Are levels of soluble C1q binding proteins in plasma/serum and synovial fluid indicative or prognostic markers in the course of Rheumatoid Arthritis and SLE? Development of pathway specific assays, to monitor activity of complement activation complexes in human and murine serum/plasma samples.

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By

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"Επειδη το ειδεναι και το επισθασθαι συμβαινει περι πασας τας μεθοδους, ων εισιν αρχαι η αιτια η στοιχεια, εκ του ταυτα γνωριζειν (τοτε γαρ οιομεθα γιγνωσκειν εκαστον οταν τα αιτια γνωρισωμεν τα πρωτα και τας αρχας τας πρωτας και μεχρι των στοιχειων), δηλον οτι και της περι φυσεως επιστημης πειρατεον διορισασθαι πρωτον τα περι τας αρχας"

Αριστοτελης, Φυσικα, Βιβλιο 1, 184: α10-16

[In all methods knowledge and science occur; these can be principles, or causes, or facts (one can only say that has knowledge and science when one knows the first causes, the first principles and all the facts) and it is obvious that to know the science of nature one has to attempt to know first of all the principles.]

[Aristotle, Physics, Book 1, 184: α10-16]

This thesis is dedicated to my mother

Mrs Ολγα Γαλανοπουλου

Abstract

The aims of my PhD project were a) to determine the levels of C1q-binding proteins (gC1qBP and calreticulin) in (i) normal serum, (ii) serum/plasma samples of patients diagnosed with rheumatoid arthritis and systemic lupus erythematosus and (iii) synovial fluid samples of patients diagnosed with rheumatoid arthritis. For this, coding cDNA sequences for gC1qBP and calreticulin were cloned and expressed recombinant proteins were purified and used to develop functional ELISA assays. It was observed that calreticulin levels were significantly increased in the synovial fluid samples of patients diagnosed with rheumatoid arthritis as compared to synovial fluid samples of patients diagnosed with osteoarthritis. gC1qBP was found to be significantly increased in the serum samples of patients diagnosed with systemic lupus erythematosus and decreased in synovial fluid samples of patients diagnosed with rheumatoid arthritis, as compared to serum samples of healthy control individuals.

b) to establish functional assays to measure complement activation in human, murine and guinea pig sera. I investigated for potential MBL ligands and demonstrated MBL binding and lectin pathway activation using the pneumococcal vaccine Pneumovax II and *Acanthamoeba* (whole cells) trophozoites and cysts. CR1 has been described as a ligand for MBL. I tested this hypothesis using functional ELISA assays. In contrast to conclusions in a previous report of another research group, my results show that soluble CR1 binds to C1q but not to MBL. The results presented in my thesis also provide strong evidence that CR1 appears not to interact directly with MBL or MBL-mediated lectin pathway activation as the addition of soluble CR1 to my lectin pathway activation assay has no effect whatsoever on C4 cleavage. Nevertheless, sCR1 regulates lectin pathway activation further downstream and significantly inhibits lectin pathway mediated C3 cleavage, most likely by the decay accelerating and co-factor activity of sCR1 in the factor I-mediated inactivation of either C4b or C3b.

Using the C3 cleavage assay and murine strains deficient in C1q, factor B and factor B/C2, I showed the importance of the amplification loop of the alternative pathway in complement activation.

Finally, using the C3 cleavage assay with guinea pig serum deficient in complement component C4, it was demonstrated that MASP-1 does not appear to be an effective enzyme in cleaving C3, as proposed by others.

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I would like to thank my mother Mrs Olga Galanopoulou and my brother Mr. Andreas Tzimas for their support and patience during the past years.

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Abbreviations

ANA	antinuclear antibody
AP	alkaline phosphatase
AP (1 and 2)	transcription factor activating protein
APC	antigen presenting cell
APS	ammonium persulphate
BC	conglutinin
Bf-/-	homozygous factor B-deficient mice
C1q a-/-	homozygous C1q-deficient mice
C1-Inh	C1 inhibitor
C1qRp	C1q receptor for phagocytosis
C3aR	C3a receptor
C5aR	C5a receptor
C4BP	C4 binding protein
cC1qR	receptor for collagenous region of C1q
CCP	complement control protein
CD	clusters of differentiation
cDNA	complementary DNA
CL-43	collectin-43
CL-L1	collectin liver 1
CR (1, 2, 3 and 4)	complement receptor (1, 2, 3 and 4)
CRD	carbohydrate recognition domain
CRT	calreticulin
CTL	cytotoxic T lymphocytes
DAF	decay accelerating factor
dNTP	deoxynucleotide triphosphate
dsDNA	double-standed deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
Erp-57	ER protein 57
FDC	follicular dendritic cell
gC1qBP	protein binding to the globular region of C1q

gC1qR	receptor for the globular region of C1q
HK	high molecular weight kininogen
HLA	human leukocyte antigen
HRP	horse radish peroxidase
hsp	heat shock protein
ICAM-1	intracellular adhesion molecule-1
IC	immune complex
IL	interleukin
INF	interferon
IPTG	isopropylthio-β-D-galactoside
kb	kilobase
kDa	kilodalton
LPS	lipopolysaccharide
LT-α	lymphotoxin α
MBL	mannan-binding lectin
MAC	membrane attack complex
MASPs	MBL-associated serine proteases
MCP	membrane cofactor protein
NK cell	natural killer cell
OA	osteoarthritis
OD	optical density
PBS	phosphate-buffered saline (see section 2.4.1)
PCR	polymerase chain reaction
PDI	protein disulphide isomerase
PKC	protein kinase C
PMA	phorbol 1,2-myristate 1,3-acetate
pNPP	p-nitrophenyl phosphate
PTP	permeability transition pore
PVAXII	Pneumovax II™
RA	rheumatoid arthritis
RCA	regulation of complement activation
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLE	systemic lupus erythematosus
SP-A	lung surfactant protein-A
SP-D	lung surfactant protein-D

TAP	transporter associated with antigen processing
TBS	tris-buffered saline (see section 2.4.1)
TEMED	N,N,N',N'-tetra-methyl-ethylene diamine
Th0	naïve T lymphocytes
TIM or TM	trans-membrane protein
TNF	tumor necrosis factor
v/v	volume to volume
VnR	vitronectin receptor
w/v	weight to volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

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CHAPTER 1

Introduction

1.1 The Complement system

The following reviews were used:

- Song, W.C., M.R. Sarrias, et al., (2000). "Complement and Innate immunity". Immunopharmacology **49**: 187-198
- Hakansson, K. and K.B.M. Reid (2000). "Collectin structure: A review." Protein Science **9**: 1607-1617
- Walport, M.J. (2001). "Complement" N. Engl J Med **344** (14):1058-1066
- Eggleton P. and K.B.M. Reid (1999). "Lung surfactant proteins involved in innate immunity". Curr Opin in Immunol. **11**:28-33
- Morgan B.P. (1997). "The complement system: An overview" Methods in Molecular Biology vol. 150: Complement Methods and Protocols. Edited by: B.P Morgan © Humana Press Inc., Totowa, NJ.
- Walport, M. J., K. A. Davies, *et al.,* (1998). "C1q and systemic lupus erythematosus." Immunobiology **199**(2): 265-85.

The complement system is part of the innate immune defence and is composed of many components and receptors. Complement activation acts through a cascade of sequential activation steps. Many complement components are zymogens that are present in serum/plasma in their proenzymatic form and may be cleaved during the activation step and thereby converted in their enzymatically active state to form part of an enzyme complex that cleaves and activates other complement components further downstream of the activation cascade. The sequential activation of zymogens into their enzymatically active form allows that the activation of a small number of complement proteins at the initiation step is hugely amplified by each successive enzymatic reaction.

Complement may be activated through three different activation routes with specific activation initiation complexes for each namely, the classical complement pathway, the alternative complement pathway, and the lectin pathway (Figure 1.2). All pathways lead to the activation of C3. This key component of complement is an abundant serum/plasma glycoprotein present at concentrations ranging from 1-2 mg/ml. All three activation pathways share a common terminal effector route, which by assembly of complement components C5b, C6, C7, C8 and C9, forms the Membrane Attack Complex (MAC); a large protein complex that mediates lysis of bacterial and host cells. Because C3 is positioned between the terminal complement pathway and the three different complement activation pathways, it is considered as a central component of the complement pathways (Song, Sarrias *et al.,* 2000).

1.1.1 The classical pathway of complement activation

C1q is the recognition component of the classical pathway and initiates the classical pathway by binding to immune complexes and charge arrays such as lipid A on gram negative bacterial phospholipids and chromatin. The human C1q molecule (460 kDa) is composed of 18 polypeptide chains (6A, 6B and 6C). The A chain (223 residues), B chain (226 residues) and C chain (217 residues) each have a short N-terminal region followed by a collagen-like sequence of approximately 81 residues and a C-terminal globular region (gC1q domain) of approximately 135 residues. Comparison of the mature chains shows that there are four conserved cysteines in each chain (at positions 4, 135, 154, and 171, as per B chain numbering). The cysteines at position 4 are involved in the interchain disulphide bridges yielding the A-B and C-C subunits, the other three cysteines are considered to yield one intra-chain disulphide bond and one free thiol group per C-terminal globular region. The interchain disulphide bonding yields 6A-B dimer subunits and 3C-C dimer subunits. The collagen-like sequences in the A and B chains of an A-B subunit form a triple helical collagen like structure with the equivalent sequence in one of the C chains present in a C-C subunit. These form a structural unit of the composition ABC-CBA, which is therefore held together by both covalent and noncovalent bonds. Three of these structural units are then considered to associate, via strong noncovalent bonds in the fibril-like central portion, to yield the hexameric C1q molecule (Reid and Porter 1976; Kishore and Reid 1999).

The first component of complement, C1, is a complex of three glycoproteins; C1q, C1r, C1s. C1r and C1s are serine protease proenzymes that interact to form a Ca^{++}-dependent tetrameric complex, $C1r_2$-$C1s_2$, which makes contact with C1q. Binding of C1q to complement activators via the gC1q domain, is considered to induce a conformational change in the collagen region of C1q, which leads to the autoactivation of C1r, which in turn activates C1s. Activated C1s then cleaves component C4 into a soluble C4a fragment and a C4b fragment that will attach to the surface of the activator. Surface bound C4b acts as a docking protein for C2, which is subsequently cleaved by C1s into C2a and C2b. Surface bound C2b, together with C4b, make up the classical C3 convertase C4b2b, which cleaves C3 into C3a and C3b. C3b associates with C4b2b to form the classical C5 convertase C4b2b(3b)n that initiates the formation of the terminal activation cascade (C5b-9) by the cleavage of C5 (Law and Reid, 1995).

1.1.2 The lectin pathway

The MBL pathway is initiated by the binding of the lectin pathway specific recognition molecules MBL or ficolins to either mannose or carbohydrate structures present on microbial surfaces (bacteria, viruses and yeast). The structure of the lectin pathway

recognition molecules MBL and ficolin is similar to that of the classical pathway recognition molecule C1q. MBL and ficolins form high molecular weight oligomeric complexes of up to 18 identical chains. An MBL monomer contains a C-terminal lectin domain followed by a coiled structure termed the neck region (which is important for trimerisation) and an adjacent N-terminal collagen-like region. MBL monomers easily trimerise and form complexes of up to six trimers. There are three different members of the ficolin family that have recently been shown to serve as lectin pathway recognition molecules and may drive lectin pathway activation even in absence of MBL (see section 1.4). These are ficolin M, ficolin L and the Hakata antigen. A ficolin subunit consists of a short N-terminal domain, a collagenous domain and a C-terminal fibrinogen-like domain. The collagen-like segments assemble the ficolin subunits into multimers of trimers (mainly tetramers of trimeric subunits). These lectin pathway recognition molecules all differ considerably in respect of their affinities and carbohydrate recognition patterns and thereby may cover a large variety of microorganisms that are recognised through this route of innate immune defence.

The three serine proteases associated with all the so-far identified lectin pathway recognition molecules (i.e., MBL and ficolins) were first identified through their association with the MBL complex and were therefore termed MBL-associated serine proteases (MASPs) (i.e., MASP-1, MASP-2, and MASP-3). MASPs share an identical modular structure (composed of six structural domains) with the classical pathway-specific serine proteases C1r and C1s. These serine proteases also have in common that upon activation the single chain zymogen form is cleaved into a major N-terminal fragment (the A-chain) and a minor C-terminal fragment, which represents the serine protease domain only (B-chain). A 19-kDa MBL-associated protein of unknown function, MAp19, which consists of the first CUB domain and the epidermal growth factor-like domain of MASP-2 and therefore lacks protease activity, has been described (Stover, Thiel *et al.*, 1999; Takahashi, Endo *et al.*, 1999). MAp19 and MASP-2 are both encoded by a single structural *MASP2* gene and generated through alternative splicing/alternative polyadenylation. Likewise MASP-1 and MASP-3 are the products of a single structural *MASP1* gene and their specific mRNA transcripts are generated by alternative splicing/alternative polyadenylation (Dahl, Thiel *et al.*, 2001).

As shown for the C1 proteases C1r and C1s, activation of MASP-1 occurs through cleavage of an Arg-Ile bond adjacent to the serine protease domain (figure 1.1), which splits the enzyme into disulfide-linked A and B chains, the latter consisting of the serine protease domain (Matsushita and Fujita 1992). This Arg-Ile site is also conserved in MASP-2, and a similar cleavage appears to occur in the course of MASP-2 activation (Thiel, Vorup-Jensen *et al.*, 1997).

Based on structural similarities between C1q and MBL, as well as structural similarities between MASPs and C1r/C1s, it has been suggested that the MBL pathway of complement activation resembles the classical pathway. Within the classical pathway, C2 and C4 cleaving activities are features of C1s. By analogy to C1r, it has been predicted that at least one of the MASPs undergoes auto-activation upon carbohydrate binding of its corresponding recognition subunit (Vorup-Jensen, Petersen *et al.,* 2000). MASP-2 is so far the only lectin pathway specific serine protease that cleaves C4 and C2, whereas there is still controversy over the substrate(s) of MASP-1 and MASP-3. It has been suggested that MASP-1 is capable of directly cleaving C3 (Matsushita, Endo *et al.,* 2000). However, other studies question the physiological importance of this suggested function of MASP-1, as MASP-1 exhibits low grade turn-over (below levels of detection) when using either recombinant MASP-1 or serum and plasma of *Bf/C2-/-*, *Bf-/-* mice and *C4-/-* guinea pig in a quantitative and qualitative C3 activation assay (this work).

The MASPs may also circulate bound to ficolins (Matsushita, Endo *et al.,* 2000) and this complex may represent another mode of activation of the complement system. The ficolins are lectins that contain a collagen-like stem structure. Unlike MBL, however, the ficolins recognize carbohydrates (GlcNAc but not mannose or glucose) via a fibrinogen-like lectin domain. The two human serum ficolins, ficolin /P35 and Hakata antigen, were found to co-purify with MASPs and MAp19. Activated MASP1, MASP-2 and MAp19 were detected in immunoprecipitated ficolin preparations. The ficolin-MASP-MAp19 complexes consume C4 and C2 in a dose-dependent manner, which results in the formation of the C3 convertase; C4b2b. Direct C3 cleavage was also observed. MBL and ficolin are similar with regard to association with MASP-1, MASP-2 and MAp19.

