher solvent exposure of the first 10 residues. Indeed, comparison of the crystal structures clearly shows that this stretch of amino acids (1027-1036) lies exactly at the contact interface between the TED and MG2 domain and is mainly shielded from the solvent in C3. Upon activation, the same patch becomes solvent-exposed due to the extended positioning of the TED domain in C3b (Fig. 6). This comparison therefore supports both the large relocation of the TED domain and the smaller structural fluctuations visible in two of the three C3b structures.

3.5. Differential analysis of the available C3b structures

Based on the differences found for peptide 1037-1043 (see above), we extended our analysis and compared the distribution of all identified HDX peptides on the C3b structures by Janssen *et al.* (Janssen et al., 2006) and Abdul Ajees *et al.* (Abdul Ajees et al., 2006). The third structure by Wiesmann *et al.* (co-crystal with the CRIg receptor (Wiesmann et al., 2006)) shows a high degree of similarity with unbound C3b by Janssen and was therefore not evaluated separately. The major differences between the analyzed C3b structures are found in the CUB and TED domains. In the structure by Janssen, the CUB domain is shifted in its position but remains in a highly folded, C3-like state (Fig. 5B). The TED domain undergoes a number of small but significant conformational adaptations and is therefore different between C3 and C3b (Fig. 5A). In the structure by Abdul

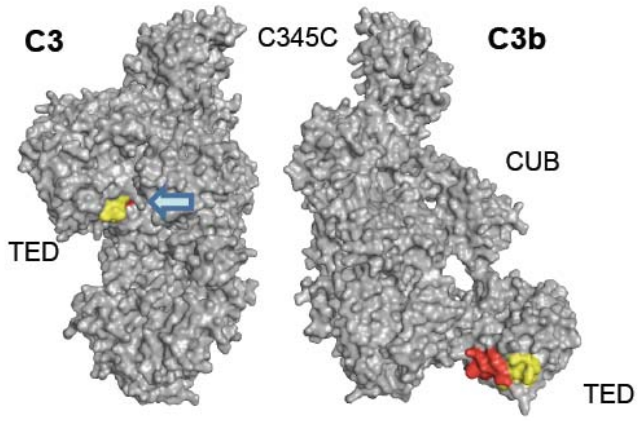

Fig. 6. Detailed structural analysis of peptides 1037-1043 (MALDI) and 1027-1044 (ESI). The diverging HDX results for the MALDI-derived peptide 1037-1043 (-10.4%) and the encompassing peptide 1027-1044 (+1.8%) from ESI analysis can be explained by the relocation of TED during C3 activation. While the common peptide stretch 1037-1043 (yellow) is similarly accessible in both C3 and C3b, the N-terminal elongation of the ESI peptide (1027-1036; red) is buried in C3b and gets exposed in C3b. As a consequence, the signal observed in ESI may be the sum of a HDX decrease (as in MALDI) and an HDX increase (for the N-terminus).

Ajees, on the other hand, the CUB domain appears in a rather unfolded state, leading to a different elongation and positioning of the TED domain (Fig. 5B). In addition, there are nearly no structural changes within the TED domain between C3 and this structural variant (Fig. 5A). The large unfolding of CUB in the latter structure should lead to a significantly enhanced solvent accessibility of this domain. However, such large changes in solvent exposure could not be confirmed by HDX-MS since most of the peptides in this region showed shifts below 10%. The only peptide with significantly enhanced HDX signal was located at the immediate interface between CUB and TED (957-968, +35.4%). Indeed, this peptide is buried in the backfolded state of TED in native C3 but becomes highly solvent exposed during CUB elongation in both C3b variants. Interestingly, peptide 957-968 is stretched out as part of the CUB arm in the structure by Janssen, whereas it is present in a more folded state as part of TED in case of C3b by Abdul Ajees (Fig. 5B). Together, these data indicate that the C3b crystal structures by Janssen and Wiesmann more closely correspond to the solution-based data we acquired using HDX-MS, especially in the CUB and TED domains.

4. Discussion

Activation and regulation of the human complement system is largely driven by a complex cascade of interactions, enzymatic cleavages, and conformational changes that unveil cryptic binding sites (Gros et al., 2007). C3 and its activated fragment C3b are at the center of this cascade and essential for both the amplification and effector functions of complement. The recent reports of the C3 and C3b crystal structures have provided detailed solid-state structures of these proteins and offered an important insight on their function on a molecular level (Abdul Ajees et al., 2006; Janssen et al., 2006; Janssen et al., 2005; Wiesmann et al., 2006). However, there are significant and fundamental differences between the available structures for C3b. Furthermore, these proteins may exhibit structural fluctuations in solution that are not apparent from their crystal structure. Therefore, we investigated the relative conformations of these two proteins in the liquid state by HDX-MS and mapped the identified peptides on the corresponding structures. Although HDX is incapable of providing an atomic-resolution structure of macromolecules, it can provide insight into protein structural dynamics in solution and supplement crystal data. While the C3b used in this study has been generated by tryptic cleavage of C3, the product is known to closely correspond to the convertase-derived C3b (Bokisch et al., 1969). Our initial experiments using MALDI revealed that many differences in deuterium uptake could be explained by the gross structural changes that are observed upon conversion of C3 to C3b (Gros et al., 2007; Janssen et al., 2006). However, sequence coverage was limited by this technology and some peptides with decreased deuterium uptake in C3b could not be explained by differences in the crystal structures. To validate the results and expand our coverage of the proteins, C3 and C3b were therefore further analyzed by ESI. With this method, the coverage of C3 could be remarkably expanded, and the combination of both techniques provided a rich peptide pool for the investigation of structural changes. In light of the often-faced size restrictions for MS-based proteins analyses (Cravello et al., 2003), the sequence coverage of 61% for the 175-kDa C3b molecule is an important achievement and demonstrates the feasibility of this method for large plasma proteins.

For most areas of the proteins, our combined HDX-MS data were in good agreement with the crystallographic structures of C3 and C3b. Significant increase of deuteration upon activation of C3 to C3b were essentially observed in the CUB, TED, MG8, and C345C domains. These areas are not only known to undergo large conformational changed during the conversion but were also attributed to carry binding sites for a series of complement factors, receptors, and regulators (Gros et al., 2007; Janssen and Gros, 2007). For example, C345C exhibits a putative binding site for factor B (Kolln et al., 2005; Kolln et al., 2004), MG8 was found important for

the binding of properdin (Daoudaki et al., 1988), and TED is described to carry several sites for complement receptor 2 (Janssen and Gros, 2007), factor H (Herbert et al., 2006; Jokiranta et al., 2006; Lambris et al., 1988), and bacterial complement evasion proteins (Hammel et al., 2007a; Hammel et al., 2007b). In addition, the CUB domain has been shown to bind factor B (O'Keefe et al., 1988) and its proper structure and orientation is considered important for the assembly of the alternative pathway C3 convertase (Gros et al., 2007). In this respect, our HDX data are highly consistent with the conformational changes upon C3 activation that were described in the crystal structures.

ESI analysis also confirmed our observation that C3 may adopt conformations in solution that result in an increased solvent exposure in a focal area around the MG3, MG6, and MG7 domains when compared to that of C3b. Specifically, discontinuous peptides 235-248, 542-560, 574-599 and 742-750 exhibited increased deuterium exchange in C3 when compared to C3b. The idea that C3 may have greater conformational flexibility in this area is intriguing and may contribute to our understanding of specific functions of C3 and C3b. Indeed, antibody studies have previously indicated that this area of the β-chain is important in factor B binding to C3b, a key step in formation of the alternative pathway convertase (Alsenz et al., 1990). In the alternative pathway of complement activation, the C3 convertase is formed from either C3b or the product of C3 thioester hydrolysis, C3(H_2O). While C3b is formed upon cleavage of C3 by the convertase, C3(H_2O) forms spontaneously in solution from non-enzymatic C3 thioester hydrolysis. Since C3b and C3(H_2O) have binding epitopes and biological functions in common, it is assumed that they also share structural features (Lambris, 1988; Sahu and Lambris, 2001). Indeed, Nishida *et al.* recently utilized electron microscopy to explore the structural transitions undergone by C3 upon activation (Nishida et al., 2006). They found that conformers of C3 that have been activated by nucleophiles (i.e. C3(N)), including C3(H_2O), have a similar general structure and domain arrangement as C3b. However, in order for C3 to form C3(N), the anaphylatoxin (ANA) and α'NT domains of C3 must move from the MG3/MG8 side of C3 to the MG7 side. Structurally, this movement requires that the ANA and α'NT domains must pass through the β-ring formed by domains MG1-MG4, and through the half-ring created by MG5-MG6 (Fig. 1A). Nishida *et al.* have hypothesized that the MG6 domain in C3 is capable of unfolding to allow this transition to occur. Our observations support this hypothesis: three of the four peptides that exhibited increased exchange in C3 when compared to C3b (542-560, 574-599 and 742-750) were partially or entirely located within the MG6 domain. Thus, it appears that the MG6 domain of C3 is unfolding or adopting a different structure during the timescale of our experiments and in doing so exposes its backbone to the solvent, leading to increased deuterium exchange. Furthermore, our data suggest that peptide 235-248 of the MG3 domain may also exhibit conformational flexibility in C3 and perhaps also play a permissive role in the formation of C3(N).

Our data interpretation is also indirectly supported by the C3b-CRIg co-crystal structure (Wiesmann et al., 2006). CRIg is a complement receptor that is found on the surfaces of resident macrophages and appears to be necessary for the clearance of C3-opsonized pathogens (Helmy et al., 2006). CRIg does not bind to native C3 but interacts with C3b and C3c. Furthermore, binding of CRIg to C3b was found to inhibit the functions of the C3 and C5 convertases of the alternative complement pathway (Katschke et al., 2007). In the co-crystal structures with C3b and C3c, CRIg was localized in the middle of the β-ring, simultaneously interacting with the MG3, MG4, MG5, MG6, and LNK domains. Interestingly, three of the peptides that exhibited decreased deuterium exchange in C3b (235-248, 542-560, 574-599) cover residues that form contacts with CRIg in the co-crystal structure. We therefore speculate that the greater organization of these peptides in C3b influences CRIg's ability to bind C3b but not C3.

The validity of the structural model of C3b by Abdul Ajees *et al.* has recently been questioned (Janssen et al., 2007). While all structures agree in an elongation and repositioning of the TED domain, which is responsible for the covalent attachment onto foreign surfaces (opsonization), the exact structural transitions at the CUB/TED interface and the relative position of TED vary considerably. The conservative changes in solvent accessibility in the CUB area, the large exposure of peptide 957-968 and the observed changes in the TED area all seem to better agree with the two structures published by Janssen *et al.* (Janssen et al., 2006) and Wiesmann *et al.* (Wiesmann et al., 2006), respectively, than with the C3b structure by Abdul Ajees *et al.* (Abdul Ajees et al., 2006). In their reply to the recent controversy (Janssen et al., 2007), Abdul Ajees *et al.* suggest that their structure may represent a different physiological stage in the activation process of C3b, in which CUB is further unfolded compared to the other structure. On the other hand, a rather large gap in the crystal lattice of 2HR0 may also indicate the presence of an impurity caused by a regulator of complement activation, which may have induced additional cleavage (Janssen et al., 2007). While further investigations and additional experiments are required to confirm the true solvent structure of active C3b, our HDX data generally show preferential agreement with the structures published by Janssen *et al.* and Wiesmann *et al.*

Finally, the comparison of the current results for C3b with our previous data for C3(H_2O) (Winters et al., 2005) provides further support for shared structural elements between these two proteins. In the prior analysis of C3(H_2O), a >10% decrease in deuterium exchange (in going from C3 to C3(H_2O)) was observed for peptides 235-248, 542-566, 1108-1121 and 1108-1123. Analysis of

the relevant peptides for C3b, described herein, also showed a decrease in deuterium exchange of >10% for peptides 235-248 and 550-558 and a smaller decrease for peptides 1108-1121 and 1108-1123. A similar pattern was observed for the peptides that exhibited an increase in deuterium exchange. Most of the peptides that exhibited a >10% increase in deuterium exchange in going from C3 to C3(H_2O) (922-945, 956-968, 957-968, 1027-1036, 1189-1206, 1189-1209, 1237-1248 and 1237-1251) also showed a similar change for the conversion to C3b. The two only exceptions were the MALDI-derived peptide 922-945 (no significant change) and the ESI-derived peptide 1029-1044 (minimal increase in deuteration). This high agreement in the deuteration patterns provides more evidence for a structural similarity between these two related proteins and cross-validates the experimental method.

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Chapter 6

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B lymphocytes and macrophages release cell membrane deposited C3-fragments on exosomes with T cell response-enhancing capacity

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B lymphocytes and macrophages release cell membrane deposited C3-fragments on exosomes with T cell response-enhancing capacity☆

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Abstract

Recently exosomes have been shown to play important roles in several immune phenomena. These small vesicles contain MHC proteins along with co-stimulatory and adhesion molecules, and mediate antigen presentation to T cells. In the present study we show that upon incubation with autologous serum, murine macrophages and B cells – but not T lymphocytes – fix C3-fragments covalently to the cell membrane and release them on exosomes in a time dependent fashion. While in the case of human B lymphocytes CR2 has been shown to serve as the main C3b-acceptor site, here we clearly demonstrate that cells derived from CR1/2 KO animals also have the capacity to fix C3b covalently. This finding points to a major difference between human and murine systems, and suggests the existence of additional acceptor sites on the cell membrane. Here we show that C3-fragment containing exosomes derived from OVA loaded antigen presenting cells induce a significantly elevated T cell response in the presence of suboptimal antigen stimulus. These data reveal a novel function of cell surface-deposited C3-fragments and provide further evidence for the role of exosomes secreted by antigen presenting cells. Since fixation of C3b to plasma membranes can be substantial in the presence of pathogens; moreover tumor cells are also known to activate the complement system resulting in complement-deposition, C3-carrying exosomes released by these cells may play an important immunomodulatory role *in vivo*, as well.

Keywords: B cells; Macrophages; Complement; Antigen presentation

1. Introduction

The third component of the complement system has surprisingly diverse roles in the body ranging from participating in the elimination of invading pathogens and shaping the immune response, to the development of organs in the host (Barrington et al., 2001; Mastellos and Lambris, 2002). It has been known for long that following complement activation C3b, iC3b and C3d fragments, covalently bound to antigens serve not only as opsonins, but provide co-stimulatory signals for the cells of the acquired immune response as well (reviewed in Erdei et al., 2003; Nielsen and Leslie, 2002; Sahu and Lambris, 2001). Upon incubation in homologous serum malignant tumor B cell lines (Budzko et al., 1976; Mold et al., 1988; Praz and Lesavre, 1983), normal B cells (Erdei et al., 1983; Nielsen et al., 2002), monocytes and polymorphonuclear leukocytes (Marquart et al., 1994) were shown to fix C3b on the cell membrane. Deposition of C3-fragments occurs not only *in vitro*, but also under physiological conditions, as freshly isolated normal human cells have been demonstrated to carry C3dg on their surface (Marquart et al., 1994). Importantly, deposition of the complement fragments

Abbreviations: CR1, complement receptor type 1; CR2, complement receptor type 2; C3, third complement component; C3bi, C3b, C3d, proteolytic fragments of C3; MA, methylamine; MVB, multivesicular bodies; PE, phosphtaydilethanolamine.

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does not affect the viability of the cells under physiological conditions, and the human lymphoblastoid Raji cell line carrying C3-fragments was shown to survive for weeks (Praz and Lesavre, 1983). Cell surface bound C3-fragments were found to exert an additional important function, namely the enhancement of the antigen presenting capacity of B lymphocytes and macrophages (Kerekes et al., 1998, 2001).

Plasma membrane-bound C3-fragments might either be ingested or released by the cells. In the latter case they might be involved in various immunological processes; however this possibility has not been studied so far. Regarding the elimination of the membrane attack complex (MAC) to protect nucleated cells from complement-mediated lysis, the process of ectocytosis and endocytosis have been suggested (Pilzer et al., 2005). While ectosomes (vesicles of approximately 0.5–2 μm) are directly released from the cells, exosomes (particles of 40–100 nm) are generated by a more complex cellular process. Secretion of exosomes was originally proposed as a mechanism to remove obsolete protein from the plasma membrane (Pan and Johnstone, 1983). These small membrane vesicles are formed by inward budding of endosomal membranes generating multivesicular bodies (MVB), the subcellular site for peptide loading to MHCII, and are released following fusion of the MVB with the plasma membrane. Production of exosomes has been described for several immune cells including B lymphocytes, macrophages, dendritic cells and mastocytes (reviewed in Li et al., 2006). These vesicles contain MHC proteins along with co-stimulatory and adhesion molecules; therefore they may have a role in antigen presentation to T cells. Exosomes derived from dendritic cells and mastocytes were shown to present antigens to T cells and to generate anti-tumor response *in vivo* (reviewed in Mignot et al., 2006).

Exosomes are present in human plasma (Caby et al., 2005) thus, they may serve as carriers for various molecules. Here we demonstrate that these small vesicles, which are released by serum-treated B cells and macrophages, contain complement C3-derived fragments, as well. Regarding the function of C3-fragment containing exosomes, we show that particles derived from antigen-pulsed cells significantly enhance the proliferation of T lymphocytes in the presence of naïve antigen presenting cells. As both virus-infected and tumor cells are known to bind C3-fragments covalently, moreover C3-deposition to APC have been shown to enhance the activation of antigen specific T cells, exosomes released by these cells might contribute to the development of immune responses *in vivo*, as well.

2. Materials and methods

2.1. Mice

8 to 12 weeks old BALB/c, C3 KO (Circolo et al., 1999) and CR1/2 KO (Molina et al., 1996) mice (kind gift of Matyas Sandor (UW-Madison, USA) were housed under pathogen-free condition in our animal facility. All animal experiments were in accordance with national regulations and were authorized by the ethical committee of the institute. Serum collected from BALB/c or C3 KO mice were freshly aliquoted and kept at −70 °C until use.

2.2. Serum-treatment of the cells and flow cytometric analysis

3×10^5 mouse B cell-lines 2PK3, A20, P388D1 macrophages and splenocytes derived from BALB/c or CR1/2KO mice were incubated in BALB/c or C3 KO serum. Sera were obtained from freshly drawn blood of the animals and diluted ten times in FCS-free medium RPMI 1640 for 1 h at 37 °C. In certain experiments methylamine (MA) treated serum (Kerekes et al., 1998) serum was used. After washing three times, the cells were incubated with FITC conjugated $F(ab)_2$ fragment of polyclonal goat anti-mouse C3 antibody (MP Biomedicals) for 20 min at 4 °C, then washed again. Lymphocytes were separated from non-lymphoid cells by their low FSC and SSC signal, and anti-mouse CD45R/B220-PE-Cy5 (BD Pharmingen) was used to distinguish B cells from T cells in the splenocyte suspension. 1×10^4 cells were acquired in a Becton–Dickinson FACSCalibur flow cytometer and data were analyzed by FCS Express III software (De Novo Software). To demonstrate the magnitude of C3 deposition, relative Gmean (R_{Gmean}) was calculated, which is the quotient of Gmean values of BALB/c serum-treated cells and the negative control (i.e. C3 KO serum treated cells).