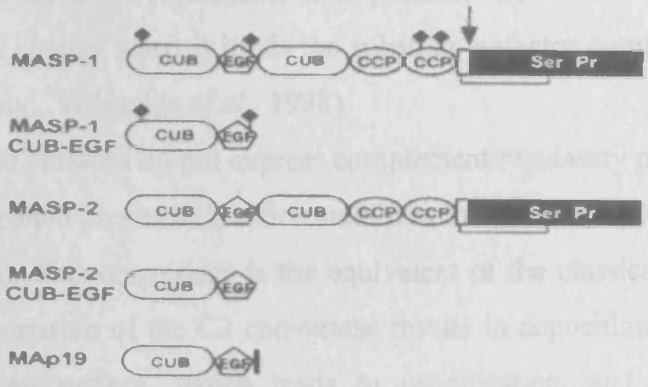


Figure 1.1 Modular structures of the MBL associated serine proteases (MASP-1, MASP-2 and Map19). Ser Pr, serine protease domain, CUB, N-terminal module originally found in C1r/C1s, EGF, epidermal growth factor–like molecule, CCP, complement control protein modules. The arrow idicates the Arg-Ile bond cleaved upon activation of MASP-1. ♦, N-linked oligosaccharides. ▮, specific EQSL sequence at the C-terminal end of MAp19.

1.1.3 The Alternative complement pathway

The alternative complement pathway may be activated on many microbial surfaces in the absence of specific antibody, and it leads to the formation of a distinct C3 convertase designated C3bBb. The alternative pathway does not have a defined recognition molecule similar to C1q or MBL/ficolins of the classical and lectin pathways, respectively. Its activation is initiated through spontaneous hydrolysis of C3 to give C3 (H_2O), which binds factor B and enables bound factor B to be cleaved by factor D. The resulting C3 convertase cleaves C3 to give C3a and C3b, which can attach to host cells or pathogen surfaces (Morgan, B.P., 1997).

Factor B associates with bound C3b to form the C3bB complex. Factor D cleaves Factor B in this complex to form Ba and Bb, forming the protease C3bBb. Factor D will not cleave Factor B unless it is associated with C3b (Arlaud, Volanakis *et al.,* 1998). Isolated Factor D is capable of cleaving low-molecular-mass substrates, but has negligible proteolytic activity. It is thought that binding to the C3bB complex is required to align the active-site His, Asp and Ser residues of Factor D for optimal activity (Arlaud, Volanakis *et al.,* 1998; Jing, Macon *et al.,* 1999). If C3bBb forms on the surface of host cells, it is rapidly inactivated by complement-regulatory proteins expressed by the host cells. These are, the complement receptor 1 (CR1 or CD35), decay-accelerating factor (DAF), membrane cofactor protein (MCP) and the soluble factor H. C3b bound to H, CR1 and MCP, is cleaved by factor I to yield inactive C3b (iC3b). Factor I cleaves its substrates C3b and C4b only when they are bound to a cofactor protein. Factor I interacts weakly with C3b and Factor H, and C3b and Factor H also interact directly in this complex. There is evidence for conformational change in C3b when it binds to Factor H, and conformational change in Factor I when it binds to both components of the C3b-H complex. It is possible that Factor I, like Factor D, undergoes conformational change when it binds the substrate-cofactor complex, to align the active site optimally (Arlaud, Volanakis *et al.,* 1998).

Bacterial surfaces do not express complement regulatory proteins and favor binding of Factor P (also called properdin), which stabilizes the C3bBb convertase's activity (Schwaeble and Reid 1999). This convertase is the equivalent of the classical/lectin pathway convertase C4b2b. The formation of the C3 convertase results in deposition of many molecules of C3b on the pathogen surface, which leads to opsonisation, and activation of the terminal complement components.

1.1.4 The terminal complement pathway

The terminal complement pathway assembles MAC and consists of complement components C5 to C9. C5 must be bound to C3b before the C5 convertase enzyme can cleave

it. The classical pathway C5 convertase enzyme is a trimolecular complex, C4b2b3b in which the C3b is covalently bound to C4b. C5 binds selectively to the convertase because it has a higher binding constant to C3b when it is bound to C4b, than for C3b, which is bound to other surface molecules (Jenaway, Walport *et al.,* 2001). The alternative pathway C5 convertase enzyme is also a trimolecular complex, C3bBb3b, in which one C3b is covalently bound to the other. Cleavage of C5 releases the peptide fragment C5a, which is a potent anaphylatoxin and a chemotactic factor (Jenaway, Walport *et al.,* 2001).

The remainder of the formation of the MAC is non-enzymatic. C5b binds C6, forming C5b6, and this then binds C7 to form a C5b67 complex. The binding to C7 marks the transition of the complex from a hydrophilic to a hydrophobic state that inserts into lipid bilayers. C8 then binds to this complex, followed by a stepwise addition of up to 14 C9 monomers, resulting in the formation of a lytic 'plug' or pore-forming molecule (Jenaway, Walport *et al.,* 2001). Although a small amount of lysis occurs when C8 binds to C5b67, it is the polymerised C9 that causes the majority of lysis (Jenaway, Walport *et al.,* 2001).

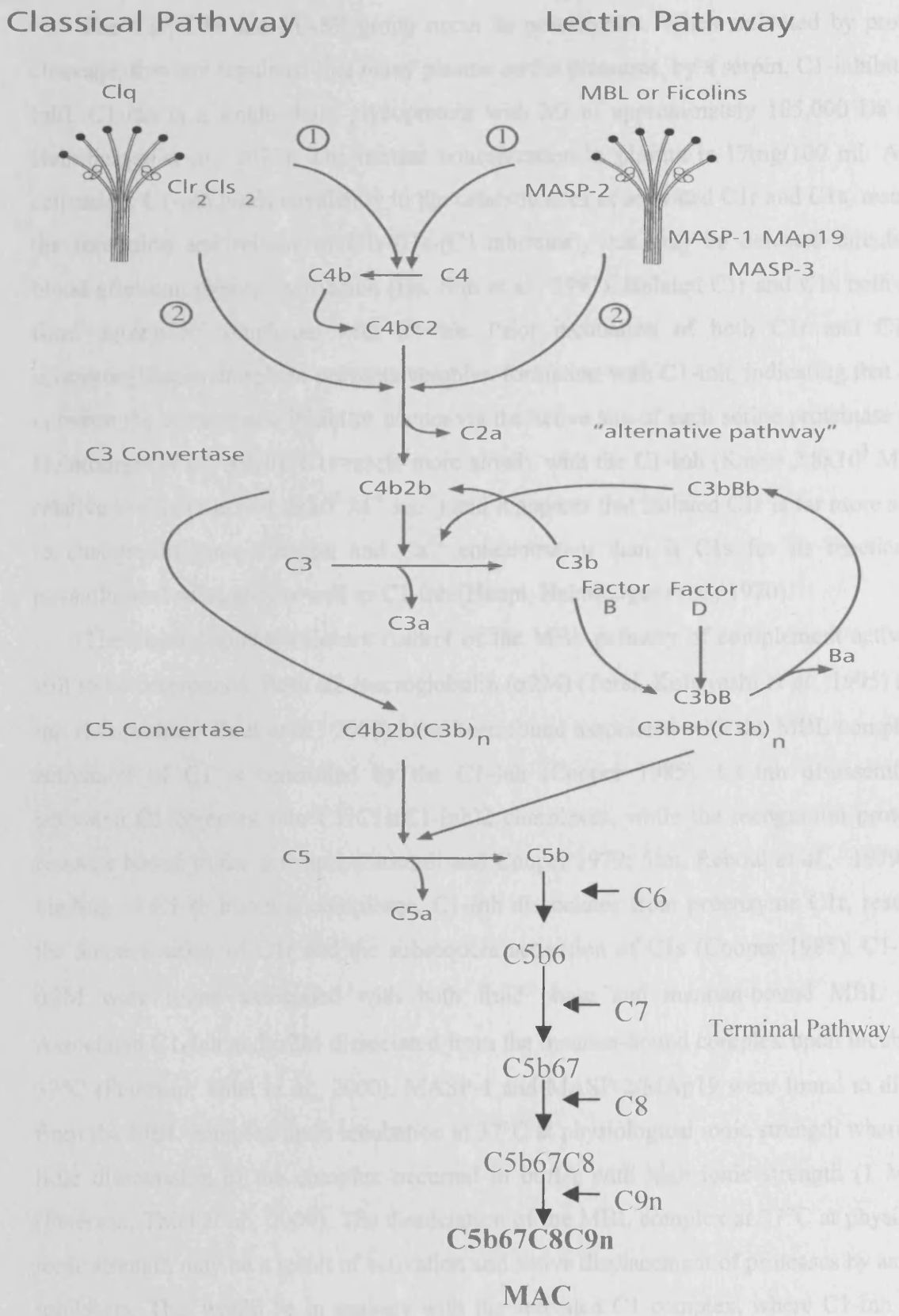


Figure 1.2 Overview of the complement pathways

1.2 Regulation of the complement system

The C1r, C1s and MASP group occur as proenzymes. When activated by proteolytic cleavage, they are regulated like many plasma serine proteases, by a serpin, C1-inhibitor (C1-Inh). C1-inh is a single-chain glycoprotein with Mr of approximately 105,000 Da (Haupt, Heimburger *et al.,* 1970). The normal concentration in plasma is 17mg/100 ml. After C1 activation, C1-inh binds covalently to the catalytic sites of activated C1r and C1s, resulting in the formation and release of C1r-C1s-(C1-inhibitor)$_2$ that may be detected circulating in blood after complement activation (He, Sim et al., 1997). Isolated C1r and C1s both react to form equimolar complexes with C1-Inh. Prior incubation of both C1r and C1s with diisopropylfluorophosphate prevents complex formation with C1-Inh, indicating that binding between the enzyme and inhibitor occurs via the active site of each serine proteinase (Haupt, Heimburger *et al.,* 1970). C1r reacts more slowly with the C1-Inh (Kass= $2.8x10^3$ M^{-1} sec^{-1}) relative to C1s (Kass= $1.2x10^4$ M^{-1} sec^{-1}) and it appears that isolated C1r is far more sensitive to changes in ionic strength and Ca^{+2} concentration than is C1s for its reactions with physiological substrates as well as C1-Inh (Haupt, Heimburger *et al.,* 1970).

The physiologically relevant control of the MBL pathway of complement activation is still to be determined. Both α2-macroglobulin (α2M) (Terai, Kobayashi *et al.,* 1995) and C1-Inh (Matsushita, Thiel *et al.,* 2000) have been found associated with the MBL complex. The activation of C1 is controlled by the C1-Inh (Cooper 1985). C1-Inh disassembles the activated C1 complex into C1rC1s(C1-Inh)2 complexes, while the recognition protein C1q remains bound to the activator (Ziccardi and Cooper 1979; Sim, Reboul *et al.,* 1979). Upon binding of C1 to immune complexes, C1-Inh dissociates from proenzyme C1r, resulting in the autoactivation of C1r and the subsequent activation of C1s (Cooper 1985). C1-Inh and α2M were found associated with both fluid phase and mannan-bound MBL at 4°C. Associated C1-Inh and α2M dissociated from the mannan-bound complex upon incubation at 37°C (Petersen, Thiel *et al.,* 2000). MASP-1 and MASP-2/MAp19 were found to dissociate from the MBL complex upon incubation at 37°C at physiological ionic strength whereas only little dissociation of the complex occurred in buffer with high ionic strength (1 M NaCl) (Petersen, Thiel *et al.,* 2000). The dissociation of the MBL complex at 37°C at physiological ionic strength may be a result of activation and active displacement of proteases by associated inhibitors. This would be in analogy with the activated C1 complex, where C1-Inh actively displaces C1r and C1s.

A number of synthetic inhibitors were found to differ in their activities towards complement activation via the MBL pathway and the classical pathway (Petersen, Thiel *et al.,* 2000). The difference in sensitivity to various inhibitors and the influence of high ionic

strength on the complexes indicate that the activation and control of the MBL pathway differ from that of the classical pathway.

C4b-binding protein (C4BP), DAF and CR1 are potent inhibitors of the classical pathway of complement. C4BP and CR1 are thought to bind C4b and upon binding, C4b is cleaved by factor I to inactive forms C4d and C4c. Structural analysis of 3D model of the C4BP alpha-chain suggested that a cluster of positively charged amino acids at the interface between complement control protein (CCP) modules 1 and 2 could be involved in ligand binding (Blom, Berggard *et al.*, 2000). Using C4BP mutants, where the positively charged amino acids were changed to glutamines, it was found that all of these displayed lower apparent affinity for C4b and that the site was also a specific heparin-binding site (Blom, Berggard *et al.*, 2000). The mutants demonstrated decreased ability to serve as factor I cofactors in the inactivation of C4b. Also, their capacity to prevent the assembly of C3-convertase and to accelerate its decay was decreased (Blom, Berggard *et al.*, 2000).

The alternative pathway is controlled by the action of inhibitory proteins present on membranes and in plasma. When C3b is deposited on a non-self surface or on immune complexes, the tight control of the pathway is interrupted, leading to activation and elimination by phagocytosis or lysis by the terminal complement components (Law and Reid, 1995). Activation of the alternative pathway in serum is effectively limited by the short half-life of active C3b. In the fluid phase C3b binds soluble Factor H and is further reduced by Factor I into inactive C3b. Membrane bound C3b is inactivated by Factor I using CR1, MCP or DAF as a cofactor. Factor H, CR1, MCP and DAF all destabilize the alternative pathway C3 and C5 convertases (see section 1.1.3).

In contrast, properdin stabilizes the alternative pathway C3 and C5 convertases by binding to C3b in C3bBb, and is the only known positive regulator of complement (see section 1.1.3).

1.3 C1q and Collectins

C1q is structurally similar to the collectins, a family of C-type lectins involved in the innate immune system. The collectins represent a nonclonal and innate host defence system, which is functional in the absence of, and prior to, the development of the adaptive antibody based immune system. Six different collectins have been characterized so far. Lung surfactant proteins A and D (SP-A, SP-D) are found in the pulmonary surfactant on the epithelial lining of the lungs where they provide protection against invading pathogens and allergens. The mannan-binding lectin (MBL) is found in serum and is produced by the liver. In some animal species, but not in man, there are two forms of MBL, MBL-A, which is found in serum, and MBL-C, which is found in the liver. Conglutinin (BC) and collectin 43 (CL-43), are bovine serum proteins more closely related to SP-D than to MBL. In addition, an intracellular

collectin of unknown function, collectin liver 1 (CL-L1) was discovered in liver cells (Ohtani, Suzuki *et al.,* 1999).

1.3.1 Structure of Collectins.

The collectin monomers are composed of four different regions: an N-terminal region, a collagenous region, an α-helical coiled-coil and a C-terminal lectin domain (Figure 1.3 A). The presence of a collagen-like region in these molecules imposes a trimeric structure. This is a prerequisite for their proper function; the carbohydrate affinity of a single collectin carbohydrate recognition domain (CRD) is weak but the trimeric organization permits a trivalent and hence stronger interaction between collectin and carbohydrate-containing target surface. In most of the collectins, these trimers are further assembled into larger entities, which enables them to cross-link several target particles and perhaps also to interact simultaneously with target and with host cells (Hakansson and Reid 2000).

The N-terminal region is defined as the segment N-terminal to the first collagenous triple-helix residue. This relatively cysteine-rich region stabilizes the trimers through disulfide bridging (Holmskov, Laursen *et al.,* 1995) and links them together in the collectin oligomers. There seems to be no overall homology between the collectins in this part of the molecule. The collectins can nonetheless be divided into four different groups with related N-terminal regions. The N-terminal sequences of SP-D, conglutinin, and collectin-43 are clearly related and of similar length, i.e., 25–28 amino acids, and include two cysteine residues. The N-terminal region of SP-A is much shorter and due to variation in signal peptidase cleavage, is not homogenous; there are two isoforms with seven or ten residues containing one or two cysteines, respectively (Elhalwagi, Damodarasamy *et al.,* 1997). The third group consists of mannan-binding lectins A and C, which have distinct, yet related, N-terminal regions, each approximately 20 amino acids long.

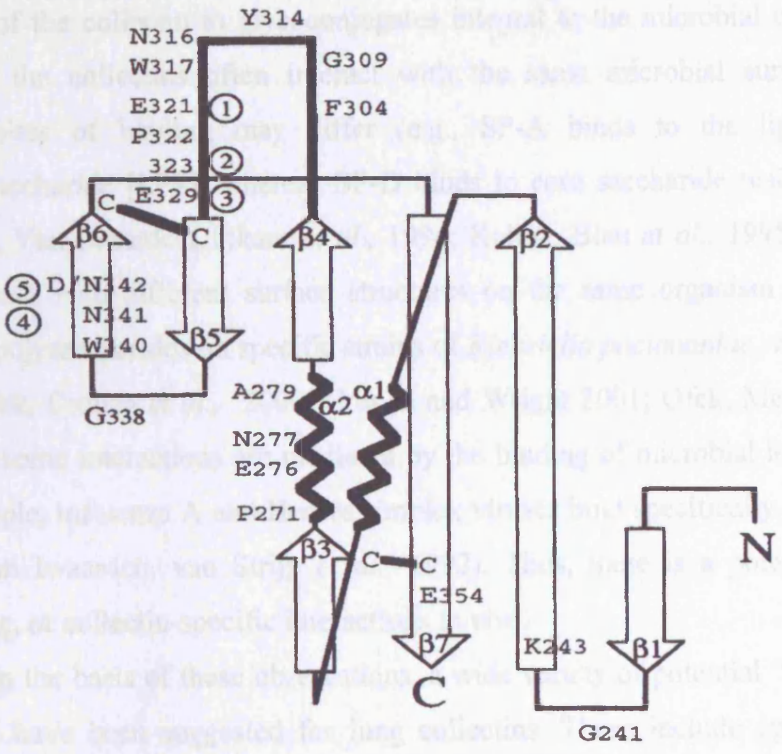
The repetitive amino acid sequence Gly-X-Y is typical of collagen (-like) polypeptide. X and Y can be any amino acid sequence but are frequently prolines or hydroxyprolines. Each of the three chains forms a left-handed polyproline II like helix and are coiled around each other in a right-handed manner with the glycine residues in the interior of the superhelix. Interchain hydrogen bonds between N-H groups of glycine and the C=O groups of the amino acid in X position stabilise the structure, and since there are no interchain hydrogen bonds the collagen helix can exist only as a trimer. The collagen triple helix is surrounded by a network of water molecules that interacts with most polar groups and all residues are exposed to the solvent, either by the side chains or carbonyl groups. This hydration is further enhanced by hydroxylation of some of the prolines in the Y position without which collagen is unstable at biological temperature.

The triple helix appears to be ideal as a cross linker between different domains due to its high tensile strength, stability, and relative resistance to proteolysis. In addition a triple helical region can also mediate binding interactions with other macromolecules as has been shown for C1q and the macrophage scavenger receptor (Hoppe and Reid 1994). The collagenous region in MBL contains the binding site for its associated serine proteases (MASP-1 and MASP-2) through which the complement cascade reactions are triggered (Matsushita and Fujita 1992) (Thiel, Vorup-Jensen *et al.,* 1997). SP-A and the other collectins bind to their macrophage receptor through the collagenous region.

An α-helical coiled-coil can often be recognized from the amino acid sequence by its characteristic heptad repeat pattern a-b-c-d-e-f-g, where residues "a" and "d" are hydrophobic amino acids. The structure of several coiled-coils, including the structure of trimeric MBL and SP-D fragments comprising the coiled-coil region and the lectin domains (Sheriff, Chang *et al.,* 1994; Weis and Drickamer 1994; Hakansson, Lim *et al.,* 1999) and the related tetranectin molecule (Nielsen, Kastrup *et al.,* 1997), are known from X-ray crystallography. The α-helices make 8–9 turns and there are several violations against the heptad rule, i.e., hydrophilic and even charged residues can be found in some of the "a" and "d" positions. The heptad pattern is further obscured by position "g" in the fourth and position "c" in the fifth heptad, which are involved in hydrophobic interactions with the lectin domain of the collectins. In tetranectin, a cysteine residue that is disulfide bonded to the C-type lectin domain occupies one of these positions. A proline residue with a cis peptide bond abruptly terminates the helical region in SP-D.

The C-type lectin domain is mainly composed of two anti-parallel β-sheets; one of these is four-stranded and the other five-stranded. The four-stranded β–sheet is found at the N-terminal part of the domain and is flanked by two helices. In the trimeric collectins, this sheet is interacting with the α-helical coiled-coil. The five-stranded β-sheet is rather distorted and more remotely located away from the trimer center. This β-sheet, together with some of the loop structure, makes up the carbohydrate binding ligand site. Two of the β-strands (β2 and β7) are relatively long and participate in both sheets. One of the disulfide bridges anchors helix α1 to strand β7 of the four-stranded β-sheet, the other ties together the two most peripheral strands (β5 and β6) of the five-stranded β-sheet. The calcium binding amino acid ligands are found at or close to these two strands (Figure 1.3 B) and this disulfide bridge probably plays an important role in stabilizing the structure around the functional carbohydrate-binding site.

A



B

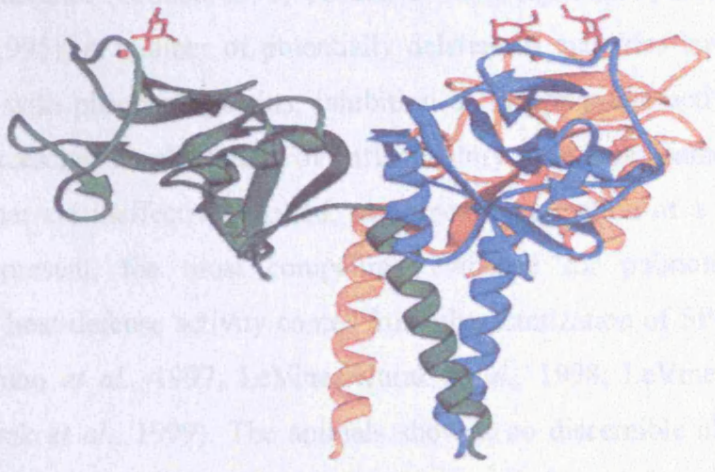


Figure 1.3 A: Two-dimensional representation of the C-type lectin fold. The β-strands and the α-helices have been numbered β1-β7 and α1-α2, respectively. The amino acids that are coordinated to the carbohydrate binding calcium ion are denoted with encircled numbers 1-5. **B:** The trimeric structure of the neck-CRD fragment of human MBL. The red colour represents the positions of the carbohydrate binding sites together with the calcium ions (adapted from Hakansson, K. and K.B.M. Reid (2000). Protein Science 9: 1607-1617).

1.3.2 Host defence Roles of Collectins

Interactions of collectins with microorganisms involve specific binding of the lectin domains of the collectin to glycoconjugates integral to the microbial cell wall or envelope. Although the collectins often interact with the same microbial surface structures, their specific sites of binding may differ (e.g., SP-A binds to the lipid A domain of a lipopolysaccharide [LPS], whereas SP-D binds to core saccharide residues) (Kuan, Rust *et al.,* 1992; Van Iwaarden, Pikaar *et al.,* 1994; Kalina, Blau *et al.,* 1995) The collectins may also interact with different surface structures on the same organism (e.g., SP-A binds to capsular polysaccharides on specific strains of *Klebsiella pneumoniae*, whereas SP-D binds to LPS) (Ofek, Crouch *et al.,* 2000; Crouch and Wright 2001; Ofek, Mesika *et al.,* 2001). In addition, some interactions are mediated by the binding of microbial lectins to the collectin. For example, influenza A and Herpes simplex viruses bind specifically to N-linked sugars on SP-A (van Iwaarden, van Strijp *et al.,* 1992). Thus, there is a potential for cooperative, competing, or collectin-specific interactions *in vivo*.

On the basis of these observations, a wide variety of potential "antibacterial" defense functions have been suggested for lung collectins. These include opsonization, microbial agglutination with effects on cellular uptake or mucociliary clearance, microbial recognition, modulation of leukocyte activation, detoxification or presentation of endotoxin, regulation of cytokine production, phagocyte recruitment, and interference with bacterial growth or epithelial adherence (Crouch 1998; Crouch 1998; Wright 1997; Reid, Colomb *et al.,* 1998; van Golde 1995). A number of potentially deleterious activities have also been suggested: competition with phagocyte lectins, inhibition or inappropriate activation of LPS-activated defences, excessive amplification of inflammatory reactions, enhanced internalization of organisms that are ineffectively killed, and air-space retention of a microorganism (Crouch 1998). At present, the most compelling evidence for pulmonary collectin-mediated, antibacterial host-defense activity comes from characterization of SP-A–null transgenic mice (LeVine, Bruno *et al.,* 1997; LeVine, Kurak *et al.,* 1998; LeVine, Gwozdz *et al.,* 1999; LeVine, Kurak *et al.,* 1999). The animals showed no discernible abnormality in surfactant function or homeostasis (Ikegami *et al.,* 1998). Host-defense defects have been observed after microbial challenge. For example, SP-A–null mice show increased proliferation and dissemination and decreased phagocytosis of *Group B streptococci* (*GBS*) strains, and decreased clearance of *Staphylococcus aureus* and *Pseudomonas aeruginosa* after intra-tracheal inoculation.

SP-D is known to interact with the LPS of gram-negative bacteria, can mediate bacterial agglutination, and has been shown to enhance the phagocytosis of gram-negative and gram-positive bacteria by human neutrophils in vitro (Kuan, Rust *et al.,* 1992; Hartshorn,

Crouch *et al.,* 1998). Pikaar and coworkers reported that SP-D does not enhance the internalization of a laboratory strain of *E. coli* that can be aggregated by SP-D (Pikaar, Voorhout *et al.,* 1995). Restrepo and coworkers observed lectin-dependent binding of SP-D to two strains of *P. aeruginosa*, one mucoid and one non mucoid (Restrepo, Dong *et al.,* 1999) as well as an approximately two-fold, concentration-dependent enhancement of internalization. Opsonization experiments with *H. influenzae*, in which the organisms were preincubated with SP-D and washed before their addition to the cells, resulted in comparable enhancement in phagocytosis. However, there was no increase in the uptake of non-opsonized or opsonized organisms when the macrophages were attached to SP-D coated slides. Internalization of the organism was accompanied by a significant decrease in colony-forming units for the mucoid, but not the non-mucoid strain, suggesting that the non-mucoid organisms were internalized but not killed. Interestingly, SP-D did not aggregate either strain, despite efficient aggregation of control *E. coli* (Restrepo, Dong *et al.,* 1999). The above studies provide evidence for SP-D lectin-dependent, receptor-mediated opsonization and killing of a mucoid strain of *P. aeruginosa.*

Following the initial finding that MBL was involved in C4-dependent complement activation, it was shown that the binding of MBL to the rough strains of *E. coli* (K-12 and B) promoted complement-dependent bacterial killing. MBL binding was inhibited by mannose sugars, indicating that MBL bound to the carbohydrate determinants of the cell wall lipopolysaccharide of the bacteria, and killing of the bacteria was shown to depend on the presence of complement component C4 (Eggleton and Reid 1999).

Previous studies have shown that both native and recombinant human MBL bind to wild-type virulent *Salmonella montevideo* that expresses a mannose-rich lipopolysaccharide. Interaction with MBL results in opsonization and killing by phagocytes. Kuhlman (Kuhlman, Joiner *et al.,* 1989) showed that low concentration of MBL (less than 10 micrograms/ml) markedly enhanced complement deposition via the alternative complement pathway on *S. montevideo*. A mutant bacterial strain that did not express the mannose-rich lipopolysaccharide was not opsonised by MBL. In the presence of MBL the C3 bound to *S. montevideo* during incubation in serum was in the form of C3b and iC3b at a ratio of 1:2. The presence of *S. montevideo* with MBL rendered this normally serum resistant organism susceptible to complement-mediated killing. These results emphasize that MBL and complement cooperate in first line defence of the non-immune host.

Clinical isolates of bacteria from patients with meningitis were examined for MBL binding, which was compared with that of non-capsulate strains of the same bacterial species. Non-capsulate *Lysteria monocytogenes*, *Haemophilus influenzae*, *Neisseria meningitidis*, *N. cinera* and *N. subflava* bound large amounts of MBL. *E.coli, Streptococcus suis,*

Streptococcus pneumoniae and *N. meningitidis* serogroup A showed an intermediate MBL binding capacity, while capsulate *N. meningitidis*, *H. influenzae* type b and *Streptococcus agalactiae* exhibited a low MBL binding capacity. The presence of a capsule in the majority of pathogens causing bacterial meningitis protects them from binding by MBL (van Emmerik, Kuijper *et al.*, 1994).

Lipoteichoic acids from the cell wall of Gram-positive bacteria are also ligands for MBL. Lipoteichoic acids of *Micrococcus* and *Enterococcus* species containing mono-, di- and oligoglucosyl moieties showed highest binding to MBL, whereas lipoteichoic acids from *Streptococcus pyogenes* and *Staphylococcus aureus* lacking terminal sugars or those from *Listeria* and *Lactococcus* species containing galactosyl moieties, showed little binding to MBL (Polotsky, Fischer *et al.*, 1996).

1.4 Human MBL deficiencies

There are three MBL gene mutations described to date. A single point mutation in codon 54 of exon 1 of the MBL gene results in an aspartic substitution for glycine in the collagenous region of the protein (Sumiya, Super *et al.*, 1991). Crysallographic studies have confirmed that such substitutions of axial glycines distort the secondary structure of the collagenous helix. The mutant protein fails to polymerise and approximately 95% of such protein is degraded. The codon 54 mutation is relatively common in the British population. Individuals homozygous for the mutation have profoundly low MBL levels (<10ng/ml), heterozygotes have median levels of 358ng/ml and wild type homozygous individuals have median levels of 1630ng/ml. A second single point mutation in codon 57 of the MBL gene results in a glutamic acid substitution for glycine. This allele was identified in Sub-Saharan Africans and is rare in Eurasian populations (Lipscombe, Lau *et al.*, 1992; Lipscombe, Sumiya *et al.*, 1992). A third mutation results in a substitution of cysteine for arginine in codon 52 (Madsen, Garred *et al.*, 1994). All three mutations described above, influence the stability of the collagenous triple helix, heterozygous individuals have only a 1 in 8 probability of selecting three normal peptide chains during biosynthesis and therefore these individuals have protein levels which are approximately 1/8 normal.

1.5 Complement receptors and binding proteins

A primary function of the complement cell surface receptors is to enhance the responsiveness of the innate immune system in destroying and removing proteins, microorganisms and other foreign material as well as cellular debris from the circulation. A number of complement receptors have been found on specialized cell types and characterised at the molecular and biochemical level. These include complement receptor 1 (CR1, CD35),

complement receptor 2 (CR2, CD21), complement receptor 3 (CR3, CD11b/CD18), complement receptor 4 (CR4, CD11c/CD18), C3a receptor and the C5a receptor (CD88) (Morgan 1997). CR1 and CR3 are especially important in inducing phagocytosis of bacteria with complement components on their surface. CR2 is found mainly on B cells, where it is also part of the B-cell co-receptor complex and the receptor by which the Epstein-Barr virus selectively infects B cells, causing mononucleosis. CR3 and CR4 are integrins; CR3 is known to be important for leukocyte adhesion and migration, while CR4 is only known to function in phagocytic responses (Morgan 1997). The C5a and C3a receptors are seven TM-G protein-coupled receptors. They are present on endothelial cells, mast cells, and phagocytes and they activate G-protein (Morgan 1997).