2.3. Visualization of deposited C3 fragments with confocal laser scanning microscope

Cells were treated with serum then incubated with FITC conjugated polyclonal goat anti-mouse C3 $F(ab')_2$ antibody as described above. Following 40 min incubation in medium, the cell membrane was labeled with Alexa 566 conjugated cholera toxin subunit B (Invitrogen) and the nucleus was stained with DRAQ5 (Biostatus). Labeled cells were placed onto 0.15 mm thin coverslips and assayed by Olympus Fluoview 500 confocal microscope (Hamburg, Germany), using 60×, high N.A. oil immersion objective.

2.4. Measurement of the amount of C3 in the supernatant of cultured cells by ELISA

ELISA plates (Greiner bio-one) were coated with polyclonal goat anti-mouse C3 $F(ab')_2$ (MP Biomedicals) at 4 °C, overnight. Following washing three times with PBS containing 0.05% Tween 20, 50 μl, the supernatant of various samples was added in triplicates and plates were incubated at room temperature for 1 h. After washing the plates with PBS containing 0.05% Tween 20, HRP-conjugated goat–anti-mouse C3 (MP Biomedicals) was added to the wells and the reaction was developed by 3,3′,5,5′-tetramethyl-benzidine (TMB) (Sigma). Optical density of the samples was measured at 450 nm.

2.5. Isolation and visualization of exosomes

Cells were separated after treatment by centrifugation at $300 \times g$ for 8 min. Cell-free supernatant was filtered through

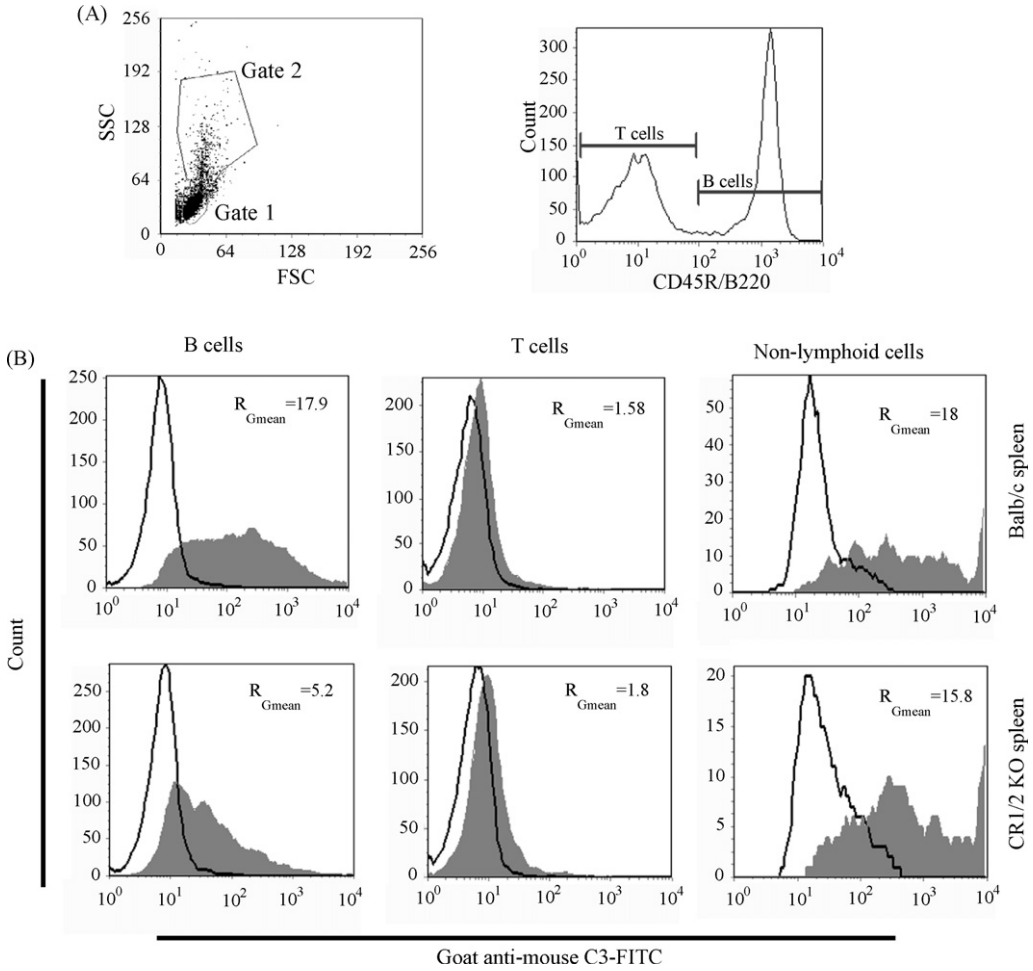

Fig. 1. Mouse B lymphocytes and non-lymphoid splenocytes of both wilde type and CR1/2 KO mice activate the complement system and fix C3-fragments. C3-fragment deposition on splenocytes treated with the serum of BALB/c (shaded histogram) or C3 KO mice (thick lines), was measured by cytofluorimetry using FITC-labeled goat anti-mouse C3. Lymphocytes (gate 1) were separated from non-lymphoid cells (gate 2) by their low FSC and SSC signal. B cells were identified by the presence of CD45R/B220 (panel A). C3-deposition on cells derived form BALB/c mice (upper row, panel B); CR1/2 KO mice (lower row, panel B). One representative experiment of 3 is shown.

a 0.22 μm cut-off size strainer to remove cell debris. Exosomes were separated as described (Raposo et al., 1996; Thery et al., 2001), employing centrifugation at 70,000 × *g* for 1 h in a Beckmann Optima™ TL ultracentrifuge. The supernatant was cleared off and the lower 20 μl exosome-containing fraction was used in further experiments. For negative staining, a drop from the exosome fraction was applied to Formvar/carbon-coated glow-discharged copper grids for 30 s. The solution was then removed and the grid stained with one drop of freshly filtered 1% aqueous uranyl acetate for 30 s. The excess stain was removed by blotting with filter paper.

2.6. Immunoelectron microscopy

3×10^6 A20 cells were treated with serum as it is described above. For immunogold labelling samples were prepared according to the method of Locke (1994) to preserve the antigenicity of the proteins in ultra-thin sections. Immunogold labelling was performed by following a procedure simplified from that of Leung et al. (1989). For visualization of C3-fragments on the surface of exosomes FITC conjugated goat anti-mouse C3 $F(ab')_2$ antibody and 10 nm gold-conjugated mouse anti-FITC antibody (Electron Microscopy Sciences) were used. Sections were viewed in a JEOL JEM100 CXII electron microscope.

2.7. Antigen-presentation assay

T cells were isolated from the lymph nodes of BALB/c mice injected with OVA (100 μg/animal) in complete Freund's adjuvant (Sigma) ten days before the assay. B cells were depleted from the suspension by panning; using anti-mouse IgM coated Petri dishes. The purity of T cells was always >97%. For the cell culture medium RPMI1640, supplemented with 5% FCS and 0.1 M mercaptoethanol was used.

As APC, the B cell line A20 was used. Cells were pulsed with a suboptimal dose of OVA at the density of 2×10^6 cells/ml or as a control, in medium only, at 37 °C for 2 h. After washing, cells were incubated with Balb/c serum or medium, as described

above. Exosomes were isolated from the supernatant of cells after culturing at 37 °C for 20 h.

To study the effect of exosomes on antigen-presentation, the exosome fraction was added to mitomycin C (Sigma) treated A20 cells ($1–5 \times 10^3$ well^{-1}) and OVA-specific T cells (2×10^5 well^{-1}). After culturing for 4 days [^{3}H]thymidine-pulsed samples were harvested and measured. Two-tailed Student test was used for statistic analysis.

3. Results

3.1. Murine non-lymphoid splenocytes and B lymphocytes but not T cells activate the complement system, resulting in C3-fragment deposition on the plasma membrane; CR1/2 is not the exclusive covalent binding site for C3b

Human B cells and B lymphoblastoid cell lines have been known for long to activate the alternative pathway of complement, resulting in C3-fragment deposition on the cell membrane (Budzko et al., 1976; Erdei et al., 1983; Mold et al., 1988; Praz and Lesavre, 1983). The process is mediated via complement receptor type 2 (CR2, CD21), with complement receptor type 1 (CR1, CD35) playing a subsidiary role (Mold et al., 1988; Nielsen et al., 2002). Since it is not known which type of murine cells are able to fix C3-fragments, moreover, in contrast to the human system, mouse CR1 and CR2 are encoded by the same gene, we aimed to clarify how complement deposition takes place in mouse systems and whether CR1/2 are involved. To this end spleen cells were isolated from BALB/c and CR1/2 KO mice and cell-bound C3-fragments were assessed by flow cytometry. As demonstrated in Fig. 1, B lymphocytes and non-lymphoid cells fix C3-fragments when incubated with normal mouse serum, while T cells do not. As shown in the figure, treatment of splenocytes with the serum of C3 KO animals does not allow C3-fragment deposition to the surface of any of these cell types. Similarly to the human system, and in good agreement with our earlier observation (Mastellos et al., 2004), B cells derived from BALB/c mice show substantial C3-fragment deposition. However, here we demonstrate that B cells of the CR1/2 KO animals are also able to fix C3-fragments, suggesting that CR1/2 is not the exclusive binding site for the activated C3-fragments on mouse B lymphocytes. Interestingly, non-lymphoid cells derived from either normal or CR1/2 KO mice as well fix C3-fragments with almost the same intensity (R_{Gmean} 18 versus 15.8, respectively). Since these cells are mainly macrophages and dendritic cells lacking CR1/2, our results strongly suggest the presence of so-far unidentified C3-acceptor sites on the surface of non-lymphoid cells, as well.

3.2. C3-fragments are fixed covalently and appear in patches in the cell membrane

Based on our earlier (Kerekes et al., 1998) and present results (Fig. 2A) we used the mouse B cell lines 2PK3, A20 and the macrophage line P388D1 for further studies. As seen in Fig. 2A, when cells were incubated with fresh serum of C3 KO animals, no C3-fragment deposition could be observed. In order to investigate whether C3b is fixed covalently to the membrane of these cells, MA-treated serum was used. MA, the nucleophile reagent is known to destroy the active thioester group of nascent C3b, consequently these fragments will not be able to bind covalently to their target (Law et al., 1979). As shown in Fig. 2A, fixation of C3 did not occur in this case, proving that C3-fragments detected after incubation of the cells with intact, autologous fresh serum are fixed to the cell membrane covalently.

Cell surface deposited C3-fragments were also visualized with confocal laser scanning microscope. Fig. 2B shows that the distribution of C3 fragments is not stochastic, but rather concentrated in small patches on the surface of A20 cells. Experiments with 2PK3 cells and P388D1 macrophages gave similar results (data not shown).

3.3. Time-dependent release of C3-fragments from B cells and macrophages

Next we aimed to clarify the fate of deposited C3-fragments. To this end serum-treated cells were incubated at 37 °C and the amount of cell surface bound fragments on one hand, and the quantity of released C3-fragments on the other, were assessed at the indicated times. In the control sample cells were treated with C3 KO serum. Panel A of Fig. 3 shows that the amount of membrane-bound C3-fragments decreases by time both on B cells and macrophages. In the case of B lymphocytes the majority of complement fragments disappear from the cell surface in 10 h, as it is clearly seen in panel B of Fig. 3, demonstrating changes in the relative fluorescence intensity of the samples. This process is much faster on macrophages – i.e. a major decrease of membrane-bound C3-fragments can be observed already after 5 h – most probably due to the more intensive membrane transport events of this cell type.

To find out whether C3-fragments are ingested or released by the cells, the C3-content of the supernatants taken at the indicated time-points was measured by ELISA. As seen in panel C of Fig. 3, the amount of C3-fragments in the supernatant of both cell-type increases by time. It has to be noted that when P388D1 cells are treated with C3 KO serum, a slowly but continuously increasing amount of C3 can be detected after incubation for longer periods (panel C of Fig. 3), due to the released complement protein synthesized by the macrophages themselves (Nathan, 1987).

3.4. C3-fragments released by the cells are constituents of exosomes

In further experiments we set out to study whether C3-fragments are released by the cells in soluble form or they are present in the small vesicles which are known to be secreted continuously by B cells and macrophages (Denzer et al., 2000b; Raposo et al., 1996). To this end we isolated exosomes by ultracentrifugation from the cell-free supernatant of the serum-treated cells after 20 h, as it is described in Section 2. The C3-content of both the pellet and supernatant was measured by ELISA. Panel A of Fig. 4 shows that the vast majority of C3

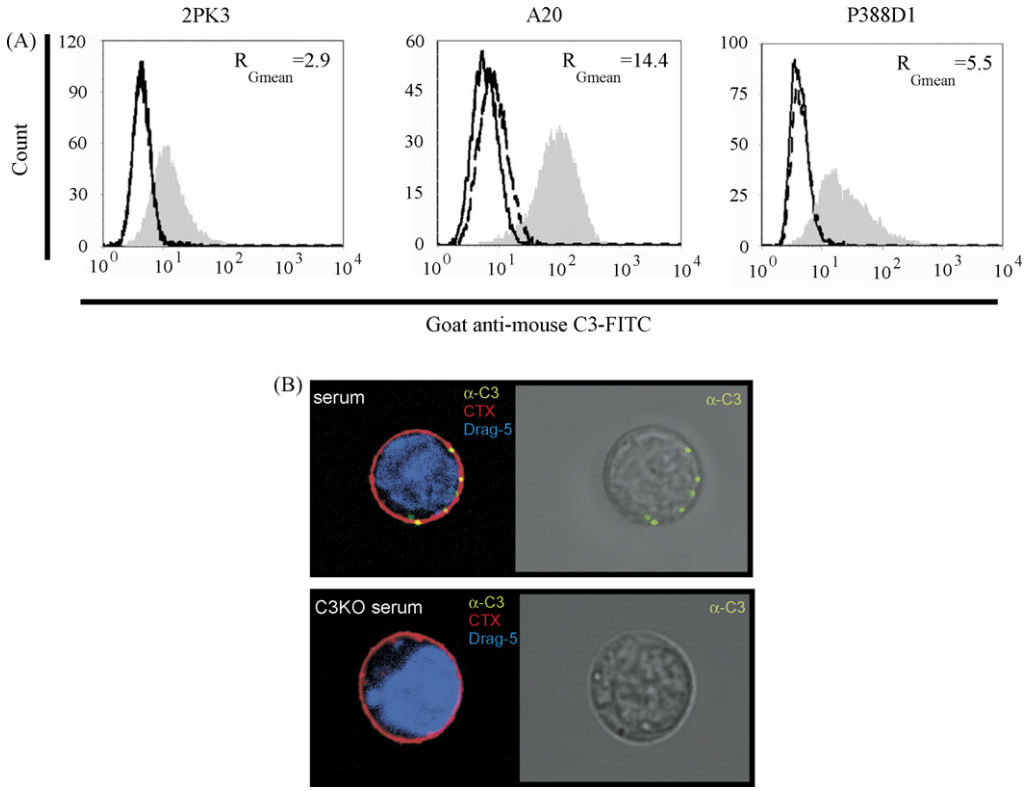

Fig. 2. Covalent binding of C3-fragments to mouse B cell lines and P388D1 macrophages; patchy distribution of the complement protein on the cell membrane. (Panel A) C3-deposition on serum-treated 2PK3, A20 and P388D1 cells was measured by cytofluorimetry using FITC-labeled goat anti-mouse C3. Cells were treated with fresh serum of Balb/c (shaded histogram), or C3 KO (solid line) mice or with MA-treated serum (dashed line). (Panel B) C3-deposition on serum-treated A20 cells. The nucleus (blue), the cell membrane (red) and deposited C3-fragments (green) were visualized by confocal laser scanning microscope. One representative experiment of 3 is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

could be detected in the exosome-fraction, excluding the possibility that the complement fragments are cleaved off or shed by the cells in soluble form.

The presence of exosomes in the pellet was confirmed by electron microscopy. As shown in Fig. 4B. small vesicles of 50–80 nm diameter were identified in the pellet but not in the supernatant of the ultracentrifuged samples derived from A20 B cells. We confirmed the presence of C3 on the surface of the small vesicles in the multivesicular bodies by immunocytochemistry of ultrathin sections of serum-treated cells (Fig. 4C).

3.5. C3-fragment containing exosomes enhance antigen presentation

Exosomes released by dendritic cells and B cells are known to carry MHC-, co-stimulatory- and adhesion-molecules, which are involved in the process of antigen presentation (Clayton et al., 2001; Escola et al., 1998; Raposo et al., 1996). Moreover, earlier we have demonstrated that APC-bound C3-fragments significantly enhance stimulation of antigen specific T cells, particularly at suboptimal antigen doses (Kerekes et al., 1998). Since our present results show that exosomes released by serum-treated cells contain C3-fragments, in further studies their possible effect on antigen presentation was studied. For the experiments we isolated the 40–100-nm-sized membrane vesicles from the supernatant of serum-treated A20 cells pulsed with a suboptimal antigen-dose and set up the antigen presentation assay using these exosomes in the presence of naïve A20 cells. Proliferation of T cells isolated from the lymph-node of OVA-immunized mice was measured by [^{3}H]thymidine uptake. Fig. 5 shows that exosomes derived from normal serum-treated APC significantly increase the efficiency of antigen presentation compared to exosomes derived from APC incubated with C3 KO serum. This result points to the role of C3 in the enhancement of exosome mediated antigen presentation.