In 1977, Sobel and co-workers observed saturable and concentration-dependent binding of C1q to B-lymphocytes and other cells of the lymphoblastoid lineage (Sobel, Bokisch *et al.,* 1975). Since then a number of researchers have observed that C1q is also capable of modulating diverse cellular responses, and this has resulted in an intense search for C1q binding proteins, which may be C1q receptors. A number of intracellular and cell surface proteins have been found to interact with C1q.

1.5.1 C3 Receptors

CR1 and CR2 are members of the RCA family of proteins and both are type I membrane proteins whose extracellular portions consist entirely of complement control protein (CCP) repeats (Jenaway, Walport *et al.,* 2001). CR1 has two binding sites for C3b and one for C4b. CR2 has a single binding site which recognises iC3b, C3dg and C3d. CR1 is expressed on neutrophils, monocytes, and macrophages, where it mediates the phagocytosis of appropriately opsonised particles, and it is also present on erythrocytes where it mediates transport of immune complexes (Tas, Klickstein *et al.,* 1999). Furthermore CR1 is one of the several cofactors that Factor I can utilise to inactivate membrane bound C3b (Tas, Klickstein *et al.,* 1999). CR2 is located on phagocytes, follicular dendritic cells, glial cells, B cells and certain epithelial cells. CR2 is found on the B cells as part of a co-receptor complex that can augment the signal received through the antigen-specific immunoglobulin receptor (Jenaway, Walport *et al.,* 2001). Thus a B cell whose antigen receptor is specific for a given pathogen will receive a strongly augmented signal on binding this pathogen if it is also coated with C3dg.

CR3 and CR4 belong to a group of adhesion molecules known as integrins. CR3 and CR4 are expressed on macrophages, monocytes, and polymorphonuclear leukocytes (Jenaway, Walport *et al.,* 2001). CR3 is expressed on dendritic cells and CR4 is expressed on follicular dendritic cells (FDC) (Jenaway, Walport *et al.,* 2001). CR3 and CR4 are the

principal opsonic receptors for iC3b. Besides iC3b, CR3 also binds to ICAM-1, to fibronectin, to LPS and two proteins of the blood clotting system, factor X and fibrinogen (Jenaway, Walport *et al.*, 2001).

1.5.2 Anaphylatoxin Receptors

The small complement fragments C3a, C4a and C5a act on specific receptors to produce local inflammatory responses. When produced in large amounts or injected systemically, they induce a generalised circulatory collapse, which is termed anaphylactic shock and these small complement fragments are often referred to as anaphylatoxins.

C3a and C5a mediate cellular responses through two distinct but related receptors, C3aR and C5aR (CD88). Early functional and cross-linking studies using purified factors indicated that C5a receptors exist on neutrophils (Chenoweth and Hugli 1978), neutrophils (van Epps and Chenoweth 1984) (Chenoweth, Goodman *et al.*, 1982), basophils (Schulman, Post *et al.*, 1988), and eosinophils (Gerard, Hodges *et al.*, 1989), as well as platelets and mast cells from certain species (Fukuoka and Hugli 1990). C5aR is expressed on myeloid cell lines, such as U937 and HL-60, but only after these cells are induced to differentiate to a more mature stage of development. It has also been shown that C5aR is expressed on liver parenchymal cells, lung vascular smooth muscle, lung and umbilical vascular endothelial cells, bronchial and alveolar epithelial cells, as well as astrocytes and microglial cells (Haviland, McCoy *et al.*, 1995; Lacy, Jones *et al.*, 1995; Gasque, Singhrao *et al.*, 1997). Tissue distribution of C5aR was most predominant in (human) heart, lung, spleen, spinal cord, and in many regions of the brain. The C5a receptors are distributed much more widely than previously believed in terms of both cell and tissue type, and these receptors are present not just on myeloid inflammatory cells but on a variety of tissue cells.

The C3a receptor has been demonstrated on guinea pig platelets (Fukuoka and Hugli 1988), rat mast cells (Fukuoka and Hugli 1990), human alveolar macrophages, neutrophils, basophils (Glovsky, Hugli *et al.*, 1979) and eosinophils (Daffern, Pfeifer *et al.*, 1995). Flow cytometry identified C3aR on peripheral monocytes and umbilical vein endothelial cells, as well as the Raji cell line and differentiated HL-60 and U-937 monocytic cell lines (Roglic, Prossnitz *et al.*, 1996). Northern blot analysis showed high levels of mRNA for C3aR in human lung and spleen, with lower levels in heart, placenta, kidney, thymus, testis, ovaries, small intestine, colon, and several regions of the brain (Ames, Li *et al.*, 1996). Observations of C3a as a chemotactic factor for eosinophils and perhaps basophils, but not neutrophils, suggests a specialised role for C3a in inflammatory responses involving these cell types (Daffern, Pfeifer *et al.*, 1995).

Cellular responses to the anaphylatoxin C5a reflect the prominent pro-inflammatory character of the molecule. The property most closely identified with C5a is the potent chemotactic activity for granulocytes, particularly neutrophils and eosinophils (Fernandez, Henson *et al.,* 1978). C5a is also a potent activator of inflammatory mediator release from all granulocytes (Takafuji *et al.,* 1994). In neutrophils and eosinophils, C5a induces release of all known classes of secretory granules leading to extracellular liberation of a wide range of inflammatory mediators including elastase, peroxidase, glucoronidase and lactoferrin in neutrophils (Takafuji, Tadokoro *et al.,* 1994). C5a also activates the NADPH-oxidase pathway in granulocytes, leading to an oxidative burst (Elsner, Oppermann *et al.,* 1994). C5a has been shown to act as a pro-adhesive stimulus for granulocytes and has been shown to increase adherence of neutrophils to cultured endothelial cells (Neeley, Hamann *et al.,* 1993).

The effects of C3a appear to be much more selective in terms of cellular responsiveness and less specifically pro-inflammatory, depending on the cell type. C3a is chemotactic for eosinophils and can induce both granule release and an oxidative burst (Takafuji, Tadokoro *et al.,* 1994); (Elsner, Oppermann *et al.,* 1994). C3a, like C5a, also upregulates expression of β2 integrins and induces shedding of L-lectin for eosinophils. C3a is at least 10-fold less potent than C5a in inducing these responses. C3a is also an effective activator of mast cells, leading to a full profile of mediator release (Cochrane and Muller-Eberhard 1968). In fact most of the immediate tissue and *in vivo* effects of C3a are consistent with its role as a mast cell activator.

1.5.3 C1q binding proteins and C1q receptors

C1q binds to a number of different cell types and triggers a variety of cellular responses, including phagocytosis, enhanced microbial killing by phagocytes, induction of chemotaxis and stimulation of increased oxidative metabolism. This has focused attention on both the head and tail regions of C1q as possible ligands for cell surface C1q binding proteins and receptors. The collagen-like tail regions of C1q have been proposed to bind to a number of proteins including C1qRp, C1qRO2, calreticulin (also termed cC1qR and collectin receptor) and CR1. The globular heads region of C1q has been observed to bind to the globular head C1q binding protein (gC1qBP).

1.5.3.1 Calreticulin-C1qR (collectin receptor)

Calreticulin (as a C1q-binding protein) was first isolated from detergent lysates of Raji and U937 cells, and human tonsil lymphocytes (Erdei and Reid 1988). The protein was purified using C1q-Sepharose affinity chromatography and eluted with a salt gradient. A protein of approximately 60 kDa showing C1q binding specificity was eluted between 45 and

80 mM NaCl. It was shown that this protein was synthesised by both Raji and U937 cells and that it was expressed on the surface of the cells. The binding of this protein to immobilised C1q was shown to be saturable and was inhibited by preincubation with soluble C1q or the collagen stalks of C1q (Erdei and Reid 1988).

Malhotra and colleagues (Malhotra, Reid *et al.,* 1988) also reported purification of calreticulin from U937 cell lysate using affinity chromatography. In the affinity purification procedure the detergent lysate was incubated with C1q-Sepharose in low ionic strength buffer and calreticulin-containing fractions were eluted with 50 mM NaCl in the loading buffer. The calreticulin-containing fractions were further purified by ion exchange chromatography and gel filtration. This showed that the protein purified consists of two components, one basic that does not bind to Mono Q and one acidic that binds. Both components showed identical mobility on SDS-PAGE. Only the acidic component was found to bind specifically to immobilised C1q and was further characterised as a C1q receptor (Malhotra, Reid *et al.,* 1988). The role of the basic component is not known, as it does not show any interaction with C1q. In 1993, Daha and colleagues (Daha, Dunn *et al.,* 1993) reported isolation of the same protein from human umbilical vein endothelium.

The calreticulin gene

The calreticulin gene consists of nine exons and spans approximately 3.6kb and 4.6kb of human and mouse genomic DNA, respectively (McCauliffe, Yang *et al.,* 1992; Waser, Mesaeli *et al.,* 1997). Human and mouse genes have been localised to chromosomes 19 and 8 respectively (McCauliffe, Yang *et al.,* 1992; Rooke, Briquet-Laugier *et al.,* 1997). The nucleotide sequences of the mouse and the human gene show greater than 70% identity, with the exception of introns 3 and 6 (McCauliffe, Yang *et al.,* 1992; Waser, Mesaeli *et al.,* 1997), indicating a strong evolutionary conservation of the gene. In the mouse gene these introns are approximately twice the size of the corresponding introns in the human gene (McCauliffe, Yang *et al.,* 1992; Waser, Mesaeli *et al.,* 1997).

The promoter of the mouse and human calreticulin genes contain several putative regulatory sites, including AP-1 and AP-2 sites, GC-rich areas, including an Sp1 site, an H4TF-1 site, and four CCAAT sequences (McCauliffe, Yang *et al.,* 1992; Waser, Mesaeli *et al.,* 1997). AP-2 and H4TF-1 recognition sequences are typically found in genes that are active during cellular proliferation. Several poly (G) sequences, including GGGNNGGG motifs, are also found in the calreticulin promoter and other ER proteins. These motifs may play a role in regulation of the expression of luminal ER proteins and in ER stress-dependent activation of the calreticulin gene (Llewellyn, Sheikh *et al.,* 1995).

Depletion of Ca^{+2} stores induces activation of the calreticulin promoter followed by increase in calreticulin mRNA and protein levels (Llewellyn, Sheikh *et al.,* 1995). The calreticulin promoter is activated by Zn^{+2} and heat shock. Expression of calreticulin is also induced by viral infection, by amino acid deprivation and in stimulated cytotoxic T cells, indicating that the calreticulin gene is activated by a variety of chemical and biological stresses and it might affect numerous biological and pathophysiological conditions.

Structure of Calreticulin.

Calreticulin is a 46-kDa protein with an N-terminal cleavage amino acid signal sequence and a C-terminal KDEL ER retrieval signal. These specific amino acid sequences are responsible for targeting and retention of calreticulin in the ER lumen. Depending on species, calreticulin may have one or more potential glycosylation sites. The glycosylation pattern of the protein seems to be heterogeneous and does not appear to be a conserved property of the protein. The glycosylation of calreticulin seems to be more common in plant than in animal cells (Navazio, Baldan *et al.,* 1996). Heat shock may trigger glycosylation (Jethmalani and Henle 1998), 1998, (Jethmalani and Henle 1994), however, the functional consequence of this stress-induced glycosylation is not known. Calreticulin has three cysteine residues, and all of them are located in the N-domain of the protein. Two out of three cysteine residues found in the protein form a disulphide bridge (Matsuoka, Seta *et al.,* 1994), which may be important for proper folding of the N-terminal region of the protein.

Structural predictions of calreticulin suggest that the protein has at least three domains. The N-terminal part of the protein, encompassing the N- and P-domain of calreticulin, has the most conserved amino acid sequence. Examination of the intron-exon organisation of the calreticulin gene suggests that the central P-domain of the protein may be encoded by exons 5, 6 and 7, whereas the first four exons and the last two exons may encode the N- and C-domain of the protein respectively, (Michalak, Corbett *et al.,* 1999). The S-domain, a subfragment that spans the N- and P- domain intersection, has C1q binding properties (Stuart, Lynch *et al.,* 1996).

The N-terminal half of the molecule is predicted to be a highly folded globular structure containing eight anti-parallel β-strands connected by protein loops. The amino acid sequence of the N-domain is highly conserved in all calreticulins (Michalak, Burns *et al.,* 1996). The N-domain binds Zn^{+2} and involves four of the histidine residues found in this domain (Baksh, Spamer *et al.,* 1995). The N-domain interacts with the DNA-binding domain of the glucocorticoid receptor *in vitro* (Burns, Duggan *et al.,* 1994), with rubella virus RNA (Nakhasi, Singh *et al.,* 1994; Singh, Atreya *et al.,* 1994; Atreya, Singh *et al.,* 1995), with a-integrin (Rojiani, Finlay *et al.,* 1991) and with protein disulphide-isomerase (PDI) and ER

protein 57 (Erp57) (Baksh, Spamer *et al.,* 1995; Corbett, Oikawa *et al.,* 1999). Interaction of this region of calreticulin with PDI inhibits PDI chaperone function, but enhances Erp57 activity. These protein-protein interactions are regulated by binding of Ca^{+2} to the C-domain of calreticulin (Corbett, Oikawa *et al.,* 1999). The N-domain also inhibits proliferation of endothelial cells and suppresses angiogenesis (Pike, Yao *et al.,* 1998).

The P-domain of calreticulin comprises a proline-rich sequence with three repeats of the amino acid sequence PXXIXDPDAXKPEDWDE (repeat A) followed by three repeats of the sequence GXWXPPXIXNPXYX (repeat B) (Michalak, Corbett *et al.,* 1999). This region of the protein binds Ca^{+2} with high affinity, and the repeats may be essential for the high-affinity Ca^{+2} binding of calreticulin. More importantly repeats A and B, are critical for the lectin-like chaperone activity of calreticulin. The P-domain of calreticulin interacts with PDI (Baksh, Spamer *et al.,* 1995; Corbett, Oikawa *et al.,* 1999) and perforin (Andrin, Pinkoski *et al.,* 1998), (Fraser, Michalak *et al.,* 1998), a component of cytotoxic T-cell granules. The P-domain has lectin-like activity and amino acid sequence similarities to other Ca^{+2}-binding chaperones, including calnexin, calmegin, and CALNUC, a Golgi Ca^{+2}-binding protein.

The C-terminal region of calreticulin is highly acidic and terminates with the KDEL ER retrieval sequence. This domain of calreticulin binds over 25 mol of Ca^{+2} /mol of protein (Baksh and Michalak 1991). Ca^{+2} binding to this domain of the protein plays a regulatory role in the control of calreticulin interaction with PDI, Erp57 and perhaps other chaperones (Corbett, Oikawa *et al.,* 1999).

The S-domain is a calreticulin region, which encompasses a peptide sequence that has limited homology to the CUB domains of C1r or C1s, and has overall sequence similarity with CUB domains (Stuart, Lynch *et al.,* 1996; Stuart, Lynch *et al.,* 1997). In a C1-dependent haemolytic assay calreticulin domains were tested for their potential to inhibit complement activation (Stuart, Lynch *et al.,* 1996; this work]. Strong inhibitory activity was observed for the recombinant S-domain. It was also shown that C1q efficiently binds to the N-domain and to a lesser extent to the P-domain. No C1q binding activity was detected for the C domain. These data indicated that the C1q binding site of C1qR/CaR is localised on the S-domain (Stuart, Lynch *et al.,* 1996).

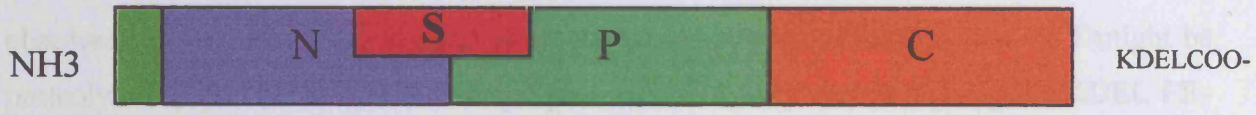


Figure 1.4 A schematic representation of the calreticulin domains.

Cellular localisation of Calreticulin.

Calreticulin is widely distributed in cells, and appears to be associated with the endoplasmic reticulum. Numerous studies confirmed ER localisation of calreticulin in many diverse species, including plants. The protein has also been localised to the cytoplasmic granules of the cytotoxic T-cell (Andrin, Pinkoski *et al.,* 1998; Fraser, Michalak *et al.,* 1998; Dupuis, Schaerer *et al.,* 1993), sperm acrosomes (Nakamura, Moriya *et al.,* 1993) and tick saliva (Jaworski, Higgins *et al.,* 1996). It does not contain a transmembrane domain within its sequence, but has been shown to be detectable on the cell surface (Sim, Moestrup *et al.,* 1998). It has been found as a C1q-binding protein on the surfaces of tonsil B lymphocytes, Raji cells, U937 cells and blood vessel endothelium. Calreticulin has been characterised as a surface laminin-binding protein of mouse melanoma cells (White, Zhu *et al.,* 1995), a surface receptor for fibrinogen fragments on human fibroblasts (Gray, Park *et al.,* 1995), and a C1q receptor downregulated by PMA, on the surface of neutrophils and HL-60 cells (Eggleton, Ghebrehiwet *et al.,* 1995). Immunohistochemistry with anti-C1qR antibodies shows that it is present on the surface of amniotic and pulmonary epithelium (Malhotra, Haurum *et al.,* 1993; Malhotra, Willis *et al.,* 1994).

A major question is how CRT moves from the ER to the outside of the cell. Although CRT contains the ER-retrieval sequence KDEL at its C-terminus, the protein has been identified on the surface of a wide variety of cells where it has been implicated in a variety of functions. The important issue of how CRT escapes ER retention and is translocated to the cell surface remains to be answered. However, there is now evidence localizing CRT to the secretory pathway from studies on plant cells (Borisjuk, Sitailo *et al.,* 1998), B16 mouse melanoma cells, rat hepatocytes (Zuber, Spiro *et al.,* 2000) and also the neuronal cell line NG-108-15 (Xiao, Chung *et al.,* 1999). In the neuronal cell line, a number of ER-resident proteins containing the KDEL sequence have been identified at the cell surface by surface-biotinylation, including CRT and protein disulfide isomerase (ER-lumenal thiol reductase) (Xiao, Chung *et al.,* 1999). Several theories could explain the transport of ER proteins to the cell surface. The proteins might be expressed in different isoforms that do not contain the ER-retrieval sequence. In support of this idea, CRT detected on the surface of NG-108-15 cells is not recognized by antibodies against KDEL (Xiao, Chung *et al.,* 1999); however, this could be because the KDEL receptor is transported to the surface in complex with CRT, as was observed in B16 cells (Zhu, Zelinka *et al.,* 1997). Another possibility is that CRT might be proteolytically processed by ER-lumenal proteases to a form that is missing the KDEL ER-retrieval sequence, given that, under ER-lumenal Ca^{+2} concentrations, the C-domain of the protein containing the KDEL amino acid sequence is susceptible to proteolysis (Corbett and Michalak 2000). Saturation of the ER-retention machinery could also play a role (Crofts,

Leborgne-Castel *et al.,* 1999). This could be especially relevant in studies where CRT expression is unregulated, such as in activated T-cells, where the protein is targeted to the cytotoxic T-lymphocyte (CTL) granules (Burns, Littlefield *et al.,* 1992). Interaction between a CTL and its target cells stimulates release of granule contents, including CRT, into the extracellular space, providing one mechanism for extracellular targeting of the protein. Finally, protein glycosylation might play a role as experiments carried out in Chinese hamster ovary cells have shown that CRT becomes glycosylated under conditions of stress, resulting in the redistribution of the protein within the cell (Jethmalani and Henle 1997).

Calreticulin appears to be strongly bound to the cell surface. The role of CRT at the cell surface is unclear. CRT does not possess a transmembrane domain, but it clearly orchestrates a number of cellular events from the cell surface, including cellular adhesion and migration. CRT might modulate cell adhesion from within the cell through an interaction with integrin tails or through regulation of focal-adhesion-associated proteins as well as modulation of cytosolic phosphotyrosine levels. Another possibility is that CRT can modulate cell adhesion from outside the cell. CRT has been demonstrated to bind to the extracellular matrix proteins fibrinogen (Gray, Park *et al.,* 1995) and laminin (White, Zhu *et al.,* 1995), and it has been reported that cell-surface CRT can complex with integrins (Zhu, Zelinka *et al.,* 1997). Antibodies against CRT can prevent spreading of B16 cells (White *et al.,*, 1995) and block neurite formation in differentiating NG-108-15 cells (Xiao, Chung *et al.,* 1999).

Previous work on endothelial cells has shown that CRT, or fragments of the protein, inhibits angiogenesis and suppresses tumor growth (Pike, Yao *et al.,* 1998). Tumor growth and metastasis formation is dependent on an adequate blood supply, and therefore the generation of new blood vessels (angiogenesis) is essential for the progression of a tumor. CRT disrupts this process by specifically inhibiting the proliferation of endothelial cells (Pike, Yao *et al.,* 1999).

An alternative function of cell-surface CRT might be in regulating responses of the immune system. CRT is present in the CTL granules, where it has been proposed to prevent perforin from forming pores in the granule membrane, either by Ca^{+2} –chelation (Dupuis, Schaerer *et al.,* 1993) or direct interactions with perforin (Andrin, Pinkoski *et al.,* 1998). However, more recent work has suggested that CRT has a more active role in preventing autolysis of the lymphocyte by binding directly to the cell surface (Fraser, Karimi *et al.,* 2000). Experiments performed on erythrocytes showed that CRT bound to the membrane, where it prevented the insertion of perforin and hence prevented cell lysis. Furthermore, inhibition of lysis was dependent neither on a direct interaction between CRT and perforin nor on the ability of CRT to chelate Ca^{+2} (Fraser, Karimi *et al.,* 2000). Finally, cell-surface CRT has been implicated in the upregulation of the immune system. An anti-microbial

peptide, which activates neutrophils, has been shown to act through interactions with cell-surface CRT (Cho, Homma *et al.*, 1999).

CRT has also been implicated in a number of pathological processes. Autoantibodies against CRT have been found in approximately 40% of systemic lupus erythematosus (SLE) patients, and a number of patients with secondary Sjögren's syndrome (Eggleton, Ward *et al.*, 2000). Other than in SLE, antibodies to CRT have been detected in patients with rheumatoid arthritis (Verreck, Elferink *et al.*, 1995), coeliac disease (Tuckova, Karska *et al.*, 1997), complete congenital heart block (Orth, Dorner *et al.*, 1996) and halothane hepatitis (Gut, Christen *et al.*, 1993).

1.5.3.2 gC1qR/p33 – The C1q-binding protein (gC1qBP)

gC1qBP was first isolated as a candidate cell surface C1q receptor for the globular heads of C1q (gC1qR) from a membrane preparation of Raji cells (Ghebrehiwet, Lim *et al.*, 1994), (Ghebrehiwet and Peerschke 1998). A full- length cDNA of gC1qBP has been isolated using oligonucleotides derived from a partial peptide sequence of the gC1qR candidate protein that was isolated by C1q affinity chromatography using cell extracts of the B cell line Raji. The cDNA encodes a pre-pro-protein of 282 residues from which a 73-residue-long N-terminal segment is removed by site-specific cleavage to generate the mature gC1qBP (Ghebrehiwet, Lim *et al.*, 1994). The mature protein comprises 209 amino acid residues and represents a highly acidic protein (28 Glu and 20 Asp residues) that is devoid of a typical hydrophobic transmembrane-spanning region (Ghebrehiwet, Lim *et al.*, 1994). Mature gC1qBP contains only one cysteine residue at amino acid position 186 and thus does not have any intra-disulphide bonding but may allow it to form dimers and tetramers as is often seen on SDS-PAGE (Ghebrehiwet and Peerschke 1998). The mature protein is preceded by a 60 residue-long hydrophobic stretch containing five cysteines, which in turn is preceded by a 13-residue long leader peptide, which probably contains a signal peptide. The mature protein is presumed to be generated by a site-specific cleavage and removal during post-translational processing (Ghebrehiwet and Peerschke 1998). The precise function of the 60 residues immediately preceding the mature protein has not been determined as yet but is predicted to play role in cellular translocation (Ghebrehiwet and Peerschke 1998). Before its assumed role as a specific cell surface receptor for C1q, the sequence of the proposed receptor for gC1q was determined by another, unrelated approach where the same molecule was isolated by its tendency to co-purify with the nuclear pre-mRNA splicing factor SF2/ASF, and termed p33 (Krainer, Mayeda *et al.*, 1991). It has become apparent that p33 or gC1qBP is a conserved eukaryotic protein. Homologous genes have been identified in a number of eukaryotic species, ranging from fungi to mammals, as a result of both biochemical studies and genome

sequencing efforts. It has been reported that p33 can interact with many viral proteins, including HIV-1 Tat (Desai, Loewenstein *et al.,* 1991; Fridell, Harding *et al.,* 1995) and Rev (Luo, Yu *et al.,* 1994; Tange, Jensen *et al.,* 1996), EBNA-1 of Epstein-Barr virus (Wang, Finan *et al.,* 1997), ORF P of herpes simplex virus (Bruni and Roizman, 1996), core protein V of adenovirus (Matthews and Russell 1998), laminin B receptor protein (p58) (Simos and Georgatos 1994), transcription factor TFIIB (Yu, Loewenstein *et al.,* 1995), complement component C1q (Ghebrehiwet, Lim *et al.,* 1994), kininogen (Herwald, Dedio *et al.,* 1996) and vitronectin (Lim, Reid *et al.,* 1996).

Gene structure

The human gC1qBP gene was assigned to human chromosome 17q13.3 (Guo, Weremowicz *et al.,* 1997). High degree of amino acid identity exists between the human, rat and mouse gC1qBP cDNA sequences (Lynch, Reid *et al.,* 1997). The human and mouse gC1qR genes are similar in their exon/intron organisation comprising of six exons and five intron each within a total length of approximately 6 kb of DNA. The first exon encodes a long stretch of 70 amino acids residues including the putative signal peptide and 4 amino acid residues found in the mature protein. Exons 2-5 encode four hydrophilic domains whereas exon 6 encodes a domain, which is more or less neutral. The putative binding sites for C1q (Ghebrehiwet, Lu *et al.,* 1996), cC1qR (Ghebrehiwet, Lu *et al.,* 1997) and vitronectin (Lim, Reid *et al.,* 1996) have been identified in non-overlapping regions of the domain encoded by exon 2 whereas the site for high molecular weight kininogen (HK) and factor XII has been located in the domain encoded by exon 5 (Joseph, Ghebrehiwet *et al.,* 1996). The gC1qR molecule contains an HIV-1 Tat binding site in a domain that is encoded by exon 6 (Ghebrehiwet, Lu *et al.,* 1996). In addition, there is a single tyrosine kinase recognition consensus signal in exon 6. Sp1 sites in the upstream region close to the transcription start site are suggested to be very important for the promoter activity of the gC1qR gene. Tye and coworkers (Tye, Ghebrehiwet *et al.,* 2001) recently showed that one out of these seven GC-rich Sp1 sites (-96 to 76) binds specifically to cell line PANC-1 nuclear proteins in gel mobility shift assays. One of these nuclear factors was further proved to be Sp1 binding factor in supershift assays employing anti-Sp1 antibodies (Tye, Ghebrehiwet *et al.,* 2001). These results showed that binding of Sp1 to the SP1 binding site located at around 80 bp upstream to the translation initiation codon might play an important role in transcription control in human gC1qBP gene.

The three dimensional structure of gC1qR was revealed by X-ray crystallography. The mature protein molecule has one N-terminal α-helix followed by seven consecutive antiparallel β-strands and two C-terminal α-helices (Jiang, Zhang *et al.,* 1999). Three

molecules form a doughnut shape quaternary structure with an internal channel of 10 A diameter. By aligning the exon boundaries and the three dimensional structure, it is observed that: exon 1 encodes the first 77 amino acid residues containing the mitochondria-targeting sequence (Dedio, Jahnen-Dechent *et al.,* 1998); exon 2 encodes the N-terminal α-helix and the first two β-strands; exon 3 encodes β-strand 3; exon 4 encodes β-strands 4 and 5; exon 5 encodes β-strands 6 and 7 and the last exon encodes for the C-terminal α-helices.

Cellular localisation and functions of gC1qBP

In the original paper (Ghebrehiwet, Lim *et al.,* 1994) immunofluorescence studies have indicated the surface expression of p33/gC1qR on B cells, mast cells, neutrophils, platelets and endothelial cells. A subsequent paper claimed that it was also abundantly expressed on the surface of neutrophils (Eggleton, Ghebrehiwet *et al.,* 1995). Later, more controlled studies, however, demonstrated that gC1qR is absent from the cell surface and can therefore not act as a cell surface receptor for C1q (van den Berg, Prins *et al.,* 1997). This has been confirmed by Dedio and coworkers who showed that the candidate C1q receptor gC1qR is associated with the vesicular fraction of endothelial cells (Dedio and Muller-Esterl 1996).

By using a fusion protein of gC1qR residues 1-81 or 1-33 with the N-terminus of the marker green fluorescent protein it was demonstrated that gC1qR is localized in mitochondria and colocalises with mitochondrial pyruvate dehydrogenase of EA.hy926 cells (Dedio, Jahnen-Dechent *et al.,* 1998). Using authentic mitochondrial sequences Dedio and coworkers found that p33/gC1qR may be associated with the matrix and/or the inner membrane of mitochondria. This hypothesis was supported by the finding that p33/gC1qR was detected in bovine liver mitoplasts, i.e., mitochondria devoid of an intact outer membrane (Dedio, Jahnen-Dechent *et al.,* 1998).

As gC1qR is absent on the cell surface membrane and can therefore not act as a cell surface C1q receptor, I will continue to use the term C1qBP (for C1q binding protein) to describe this molecule.

The findings of Dedio et al (Dedio, Jahnen-Dechent *et al.,* 1998) do not exclude the possibility that gC1qBP, like many other proteins, could be exported from the mitochrondria by an unknown mechanism. While predominantly found associated intracellularly with the mitochondria and the nucleus, the presence of gC1qBP on the cell surface remains controversial.