4. Discussion

APC – such as B lymphocytes and macrophages – are able to activate the alternative pathway of complement, which leads to covalent deposition of C3-fragments to the cell surface (Erdei et al., 1983; Kerekes et al., 1998; Marquart et al., 1994; Mold et al., 1988; Praz and Lesavre, 1983). The activation of the autologous complement system does not lead to lysis under non-pathogenic conditions, on the contrary, Raji cells for example, were shown to survive for weeks after serum treatment (Praz and Lesavre, 1983). A primary acceptor site for C3b on the surface of human peripheral B lymphocytes is complement receptor type 2, as more than 95% of C3 deposition was abolished when the cells were pretreated with CR2-specific antibody (Marquart et al., 1994). CR2 was confirmed as being the primary binding site for C3-fragments on Raji cells, as well (Mold et al., 1988). The

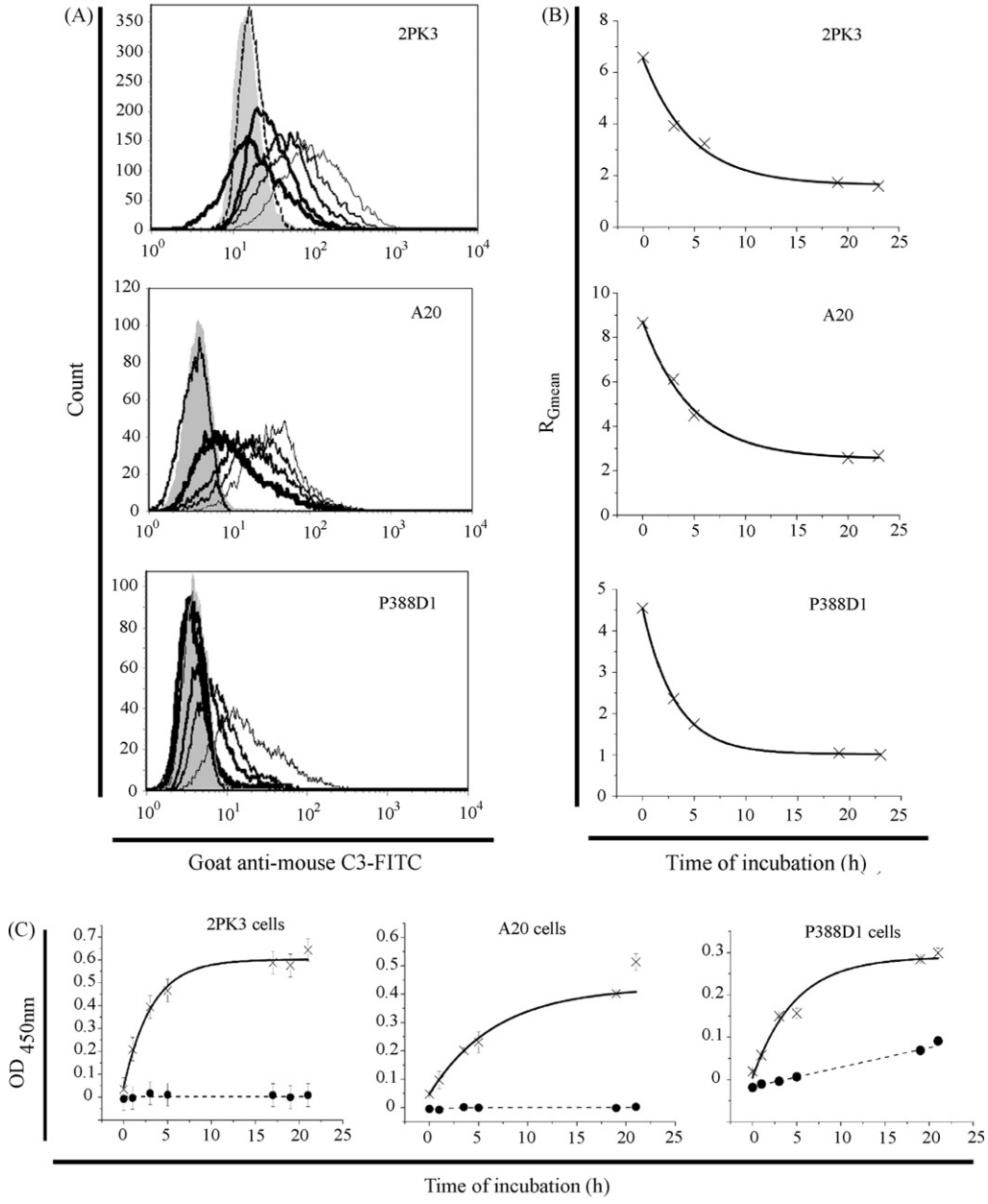

Fig. 3. Time-kinetics of C3-release from serum treated B cells and macrophages. (Panel A) C3-deposition on 2PK3, A20 and P388D1 cells as measured by cytofluorimetry. Cells were incubated with the serum of BALB/c mice for 1 h at 37 °C, washed, and kept in unsupplemented medium for different time-points. The thickness of the lines represents the length of incubation as indicated on the diagrams of panel B. Shaded histogram: C3 KO serum treatment; dashed line: autofluorescence. (Panel B) Relative Gmean ($Gmean_{Balb/c}/Gmean_{C3\ KO}$) values as a function of incubation time. (Panel C) C3-content of the cells' supernatant measured by sandwich ELISA. Time-dependent release of C3 in the supernatant of the cells treated with the serum of BALB/c mice (thick line), and with the serum of C3 KO animals (dashed line). One representative experiment of 3 is shown.

covalent binding site for activated C3 has been shown to be different from the interaction site of C3d, the natural ligand of CR2 (Marquart et al., 1994; Olesen et al., 1998). Our results clearly demonstrate that in contrast to human B cells, CR1/2 expressed on murine B lymphocytes has a much smaller contribution to the covalent fixation of activated C3. Fig. 1 shows that serum-treatment results in a substantial C3-fragment deposition on spleen B cells even in the absence of CR1/2—i.e. when cells derived from CR1/2 deficient animals were employed. As murine macrophages do not express CR1/2 on their surface (Martin and Weis, 1993), our finding that non-lymphoid cells (Fig. 1) and the macrophage line P388D1 (Fig. 2) are also able to bind activated C3 covalently, strongly suggest the presence of a yet unknown acceptor-site on the plasma membrane of these cells. Marquart et al. (1994) published similar findings in the case of the CR2-negative human monocytes and granulocytes, again suggesting that not only this molecule can serve as a C3b-acceptor on the cell surface. As our confocal laser scanning microscopic results show, covalent deposition of C3b is not diffused (Fig. 2B). The patchy distribution may suggest that certain areas of the cell membrane are prone to interact with the activated complement component, and C3b-fragments initially deposited

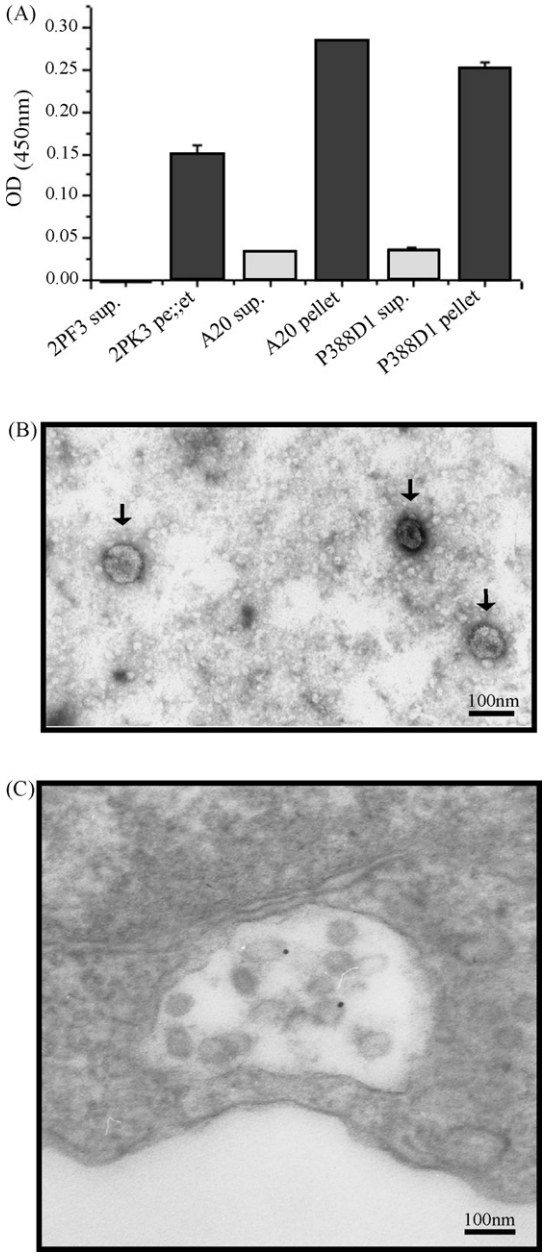

Fig. 4. Exosomes released by serum-treated cells carry C3-fragments. (Panel A) C3-content of fractions obtained after ultracentrifugation of supernatants of serum-treated 2PK3, A20 and P388D1 cells. Grey column: exosome-free supernatant; black column: exosome-containing fraction. (Panel B) Exosomes of A20 cells visualized by electronmicroscopy. (Panel C) Detection of C3-fragments on the surface of exosomes packed in the multivesicular bodies of serum-treated A20 cells. For the immunocytochemical staining of the ultrathin sections FITC conjugated goat anti-mouse C3 $F(ab')_2$ antibody and 10 nm gold-conjugated mouse anti-FITC antibody was used. One representative experiment is shown.

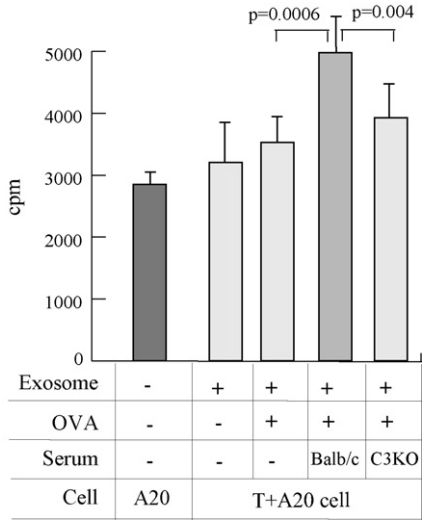

Fig. 5. C3-fragment carrying exosomes enhance antigen presentation. Exosomes derived from APC pulsed with suboptimal amount of antigen and treated with normal or C3 KO serum were mixed with naïve A20 cells and OVA specific T cells. T cell proliferation was measured by [^{3}H]thymidine uptake. One representative experiment of 3 is shown.

to these sites readily gather further fragments by the activity of the in situ formed alternative pathway C3-convertases. Interestingly, normal T lymphocytes do not activate the complement system (Fig. 1), which can be a result of the lack of activator or acceptor molecules on the cell surface, or alternatively might be due to the different expression pattern of complement control proteins on these cells. Our present data and those of Marquart et al. (1994), clearly show that C3-deposition is cell type specific, and professional APC – such as B cells and macrophages – posses the ability to fix C3-fragments covalently, in contrast to T lymphocytes. This finding points to major differences in the capacity of various cell membrane constituents to activate and fix C3-fragments covalently-, despite the fact that chemically, nascent C3b fragments can bind to almost any hydroxil-groups via their active thioester group (Law et al., 1979). Thus, a so far unidentified cell membrane constituent must be present on the surface of CR1/2 negative cells, which serves as an activator of the complement cascade and as an acceptor for nascent C3b. Identification of this molecule would be important to get more insight into the physiology of immunecompetent cells, particularly due to the lack of this structure in T cells.

As it is known that under physiological conditions membrane vesicles are released from several cell types (Beaudoin and Grondin, 1991), the fate and further function of deposited C3 fragments was in the focus of our study. Here we demonstrate that C3-carrying exosomes are secreted by serum-treated B lymphocytes and macrophages. We show that these cells remove nearly all plasma membrane-bound C3-fragments within a few hours after deposition. Under physiological conditions however, complement activation may take place on the surface of exosomes, as well, generating C3-opsonized particles. Mold et al. have shown that liposomes are able to activate the alternative pathway when phosphatidylethanolamine (PE) is exposed on their surfaces (Wang et al., 1993; Mold, 1989). On live cells PE distribution is asymmetric between the two sides of the plasma membrane, and most of it is concentrated in the inner side (Seigneuret and Devaux, 1984). In the case of exosomes however, there is a symmetric distribution (Laulagnier et al., 2004), consequently PE present in the outer leaflet of the membrane might be able to activate the complement system. It is also known that the exosome limiting membrane contains complement regulatory proteins (Clayton et al., 2001), which might also affect

C3 deposition on their surface. Supporting our results, recently Koppler et al. (2006) reported the complement-dependent transfer of the bigger size (200–1000 nm) microparticles to human B cells, directing the activation of the cells towards an anti-inflamatory phenotype.

Exosomes have been shown to play an important role in several immune phenomena (Li et al., 2006; Mignot et al., 2006). These small, 40–90 nm diameter vesicles are formed in MVB (Pan et al., 1985) which is the subcellular site for peptide loading onto MHCII (Harding and Geuze, 1993). Exosomes were detected in the blood (Caby et al., 2005) and in bronchoalveolar lavage fluid (Admyre et al., 2003) suggesting that they may serve as messengers between cells in the body (Denzer et al., 2000a). B cell-derived vesicles were shown to contain several molecules which are important for antigen presentation; such as MHC classes I and II molecules, B7.1 (CD80), B7.2 (CD86) co-stimulatory molecules, tetraspan proteins (CD63, CD81, CD82) (Escola et al., 1998) and ICAM-1 (CD54) adhesion molecules on their surface (Clayton et al., 2001; Raposo et al., 1996). We now add C3-fragments to the list of exosomal proteins which are released by APC.

There are several possible ways for exosomes to interact with various cells and influence different functions of the target. As these small vesicles have been shown to induce antigen specific, MHCII restricted T cell response *in vitro* (Raposo et al., 1996) moreover, APC-deposited C3 fragments are known to facilitate antigen presentation (Erdei et al., 1992; Kerekes et al., 1998, 2001), we assumed that exosomes carrying C3-fragments might increase the efficiency of antigen presentation. In the present study we show that C3-fragment containing exosomes derived from APC loaded with a suboptimal antigen dose – i.e. employing a stimulus which most probably occurs *in vivo*, as well – induce a significantly stronger OVA-specific T cell response than exosomes without the complement fragments (Fig. 5). The same process may take place under physiological conditions, since covalent deposition of C3-fragments on the surface of APC occurs *in vivo*, as well (Kerekes et al., 2001; Marquart et al., 1994). Morevover, tumor cells as well as virus-infected cells may also require the ability to activate the alternative pathway (Cooper and Oldstone, 1983; Mignot et al., 2006) and consequently may release C3-containing exosomes which contribute to the generation of an effective immune response.

Another known physiological target for B cell derived exosomes is the follicular dendritic cell, that does not produce MHCII (Denzer et al., 2000b). As these cells express CR1 and CR2 receptors as well on their surface – which are known to play a crucial role in the generation of normal humoral immune response (Fang et al., 1998) – they may bind C3-carrying exosomes more efficiently than vesicles lacking this ligand. This complement-mediated enhancement may be important in the follicular dendritic cell guided affinity maturation and Ig isotype switching of B cell clones during the germinal center reaction.

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Chapter 7

Krisztián Papp, Zsuzsanna Szekeres, Nóra Terényi, Andrea Isaák, Anna Erdei, József Prechl

On-chip complement activation adds an extra dimension to antigen microarrays

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On-chip Complement Activation Adds an Extra Dimension to Antigen Microarrays*

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Antibody profiling on antigen microarrays helps us in understanding the complexity of responses of the adaptive immune system. The technique, however, neglects another, evolutionarily more ancient apparatus, the complement system, which is capable of both recognizing and eliminating antigen and serves to provide innate defense for the organism while cooperating with antibodies on multiple levels. Complement components interact with both foreign substances and self molecules, including antibodies, and initiate a cascade of proteolytic cleavages that lead to the covalent attachment of complement components to molecules in nanometer proximity. By refining the conditions of antibody profiling on antigen arrays we made use of this molecular tagging to identify antigens that activate the complement system. Antigen arrays were incubated with serum under conditions that favor complement activation, and the deposited complement C3 fragments were detected by fluorescently labeled antibodies. We used genetically C3-deficient mice or inhibition of the complement cascade to prove that the technique requires complement activation for the binding of C3 to features of the array. We demonstrate that antigens on the array can initiate complement activation both by antibody-dependent or -independent ways. Using two-color detection, antibody and complement binding to the relevant spots was measured simultaneously. The effect of adjuvants on the quality of the immune response and binding of autoantibodies to DNA with concomitant complement activation in the serum of mice suffering from systemic autoimmune disease was readily measurable by this new method. We propose that measurement of complement deposition on antigen microarrays supplements information from antibody binding measurements and provides an extra, immune function-related fingerprint of the tested serum. ***Molecular & Cellular Proteomics 6: 133–140, 2007.***

The immune system is traditionally divided into innate and adaptive arms. Although these two arms are highly interconnected and mutually interactive, we generally regard antibodies as components of the clonal adaptive immune response, whereas the complement system functions as a non-clonal innate mechanism for maintaining body integrity. Antibodies can be beneficial or deleterious: those against infectious agents can protect from disease, whereas antibodies against self molecules can induce autoimmune reactions. Because antibodies have identifiable target molecules they became the cornerstone for diagnosing states of disease. In the last decade the importance of innate immunity was reconsidered, revealing that it has decisive roles both at the initiation and effector phase of the immune response (1, 2). Yet modern high throughput technologies have not yet been applied for the analysis of the complement system.

Current multiplex assays for profiling serum immune reactivity utilize only antibody binding as readout. Antigens spotted in microarray format have been used for measuring serum antibodies against microbial components (3, 4) or whole microbes (5), allergens (6–8), and self structures (9–13). These assays are useful for simultaneously identifying several antigen-antibody interactions and therefore help to draw a more complete picture about the immune status of the tested individual. This is, however, a still picture because the presence of an antibody with a particular specificity does not necessarily mean disease or immunity to infection. Certain aspects on the level of a particular antibody molecule, like affinity and isotype, and others on the level of the organism, like overall concentration of antibodies against a target, relative concentration of antibodies with different isotypes, and structural arrangement of the recognized epitopes on the target, together determine the ultimate functional effects of antibodies. One of these functional effects is the initiation of complement activation.

The complement system comprises about 30 proteins, including soluble and membrane-bound components. Certain recognition molecules are able to initiate the complement cascade of proteolytic cleavages, leading to the generation of complement protein fragments with various biological activities. Three pathways of complement activation have been described: the classical, lectin, and alternative pathways. The classical pathway can be initiated by antigen-bound antibodies (14) and other molecules that bind C1q, like C-reactive protein or serum amyloid P protein (15). The lectin pathway relies on the mannose binding lectin and ficolins, molecules that recognize carbohydrate patterns (16–18). The alternative

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pathway is capable of autoactivation; therefore surfaces that lack or are unable to bind complement regulatory proteins promote its activation (19). Importantly all three pathways converge at the point of complement C3 cleavage and activation, a point where an amplification loop insures the generation of sufficient amounts of C3 fragments. Complement component C3 is additionally one of the most abundant serum proteins after albumin and immunoglobulins. Thus, whichever pathway initiates the cascade, C3 fragments will be generated with high efficiency. The initial products of C3 cleavage are a small peptide, C3a, with inflammatory properties and a bigger fragment, C3b, with proteolytic activity as part of a complement convertase enzyme complex. Upon cleavage, an extremely reactive thioester group of the C3 molecule becomes exposed (20) and reacts with a nearby hydroxyl or amine group (21). As a result, the molecules at the site of complement activation will be covalently tagged with the C3 activation and degradation fragments C3b, iC3b, and C3d.