Most of the reported interactions of p33 should take place outside of mitochondria. For p33 to interact with the globular head of C1q (Ghebrehiwet, Lim *et al.,* 1994), H-kininogen (Herwald, Dedio *et al.,* 1996), vitronectin (Lim, Reid *et al.,* 1996), and hyaluronic acid (Deb and Datta 1996), it should be located on the extracellular side of the plasma

membrane or in the extracellular matrix. Small amounts of cell surface or plasma p33 may actually originate from lysed cells. An interesting feature of p33 is its high negative-charge density and asymmetric charge distribution, which suggest a possible mode of association with the inner mitochondrial membrane (Jiang, Zhang *et al.,* 1999). An interesting question is whether the externalization of p33 is the result of an apoptotic process (Jiang, Zhang *et al.,* 1999). The mitochondrial inner membrane has a large conductance channel, known as the permeability transition pore (PTP) (Bernardi, Broekemeier *et al.,* 1994). The PTP allows free diffusion of small molecular-weight solute molecules. Furthermore, high Ca^{+2} concentration appears to promote pore opening. This process plays an important role in the programmed cell death (Petit, Susin *et al.,* 1996; Green and Reed 1998). It has been suggested that p33 may participate in apoptosis by interacting with the matrix side of the internal membrane component of PTP and regulating the pore opening in a divalent-metal concentration-dependent manner (Jiang, Zhang *et al.,* 1999). The negatively charged side of p33 may potentially bind to the inner mitochondrial membrane in the presence of divalent metal ions, as negatively charged phospholipids can chelate divalent cations. If p33 is indeed localized proximally to the inner mitochondrial membrane, the central channel in the p33 trimer may have important functions. The channel is large enough to allow passage of a particle of moderate size. Depending on the degree of blockage by the loops connecting β6 and β7, which may undergo conformational changes, the opening would have a diameter ranging from 10 to 20 Å (Jiang, Zhang *et al.,* 1999). This channel size would correspond to a range of molecular mass cutoffs for globular molecules of about 0.4–3 kDa, assuming an average density of about 1.35 g/cm^3 (average protein density). The inner mitochondrial membrane has various types of pores of this size for protein import and exchange of solute molecules. Protein import into mitochondria has been extensively studied in recent years, and a number of trans-membrane proteins (TIMs) have been shown to be involved in protein import through the inner membrane (Neupert 1997). However, there is so far no evidence of the involvement of p33 in mitochondrial protein import or of its interaction with TIMs.

p33 is capable of interactions with numerous viral proteins and many of these reported interactions take place in the nucleus. It is now clear that p33 is mainly localized in mitochondria at steady state and under normal circumstances. However, localization of a small fraction of p33 in the nucleus and/or dynamic shuttling of p33 between mitochondria and the nucleus remain possible. In fact, some nuclear localization has been reported by using different fixation procedures or different cell lines (Matthews and Russell 1998). More strikingly, it was observed that the localization of p33 in HeLa cells is altered during adenovirus infection (Matthews and Russell 1998). This finding led to the suggestion that p33 might be a part of a continuous nucleus–mitochondrion network that allows shuttling of

macromolecules, and that adenovirus might take advantage of this host process to deliver its genome to the nucleus (Matthews and Russell 1998). The validity of this hypothesis remains to be tested.

1.5.3.3 C1qRp (CD93)

C1q affinity chromatography in which C1q collagen tails were coupled to Sepharose was used to isolate another C1q receptor candidate of a mol. wt of 126,000 Da and termed C1qRp and it was thought to be involved in the enhancement of phagocytosis, but not the C1q mediated oxidative burst. The molecule was cloned by a panning cDNA cloning procedure, using a monoclonal IgM (R3) antibody specific for C1qRp (Nepomuceno, Henschen-Edman *et al.,* 1997). The cDNA sequence contains an open reading frame that encodes a signal peptide of 21 amino acids followed by a mature protein of 631 residues. While the predicted molecular mass of the protein is only 66.5 kDa, extensive O-linked glycosylation has been shown to account for a substantial proportion of the aberrant migration behavior in SDS-PAGE (Nepomuceno, Ruiz *et al.,* 1999). The primary structure predicted from the cDNA sequence consists of a C-terminal 47 residue cytoplasmic tail that contains the tyrosine kinase recognition motif, RAMENQY. This is followed by a 25 amino acid transmembrane domain, with the majority of the N-terminal region of the protein (559 amino acids) positioned extracellularly. The portion of the protein juxtaposed to the membrane is a serine/threonine-rich-mucin-like domain that serves to extend the N-terminal region of the molecule away from the surface of the cell, thus being more accessible for ligand interaction. This region is followed by five epidermal growth factor (EGF)-like modules, ranging between 40 and 43 amino acids in length. These EGF-like domains are capable of binding calcium and are found in a number of proteins of diverse function, where they may serve to stabilize protein-protein interactions. Finally, the N-terminal domain shows sequence identity to the carbohydrate recognition domain (CRD) found in the C-type lectins. These CRDs are found in membrane receptors thought to regulate endocytosis, including the human mannose macrophage receptor (Ezekowitz, Sastry *et al.,* 1990).

There are a number of cell surface molecules present on phagocytes that enhance the uptake of microorganisms, IC and antigens, including CR1 and Fc receptors. Early studies employing collagen-like fragments of C1q demonstrated that this region of C1q interacting with the cell surface functioned as a ligand to enhance FcR-mediated phagocytosis in human monocytes and macrophages (Bobak, Gaither *et al.,* 1987). A large number of cell surface associated proteins bind to C1q, which has made it difficult to identify the specific protein responsible for enhanced phagocytosis. C1qRp has been reported to be present on phagocytic

cells (macrophages and granulocytes) and endothelial cells and a role of C1qRp in stimulating phagocytosis has been proposed (Nepomuceno, Henschen-Edman *et al.*, 1997).

Phagocytosis is a major host defense mechanism by which potential deleterious material (both pathogenic organisms and cellular debris) is cleared from circulation and tissue and made accessible to inactivation. The phagocytic capacity or potential of a cell can be modulated by cytokines that trigger cellular differentiation (Gresham, Clement *et al.*, 1986; Sampson, Heuser *et al.*, 1991) or by other activation ligands. This activation is probably relevant at sites of inflammation where MBL, SPA and C1q, and/or other regulatory molecules may accumulate and enhance the phagocytotic capacity of acute inflammatory cells. Nepomuceno and coworkers (Nepomuceno, Henschen-Edman *et al.*, 1997) suggested that C1qRp present on the surface of these cells might mediate phagocytosis. However, C1qRp has been identified on NK cells and dendritic cells (Dean, McGreal *et al.*, 2000; Lovik, Vaage *et al.*, 2000). Because NK cells are non-phagocytotic cells, it is most likely that C1qRp has other functions in these non-phagocytotic cells (Lovik, Vaage *et al.*, 2000).

Petrenko and colleagues (Petrenko, Beavis *et al.*, 1999) identified and characterized the stem cell antigen AA4 and it was found to be homologous to the human C1qRp. In human monocytes, CD43 (ICAM-1) was identified as the major component in the C1q-binding complex that includes C1qRp (Guan, Burgess *et al.*, 1991). Therefore C1qRp may be an element of a larger receptor complex that binds C1q. The high degree of sequence conservation found within C1qRp and AA4 suggests that C1qRp is a homolog of AA4 and may be involved in some aspects of hematopoietic and vascular development in humans (Petrenko, Beavis *et al.*, 1999).

Finally, it was recently reported that C1qRp is present in endothelial cells (Fonseca, Carpenter *et al.*, 2001). Endothelial cells have the ability to bind immune complexes, phagocytose pathogens and clear apoptotic cells and it remains to be seen whether the interaction of ligand with C1qRp promotes a pro-inflammatory response.

As this interesting molecule apparently is neither a C1q or a collectin receptor, nor a stem cell marker (Lovik, Larsen Sand *et al.*, 2001) (as it is expressed in mature cells throughout adulthood) a CD cluster nomenclature (CD93) would offer a way out of the confusion created during its history of detection.

1.5.3.4 $C1qR_{O2}$-

$C1qR_{O2}$- is a C1q receptor activity that triggers superoxidative burst in polymorphonuclear leukocytes (Ruiz, Henschen-Edman *et al.*, 1995). The production of superoxide and other oxygen intermediates, such as hydrogen peroxide and singlet oxygen, is a protective mechanism elicited by these immune cells to aid in the killing of pathogens. The

activation of this "respiratory burst" by neutrophils and macrophages can lead to local tissue destruction and is normally regulated by a complex series of signalling pathways.

The C1q-mediated stimulation of leukocytes is novel with respect to some of its features; it does not lead to a general degranulation of the leukocyte, which results in the active secretion of proteolytic enzymes and other anti-microbial components from the primary and secondary granules (Goodman and Tenner 1992). Also the C1q mediated O_2-production is not inhibited by the G-protein-sensitive inhibitor, pertussis toxin, nor has it a requirement for stable cell-cell adherence for activation of the oxidase (Goodman, Anderson *et al.*, 1995).

In a series of studies employing C1q fragments as agonists, the region of C1q responsible for triggering superoxide production has been identified as a motif flanked by amino acids 42-61 of the C-chain of the collagen-like region of C1q (Ruiz, Henschen-Edman *et al.*, 1995; Ruiz, Henschen-Edman *et al.*, 1999).

1.5.3.5 The human CR1

Human CR1 (CD35) present on leukocytes and erythrocytes recognises ICs opsonised with the activated complement components C3b and C4b, leading to their clearance by the reticuloendothelial system in liver and spleen (Cornacoff, Hebert *et al.*, 1983; Klickstein, Barbashov *et al.*, 1997). Klickstein and co workers (Klickstein, Barbashov *et al.*, 1997) showed that CR1 can bind C1q, it recognises the collagen tail portion of C1q. The function of CR1 as a proposed C1q receptor adds further to the complexity of the many biological effects of C1q mediated through different cell surface receptors. If CR1 acts as a receptor for uptake of C1q-immunoglobulin complexes, it should be expected that CR1 would function in this manner in patients with deficiencies downstream of C1q, such as patients with C4 or C2 deficiencies (Tas, Klickstein *et al.*, 1999). These patients are incapable of cleaving C4 and C3 and therefore generate C3b and C4b, which normally associate with ICs and lead to their clearance. However, it has been shown that C1q is not able to provide sufficient IC removal in these patients (Tas, Klickstein *et al.*, 1999), suggesting that the interaction of CR1 with C1q does not play a major role in complement-mediated enhancement of the clearance of ICs.

1.6 C1q and Systemic Lupus Erythematosus

1.6.1 Systemic Lupus Erythematosus: Clinical features.

Systemic lupus erythematosus (SLE) is a multi-organ disease characterised by increased autoantibody production and immune complex formation. The pathogenic relationship between the occurrences of various autoantibodies, the presence of immune complexes and the symptoms of SLE are not fully understood. One interesting aspect in the

search for such a relationship is emerging from reports of clinical features associated with hereditary deficiencies of early components within the classical complement cascade.
The main organs attacked in patients with SLE during their disease course are the skin, the vascular system, the cardiopulmonary system, the renal system, the central nervous system and the musculoskeletal system. Examples of frequently observed **skin manifestations** are photosensitivity (58%), livedo reticularis (17%), and digital skin vasculitis (13%); other clinical signs such as urticaria (6%), panniculitis (1%) and periorbital oedema are relatively rare. Regarding the **cardiovascular and pulmonary system**, the most frequent clinical signs were Raynaud's phenomenon (46%), hypertension (40%), pleuritis (35%) and pericarditis (22%). Anaemia is diagnosed at least once during the disease course in 33% of the patients. Leucocytopenia is observed in 42% and thrombocytopenia in 17% of the patients. The most frequent clinical signs related to abdominal abnormalities are hepatomegaly (17%) and splenomegaly (8%).

Renal involvement is found in 47% of the patients; the most reported abnormalities are proteinuria (32%), a decreased creatinine clearance (27%), haematuria (20%) and casts (17%). In 12% of the patients, renal involvement coincides with the presence of hypertension.

Central nervous involvement is found in 65% of the patients. In these patients a diversity of clinical signs is reported, e.g. depression (14%), seizures (9%), organic brain syndrome (9%), peripheral neuropathy (6%), cranial nerve palsy (5%), impaired consciousness (4%), psychosis (4%), aseptic meningitis (2%) and cerebellar ataxia (2%). Finally regarding the musculoskeletal system, the most frequent clinical signs are deforming arthritis (14%), myalgia (29%) and muscle weakness (24%) (Swaak, van den Brink *et al.*, 1999).

The most frequently reported clinical manifestations based on the ARA criteria at onset and diagnosis and during follow up are butterfly rash, discoid rash, oral ulcers, photosensitivity, arthritis, serositis, renal disorder, neurological disorder and haematological disorders (Swaak, van den Brink *et al.*, 1999).

1.6.2 Role of C1q in SLE

C1q levels are low in patients with SLE. There is correlation between low levels of C1q and disease activity (Walport, Davies *et al.*, 1998). The observation of the link between homozygous C1q deficiency and SLE, imply that there is a physiological activity of the early part of the classical pathway of complement activation that protects humans against the development of SLE (Walport, Davies *et al.*, 1998). C1q provides the most protection, followed by C1r and C1s, C4 and C2. The challenge is to identify the relevant physiological activity and, for this, there are two strong candidates and a weaker one. The two strong

candidates are the role of the classical pathway of complement in the processing of the immune complexes, and the role of C1q in the clearance of apoptotic cells. The third possibility is the role of C1q and the classical pathway in host defence against infection (Walport, Davies *et al.,* 1998).

The hypothesis that complement deficiency causes SLE because of impaired host defence against a causal infectious agent has no experimental support. It seems unlikely because other immunodeficiencies leading to increased bacterial infection are not associated with increased incidence of SLE and also host defence against bacteria is mediated in the complement system predominantly by C3 as opsonin (Walport, Davies *et al.,* 1998). In view of this it would be predicted that C3 deficiency should be as strongly associated with SLE as C1q deficiency. Although complement does not play an important role in the host defence against the majority of viral infections, there are some significant exceptions, such as complement-mediated enhancement of viral uptake, which is recognised as a pathogenic factor in the invasion of certain flaviviruses and of HIV (Walport, Davies *et al.,* 1998). These hypotheses have important implications in the management of disease.

1.6.3 Complement and the processing of immune complexes.

The interactions of complement with immune complexes can be considered under two headings, firstly the role of complement in the processing and clearance of immune complexes and secondly, interactions of complement with immune complexes in tissues which may provoke inflammatory injury.

Complement and Fcγ receptors are known to mediate the processing of immune complexes (IC). To explore the role of complement in the clearance and organ localization of IC, Nash *et al.,* (Nash, Taylor *et al.,* 2001) analysed the processing of radiolabelled preformed IC, injected intravenously in mice deficient in the classical pathway of complement caused by a targeted disruption of the C1qA-chain gene (Botto 1998). It was shown that the liver and spleen were the main sites of IC uptake in all mice and that the splenic uptake of IC was significantly reduced in the C1q-deficient mice compared with the control mice. C1q-deficient mice also exhibited an initial accelerated hepatic uptake of IC similar to that seen in human subjects with hypocomplementaemia. The hepatic localization of IC at later time points was similar in both groups of mice (Nash, Taylor *et al.,* 2001). These data confirm that the classical pathway of complement plays an important role in the appropriate processing of IC in vivo.

The role of complement in promoting tissue injury when bound to immune complexes is very important in the development of an autoimmune response. This hypothesis provides the explanation for the link between complement deficiency and the development of SLE. In

the absence of complement, immune complexes may escape clearance by the mononuclear phagocytic system and end up in tissues where they trigger an inflammatory response, with the release of autoantigens, leading to development of an autoimmune response (Walport, Davies *et al.,* 1998). In support of this hypothesis is abundant evidence showing abnormalities of immune complex processing in the presence of hypocomplementaemia. In humans the clearance *in vivo* of large, complement-fixing soluble immune complexes has been studied by intravenous injection of a number of different model immune complexes, including IgG aggregates (Lobatto, Daha *et al.,* 1988) tetanus toxoid-containing complexes (Schifferli, Ng *et al.,* 1988) and anti-Hepatitis B surface antigen complexes (Davies, Erlendsson *et al.,* 1993). It was shown that within the circulation immune complexes bound rapidly to erythrocyte complement receptor type 1 (CR1) and binding was correlated with CR1 numbers and with levels of complement activity (Walport, Davies *et al.,* 1998). The major sites of clearance from the circulation were the spleen and the liver. The initial rate of clearance from the circulation of immune complexes by the liver was found to be faster in hypocomplementaemic patients. However, in hypocomplementaemic patients, immune complexes were retained less efficiently by the liver and were slowly released back into the circulation. The explanation for these findings is that in the absence of normal complement activation, immune complexes remained larger and were retained efficiently by hepatic Fc receptor-bearing cells, accounting for the accelerated uptake. The release back into the circulation may be explained by the inefficient internalisation of immune complexes bound by Fc receptors alone, in comparison with immune complexes ligating both Fc and complement receptors, which may promote more efficient internalisation of immune complexes, followed by immune complex catabolism (Walport, Davies *et al.,* 1998).

On the contrary, immune complex processing by the spleen was reduced in hypocomplementaemic patients (Walport, Davies *et al.,* 1998). This can be related to the mode of delivery of complexes to the fixed mononuclear phagocytic system. In the spleen, the anatomical organisation of the organ favours the uptake of small particles. The haematocrit in the spleen is relatively high compared with major vessels, and splenic macrophages have been shown to play an important role in the processing of IgG-coated red cells. It might therefore be expected that immune complexes bound to red cell CR1 would be selectively processed in the spleen, whereas complexes presented in the fluid phase would be processed in the liver. The above finding show that immune complex processing is abnormal in patients with hypocomplementaemia and that ineffective immune complex clearance could cause tissue injury and stimulate autoantibody response.

1.6.4 C1q and apoptosis

The process of programmed cell death, or apoptosis, is a normal physiologic process that is important in maintaining homeostasis. Apoptotic cells are characterised by cell shrinkage, collapse of the nucleus and cytoplasmic blebbing. Plasma membranes undergo anionic phospholipid exposure and modification of carbohydrate moieties. The resulting cellular fragments, or apoptotic bodies are subjected to rapid receptor-mediated ingestion by macrophages or resident tissue phagocytes without the inflammatory changes, which accompany necrosis. Some of the receptor pathways in the processing of apoptotic cells have been elucidated. For example the phagocytosis of human apoptotic neutrophils by monocyte-derived macrophages involves a charge-sensitive recognition that is mediated by the macrophage αvβ3 integrin, the vitronectin receptor (VnR) (Savill, Dransfield *et al.,* 1990). Murine inflammatory macrophages can recognise apoptotic lymphocytes, thymocytes and neutrophils with the involvement of a macrophage phosphatidylserine receptor. Phosphatidylserine is normally found in the inner surface of the cell membrane and when the cells become apoptotic it is translocated to the external leaflet of the plasma membrane and binds the phosphatidylserine receptor (Fadok, Savill *et al.,* 1992). There appear to be several mechanisms of uptake of apoptotic cells and it seems that different pathways of clearance may operate in different tissues.

In addition the last few years it has become apparent that cells undergoing apoptosis, e.g. keratinocytes after exposure to UV light, generate discrete sub-cellular structures referred to as surface blebs, which contain either nuclear or cytoplasmic constituents exclusively many of which are targeted by autoantibodies in SLE. These packages have been shown to contain high concentrations of autoantigens, and there is also evidence that the self-antigens within the blebs may become altered by several mechanisms. These include cleavage by intracellular proteases that become activated during the apoptotic process and selective phosphorylation by stress activated protein kinases (Utz, Hottelet *et al.,* 1997). In addition, it has been demonstrated that when apoptosis of human keratinocytes is induced by viral infection, viral antigens and autoantigens co-cluster in specific subsets within the surface blebs, and may present a novel challenge to self tolerance if not cleared and processed properly (Rosen, Casciola-Rosen *et al.,* 1995).

The immunogenic potential of the apoptotic cells has been demonstrated by several groups (Albert, Sauter *et al.,* 1998; Rovere, Sabbadini *et al.,* 1999; Henry, Bretaudeau *et al.,* 1999; Mevorach, Zhou *et al.,* 1998). Albert *et al.,* showed that dendritic cells acquire influenza-specific antigens from apoptotic influenza-virus infected macrophages and can prime virus-specific CTL responses (Albert, Sauter *et al.,* 1998). It has also been shown that dendritic cells can present tumour antigens derived from apoptotic tumour cells (Henry,

Bretaudeau *et al.,* 1999; Rovere, Sabbadini *et al.,* 1999). Immunisation of mice with large numbers of apoptotic thymocytes results in the generation of autoantibodies (Mevorach, Zhou *et al.,* 1998) demonstrating that immune tolerance can be broken by apoptotic cells. These data indicate that apoptotic cells constitute a pool of potentially immunogenic self-antigen that must be handled properly to avoid autoimmunization.

Numerous efforts have been made to classify individuals with SLE into subsets based upon clinical, serological, and immunogenetic phenotypes. Undoubtedly, SLE represents a group of disorders that can result from different combinations of genetic and environmental factors. The concordance rate of SLE among monozygotic twins is approximately 50%. In contrast, a mutation at the C1q locus that results in a complete deficiency of classical pathway function essentially guarantees that SLE will result. Either this genetic defect must be sufficient to cause the disease, or its acts in combination with a common somatic genetic event or following exposure to a common environmental agent, such as sunlight or a ubiquitous virus. C1q must participate in a specific molecular pathway critical for the maintenance of immune tolerance.

Taylor *et al.,* (Taylor, Carugati *et al.,* 2000), used complement deficient mice to determine the relative contribution of different complement proteins to the phagocytosis of apoptotic cells *in vivo.* When apoptotic Jurkat human T cells were used, C1q and C4 deficient mice showed a delay in the clearance of these apoptotic cells. This indicates that opsonisation with C3 via classical pathway activation was the most likely mediator of clearance in this model. When apoptotic murine thymocytes were used instead of apoptotic human cells, the C4-deficient mice showed a defect in the phagocytic uptake of apoptotic murine cells by inflammatory macrophages but this defect was not as severe as that seen in the C1q-deficient mice.

To address the role of C3 in the phagocytosis of apoptotic cells, resident peritoneal macrophages, as opposed to inflammatory macrophages were studied *in vivo.* C1q-deficient mice were the only complement-deficient animals to exhibit a defect in the phagocytosis of apoptotic cells by resident peritoneal macrophages suggesting that C1q is the complement protein with the predominant role in the physiological phagocytosis of apoptotic cells *in vivo* in the absence of inflammation. However there was a role for C4 in the clearance of apoptotic syngeneic thymocytes by inflammatory macrophages. The above findings suggest that C1q and C4 may mediate the uptake of apoptotic cells by different mechanisms, either involving separate receptors or a single receptor that is only activated for uptake of C4/C3-coated cells on inflammatory macrophages. The lack of a role for C4 and C3 in the phagocytosis of apoptotic cells by resident peritoneal macrophages could be related to the activation state of the macrophages, as it has been shown that murine resident macrophages are unable to

phagocytose C3-coated particles without the presence of additional stimuli such as thioglycollate elicitation (Shaw and Griffin 1984). However these resident macrophages may be able to utilise C1q as an opsonin presumably via interaction with one of the candidate C1q receptors described in section 1.4.3 (Ghebrehiwet, Silvestri *et al.,* 1984; Peerschke, Reid *et al.,* 1994; Klickstein, Barbashov *et al.,* 1997; Sim, Moestrup *et al.,* 1998).

This is supported by the independent observation that C1q-deficient macrophages from three C1q-deficient humans with SLE showed a defect in the phagocytotic uptake of apoptotic cells in vitro (Taylor, Carugati *et al.,* 2000). This defect was corrected in a dose-dependent manner, using purified C1q protein. Collectively, the above findings demonstrate an important role of the early proteins of the classical pathway in the clearance of apoptotic cells.

1.7 Rheumatoid Arthritis, a chronic inflammatory disease

1.7.1 Pathogenesis of Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common chronic inflammatory and destructive arthropathy. It is characterised by progressive joint damage that is mediated by several mechanisms. Early erosion of cartilage and bone is associated with the formation of a proliferating pannus. The interface between pannus and cartilage is occupied predominantly by activated macrophages and synovial fibroblasts. The inflammatory process is usually tightly regulated, involving both mediators that initiate and maintain inflammation and mediators that shut the process down. In states of chronic inflammation, an imbalance between the two mediators leaves inflammation unchecked, resulting in cellular damage. In the case of RA, this damage is manifested by the destruction of cartilage and bone. The synovial membrane in patients with RA is characterised by hyperplasia, increased vascularity and an infiltrate of vascular cells, primarily CD4+T cells, which are the main orchestrator of cell-mediated immunoresponses. In genetic studies, RA is strongly linked to the major-histocompatibility-complex class II antigens HLA-DRB10404, DRB10401 and DRB10410 (Lanchbury 1992; Snijders, Elferink *et al.,* 2001). The main function of HLA class II molecules is to present antigenic peptides to CD4+T cells, which strongly suggests that rheumatoid arthritis is caused by an unidentified arthritogen antigen (Gregersen, Silver *et al.,* 1987). The antigen could be either an exogenous antigen, such as a viral protein, or an endogenous protein such us citrullinated proteins (Baeten, Peene *et al.,* 2001).

1.7.2 Mediators of Inflammation in Rheumatoid Arthritis

Upon activation CD4+T cells stimulate monocytes, macrophages and synovial fibroblasts to produce the cytokines interleukin-1, interleukin-6, and TNFα. TNFα and

interleukin-1 are potent stimulators of mesenchymal cells, such as synovial fibroblasts, osteoblasts and chondrocytes that release tissue-destroying matrix metalloproteinases (Shingu, Nagai *et al.*, 1993). Interleukin-1 and TNFα also inhibit the production of tissue inhibitors of metalloproteinases by synovial fibroblasts (Shingu, Nagai *et al.*, 1993). These dual actions are thought to lead to joint damage. Furthermore, activated CD+4T cells also stimulate B cells, through cell surface contact and through the binding of aLb2 integrin, CD154 (CD40 ligand), and CD28, to produce immunoglobulins, including rheumatoid factor. The precise pathogenic role of rheumatoid factors is not known, but they are thought to be involved in the activation of complement through the formation of immune complexes (Choy and Panayi 2001). Activated CD4+T cells express osteoprotegerin that stimulate osteoclastogenesis. Such activated T cells caused joint damage in an animal model of RA (Kong, Feige *et al.*, 1999).

1.7.2.1 The role of TNFα in inflammation

TNFα is a potent cytokine that exerts diverse effects by stimulating a variety of cells. It is a soluble 17-kDa protein that is composed of three identical subunits. It is produced by mainly monocytes and macrophages, but also by B cells, T cells, and fibroblasts. Newly synthesized TNFα is inserted into the cell membrane and then it is released through the cleavage of its membrane-anchoring domain by a serine metalloproteinase (Black, Rauch *et al.*, 1997). Therefore, TNFα secretion might be suppressed by inhibitors of this enzyme (McGeehan, Becherer *et al.*, 1994). TNFα is an autocrine stimulator as well as a potent paracrine inducer of other inflammatory cytokines including interleukin-1, interleukin-6, interleukin-8 and granulocyte-macrophage colony-stimulating factor (Nawroth, Bank *et al.*, 1986; Haworth, Brennan *et al.*, 1991; Butler, Maini *et al.*, 1995). TNFα also promotes inflammation by stimulating fibroblasts to express adhesion molecules, such as intercellular adhesion molecule-1 (Chin, Winterrowd *et al.*, 1990). These adhesion molecules interact with their ligands on the surface of leukocytes resulting in increased transport of leukocytes into inflammatory sites, including the joints in RA patients. In cultures of synovial cells from RA patients, blocking TNFα with antibodies significantly reduced the production of interleukin-1, interleukin-6, interleukin-8 and granulocyte-macrophage colony-stimulating factor (Butler, Maini *et al.*, 1995). Therefore blocking TNFα may have a more global effect on inflammation than blocking of other cytokines that are present in high concentrations in synovial fluids, such as interleukin-1.

In transgenic mice that over-expressed human TNFα gene, an inflammatory and destructive polyarthritis similar to rheumatoid arthritis spontaneously developed (Keffer,

Probert *et al.,* 1991). Pre-treatment of these animals with a monoclonal antibody against TNFα prevented the development of arthritis. Blocking TNFα with a soluble TNF-receptor fusion protein or with monoclonal antibodies also decreased disease activity in mice and in humans with type II collagen-induced arthritis (Wooley 1991).

1.7.2.2 The role of Interleukin-1 and interleukin-6 in inflammation

Interleukin-1 is mainly produced by monocytes and macrophages, but is also produced by endothelial cells, B cells, and activated T cells (Koch, Kunkel *et al.,* 1995). Interleukin-1 binds to two types of cell-surface receptors. Type I receptors are found in low numbers in many cells, have a cytoplasmic tail and are capable of intracellular signalling (Sims, Gayle *et al.,* 1993). Type II receptors are decoy receptors: they are found on neutrophils, monocytes and B cells where they bind circulating Interleukin-1 but do not deliver any intracellular signals (Colotta, Re *et al.,* 1993). Soluble forms of both types of interleukin-1 receptor compete with cell-surface receptors, thereby decreasing interleukin-1-mediated activation of cells. In addition, a naturally occurring antagonist, interleukin-1-receptor antagonist, binds the type I receptor with high affinity without triggering a signal, thus providing another mechanism for the inhibition of interleukin-1 activity (Stevenson, MacLeod *et al.,* 1995).

In a rabbit model, interleukin-1 was injected into the knee joints of the rabbits and this resulted in degradation of cartilage (Pettipher, Higgs *et al.,* 1986). On the contrary, injection of antibodies against interleukin-1 ameliorates collagen-induced arthritis in mice and decreases damage to cartilage (Joosten, Helsen *et al.,* 1996). Like TNFα, Interleukin-1 may cause damage by stimulating the release of matrix metalloproteinases from fibroblast and chondrocytes (MacNaul, Hutchinson *et al.,* 1990). The concentrations of Interleukin-1-receptor antagonist are high in the synovial fluid of RA patients, but not high enough to suppress inflammation (Chomarat, Vannier *et al.,* 1995).

Interleukin-6 is a pleiotropic inflammatory cytokine produced by T cells, monocytes, macrophages, and synovial fibroblasts (Van Snick 1990). Interleukin-6 was originally identified as a factor that induces the final maturation of B cells into plasma cells. Interleukin-6 is involved in diverse biologic processes, such as the activation of T cells, the induction of the acute phase response, the stimulation of the growth and differentiation of haematopoietic precursor cells and finally the proliferation of synovial fibroblasts (Van Snick 1990).

Interleukin-1 and TNFα stimulate the expression of adhesion molecules on endothelial cells and increase the recruitment of neutrophils into the joints. Neutrophils release elastase and proteases, which degrade proteoglycan in the superficial layer of cartilage (Moore, Iwamura *et al.,* 1993). The depletion of proteoglycan enables immune complexes to

precipitate in the superficial layer of the collagens and exposes chondrocytes. Chondrocytes and synovial fibroblasts release matrix metalloproteinases when stimulated by interleukin-1, TNFα, or activated CD4+ T cells. Matrix metalloproteinases are enzymes that degrade connective tissue matrix and are thought to be the main mediators of joint damage in rheumatoid arthritis.

CHAPTER 2

Materials and Methods

Abbreviations and constituents of buffers are given in section 2.4

2.1. DNA Methods

2.1.1 Polymerase Chain Reaction

The polymerase chain reaction is used to amplify a segment of DNA that lies between two regions of known sequence. Two synthetic oligonucleotide primers are synthesised; one is complementary to the antisense strand at the 5' end of the region to be amplified, and the other is complementary to the sense strand at the 3' end of the region to be amplified. Template DNA is mixed with a thermostable DNA polymerase and a large molar excess of each of the primers and the four dNTP's. The reaction mixture is first heated to 95°C to denature the template, then cooled to a temperature that permits the primers to anneal to their target sequences (TA) and finally incubated at a temperature that is optimal for DNA synthesis. The cycle of denaturation, annealing and DNA synthesis is repeated 25-30 times using an automated thermal cycler. Because the products of each cycle serve as templates for the next, each cycle essentially doubles the amount of amplified DNA and the reaction proceeds exponentially. Thirty cycles of amplification result in a theoretical magnification of 10^9.