To obtain additional, function-related information from antigen arrays we have developed a reverse immunoassay technique that allows the detection of complement activation by the features of the array. This technique takes advantage of the deposition of complement C3 fragments to the molecules that initiate the complement cascade. We have characterized the system with respect to sensitivity and specificity and worked out proper controls to allow for semiquantitative measurements. We show that the technique, in combination with antibody profiling measurements, can be used to provide additional functional information beyond antibody binding.

EXPERIMENTAL PROCEDURES

Collecting Serum Samples for Complement Deposition Measurements—BALB/c and C57/B6 mice were bred at the animal facility of the university and used at the age of 6–8 weeks. Serum from MRL/lpr mice was generously provided by Nikolina Mihaylova (Bulgarian Academy of Sciences, Sofia, Bulgaria); serum from C3-deficient (22) and JHD animals were a kind gift from Matyas Sandor (University of Wisconsin-Madison). For obtaining immune sera groups of three mice were immunized by injecting 100 μg of antigen alone or mixed with complete Freund's adjuvant (Sigma) in a total volume of 200 μl, 100 μl subcutaneously into the base of the tail and 100 μl intraperitoneally. After 2 weeks the immunization was repeated according to the same regimen except that incomplete adjuvant was used. Mice were sacrificed after 4 weeks by exsanguination, and their sera were stored at −70 °C until use. A pooled sample prepared from the individual sera within an immunization group was applied to the antigen arrays immediately after thawing.

Fabrication of the Antigen Array—All the materials for antigen spots, if not stated otherwise, were obtained from Sigma. For the preparation of zymosan, a suspension of bakers' yeast cells was boiled for 1 h and then washed with physiological salt solution. This treatment exposes the cell wall component zymosan, which is particularly efficient in initiating the lectin and alternative pathways of complement activation. A hand-held spotting device, Microcaster (Whatman), was used for arraying the antigens according to the manufacturer's instructions. The spotting pins of our contact printing arrayer were ~500 μm in diameter. Antigens were spotted onto homemade nitrocellulose-covered slides or Fastslides (Whatman) and stored at 4 °C in a sealed bag until use or for a maximum of 4 weeks. On each array were printed three to four replicate features of 1:5 serial dilutions of each antigen. The starting concentration of the antigens was 1 mg/ml in PBS containing 0.05% sodium azide.

Hybridization Conditions for Complement Activation—Dried arrays were rinsed for 15 min in PBS just before use. Arrays were then incubated with sera in a humidified chamber at 37 °C for 60 min. Serum complement activation took place with the array covered by a glass slide, which was kept at 1-mm distance from the array by plastic spacers. The amount of mouse serum required for one measurement was 600 μl or less depending on the size of the array. The reaction was terminated by washing the array with PBS. Alexa Fluor 647-conjugated detecting antibodies goat anti-mouse IgG and anti-mouse IgM were from Molecular Probes, whereas the fluorescein-conjugated goat $F(ab')_2$ fragment to mouse C3 was obtained from MP Biomedicals. This polyclonal antibody reacts with all the fragmented forms of C3 including C3b, iC3g, and C3d. The detecting antibody or the mixture of the two antibodies diluted 1:5,000 in 5% skimmed milk powder in PBS was added to the arrays, which were then incubated with gentle agitation for 30 min at room temperature in the dark. The arrays were scanned after three more 10-min washes in PBS.

Analysis of Hybridized Microarrays—In a preliminary experiment, after preparing the array for scanning as described above, digital images of individual features of the array were taken on an Olympus IX-70 inverted fluorescence microscope equipped with a DP-50 digital camera using appropriate settings for fluorescein detection. Scale was determined by using a Bürker counting chamber as standard.

Slides were scanned on a Typhoon Trio+ imager (Amersham Biosciences) following standard protocols. Laser intensity was set to provide optimal signal intensity with minimal background and without saturated pixels. Data were analyzed with ImageQuantTL (Amersham Biosciences) software. Signal intensities were calculated by subtracting background from medians of signal intensity in a spreadsheet program (Microsoft Excel). For interassay comparisons fluorescence intensities were adjusted to give comparable readings for the highest dilutions of protein LA (pLA).[1] Overlays of false color images were prepared by Adobe Photoshop software.

RESULTS

Overview of the Antibody Array—We spotted various kinds of purified or complex antigens, proteins, nucleic acids, whole mouse serum, and particulate antigens, and a goat anti-mouse C3 capture antibody on nitrocellulose-covered glass slides. Two of the antigens were potent activators of the classical pathway: a fusion protein from bacterial Ig-binding proteins (protein LA) and heat-aggregated human immunoglobulins. Zymosan, on the other hand, was used as a known activator of the lectin and the alternative pathways. Bovine and human albumins, keyhole limpet hemocyanin (KLH), lysozyme, dinitrophenol-BSA conjugates, influenza virions, and zymosan were used as foreign antigens, whereas murine albumin and DNA were used as self structures. Arrays were incubated in mouse sera at 37 °C to allow complement activation to take place. The generated C3b molecules form part of the C3 convertase complexes and permit the production of

[1] The abbreviations used are; pLA, protein LA; dsDNA, double-stranded DNA; JHD, J segment of heavy chain deleted; KLH, keyhole limpet hemocyanin.

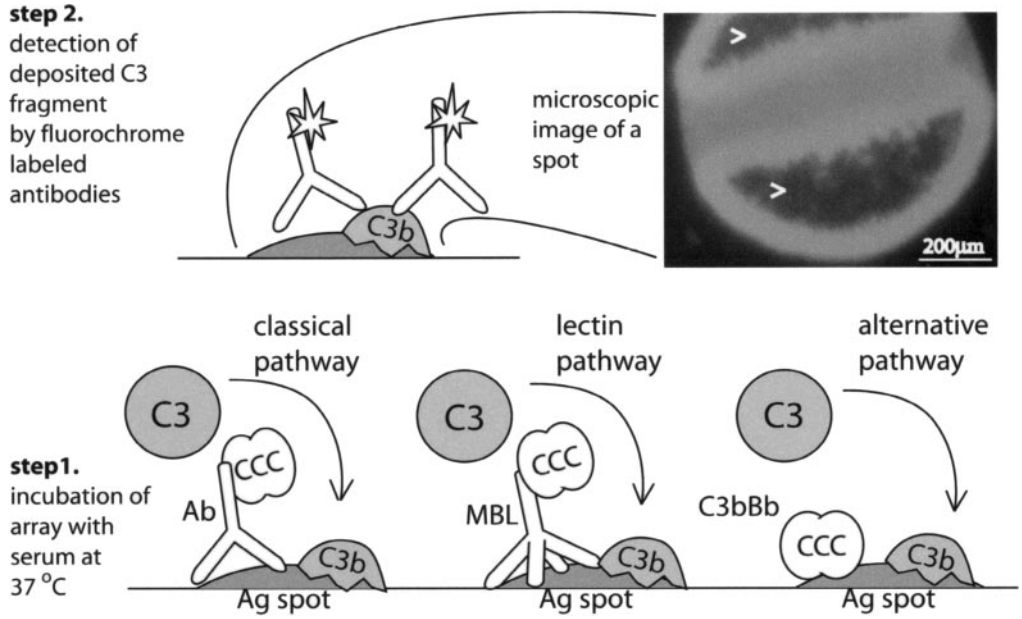

FIG. 1. **Schematic illustration and microscopic image of the C3 deposition antigen array.** Complement activation in the tested serum sample can be initiated by the binding of antibody (*Ab*) and C1q (classical pathway), lectins (*e.g.* mannose binding lectin (*MBL*)), or the alternative pathway C3 convertase complex (*C3bBb*) to antigen spots (*Ag spot*) of the array. As a result, a C3 convertase complex (*CCC*) forms on the features and generates reactive C3 fragments (*C3b*) that immediately form covalent bonds with nearby NH_2 and OH groups, including water molecules (*Step 1*). The alternative pathway convertase C3bBb forms directly on the activating surface. After washing out unbound C3, labeled antibodies are used for identifying features with deposited C3 fragments (*Step 2*). *Inset*, fluorescent microscopic image of a spot with deposited C3. Fine features imprinted by the spotting pin are readily distinguishable, confirming that practically there is no diffusion of the generated reactive C3b and fluorescence is strictly restricted to the area of printed antigen. *Arrowheads* mark shadows left by the split printing pin.

more C3b fragments. Covalent C3 fragment binding to the relevant array features was detected by fluorescently labeled antibodies as outlined in Fig. 1.

The first question to be answered was whether the short half-life of the reactive C3b component really restricts C3 deposition to the respective activating features of the array. We used a nitrocellulose-based array because earlier Western blot experience suggested that nitrocellulose was inert and suitable for incubation in fresh serum without nonspecific complement activation (23, 24). To confirm that the generated soluble C3b fragments are bound only in the area of the printed antigens we microscopically examined protein LA spots. It is evident in Fig. 1 (*inset*) that C3 signals had a sharp, high contrast border with no diffusion-derived transition of fluorescence between the spot and the background. As a result of the contact printing method the fine features (two *crescent-like shadows*) of the split printing pin clearly showed up in the image. This result suggests that diffusion of the generated soluble, reactive C3b is indeed a negligible factor on a micrometer scale, and therefore the measurement of complement C3 deposition can be applied to microarrays.

Activation of Complement and C3 Deposition on the Array—In addition to the antigens we included a dilution series of polyclonal C3-specific capture antibodies as a way to confirm the presence and compare concentrations of C3 in the serum samples. To exclude the possibility that C3 binds to the features of the array by passive adsorption we added EDTA to the serum before incubation. EDTA chelates Ca^{2+} and Mg^{2+} ions, which are indispensable for complement activation. Addition of EDTA completely prevented C3 deposition on pLA, human IgG, and zymosan; the only remaining C3 signal came from the C3 capture spots and the spotted sera containing C3 (Fig. 2). To further confirm the specificity of the system we incubated arrays with serum from genetically C3-deficient mice (22) with the result that even the capture spots became negative. The arraying and hybridization conditions were therefore suitable for the specific detection of complement activation.

Sensitivity and C3 Dependence of the Method—Sensitivity of the method, in terms of the minimal amount of C3 detectable, was established by spotting serial dilutions of C3-sufficient and -deficient serum. There was practically no background signal from the serum of C3-deficient animals (Fig. 3*A*). Assuming serum C3 concentration of normal mice as 0.5 mg/ml and an average printing volume of 50 nl, 200 pg of C3 was still detectable on the array. Relative fluorescence intensity values in the range of 0–3 $\times$ 10^7 corresponded to 0–25 ng/spot calculated C3 mass (Fig. 3*B*). Correlation coefficient of intra-assay spot-to-spot variation was less than 10% for spots containing 1 mg/ml antigen but increased at lower antigen concentrations. To assess the dependence of the method on C3 concentration we reconstituted sera of genet-

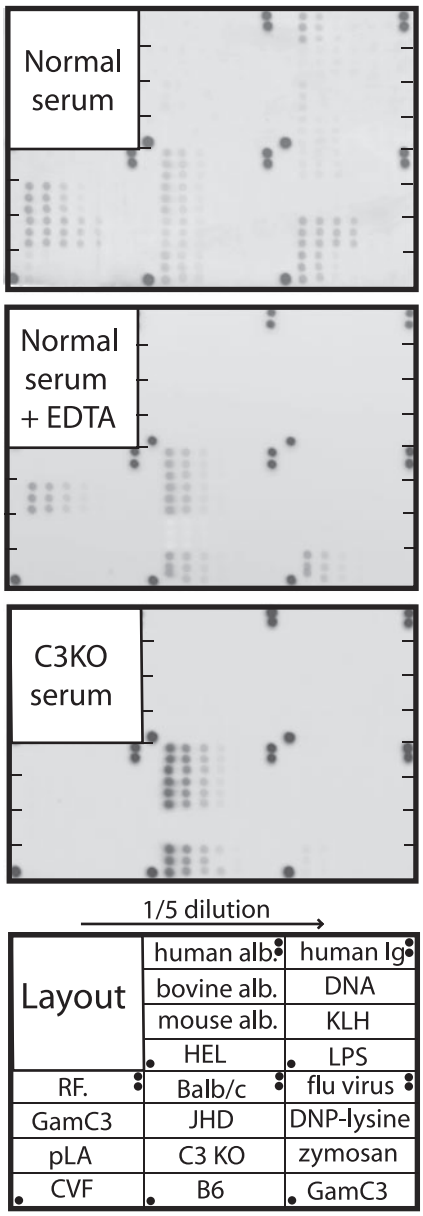

FIG. 2. **Complement deposition requires activation of the complement system.** A medium density antigen array was prepared with the components indicated in *Layout* using contact printing on a nitrocellulose surface. Orientation spots mark subarrays in the *lower left* and *upper right corners*. All antigens were spotted in six different dilutions and three parallels. The arrays were incubated in normal serum, normal serum supplemented with 20 mM EDTA, or C3-deficient serum for 1 h at 37 °C. Pictures show scanned images after labeling C3 with FITC-conjugated $F(ab')_2$ antibody fragments. *RF.*, rheumatoid factor; *GamC3*, goat anti-mouse C3; *CVF*, cobra venom factor; *alb.*, albumin; *HEL*, hen egg lysozyme; *LPS*, lipopolysaccharide; *flu*, influenza; *DNP*, dinitrophenol. Serum samples from BALB/c, JHD, C3KO, and C57/B6 mouse strains were also printed as shown.

ically C3-deficient mice with different amounts of C3-sufficient normal serum. The assay proved to work even after a hundredfold reduction in C3 concentration, although it became less sensitive in the case of zymosan-induced C3 deposition (Fig. 3*C*). It is important to note that zymosan can initiate both the lectin and alternative pathways. This two-component activation might be responsible for the observed break of the curve showing epitope density-dependent complement activating efficiency.

Two-color Microarrays for Simultaneous Detection of Antibody and C3—Individuality in serum complement immunoprofiles is expected mostly from differences in antibody repertoires. Therefore the measurement of C3 deposition is particularly interesting in combination with antibody binding measurements. To assess the possibility of concurrent determination of bound antibodies and C3 fragments we analyzed sera from mice immunized with BSA. Detection antibodies against C3 were FITC-labeled, whereas those against IgG were Alexa Fluor 647-conjugated. Whereas no C3 signal was detectable on the albumin spot after reacting with nonimmune mice serum (Fig. 2), bound C3 and IgG were readily detectable on BSA when the array was incubated in immune serum (Fig. 4). Moreover cross-reactivity against human serum albumin was also observed. Interestingly some C3 deposition was also observed on the murine albumin spots as well. No C3 deposition was detected on an unrelated protein, hen egg lysozyme. Zymosan on yeast particles is a well known activator of the lectin and alternative pathways of complement, and as Fig. 4 shows C3 deposition was indeed induced without the binding of any detectable IgG.

Characterizing Qualitative Differences of the Humoral Immune Response—The nature of antigen-specific antibodies that are generated following immunological challenge depends on the nature of the challenge itself. Thus, strong inflammatory signals promote the appearance of antibodies with the ability to sustain inflammation partly by fixing complement. To assess the potency of the C3 deposition array in discriminating the subtleties of a humoral immune response, we compared C3 deposition on KLH from sera of non-immune animals, animals immunized with protein only, and animals immunized with protein in complete Freund's adjuvant. Serum from naïve animals showed no C3 deposition on KLH, whereas significant amounts of C3 were detected, along with Ig, when sera of immunized animals were tested (Fig. 5, *A* and *B*). Of note, the difference in C3 deposition was more pronounced than in Ig binding between serum samples from animals immunized with or without complete Freund's adjuvant. The pattern of C3 deposition and IgG binding to pLA was not different in the three groups (Fig. 5, *C* and *D*).

The ability to activate the complement system influences the pathogenicity of autoantibodies. To address the use of the C3 deposition array in a murine model of systemic lupus erythematosus, we measured antibody binding and C3 fragment deposition on dsDNA spots in sera from normal and severely ill MRL/lpr mice. As expected, serum from the autoimmune animals contained antibodies against dsDNA. These antibodies activated the complement system as indicated by the deposition of C3 fragments (Fig. 5*E*).

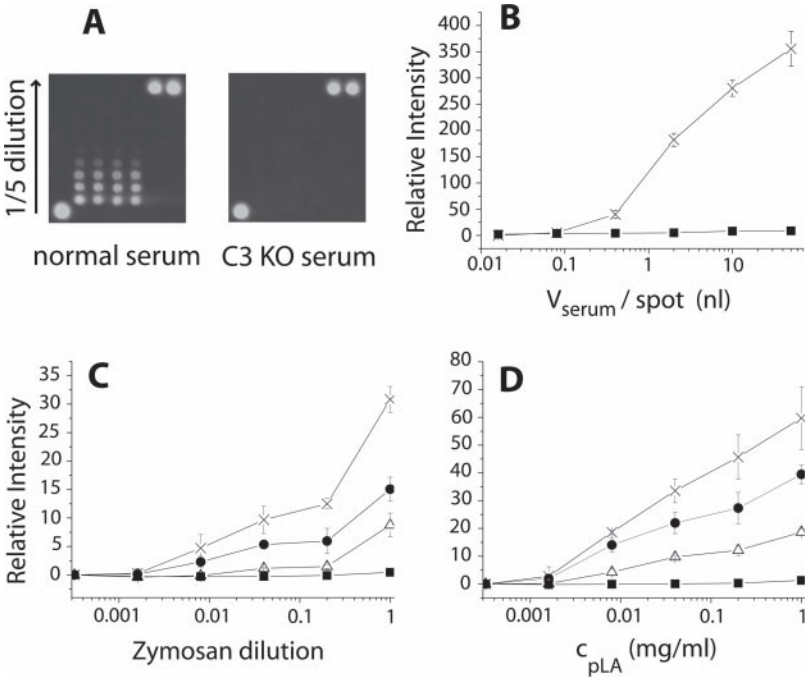

FIG. 3. **Sensitivity of detection and influence of C3 levels in the serum.** Dilutions of C3-sufficient (normal, ×) and -deficient (C3KO, ■) serum samples were spotted and developed by the C3-specific FITC conjugate (*A* and *B*). Relative fluorescence intensities were obtained from the scanned images, confirming the absence of signals from C3-deficient mouse serum and showing the sensitivity of C3 detection (*B*). C3-deficient serum (■) was reconstituted with C3-sufficient serum (×) to give 90% (●) and 1% (△) relative C3 content. Antigen arrays were incubated in these samples, and relative fluorescence intensities indicating C3 deposition on the two model antigens pLA and zymosan were obtained (*C* and *D*). Note that only the latter two diagrams show the result of C3 deposition by complement activation as opposed to printed C3 on the first two diagrams.

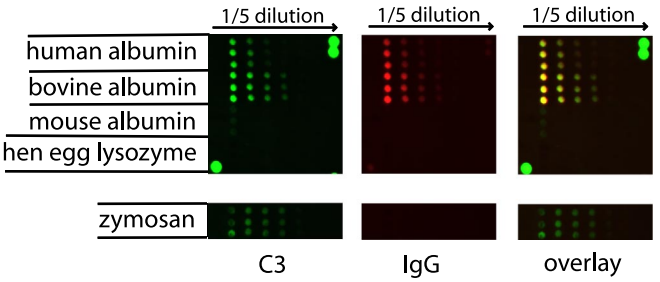

FIG. 4. **Simultaneous detection of C3 and IgG binding.** An antigen array was incubated in anti-BSA immune serum, and then deposited C3 and bound IgG were detected simultaneously with FITC- and Alexa Fluor 647-conjugated antibodies, respectively. Zymosan activated complement without antibody binding. Images are false color images of the two scans and their overlay.

DISCUSSION

A proteomics approach for characterizing the reactivity of the immune system requires identification of the repertoire of molecules that can be specifically recognized by the "detection units" of the system. The best known such unit is the antibody molecule, which can be tailored to recognize practically any structure depending on the need of the organism. Antibodies are not the only soluble detection units, however, and the mere presence of an antibody that binds a particular antigen does not necessarily have medical relevance. A further layer of humoral immune recognition is provided by the complement system. Importantly sites of complement activation are physically marked by covalently bound C3 fragments, a situation ideal for applying to solid-phase immunoassays, including protein microarrays. Antigens spotted on an array can activate the complement system by any one of the three described pathways, and the generated reactive C3 fragments will react with any neighboring molecule (including the spotted antigen and the bound antibodies) in a temporally, and therefore also spatially, confined manner. Thus, complement activation will result in the deposition of C3 only to the antigen spots that initiated the activation, marking the antigen spot clearly for identification (Fig. 1).

In this study we show for the first time that the measurement of complement activation on an antigen array is easily achievable and provides important information beyond antibody binding. Using substances that efficiently trigger complement activation in an antibody-dependent (pLA) or -independent (zymosan) way, we demonstrated that high signal to noise ratios were achievable (Fig. 3, *C* and *D*), confirming that complement activation was indeed restricted to the activating spot. Inert proteins, such as albumin or lysozyme, did not give any signal in normal sera, supporting the specificity of the technique. Note, however, that KLH, a relatively huge aggregated protein of 10^5–10^7 daltons, induced C3 deposition even in the sera of nonimmune animals (Figs. 2 and 5). The most likely cause for this activation is the presence of complement-activating natural antibodies against KLH in mouse serum as it has been described in humans (25). The binding of these antibodies is probably below the detection limit, similar to the undetectable binding of BSA-specific antibodies to murine serum albumin in Fig. 4 where C3 deposition was observed without IgG signal.

In the presence of the chelator molecule EDTA, which blocks all three pathways of complement activation, no C3 deposition was observed (Fig. 2). Thus, C3 was not passively adsorbed but was deposited by covalent attachment exclusively as the result of an active complement pathway. In addition to its covalent linkage to the array features, detecting C3 fragments as a readout of complement activation is also

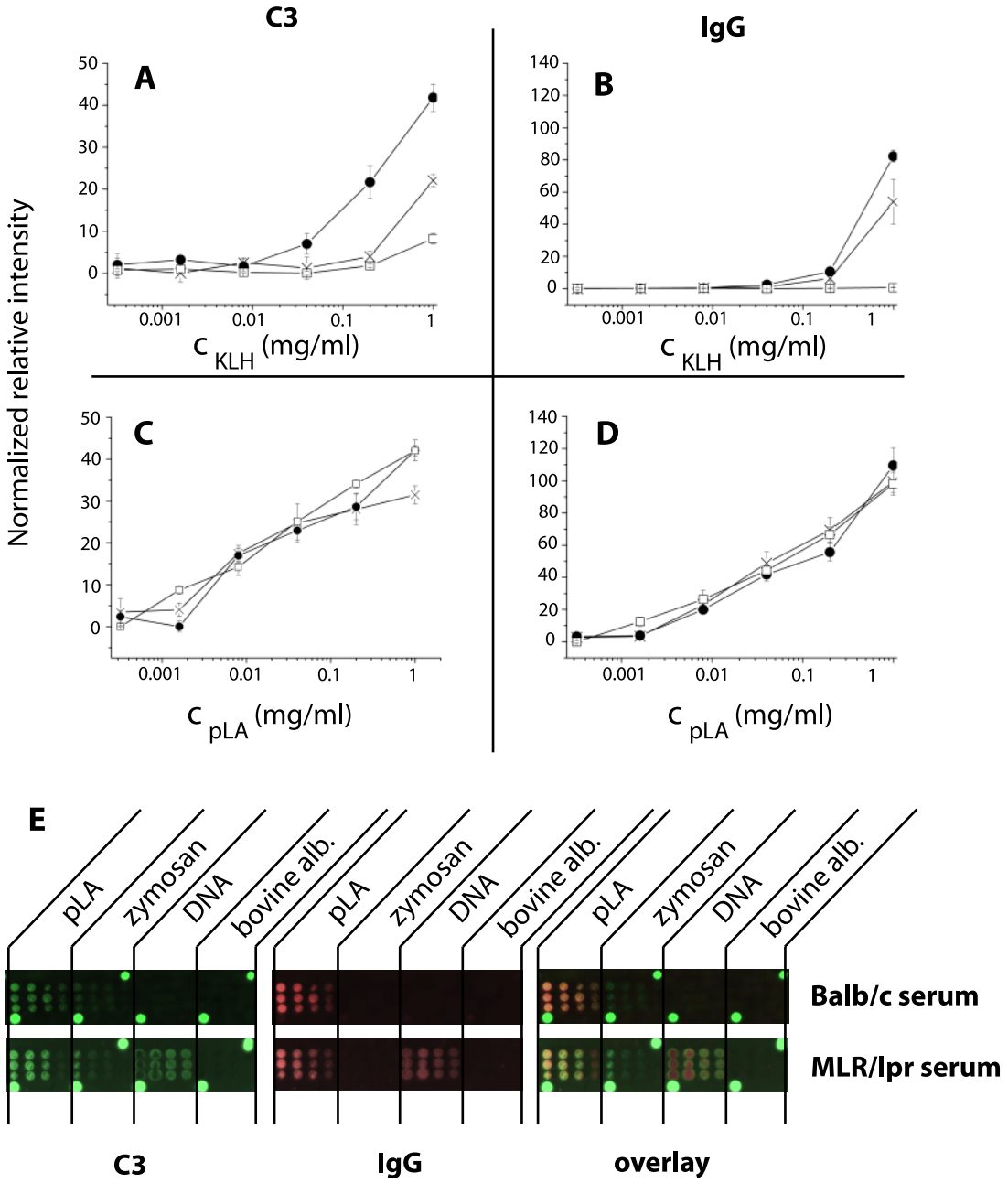

FIG. 5. **Qualitative differences in serum antibody response as reflected by C3 deposition.** *A–D*, antigen arrays were incubated in sera from three different groups of mice: nonimmune (□), KLH-immunized (×), and KLH + complete Freund's adjuvant-immunized (●) animals. Diagrams show fluorescence intensities of deposited C3 (*A* and *C*) or bound IgG (*B* and *D*) on KLH (*A* and *B*) or pLA (*C* and *D*) spots. *E*, the ability of autoantibodies against dsDNA to activate complement was tested by incubating the arrays in serum from normal or MRL/lpr mice. Shown are false color images of the fluorescein and Alexa Fluor 647 scans and their overlay. *GamC3*, goat anti-mouse C3; *alb.*, albumin; *mIgG*, mouse IgG.

advantageous because of the presence of an amplification loop (19): a single C3 convertase can generate several more convertases, which in turn cleave more and more C3 molecules. The process is comparable to the tyramide signal amplification method (26) that is used for increasing the sensitivity of immunoassays and *in situ* hybridizations.

In view of the fact that healthy individuals possess functionally equivalent amounts of complement proteins, most of the individual variability is expected to originate from antibody-dependent complement activation. In our experiments, as expected, complement activating properties of the antibodies were influenced by the immunization protocol: C3 deposition was more pronounced in the serum of animals immunized with inflammation-inducing adjuvants (Fig. 5). This result is in

agreement with the known complement activating properties of the antibody isotypes produced during inflammatory conditions. In addition to epitope density and affinity of the interaction, it is the isotype (27–29) and the glycosylation status (30) of the antigen-bound antibody that determines whether complement is activated. Antibody isotypes with complement activating properties are more effective in clearing viral infections like human immunodeficiency virus (31) and more efficient in inducing self-damage (32, 33). Furthermore the relative abundance of antibodies against the same target but with distinct isotypes may determine the development of autoimmune disease (34). Simultaneous detection of more than two or three antibody isotypes is not generally achievable due to limitations in multicolor detection. Thus, contemporaneous measurement of relative contribution to antigen binding by *e.g.* IgM, IgG1, IgG2, IgG3, IgG4, and IgA is not yet feasible. Measurement of complement activating properties of bound antibodies can be a simple and meaningful alternative to solve these problems. Additionally because the test is carried out in undiluted sera, the assay more closely mimics events in the body unlike immunoassays where dilutions in reaction buffers potentially mask protein interactions.

We used dsDNA as a representative autoantigen to assess the usefulness of the C3 deposition array in an autoimmune disease model. The presence of antibodies against dsDNA is a diagnostic criterion for systemic lupus erythematosus (35), and their isotype and affinity, factors influencing complement activation, have been reported to correlate with disease severity (36, 37). Accordingly an early study showed that titers of complement-fixing dsDNA antibodies correlated with disease activity and renal damage (38). Serum from severely ill animals indeed showed both antibody binding and C3 deposition onto dsDNA in our assay (Fig. 5). The special ringlike C3 signals obtained with higher concentrations of dsDNA may have resulted from the sensitivity of complement activation to epitope density. We did not observe similar C3 signals in the case of protein antigens, so it is more likely to be a peculiar trait of dsDNA-antibody interactions.

Multiplex systems for autoantibody profiling are promising new tools for the diagnosis and classification of autoimmune diseases (13, 39) with the potential of guiding the development of treatment strategies (40). Taking into account that complement plays an important role in a range of autoimmune (33, 41, 42), inflammatory (43), ischemia-reperfusion-mediated (44), and degenerative diseases (45), we propose that the simultaneous detection of antibody binding and complement deposition in a high density antigen array format may become a clinically useful method with diagnostic and prognostic values.

The disadvantage of the complement deposition array is the need for serum samples with an intact complement system. To prevent degradation of complement proteins sera need special handling: immediate use after the drawing of blood or storage at −70 °C. As an alternative solution, when only classical pathway-mediated activation is studied, antibody binding and complement activation can be separated in time: the tested sample can be heat-inactivated, and antibody-depleted serum can be used as a complement source in two sequential incubations. Another potential drawback is that because the test is carried out with undiluted sera larger volumes might be required for measurement as compared with an ELISA test. However, as the volume of serum required for a test is still below 0.6 ml, this feature cannot be considered a real disadvantage for human applications.

CONCLUDING REMARKS

Currently there is no multiplex diagnostic method that allows the identification of complement-activating substances and antibody targets simultaneously. The technique we describe here, in addition to the potential clinical value of allowing the measurement of an essential function of antibodies in a microarray format, may also help in better understanding the interactions of the complement system on a proteomic scale.

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Chapter 8

Krisztián Papp, Zsuzsanna Szekeres, Anna Erdei, József Prechl

Two-dimensional immune profiles improve antigen microarray-based characterization of humoral immunity

Submitted

Two-dimensional immune profiles improve antigen microarray-based characterization of humoral immunity

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Abstract

Antigen arrays are becoming widely used tools for the characterization of the complexity of humoral immune responses. Current antibody profiling techniques provide modest and indirect information about the effector functions of the antibodies that bind to particular antigens. Here we introduce an antigen array-based approach for obtaining immune profiles reflecting antibody functionality. This technology relies on the parallel measurement of antibody binding and complement activation by features of the array. By comparing sera from animals immunized against the same antigen under different conditions, we show that identifying the position of an antigen in a two dimensional space, derived from antibody binding and complement deposition, permits distinction between immune profiles characterized by diverse antibody isotype distributions. Additionally, the technology provides a biologically interpretable graphical representation of the relationship between antigen and host. Our data suggests that two-dimensional immune profiling could enrich the data obtained from proteomic scale serum profiling studies.

1 Introduction

In depth analysis of humoral immunity requires detailed characterization of the antibodies that are produced in response to immunogens. This involves, and is often restricted to, the determination of the amount and ratio of antibody isotypes and depends on the measurement of several classes and subclasses of antigen specific antibodies.

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Nonstandard abbreviations used: anti-C3, goat anti-mouse C3; anti-IgG, goat anti-mouse IgG; anti-IgM, goat anti-mouse IgM; CFA, complete Freund's adjuvant; FcγR, receptor for the crystallizable fragment of immunoglobulin G; IL-4, interleukin 4; KLH, keyhole limpet hemocyanin; KO, knock out ; pLA, protein LA; RBC, red blood cell; RFI, relative fluorescence intensity; TD, thymus dependent; TI, thymus independent; TNP, 2,4,6-trinitrophenol.

Keywords: antibody profiling, protein array, antigen microarray, complement, humoral immunity

Characterization of the contribution of antibodies with diverse isotypes to an immune response helps determining the nature of the response with respect to its duration, T helper cell bias, protectiveness or pathogenicity. Class switching is regulated by the stimuli and costimuli delivered by the immunogen and the cytokine milieu of the germinal center. Humans have 5 antibody classes (IgD, IgM, IgG, IgA, IgE) with IgG further subdivided into four subclasses (IgG1 to IgG4) as determined by the heavy chain gene usage. The same immunoglobulin classes are observed in the mouse, whose IgG subclasses (IgG1, IgG2a/c, IgG2b and IgG3) also are diversified. Importantly, IgG subclasses have different affinities for IgG Fc receptors (FcγR)[1] and dissimilar abilities to activate the complement system[2, 3], necessitating the need to determine their relative contribution to an immune response. Effector functions are also considerably influenced by the avidity[4, 5] and glycosylation[6, 7] of antibodies, but as these

properties are more cumbersome to measure they are tested less frequently.
Incubation of an array of indexed antigens with serum allows the identification of a large number of specific antibodies in the circulation, a method called antibody profiling[8]. Though antigen arrays are becoming the tools of choice for serum antibody profiling, current microarray instrumentation generally does not allow more than three parallel measurements in distinct fluorescence channels. This excludes simultaneous detection of all IgG subclasses, not speaking of other immunoglobulin classes. In an attempt to give a better view of in vivo immune complex formation and to functionally characterize array-bound antibodies we have modified experimental conditions so as to allow complement activation on the antigen arrays[9]. Complement is an innate system of detector, regulator and effector proteins, which is activated either directly by antigens or indirectly via antibodies bound to antigens, and has significant influence on the development of adaptive immunity[10, 11]. Some antibodies are particularly potent while others are ineffective at activating complement, depending on their isotype, affinity and glycosylation[2, 3, 7]. Antigens that come into contact with blood plasma are thus wrapped in varying mixtures of recognition molecules, including antibodies and complement activation products. The composition of these immune complexes both reflects earlier immunological experience and crucially influences all later steps of an immune response. Gaining insight into the nature and function of antibodies bound to a particular target on an antigen microarray would therefore extend the use of such arrays.
Using immunization protocols that induce characteristic immunity with distinct antibody isotype dominance patterns, we show that concurrent measurement of immunoglobulin binding and complement deposition on antigen microarrays is suitable for discriminating and identifying such immune responses.

2 Materials and Methods

2.1 Materials

All materials were from Sigma-Aldrich, Hungary, unless otherwise indicated. Conjugates of 2,4,6-trinitrophenol (TNP) were generated by treating keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) with trinitro-sulphobenzoic acid according to standard protocol. BSA conjugates with varying degrees of TNP content were produced by using 0.1, 0.01 and 0.001% of trinitro-sulphobenzoic acid. TNP conjugation efficiency was determined by spectrophotometry. TNP was conjugated to sheep red blood cells using 0.1% TNBA, cells were washed afterwards and used fresh. IL-4 containing supernatant[12] was produced in our laboratory; IL-4 concentration was measured by ELISA. Printed capture antibodies were heavy chain specific (μ and γ) goat anti-mouse $F(ab')_2$ fragments from Southern Biotech. Alexa-647 conjugated goat anti-mouse IgG, gamma heavy chain and light chain specific (γ+L) (Southern Biotech, AL) and FITC conjugated goat anti-mouse C3 (MP Biomedicals, OH) were used for fluorescent detection. TNP specific monoclonal antibodies H5, D10, 2.15, F4, GORK, Sp6, Hy1.2, M12 were a kind gift of Birgitta Heyman, Uppsala University. A polyclonal conjugate reacting with both kappa and lambda light-chains was created by mixing commercially available light-chain antibodies (Southern Biotech, AL) and conjugating with Alexa-647 (Invitrogen, CA).

2.2 Mouse protocol

6-8 weeks old C57/B6 mice, 5 per group, were used for immunizations. All animal experiments were in accordance with national regulations and were authorized by the ethical committee of the institute. Serum from immunoglobulin knock out (IgKO)[13] and C3 deficient (C3KO)[14] animals was a kind gift from Matyas Sandor, University of Wisconsin-Madison. TNP-Ficoll (Biosearch Technologies, CA) was administered intraperitoneally at a dose of 50 μg/mouse. Rigid, highly repetitive structures, such as carbohydrate polymers (Ficoll), induce thymus independent (TI) responses, characterized by the dominance of IgM antibodies [15]. We used TNP conjugated to a massive carrier protein, keyhole limpet hemocyanin (KLH), to evoke thymus dependent (TD) immune response. TNP-KLH, at 100 μg/mouse dosage, was injected subcutaneously and intraperitoneally alone or emulsified in complete Freund's adjuvant (CFA) or injected intravenously along with 2 μg recombinant, intraperitoneally administered IL-4. Complete Freund's adjuvant, containing mycobacterial extract, induces strong inflammation. On the contrary, administration of the antigen via the intravenous route and in the presence of an anti-inflammatory cytokine (interleukin-4) is rather tolerogenic. TNP

conjugated to sheep red blood cells (TNP-RBC) represents particulate types of antigen, with both thymus dependent and independent mechanisms involved in the immune response. $4x10^7$ cells/mouse were injected intravenously for the immunization.

For TD responses we gave booster immunizations 21 days after the primary injection, using the same formulation, except for replacing complete Freund's with incomplete adjuvant. Sera were collected at the height of the immune response, that is, 7 and 21 days following the last immunization for TI and TD responses, respectively. Isotype distribution of TNP specific antibodies was determined by ELISA and enzyme linked immunospot assay (data not shown), using isotype specific HRP-conjugated goat antibodies (Southern Biotech). For the radar chart representation optical densities derived from 1:500 serum dilutions were normalized for comparability by expressing optical densities as the percentage of the highest readings.

One individual in the TNP-KLH+IL-4 group had statistically extreme ELISA values for TNP specific IgG and therefore did not meet our inclusion criteria. Microarray results of two animals (one from the TNP-Ficoll group; one from TNP-KLH+IL-4) were not reliable and were therefore excluded from further analysis.

2.3 Antigen array data

Antigen arrays contained TNP conjugated to bovine albumin at three different ratios, with an average of 12, 2 or 0.4 TNP molecules per bovine albumin, providing various epitope densities. These conjugates were diluted in PBS containing 1 mg/ml BSA to the indicated concentrations of 1.3, 0.25 and 0.05 mg/ml. Thus, all TNP carrying features contained BSA and only the concentration of TNP was varied. Additionally, the following reference materials were printed on the slide: goat anti-mouse C3 (MP Biomedicals, OH), goat anti-mouse IgG, goat anti-mouse IgM (Southern Biotech, AL), KLH, lysozyme, BSA, protein LA (pLA), mannan, and whole murine serum. We printed these solutions in three different concentrations (1 mg/ml, 0.2 mg/ml, 0.04 mg/ml) in triplicates, using Calligrapher miniarrayer (BioRad), onto home-made nitrocellulose coated glass slides. The generation of microarray data is described elsewhere in detail[9].

Briefly, dried arrays were rinsed for 15 minutes in PBS just before use, then incubated with sera in a humidified chamber at 37°C degrees for 60 minutes. The reaction was terminated by washing the array with PBS. The mixture of the detecting antibodies diluted 1:5,000 in 5% skimmed milk powder in PBS were added to the arrays, which were then incubated with gentle agitation for 30 minutes at room temperature in the dark. For the comparison of monoclonal antibodies, shown in Figure 2, the basic method was slightly modified. Antibody concentrations were adjusted based on pilot experiments, so as to achieve antibody binding to TNP_{12}-BSA in a similar range, as assessed by pan-light chain detection. In this experiment we wanted to compare complement activating abilities of different classes and subclasses, therefore neither IgM nor IgG detection was suitable. By measuring the antibody light chains we can assume that identical fluorescence intensities imply the presence of identical numbers of antibodies. Thus, complement activation by similar numbers of antibodies can be compared. Before treating with naive serum, arrays were incubated in appropriately diluted supernatant containing anti-TNP monoclonal antibodies for 30min. The dilution was carried out in 5% BSA, 0.05% Tween 20 containing PBS. As discussed above, instead of the anti-Ig antibody, a kappa+lambda specific fluorescent conjugate was used to eliminate isotype bias.

Slides were scanned on a Typhoon Trio + Imager (Amersham Bioscience) following standard protocols. Laser intensity was set to provide optimal signal intensity with minimal background and without saturated pixels. Data were analyzed with ImageQuantTL (Amersham Bioscience) software. Signal intensities were calculated by subtracting background from medians of signal intensity in a spreadsheet program (Microsoft Excel).

Fluorescence intensity data were normalized, both for IgG and C3, to yield identical pLA derived values, assuming that antibody binding and consequent complement activation on this fusion protein of bacterial superantigens is not influenced by the immunization schemes. Correcting interassay fluorescence intensities using values obtained from capture reagent readings (goat anti-mouse IgG, goat anti-mouse C3), instead of that of pLA, did not essentially change the results (data not shown). All results were within the dynamic range of the measurement. We created overlays of false color microarray images by ImageQuantTL (GE Healthcare). Two-dimensional profiles depict Ig

and C3 signals from the three concentrations of the indicated antigen.

2.4 Statistical analysis

Data are expressed as mean +/- standard deviation. Correlations and principal components were calculated with Statistica AGA software (StatSoft, Inc.).

3 Results

3.1 Complement deposition on the array reflects properties of antigen specific antibodies

Taking advantage of two-channel fluorescent detection and multiplexicity of microarray format we measured antigen bound C3 fragments and antibodies in parallel. To confirm specificity of the technique we compared combined C3 and Ig profiles of wild type, C3 deficient, Ig deficient naïve mice and mice immunized with a model antigen, TNP. An array containing TNP-BSA conjugates with different densities of TNP moieties per BSA molecule and different concentrations of these conjugates, as well as various reference proteins, was designed for addressing TNP-specific immunity (Fig. 1A, panel layout). Sera from immunologically naïve wild type animals contain natural antibodies - mostly IgM - that can bind to high density epitopes with adequate avidity to induce moderate complement activation (Fig. 1A, panel naive). Absence of complement C3 completely abolishes (Fig. 1A, panel C3KO) while lack of antibodies diminishes this signal (Fig. 1A, panel IgKO). Thus, high densities of this antigen can initiate complement activation in an antibody independent manner. Immunization resulted in the appearance of higher affinity antibodies against TNP, as reflected by the appearance of IgG and C3 signals at lower conjugation ratios of TNP per BSA, and dilutions of these conjugates.
Next, we compared a pair of TNP specific monoclonal antibodies, one carrying a mutation that impairs C1q binding[16, 17], using our assay (Fig. 1B). The mutant version was less efficient with respect to complement activation, validating the assay for semi-quantitative measurements. We also tested complement activating ability of several other TNP-specific monoclonal antibodies (Fig. 2). By adjusting their concentrations to give similar Ig binding signals on the array, we compared C3 deposition at identical Ig values, the results being in agreement with the isotype dependence of complement activation generally[18, 19]. Notably, natural antibodies present in the naïve serum that was used as a complement source, were avidly binding and initiating complement activation at the highest antigen concentration but disappeared at the lowest antigen concentration (Fig. 2A). A monoclonal antibody with IgG2a isotype (F4) was the most potent complement activator, with IgG1 isotypes showing intermediate to low activity. It is important to note that monoclonal F4, unlike all the others, bound to TNP_{12}-BSA and TNP_2-BSA equally well (compare Fig. 2A and 2B), implying it had the highest affinity for TNP. IgG1 antibodies can initiate the alternative pathway of complement activation in addition to the classical pathway[18]. The contribution of two pathways may account for the non-linear nature of the curve representing complement activation by monoclonal D10 (Fig. 2A). The fact that the particular clone of IgM we tested was only moderately effective is partly attributable to the detection of light chains which therefore compares antibodies on a monomeric basis. Taken together, these data suggested that our assay was suitable for the characterization of the biological activities of monoclonal antibodies.

3.2 Two-dimensional antibody profiling

Next, we immunized mice using immunization schemes (see Methods) which result in characteristic distribution of antibody isotypes against the model antigen TNP. This distribution was first characterized by measuring TNP specific IgM and various IgG isotypes by ELISA (Fig. 3A). The radar chart readily reflects the diverse patterns of antigen specific antibodies achieved by the immunizations. These sera were then applied to the above described antigen array. Different patterns of Ig and C3 fragment binding were observed at different TNP conjugates (see Supplementary Figure 1), yet none of these measurements distinguished the immunization groups reliably alone. Chip-based Ig measurements showed positive correlations with all TNP specific IgG isotype levels, as determined by ELISA (Table 1). In a similar way, C3 values, with the exception of those measured at the lowest TNP densities, were positively correlated to ELISA results for IgG levels. Significant positive correlation between C3 deposition on the array and the relative amount of ELISA derived antigen specific IgM values was only observed at the lowest

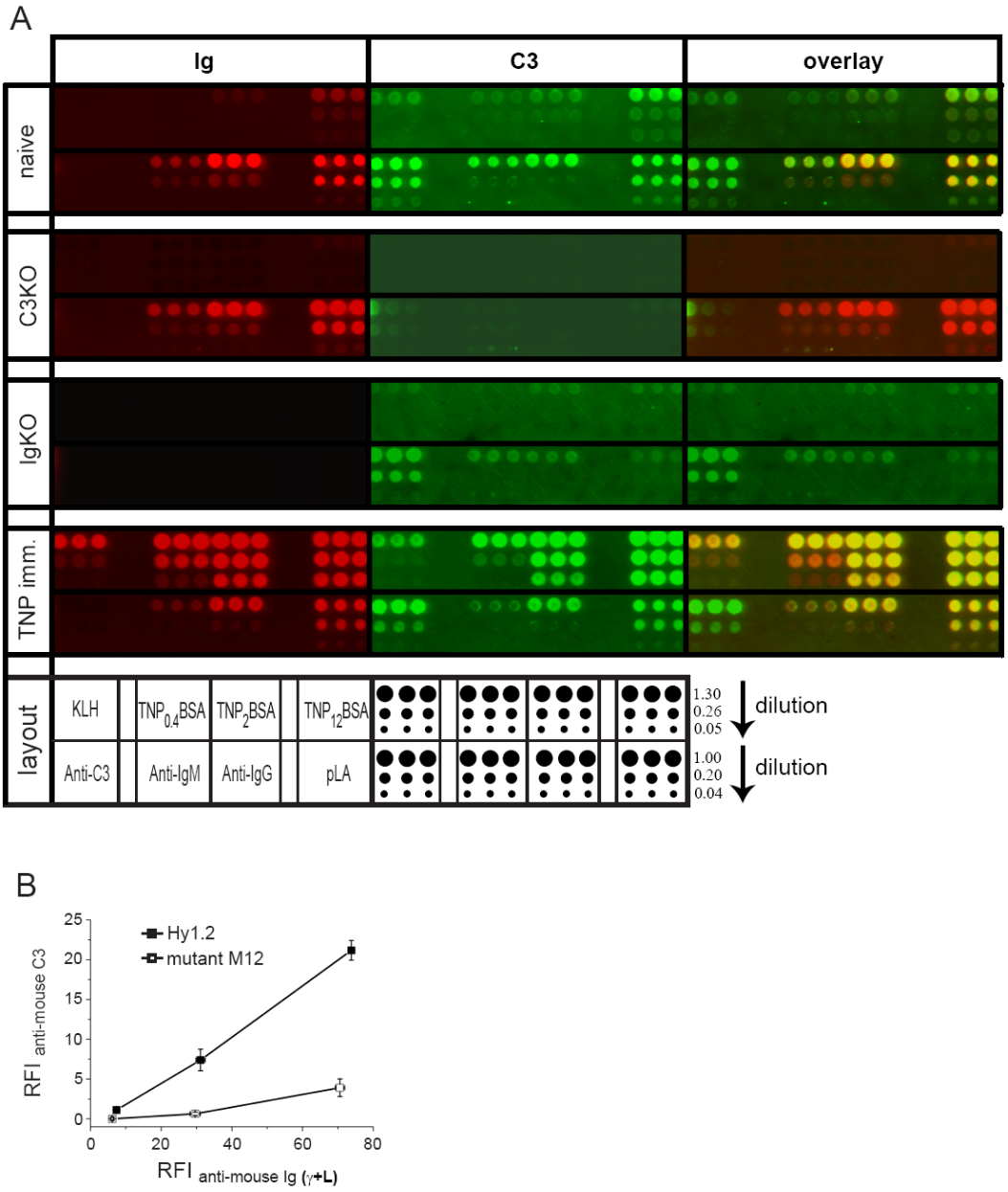

Figure 1

*Parallel detection of antibody binding and complement C3 fragment deposition. (**A**) Representative false-color images of antigen microarrays incubated in sera from naïve wild type (naïve), C3-deficient (C3KO), immunoglobulin deficient (IgKO) animals and an immunized mouse (TNP imm.) are shown, along with the layout of the subarray. We printed solutions of three different concentrations of every antigen and reference material. Additionally, three conjugates containing an average of 0.4, 2 or 12 TNP moieties per BSA were used. Polyclonal capture antibodies for mouse C3, IgM and IgG and a fusion protein of bacterial superantigens, pLA, were used for reference (see Methods). All features are in triplicates. After incubating the arrays in sera, deposited C3 fragments and bound Ig were detected by fluorescently labeled mouse C3 and Ig gamma heavy and light chain specific secondary reagents, respectively. (**B**) Comparison of wild-type Hy1.2 and mutant C1q binder monoclonal antibody M12 with respect to antibody binding and complement activation. Three data points in each curve indicate fluorescence intensities of C3 (anti-mouse C3) and Ig (anti-mouse Ig, γ+L) at 3 different concentrations of TNP_2-BSA*

concentration of $TNP_{0.4}$-BSA. C1 activation requires at least two IgG molecules whereas one IgM is still sufficient. Here, scarcely placed monomeric IgG molecules are presumably no longer able to bind C1q and cannot initiate complement activation, while C1q binding to the pentameric IgM is still potent.

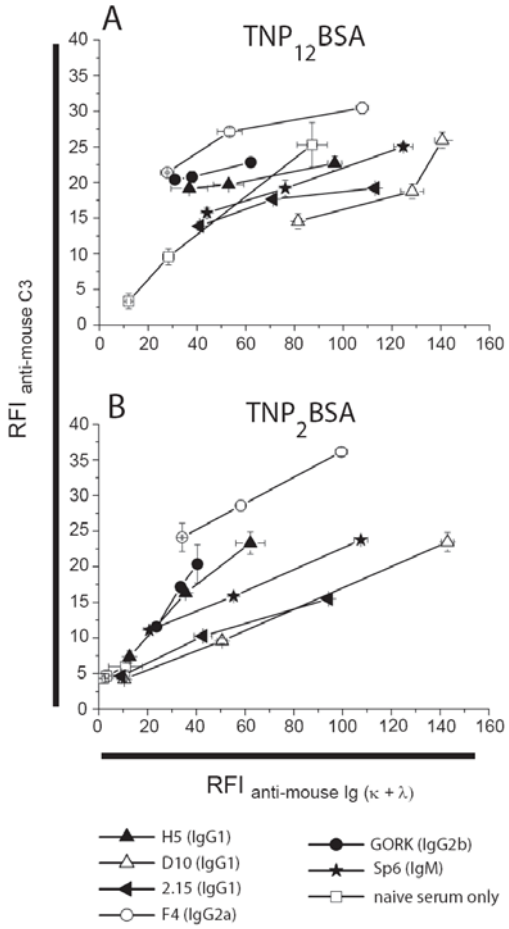

Figure 2
Comparison of the complement-activating abilities of TNP specific monoclonal antibodies. Six clones of monoclonal antibodies were applied to the TNP arrays at dilutions that were previously determined to give comparable antibody binding, as determined by pan-light chain specific detection (anti-mouse Ig, κ+λ). Fresh serum of naïve mice was applied as a complement source. Results stand for TNP_{12}-BSA (A) and TNP_2-BSA (B) binding data at three different concentrations and are representative of at least three independent experiments.

By displaying the immune responses in a two-dimensional space generated from Ig and C3 fragment binding data, we achieved to separate immunization groups in a biologically meaningful fashion (Fig. 3B). This space reflects both innate (complement C3) and adaptive (Ig) elements of a humoral response against a given antigen. Using these coordinates an antibody response that favors complement activation results in an upward shift, non-activating antibodies in the serum shift signals downward (Fig. 3C). In the case of naïve and TNP-Ficoll injected mice, IgM dominated immunity appears as potent complement activation with weak Ig binding. TNP was conjugated to keyhole limpet hemocyanin (KLH) for the induction of T-cell dependent responses and was used either alone or in combination of immunomodulatory agents. Complete Freund's adjuvant is a highly powerful inflammation inducing agent, which skews immunity towards cellular responses and promotes the appearance antibody isotypes with strong complement activating potential. This is reflected by higher C3 values of this group (TNP-KLH+CFA), as compared to those animals immunized without adjuvant (Fig. 3, B-D). To simulate tolerogenic antigen encounter we injected TNP- KLH intravenously, in the presence of IL-4. This regimen indeed resulted in a response lacking the inflammatory antibody isotype IgG2c (Fig. 3A) and an overall IgG response with poor complement activating properties (Fig. 3, B-D). Intravenous administration of TNP in particulate form, conjugated to sheep red blood cells, also showed poorer complement activating properties (Fig. 3, B-D). Displaying our results as the logarithmic ratio ($M=\log_2(C3/IgG)$) against average logarithmic intensity ($A= ½ \log_2(C3xIgG)$) of the measured parameters, as used for representing two-channel microarray data, further emphasizes the contrast between innate dominated and adaptive, non-inflammatory immune responses (Fig. 3E).

3.3 Discriminative properties of immune profiling methods

Representation of characteristic immune responses in this two-dimensional scale shows that simultaneous measurement of IgG and C3 is both suitable and sufficient for identifying these distinct immune profiles. In order to confirm the discriminatory potential of our assay and compare it with the ELISA results we calculated

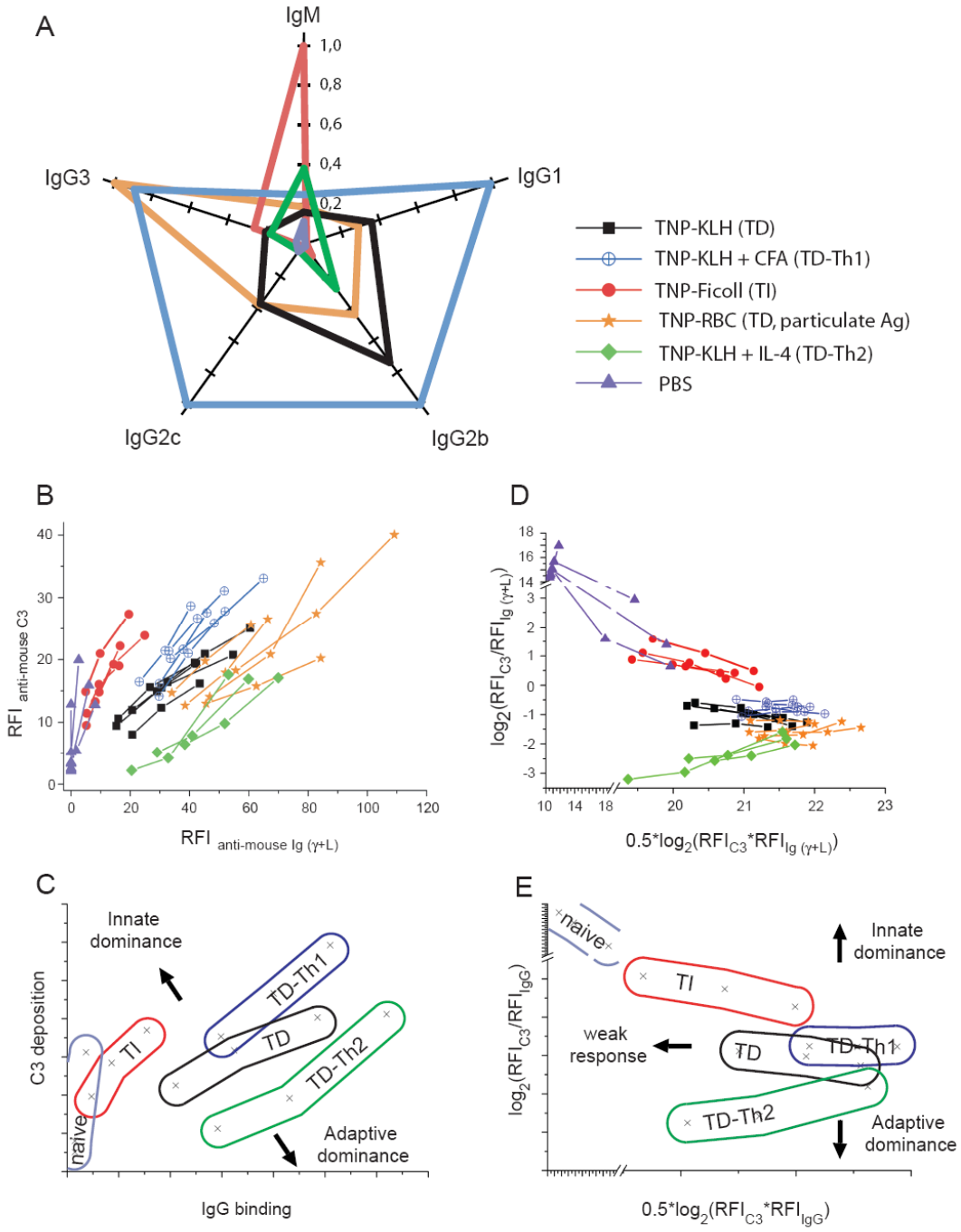

Figure 3

Isotype distribution profile of TNP specific antibodies in the immunization groups. Six groups of mice were immunized using schemes that are known to result in characteristic responses (see Methods). Thymus independent (TI) responses were induced by TNP-Ficoll, while thymus dependent (TD) responses were elicited by either soluble or particulate immunogens: TNP conjugated to a protein carrier, keyhole limpet hemocyanin (TNP-KLH) or to sheep red blood cells (TNP-RBC), respectively. TD responses were further skewed towards inflammatory reactions by complete Freund's adjuvant (CFA) or towards anti-inflammatory conditions by IL-4. Control animals received phosphate buffered saline (PBS) solution. (A)

Levels of TNP specific antibodies of the indicated isotypes were characterized by ELISA. Means of optical densities, expressed as percentage of the highest obtained values, of the respective immunization groups are shown in a radar chart. (B) Arrays, described in Figure 1, were incubated with sera of animals of the above immunization groups. IgG and C3 binding data at three different concentrations of TNP_{12}-BSA conjugates from individual sera are shown in a two-dimensional representation. Using the coordinates defined by Ig and C3 RFI values, we can simultaneously depict antibody binding and its effect on complement activation. (C) Immune responses biased towards inflammation are characterized by the appearance of antibody isotypes and glycoforms with good complement-activating properties, while tolerance and Th2 cytokines enhance the production of antibodies with poor complement activating properties. Thus, innate or adaptive dominance in the recognition of an antigen theoretically appears as an upward or downward shift, respectively, when bound C3 products and IgG define the coordinates. Enclosed areas correspond to experimental data: TI = TNP-Ficoll, TD = TNP-KLH, TD1 = TNP-KLH+CFA, TD2 = TNP-KLH+IL4.
(D, E) Graphical representation of the logarithm of RFI ratios versus average logarithmic intensities for individuals of the six immunization groups highlights the essentially different characters of natural and adaptive humoral immunity. Convergence of the curves implies that large amounts of antibody will inevitably cause complement deposition when antigen is present at high concentration, yet segregation of different immunization schemes can still be observed. Data points were derived from binding values shown in panel B, from measurements of individual sera on TNP_{12}-BSA conjugates.

principal components from the two sets of data. Results of end-point measurements from a single, optimal serum dilution were used for the comparison both for ELISA and array measurements. This analysis revealed that determination of Ig binding and C3 fragment deposition at three different concentrations of TNP_{12}-BSA on the array yielded factors which were as suitable for discriminating the immunization groups in a two-dimensional scale as determination of the five different isotypes by ELISA (Fig. 4). The first two principal components deduced from the array data account for 96% variance of the data, while 85% variance is covered by the first two components of the ELISA measurements (Fig. 4, A-B). Using these components as coordinates individuals segregated into groups according to the immunization schemes in both cases (Fig. 4, C-D).

4 Discussion

In this paper we introduce the representation of antigen-serum interactions in the dimensions of bound immunoglobulins and deposited complement C3 products, a simple and powerful solution for detailed immune profile determination on antigen arrays. Antigens attain a position in this two-dimensional space depending on their ability to bind Ig and activate the complement system. We validated this assay using a monoclonal antibody, Hy1.2, and its mutant form that is deficient in C1q binding (Fig. 1B). We also compared a set of monoclonal antibodies with different isotypes and confirmed that murine IgG2a antibodies are efficient and generally better activators of complement than IgG1 (Fig. 2A and B). It is important to stress, though, that factors other than isotype, such as affinity and glycosylation, are also known to influence complement activation. Antigen specific antibodies appear in the serum of immunologically experienced individuals, as a result of germinal center reactions that yield antibodies with increased affinity for the antigen and switched isotypes for optimal effector functions. ELISA measurements allow the precise quantitative determination of antigen specific antibodies of various isotypes but only indirectly predict functional effects. We measured total Ig in combination with C3 products to generate a functional view of the immune reactions against the antigen. During an immune response, antibodies with different immunological properties are produced against the antigen. All these antibodies can bind to the antigen, forming immune complexes with different compositions and effector functions. To model these differences, we used immunization schemes that are known to result in characteristic, immunologically distinct responses. The variable composition of the immune complexes is reflected by the isotype patterns of our immunization schemes (Fig. 3A). Our approach aims to grasp this complexity by measuring the overall functional effect of antibodies on the complement system instead of determining each component of an immune complex separately. Murine IgG subclasses are quite heterogeneous with respect to effector functions such as complement activating

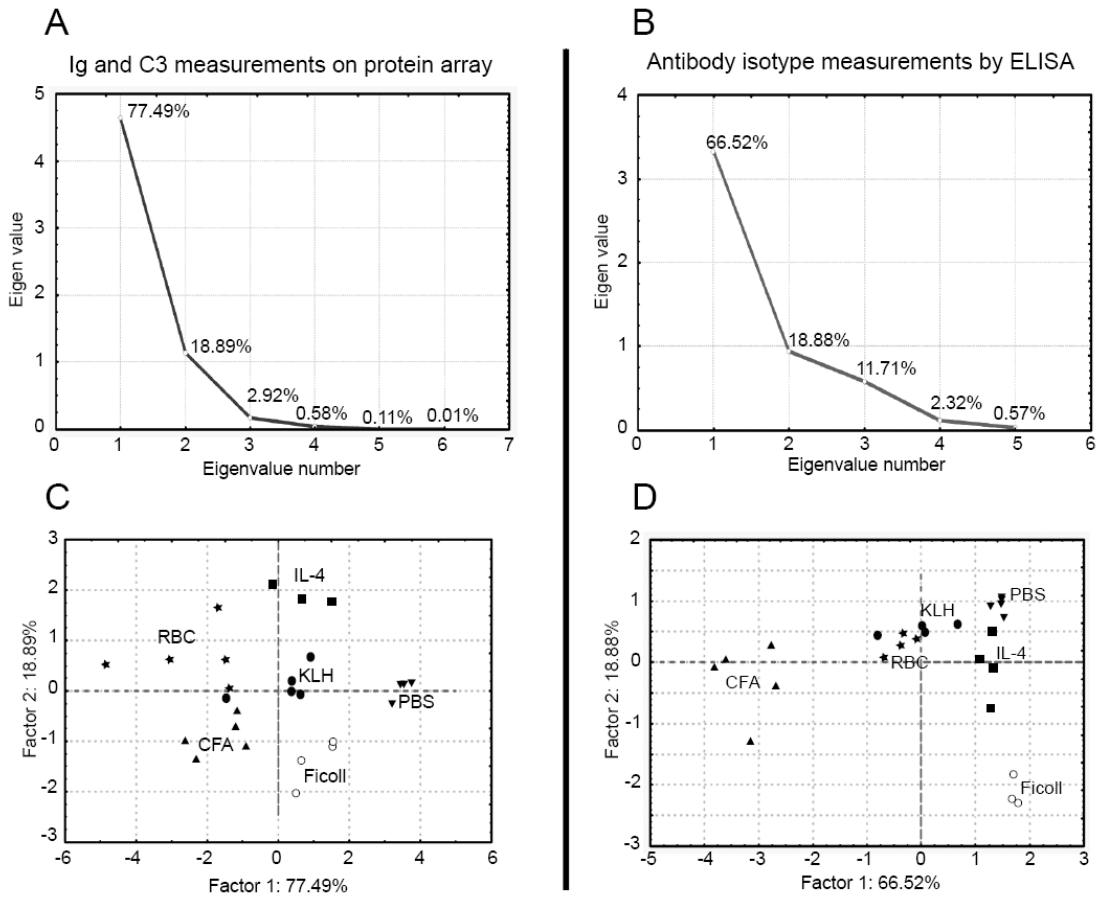

Figure 4

Principal component analysis of isotype measurements by ELISA and of two-dimensional profiling by antigen array. Scree plots represent Eigenvalues of factors of (A) array-based determinations of Ig binding and C3 deposition at three different dilutions of TNP_{12}-BSA (six variables) and (B) ELISA determinations of levels of five different TNP specific antibody isotypes. Cumulative percentage of the variance accounted for by the factors is displayed at each inflexion point. (C, D) Projections using the first two calculated factors as coordinates are shown for each set of measurements. Dots represent coordinates of values rendered to individual mice in the respective factor-planes.

properties[18, 20, 21] and FcγR binding[22]. Accordingly, antibody protectivity against infections can be determined by the dominant circulating isotype[23-26]. Humans possess a similar set of IgG antibodies with distinct effector potentials[6, 27].

For the two-dimensional characterization of sera we used a reagent which preferentially binds the heavy chains of IgG (γ chains) but also reacts with Ig light chains of all other isotypes. Therefore IgM is poorly detected on the Ig scale but is efficiently integrated into the detection of C3 products, which is justified by its biological properties. This antibody class is usually produced during the early phase of an immune response by cells that do not go through affinity maturation and do not participate in the generation of memory, similar to innate responses. Additionally IgM can activate complement with the highest efficiency of all antibody classes, this being the primary effector pathway initiated by IgM. In this study we have

Table 1
Correlation matrix[a)] of array and ELISA measurements

				ELISA				
			Conc.[b)]	IgM	IgG1	IgG2b	IgG2c	IgG3
Antigen array	Ig (γ+L)	TNP_{12}	1.3	-0.22[c)]	0.38	0.23	0.33	0.55**
			0.26	-0.24	0.44*	0.29	0.40	0.60**
			0.05	-0.26	0.48*	0.33	0.44*	0.61**
		TNP_2	1.3	-0.27	0.59**	0.45*	0.51*	0.67**
			0.26	-0.29	0.73***	0.62**	0.67***	0.75***
			0.05	-0.28	0.77***	0.69***	0.70***	0.76***
		$TNP_{0.4}$	1.3	-0.25	0.84***	0.80***	0.78***	0.68***
			0.26	-0.16	0.78***	0.79***	0.70***	0.51*
			0.05	-0.07	0.51***	0.51***	0.41	0.20
	C3	TNP_{12}	1.3	-0.01	0.61**	0.50*	0.57**	0.67**
			0.26	0.08	0.70***	0.64**	0.67**	0.62**
			0.05	0.04	0.74***	0.68***	0.70***	0.57**
		TNP_2	1.3	0.06	0.70***	0.55**	0.59**	0.67***
			0.26	-0.06	0.89***	0.85***	0.8***	0.62**
			0.05	-0.06	0.74***	0.80***	0.71***	0.42*
		$TNP_{0.4}$	1.3	0.00	0.69***	0.71***	0.62**	0.56**
			0.26	0.28	-0.12	-0.13	-0.22	0.06
			0.05	0.42*	-0.31	-0.30	-0.39	-0.15

a) Pearson's correlation coefficients are shown, unpaired measurements were omitted (n=26)
b) Concentration of the conjugate solution printed on the slide.
*c) Data are shown in grey when p>=0.05, *** p<0.001, ** p<0.01, * p<0.05.*

not considered antibodies of the IgA class, which are abundant in serum and can initiate complement activation[28]. However, these antibodies are primarily associated with mucosal immune responses and are not expected to influence our results. If required, detection of IgA should preferably be incorporated into the Ig detection channel. The same holds for IgE detection, the class associated with allergies and known to be unable to initiate complement activation.

Although antigens, depending on their biophysical and biochemical properties, may induce complement activation in immunologically naïve individuals by both antibody dependent and independent ways, immunity profoundly changes this efficiency (Fig. 1A and 3). Differences between animals which were immunized in different ways can be more subtle, underlining the importance of utilizing of antigen features with different antigen densities. Dissimilarity in complement activation for TI and TD Th2 biased immunity groups was most pronounced at lower antigen densities (Fig. 3D).

Our approach allows the direct assessment of functional properties of antibody mixtures against antigens and could therefore be used on arrays containing antigens derived from microbes[29], especially because complement has an important role in antimicrobial protection. Complement can also mediate deleterious effects of autoantibodies[30] pointing to the potential utility of this assay in combination with autoantigen arrays. Here we only followed the changes of two-dimensional immune profile against a particular model antigen under experimental conditions. When panels of

antigens are studied at a time, as in antibody profiling experiments, antigens are expected to show different antibody binding and complement activation even in immunologically naïve individuals, both because of the presence or absence of natural antibodies and their distinct intrinsic complement activating properties. In an immunologically experienced or a diseased individual, different antigens are recognized by functionally distinct antibodies of various isotypes [31] and are therefore expected to take up distinct coordinates in this two-dimensional space. Microarray-based determination of the pattern of positions of relevant antigens and monitoring of their relative movement in this space can indicate fine qualitative changes of the immune response and help observe disease or effectiveness of therapy.

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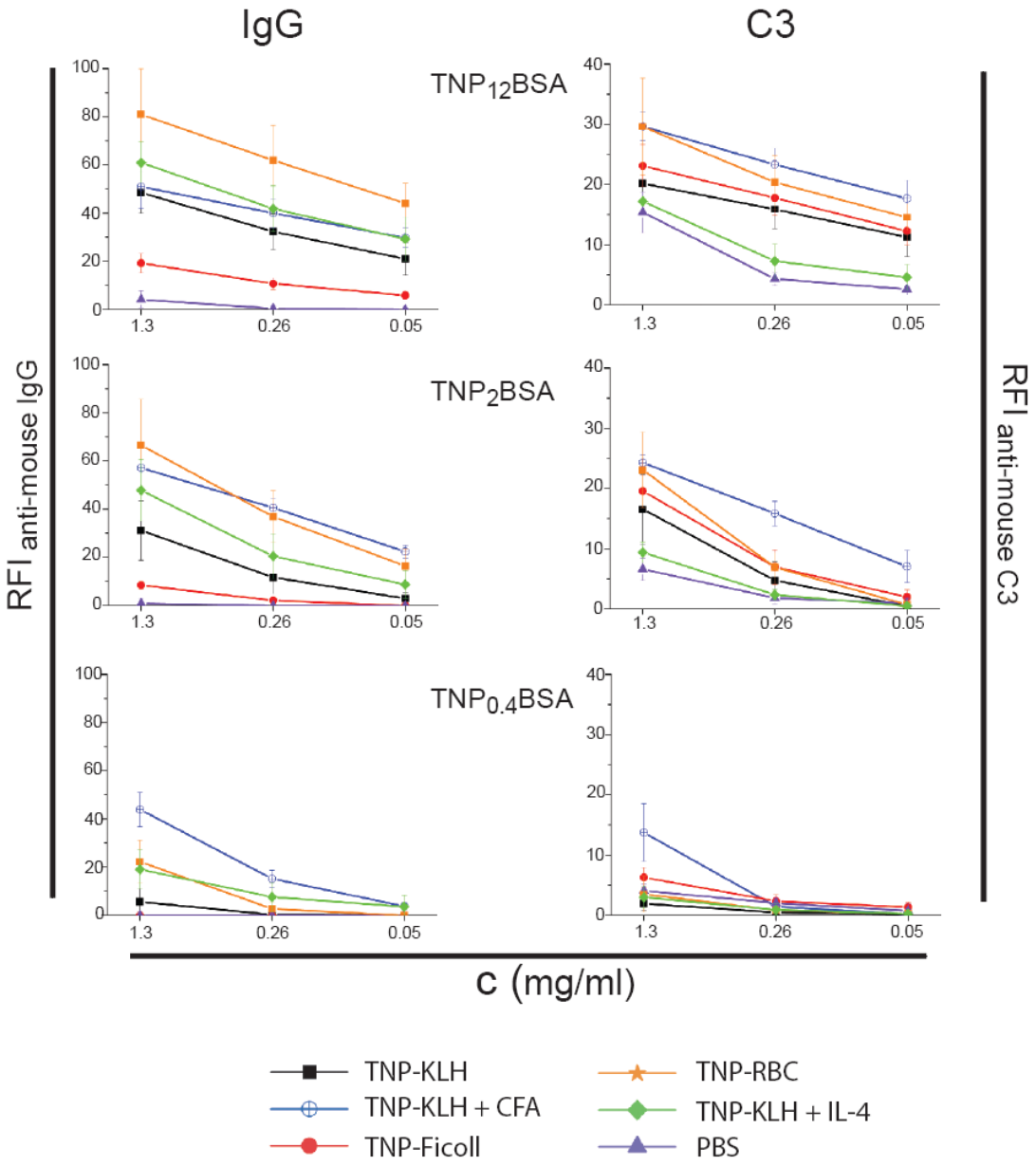

Supplementary Figure 1
Arrays, described in Figure 1, were incubated with sera from animals that were immunized with TNP under different conditions, as described in Methods. Binding of IgG and deposition of C3 fragments at different antigen densities are shown separately. Relative fluorescence intensity (RFI) values of groups are expressed as means and standard deviations.

Chapter 9

General discussion

The complement system is a fundamental element of innate immunity, composing probably the fastest activating defensive line against invading pathogens. This enzymatic cascade can destroy pathogens and activate other elements of the immune system, not only the members of innate but of acquired immunity as well. The inappropriate function of this cascade results in recurrent infections with specific pathogens (Neisseria, pyogenic bacteria) (Walport, 2001) and more frequently the appearance of autoimmune diseases, like systemic lupus erythematosus (Walport *et al.*, 1998). Based on these multiple roles, the measurement of complement activity has a value in the diagnosis of several diseases.

To better understand the function of the human complement system, the study of the more easily available animal models is feasible. Mouse is the most widely used animal model - also in research of the complement system. So far however, the field has long suffered from the lack of specific antibodies that can recognize various activation fragments of C3, which play a key role in many complement-related biological processes. To fulfill this need, we generated and characterized a panel of rat antibodies which are specific for the mouse C3 and its activation fragments (Table 1 Chapter 4). The usability of these antibodies was verified in ELISA, Western blot, and immunohistochemical methods. This work revealed that some of these antibodies are capable not only of detecting C3-fragments but also influencing its effect. Antibodies with C3b binding capacity (clones 2/11 and 3/26) dose dependently inhibited the activation of the complement system, as verified in a hemolytic assay (Figure 7 Chapter 4). This finding implies that these antibodies might inhibit the complement system in vivo as well, providing an alternative tool where blockade of the complement system is necessary, but using a C3KO animal is not suggested.

Most biological effects of complement activation are mediated via C3-derived fragments through interaction with their specific receptors. Following activation and subsequent limited proteolysis, neoepitopes are exposed on the surface of C3-fragments, which are binding sites for different receptors and complement control proteins. Probably the most fascinating part of these conformational changes occurs in the first step, when C3 convertase enzymes deliberate C3a, the anaphylatoxic peptide. The remaining larger fragment of the molecule is C3b, which binds covalently to the activating surface with its newly exposed reactive thioester group. We made an effort for the better understanding of C3-C3b conversion by studying the conformational changes of the molecule with the help of H/D exchange method combined with mass

spectrometry. Although the crystal structures of C3 (Janssen *et al.*, 2005) and C3b (Abdul *et al.*, 2006;Janssen *et al.*, 2006;Wiesmann *et al.*, 2006) molecules are solved, these data give only a static picture about the molecule while leave unanswered the question what kind of dynamic motion takes place in the fluid phase. Differences between the three crystal structures of the C3b molecule also emphasized the need of further efforts to understand in more details this crucial conformational change. By combining two types of mass spectrometry (MALDI- and ESI-MS), we not only increased the reliability of our data, but could collect information regarding 61% of the C3b molecule (Figure 1 Chapter 5). This high coverage on a large molecule, like C3 (187kDa), has never been achieved with this technique before. Based on our result, the conformation of these two molecules is identical, but nine out of the detected 82 peptides are more exposed in C3b than in C3 molecules. These peptides are situated on the α chain and distributed among four crystallographic domains: TED, CUB, C345C and MG8; exactly where binding sites of complement control proteins are located. Two overlapping peptides (1189-1206, 1189-1215) on the TED domain include a putative binding site for factor H, a complement control protein (CCP) with affinity for C3b but not C3. One peptide (1386-1422) in the MG8 domain overlaps with the properdin binding site, while a peptide within the C345C domain is contiguous with the binding site for factor B (Figure 3 Chapter 5). These data are in full agreement with the crystallographic data, and answer the question why these CCPs bind only to C3b, but not to the intact C3 molecule. In contrast, seven peptides out of the 82 show decreased solvent accessibility in the C3 molecule and these changes are not consistent with the available crystal structures (Figure 5 Chapter 5). Interestingly, three of these peptides (235-248, 542-560, 574-599) cover an area that forms contact with CRIg, a complement receptor interacting specifically with C3b and C3c. Therefore it suggests that the more compact structure of these peptides in C3b influence the binding ability of CRIg.

Next we focused our attention on the function of activated C3. A well known feature of this complement component is that following activation the internal thioesther group in the α-chain of the so-called *nascent* C3b is exposed and a newly formed metastable binding site allows the covalent attachment of the complement fragment to the OH-groups available on the activating surface. In many cases this surface is provided by invading pathogens, but C3 fragments can also fix onto the

membrane of various tissues and cells, as well. In autologous systems the deposited C3 fragments do not induce the lysis of the cells, but modify some of its functions. We were interested in the fate of cell-bound C3–fragments. We found that after treatment with fresh, autolougus serum mouse B cells, monocytes and polymorphonuclear leukocytes, but not T cells fix C3b fragments on their surface. This phenomenon occurs *in vivo,* as well (Marquart *et al.*, 1994) and the C3 fragments deposited on the surface of B lymphocytes and macrophages enhance their antigen presenting capacity (Kerekes *et al.*, 1998). With our monoclonal antibodies (except the C3a specific 3/11 clone), the deposited C3 fragments were readily detectable by cytofluorimetry (Figure 7 Chapter 4). Interestingly, the antibody produced by clone 3/26 not only recognizes covalently bound C3b, but substantially enhances deposition of the complement-fragments when present during serum treatment (Figure 8 Chapter 4). This might also occur under pathological condition, when a C3b-specific autoantibody might increase the deposition of C3-derived fragments on various cell membranes - in a similar way as nephritic factor sustains complement activation. The acceptor site for C3 deposition on the surface of the above mentioned mouse cells is not known - in contrast to human cells, where CR2 is thought to be the main acceptor site on B lymphocytes. Comparison of C3-fragment binding on B cells derived from the spleen of wild type and CR1/2 KO animal revealed, that in mice CR1/2 has much smaller contribution in this phenomenon than CR2 in humans (Figure 1 Chapter 6). Our finding that non-lymphoid cells and the macrophage cell line P388D1 are also able to fix C3 covalently, strongly suggests the presence of a yet unknown acceptor-site on murine cells. C3-fragments were visualized with confocal laser scanning microscope, and showed that these fragments concentrate in small patches on the cell surface (Figure 2 Chapter 6). We found that the cells continuously release these fragments by forming small vesicles of 50-80nm (Figure 4 Chapter 6), i.e. exosomes. These vesicles are known to carry MHC-, co-stimulatory- and adhesion-molecules on their surface and their role in the process of antigen presentation is examined extensively (Raposo *et al.*, 1996). It has been proven that exosomes containing C3-fragments increase the presentation of antigens (Figure 5 Chapter 6). We think that the effect of C3b-coated exosomes can be more pronounced *in vivo* based on the following mechanism. In germinal centers, the FDC plays an important role in affinity maturation and Ig isotype switching of B cell clones. FDCs express CR1 and CR2 receptors on their surface (Fang *et al.*, 1998), thus might accumulate C3-fragment

coated exosomes and consequently may effect various reactions in the germinal center.

The very labile and reactive nascent C3b fragments can bind covalently not only to the surface of various cells or microbes, but also to antigens, spotted onto an inert nitrocellulose membrane. We used this basic phenomenon for the development of a new technique, which can be used for the detection of complement activation. The field of complement measurement has long suffered from the lack of a technique which can be easily used to measure the complement activating properties of many different antigens in the same time. The chip technology brought the possibility of the detection of several hundred or even thousands of reactions simultaneously, resulting in a huge advance in the field of DNA-research and nowadays for the protein area as well. Protein arrays are already used for antibody profiling, where with this technique it is possible to detect the presence of antibodies reacting with several hundred or even thousand different antigens. With our modification, this technique became suitable for the detection of the complement activating property of the antibodies, as well. The presence of an antigen specific antibody in serum suggests that the host had an experience with that given antigen but it does not provide enough information about the effectiveness of the immune response. It is always worth to know what kind of effect can be induced by the generated antibodies. A very important feature of immunoglobulins is their complement activating property. An antibody with complement activating capability is more effective in clearing viral infections like human immunodeficiency virus (Aasa-Chapman *et al.*, 2005), but also in inducing autoimmune diseases (Azeredo da *et al.*, 2002). The effector functions of a specific antibody depend on its isotype, glycolysation and affinity. Parallel measurement of these properties could help us to assess the effect of the antibody in the host, but the determination of all of these properties one by one takes a lot of time and effort. With our technique an important function, namely complement activation caused by antigen specific antibody, is directly measurable. Using undiluted serum the *in vivo* situation can be mimicked as much as possible, so the interaction of different antibodies with the same specificity and their complement activation can take place as *in vivo*. In our protocol an array of various antigens is incubated with serum on a nitrocellulose surface, so antibodies can bind to the printed antigen and induce complement activation leading to deposition of C3-fragments (Figure 1 Chapter 7). Bound antibodies and complement fragments are detected simultaneously with the help of

fluorescently labeled detecting antibodies (Figure 4 Chapter7). As the generated active C3b fragments have a very short half-life, they do not diffuse and bind only to the initiator spot (Figure 1 Chapter 7). In consequence, many antigens can be printed and tested in a relatively small nitrocellulose chip. Nitrocellulose is an adequately inert material; it does not activate the complement system itself. The signal detected by anti-C3 antibody following serum treatment is the result of complement activation by the printed antigen; since the blockade of the complement system by EDTA results in the disappearance of the signal (Figure 2 Chapter 7). We compared the complement activating properties of eight different TNP (2,4,6-trinitrophenol) specific monoclonal antibodies. The outcome of these experiments was in good agreement with already published results, proving the reliability of our technique (Figure 2 Chapter 8). To test this technique for a mixture of TNP specific antibodies, different types of immunization regimes were used in mice. The result of the immunization was tested with ELISA method. The measurement of the concentration of 5 different isotypes of TNP specific antibodies (IgM, IgG1, IgG2b, IgG2c, IgG3) revealed that with the help of immunomodulatory agents, thymus independent, T cell dependent cellular and also tolerogen response was successfully induced in different groups of animals. The presence and the complement activating properties of TNP specific antibodies in the serum of immunized animals were measured on protein arrays. Results were visualized in two-dimensional dot-plot, where the detected IgG and C3 signals were depicted on the X and Y axes, respectively (Figure 3 Chapter 8). With the help of this graph, the different types of immune responses can be better distinguished than with the conventional ELISA where only the antibody-isotypes are measured (Figure 4 Chapter 8). Monitoring the position of the antigen in this 2D space gives information about the Th1 or Th2 dominance of the immune response.

The presence of complement activating antibodies in autoimmune diseases can be really destructive as the constantly accessible antigen induces chronic inflammation. In SLE where the presence of anti-DNA antibody is a diagnostic criteria, the complement activating properties of these antibodies correlate with disease severity (Mackworth-Young *et al.*, 1986). Testing sera from mice with SLE like syndrome also supports the notion that anti-DNA antibodies with complement activating properties are present in the blood of severely ill animals (Figure 5 Chapter 7). We propose that the simultaneous detection of antibody binding and complement activation on a high density array may become a clinically useful method with

diagnostic and prognostic values. Applying this method using human sera is in progress in our laboratory and we are convinced that the results will reveal the value of this technique in diagnosis, and classification of autoimmune diseases.

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Summary

The complement system is a fundamental element of humoral innate immunity that comprises the first protection line against pathogens. It contains more than 30 proteins setting up an orchestrated enzyme cascade with multiple immunological effects. In this study we focused on the structure and function of the third complement component (C3) which is a key molecule in the cascade and via its activation fragments it plays multiple roles in several immune phenomena.

In the first step monoclonal antibodies against mouse C3 were generated. Two of them are not only applicable for the detection of various C3 fragments, but also can block complement activation. The usefulness of these antibodies was confirmed in ELISA, Western-blot, cytofluorimetry and immunhistochemistry, as well.

Next we concentrated on structural changes occurring after C3 activation, as this process makes possible the binding of C3-fragments to the activating surfaces and receptors. The fluid phase formation of C3 and C3b was compared by H/D exchange coupled with mass spectrometry. Some of our data were in good agreement with x-ray crystallographic results, confirming that the binding sites of factor H, factor B and properdin are more buried in C3 than in C3b. In contrast to the crystal structure data however,, we found three peptides forming a cluster of loops that are more exposed in C3, which may influence the ability of CRIg to bind C3b but not C3.

These conformational changes make possible the exposure of the active thioester group and the covalent binding of C3b to pathogens and self cells. C3-fragments deposited onto APCs are known to enhance antigen presentation. Our results revealed that C3-fragments concentrate in small patches on the cell membrane and their amount decreases in time, as the cells release them by forming exosomes. These C3 fragment containing exosomes were shown to increase their antigen presenting capacity in the presence of naive APC.

Following complement activation C3b fragments bind covalently also to antigens, spotted on nitrocellulose surface. Based on this phenomenon we developed a protein array-based technique for the measurement of complement activation of numerous antigens in a single reaction volume. Simultaneous assessment of the presence and the complement activating properties of antigen specific antibodies in serum makes possible to depict these data in a single 2D graph. This provides a biologically interpretable graphical representation of the relationship between antigen and host.

Hungarian summary – Összefoglalás

A veleszületett immunrendszernek szerves komponense a komplement-kaszkád, ami azonnali védelmet nyújt számtalan kórokozóval szemben. A komplementrendszert több mint 30 összehangoltan működő fehérje alkotja. Vizsagálataink során a harmadik komponensre (C3) koncentráltunk, melynek fragmentumai számos immunfolyamatban játszanak fontos szerepet.

Első lépésként, munkacsoportunk az egér C3 molekulájával reagáló monoklonális ellenanyagokat állított elő. Az antitesteket széleskörűen jellemeztük ELISA, FACS, Wester-blott és szövetfestési módszerrel, és megállapítottuk, hogy az előállított antitestek jól használhatók számos különböző kísérlethez.

A C3 molekula aktivációjakor bekövetkező konformációs változások nyomon követésére a H/D kicserélődés módszerét alkalmaztuk. Eredményeink, összhangban a röntgenkrisztalográfiás adatokkal, azt mutatják, hogy a H- és a B-faktor, valamint a properdin kötőhelye rejtettebb pozícióban van a C3 molekulában, mint a C3b fragmentumban. A korábbi eredményekkel szemben viszont találtunk egy területet, mely a C3b fragmentumban rendezettebb és így hozzájárulhat a CRIg kötődéshez.

A C3 molekula hasítása és a C3b fragmentumok létrejötte feltétlenül szükséges ahhoz, hogy a molekula kovalensen kötődjön a kórokozókhoz, így elősegítve azok elpusztítását. Ismert, hogy a C3b molekula képes az antigénprezentáló sejtek felszínére is lerakódni, így segítve az antigén-specifikus T-sejtek proliferációját. Munkánk során követtük a sejtmembránhoz kötődő C3- fragmentumok sorsát, és kimutattuk, hogy ezek a molekulák exosomák felszínén válnak le a sejtekről. Bizonyítottuk azt is, hogy ezen kis vezikulumok fokozzák az antigénbemutatás hatékonyságát naiv APC jelenlétében.

A komplementrendszer aktiválódása során keletkező C3b fragmentumok képesek kovalensen kötődni a nitrocellulóz felszínre nyomtatott antigénekhez is. Ezt a jelenséget alapul véve munkacsoportunk továbbfejlesztette az egyre nagyobb tért hódító fehérje chip technikát, és így lehetőségünk van arra, hogy egyidejűleg határozzuk meg az adott antigénnel specifikusan reagáló autoantitestek mennyiségét és azok komplementaktiváló képességét. Munkánk során bizonyítottuk, hogy a kötődő ellenanyag mennyiségének és komplement aktiváló képességének egy grafikonon való ábrázolása segít a szervezet és antigén közötti köcsönhatás meghatározásában.

Abbrevations

α2-m	α2-macroglobulin
ANA	ANAphylatoxin domain
C1-9	Complement component 1-9
C1inh	C1 inhibitor
C3aR	C3a Receptor
C4bp	Complement component 4 binding protein
CCP	Complement Control Protein
CR1-4	Complement Receptor 1-4
CRIg	Complement Receptor of the Immunoglobulin superfamily
Crry	Complement Receptor 1 Related protein
DAF:	Decay-Accelerating Factor
DC	Dendritic Cell
ELISA	Enzyme-Linked ImmunoSorbent Assay
ESI	ElectroSpray Ionization
FDC	Follicular Dendritic Cell
HRF	Homologous Restriction Factor
MAC	Membrane Attack Complex
MALDI	Matrix-Assisted Laser Desorption/Ionization
MASP1	Mannose-binding lectin-Associated Serine Protease 1
MCP	Membrane Cofactor Protein
MG 1-8	MacroGlobulin domain 1-8
MIRL	Membrane Inhibitor of Reactive Lysis
MS	Mass Spectrometry
SLE	Systemic Lupus Erythematosus
TED	ThioEster-containing Domain
TNP	2,4,6 TriNitroPhenol

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List of publications

Papers:

1. Dimitrios Mastellos, József Prechl, Glória László, Krisztián Papp, Eszter Oláh, Emelia Argyropoulos, Silvia Franchini, Ruxandra Tudoran, Maciej Markiewski, John D. Lambris, Anna Erdei: **"Novel monoclonal antibodies against mouse C3 interfering with complement activation: description of fine specificity and applications to various immunoassays"** Molecular Immunology 2004. 40:1213-21. IF: 3.20

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3. Krisztián Papp, Péter Végh, József Prechl, Krisztina Kerekes, János Kovács, György Csikós, Zsuzsa Bajtay, Anna Erdei: **"B lymphocytes and macrophages release cell membrane deposited C3-fragments on exosomes with T cell response-enhancing capacity"** Molecular Immunology 2008. 45:2343–2351 IF:4.77

4. Krisztián Papp, Zsuzsanna Szekeres, Anna Erdei, József Prechl: **"Two-dimensional immune profiles improve antigen microarray-based characterization of humoral immunity"** [submitted]

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Patent

József Prechl, Krisztián Papp: **"Measurement of complement activation products on antigen arrays"** Publication number: WO2008/007159

Oral presentations

1. Signal and Signal Processing in the Immune System, 15-19 September 2007, Balatonöszöd, Hungary

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