Modifications of the oligonucleotide primers allow restriction sites, start codons, stop codons or point mutations to be engineered into the ends of the PCR product.
Primers are 18-25 nucleotides long (excluding modifications), have 45-60% G/C content, melting temperatures (Tms) of 55-65°C and do not contain palindromic sequences. Primer pairs have approximately equal Tms and do not contain complementary sequences. Tm is calculated as 2°C per A or T base and 4°C per G or C base, excluding any mismatches or modifications.

PCR amplification was performed in a 50 µl reaction volume containing: 60pmoles of each primer, 10 nmoles of each dNTP, 1.5 mM $MgCl_2$, 20 ng of cDNA template and 2U of Taq DNA polymerase in the manufacturer's buffer. Thirty cycles of amplification were performed, each composed of 30 s at 95°C, 30 s at TA and extension time at 72°C. The initial denaturation step was prolonged at to 2 min and the final extension step to 10 min. TA was typically 5°C below the calculated Tm. PCR products were analysed by agarose gel electrophoresis (section 2.1.7). The sequences of primers used in this work are listed in the section 2.5

2.1.2 Small-scale preparation of plasmid DNA

A culture of the plasmid containing bacterial colony was set up in 5ml LB medium (section 2.4.1) and incubated overnight at 37°C in a shaking incubator (200 rpm). Following growth, 1.5ml of the culture was transferred to an Eppendorf tube and centrifuged for 3 min at 13,000rpm in a bench-top minifuge. The supernatant was discarded, another 1.5 ml of the

culture transferred and the tube centrifuged again. The pellet was resuspended in 100 µl of solution I (section 2.4.1). 200 µl of solution II were then added and the solution was mixed gently until it went clear. 150µl of solution III were then added and the tube vortexed upside down to form a white precipitate composed of protein and chromosomal DNA. The tube was centrifuged for 3 min at 13,000 rpm. After centrifugation the supernatant was removed and transferred to a fresh tube. Phenol chloroform extraction was then performed and the supernatant was transferred to a separate tube. 800µl of absolute ethanol were then added to the resultant upper phase, the solution mixed and then centrifuged for 15 min at 13,000 rpm to obtain a pellet of RNA and DNA. The supernatant was aspirated and washed in 1ml of 70% ethanol by centrifugation for 3 min at 13,000rpm. The supernatant was again aspirated and the pellet dried in a vacuum drier. The DNA was then resuspended in an appropriate amount of sterile distilled water, 1µl of RNAse (10mg/ml) added and the solution incubated at 37 °C for 15-30 min.

2.1.3 **Medium scale plasmid purification** (QIAGEN plasmid midi kit, Cat.No. 12143)

All buffers and solutions were purchased from QIAGEN

50 ml of a bacterial overnight culture were centrifuged at 10,000 x g at 4 °C, for 10 min. The bacterial pellet was resuspended in 4 ml of buffer P1 (supplied by QIAGEN), 4 ml of buffer P2 were added, the solution was mixed gently and incubated at room temperature for 5 min. 4 ml of chilled buffer P3 were then added, the solution was mixed immediately but gently and incubated on ice for 15 min. The solution was then centrifuged at 20,000 x g for 30 min at 4°C and the resultant supernatant was transferred to a fresh tube and centrifuged for 15 min at 4°C. The supernatant was then applied to the QIAGEN-tip 100 previously equilibrated with 4 ml of buffer QBT. The QIAGEN-tip 100 was washed twice with 10 ml of buffer QC, and the DNA was eluted with 5 ml of buffer QF. Finally the DNA was precipitated with 0.6 volumes of isopropanol at room-temperature as described in section 2.1.5.2, and the resultant pellet was resuspended in the appropriate volume of water.

2.1.4 Ligation

Ligation of foreign DNA molecules into vector DNA was accomplished using bacteriophage T4 ligase. This enzyme catalyses the formation of the phosphodiester bonds between neighbouring 3' hydroxyl groups and 5' phosphate ends of double stranded DNA molecules. Both blunt end ligation and cohesive ligation are possible using T4 ligase. T4 ligase works best at 37°C. However, at this temperature base pairing between the 3-4

nucleotide long protruding ends generated by restriction enzymes is very unstable, so temperatures ranging from 10- 20 °C are preferred.

Insert DNA was digested with appropriate restriction enzymes separated on an agarose gel and purified using the Ultra-Silica DNA Purification kit (section 2.1.8). Vector DNA was digested with restriction enzymes, then purified by extraction with phenol / chloroform and subsequent ethanol precipitation (section 2.1.5.1). The concentrations of purified DNA solutions were estimated by ethidium bromide stained agarose gel electrophoresis (section 2.1.7).

Ligation was done in a 10µl reaction volume containing: 50-150 ng of vector DNA, a three-fold molar excess of insert DNA and 4-6 Weiss units of T4 DNA ligase in the manufacturer's buffer. Reactions were incubated overnight at 15°C, then transformed into *E. coli* or stored at -20°C.

2.1.5 DNA Precipitation

2.1.5.1 Ethanol Precipitation

1. Add 1/10 volume 3M Na Acetate (pH4.8-5.2) to the DNA sample (<400µl per 1.5 ml microcentrifuge tube). Excess salt will interfere with the quality of the plasmid.
2. Add 2 to 3 volumes of 95% ethanol (ETOH). Mix and incubate on ice for 20 min.
3. Microcentrifuge at RT or 4°C for 30 min.
4. Carefully discard the supernatant. Wash the pellet with an equal volume of 70% ETOH (to reduce salt concentration). Centrifuge for 5 min, discard ETOH and air/vacuum dry.
5. Resuspend in TE buffer or water.

2.1.5.2 Isopropanol Precipitation

This can be useful with larger sample volumes and co-precipitates less protein.

1. As above, but add 0.6 volumes of isopropanol in place of 95% ETOH. Centrifuge immediately following isopropanol addition and wash as described above.

2.1.6 Restriction digest

Buffers for different restriction enzymes differ chiefly in the concentration of the NaCl that they contain. When DNA is to be cleaved with two restriction enzymes, the digestions can be carried out simultaneously if both enzymes work well in the same buffer.

The following procedure is for a typical reaction containing 0.2-1 µg of DNA. For digestion of larger amounts of DNA, the reaction should be scaled up appropriately.

1. Place the DNA solution in a sterile microfuge tube and mix with sufficient water to give a volume of 18 µl.

2. Add 2 µl of the appropriate 10 X restriction enzyme digestion buffer. Mix by tapping the tube.

3 Add 1-2 units of restriction enzyme and mix by tapping the tube. One unit of enzyme is defined as the amount that digests 1 µg of DNA to completion in 1 hour in the recommended buffer and at the recommended temperature (in general digestion for longer periods of time or with excess enzyme does not cause problems unless there is contamination with DNAase or exonuclease).

4. Incubate the mixture at the appropriate temperature for the required period of time.

2.1.7 Agarose Gel Electrophoresis

Molecules of linear double-stranded DNA, which tend to become oriented in an electric field in an end-on position (Fisher and Dingman 1971; Aaij and Borst 1972), migrate through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs (Helling, Goodman *et al.,* 1974). Larger molecules migrate more slowly because of greater frictional drag and because they worm their way through the pores of the gel less efficiently than smaller molecules. Heavily supercoiled DNA runs faster than linear DNA, which in turn runs faster than relaxed circular DNA. Electrophoretic mobility decreases with increasing agarose concentration, so high concentrations (>2%) are appropriate for small DNA fragments (50-500 bp) and lower concentrations (0.6-1.5%) are used for large fragments (500-10,000 bp).

Ethidium bromide, a fluorescent dye that intercalates between the staked base pairs of dsDNA is included in the gel and the gel running buffer, allowing the DNA to be visualised under UV light. The technique is sensitive enough to detect less than 10 ng of DNA.

1. Prepare slurry of 0.6 - 2% agarose in 1 X TAE and heat in a microwave oven until the agarose dissolves. Add ethidium bromide to a final concentration of 0.5 µg/ml.

2. Pour into the sealed gel-casting chamber of a proprietary horizontal gel electrophoresis apparatus. Insert the comb to form the sample wells and leave to set.

3. Remove the comb and fill the tank with enough 1 X TAE, 0.5 µg/ml ethidium bromide to cover the gel to a depth of ~2mm.

4. Mix the DNA samples (10ng to 1µg) with 0.2 volumes of DNA loading buffer and pipette into the sample wells. Include a DNA size standard. Run the gel at 2-5 V/cm (distance between the electrodes).

5. Examine on a UV transluminator. Photograph the gel.

2.1.8 Recovery of DNA from Agarose Gel Slices

Fragments of DNA that have been separated on Agarose gels were purified using the Ultra-Silica DNA Purification kit from Advanced Biotechnologies Ltd. The kit utilises a uniform size silica matrix that binds DNA in high ionic strength buffer. A slice of agarose containing DNA is cut from the gel using a scalpel, then dissolved in a solution of sodium iodide, which both denatures protein and provides the necessary high ionic strength buffer. Silica matrix is added, washed extensively, then the DNA eluted in a buffer of low ionic strength (TE or dH2O).

For most of the applications 2μg of DNA was purified and eluted in 20-30μl of TE. 5μl of purified DNA was analysed by agarose gel electrophoresis and the concentration estimated by comparing the intensity of the band with that of the DNA size markers. The yield was typically 60%.

2.1.9 Preparation and Transformation of Competent *E. coli*

Preparation of competent *E. coli*

The following procedure, which was developed by Hanahan (Hanahan 1983), can yield competent cultures of *E. coli* strains DH1, DH5, and MM294 that can be transformed at frequencies of >5 X 10^8 transformed colonies per microgram of supercoiled plasmid DNA. The maximum frequency of transformation that can be obtained routinely with most other strains of *E. coli* is approximately five- to tenfold lower.

1. Using a sterile platinum wire, streak the *E. coli* strain directly from the frozen stock onto the surface of an SOB agar plate. Incubate the plate for 16 hours at 37°C
2. Transfer one well-isolated colony into 1 ml of SOB containing 20mM $MgSO_4$. Disperse the bacteria by vortexing at moderate speed, and then dilute the culture in 30-100 ml of SOB obtaining 20 mM $MgSO_4$ in a 1-litre flask.
3. Grow the cells for 2.5-3.0 hours at 37°C until the absorption at 550 nm is 0.7 to 0.8.
4. Harvest the cells by centrifugation at 2000 x g for 10 min and discard the supernatant.
5. Resuspend the pellet in 30 ml sterile ice cold TfbI and incubate on ice for 5 - 30 min, depending on the strain chosen. 10 min works well for XL1-blue and TOP10F'.
6. Centrifuge the suspension at 2000 x g for 10 min at 4°C, then discard the supernatant and carefully resuspend the cells in 4 ml TfbII. Aliquot 0.2 ml fractions into microfuge tubes and transform immediately or freeze rapidly in a dry ice/ethanol bath and store at -80°C.

Transformation of chemically competent *E.coli*

1. Thaw competent cells slowly on ice. Add 2 - 10µl of a ligation reaction, or 0.1ng of plasmid DNA, and 2µl of 0.5 M β-mercaptoethanol (optional) to 100µl of competent cells and incubate on ice for 20 min.
2. Heat Shock at 37°C for 5 min or at 42°C for 1 min, then return to ice for a further 2 min. Add 950µl of prewarmed SOC medium and incubate at 37°C in a shaking incubator for 45 - 60 min. Plate 50µl and 150µl aliquots on selective medium and incubate overnight at 37°C.

2.1.10 Selection of Recombinant *E. coli*

Addition of antibiotics to the plating medium ensures that only cells transformed with plasmid carrying the appropriate antibiotic resistance marker will grow. Many of the vectors used encode the M15 fragment of the *LacZ* gene, which permits blue/white colour selection of recombinant plasmids. Insertion of foreign DNA into the multiple cloning sites of such vectors disrupts the M15 *LacZ* gene, preventing the formation of viable β-galactosidase. Recombinants are selected on agar containing IPTG, a non-substrate inducer of the Lac promoter, and X-gal, a colourless chromogenic compound that is hydrolysed by β-galactosidase to form a blue dye. Colonies containing plasmid with no insert appear blue, while those with recombinant plasmids usually appear white.

After ligation, transformation and selection, a number of colonies thought likely to contain recombinant plasmids are chosen for analysis by preparation of plasmid DNA (section 2.1.3) and subsequent restriction digestion (section 2.1.6).

2.1.11 DNA Sequencing

DNA was sequenced by the dideoxy chain termination procedure (Sanger method) using [α35S]dATP and the T7 Sequencing Kit from Pharmacia Biotech.

The method depends upon base-specific termination of enzyme catalysed primer-extension reactions. Single stranded DNA, or denatured ds DNA, is annealed with an oligonucleotide primer and the complementary strand is synthesised using DNA polymerase. Four separate reactions are performed all containing template, primer and the four dNTPs (one of which is radiollabeled), but each including a different dideoxy nucleotide. Dideoxynucleotides terminate synthesis, so the products of each reaction are a mixture of radiollabeled fragments all of which begin with the primer and end with the particular dideoxynucleotide present in that reaction. The four reactions are run side-by-side on a denaturing polyacrylamide gel, which is capable of separating DNA strands that differ in size by as little as one nucleotide.

After electrophoresis, the gel is dried and an autoradiograph prepared, from which the sequence can be read directly.

The polymerase chosen for sequencing is Sequenase, a form of T4 DNA polymerase that has been chemically modified and it is low in exonuclease activity. Sequenase is more efficient than the Klenow enzyme because the primer-extension reactions are performed in two stages, a "labelling" reaction and a "termination" reaction. The two stages are required because the enzyme uses dideoxynucleotides very readily. To permit synthesis of long chain-terminated fragments, dideoxynucleotides are therefore excluded during the first stage, then added for the second.

In practise Sequenase can generate chain terminated fragments more than 500bp long. These fragments are too large to separate by conventional polyacrylamide gel electrophoresis. A 40cm long polyacrylamide gel normally offers sufficient resolution to read about 200 nucleotides of sequence. By running one set of reactions for 2 or 3 different periods of time it is possible to successfully resolve bands covering 300-400 nucleotides of sequence.

Sequencing of cloned DNA normally begins with primers that are complementary to sequences present in the MCS of the vector. Such primers are commercially available. When sequencing ds DNA, the quality of the template is critical. DNA mini preps are always contaminated with RNA and small DNA fragments which serve as random primers, resulting in the appearance of "ghost" bands and artificial stops on the sequencing gel. Hence, novel DNA sequences were determined using DNA prepared using high quality DNA preparation kit (QIAGEN, section 2.1.3)

Sequencing gels were formed by casting a thin layer of polyacrylamide containing 7M Urea between two glass plates measuring ~40 cm square, separated by plastic spacers 0.4mm thick. The protocol is presented below:

1. Wash the glass plates in warm detergent, then swab with ethanol. Treat the inner surface of the larger of the two plates with a siliconising fluid (Repelcote- BDH Products) and air dry.
2. Lay one of the plates flat on the bench and arrange the two spacers along the sides. Rest the second plate on top of the spacers and clamp one side of the assembly with clips.
3. Add 50µl of TEMED and 300µl of 10% APS to 100 ml of 6% acrylamide/urea solution and mix. Raise one end of the gel and pour in the acrylamide solution. Take care to exclude air bubbles. When full, lay the mould flat on the bench. Insert the side of a comb to form a straight edge about 5 mm below the top of the mould. Allow the gel to polymerise (approx. 45 min.).
4. Assemble the gel in a vertical electrophoresis apparatus. Fill the reservoirs with 1 x TBE. Invert the comb to form the sample wells, then rinse the wells with buffer using a hypodermic syringe.

5. Connect a power supply so that the cathode is in the top reservoir. Pre-run the gel at 75 W until it reaches a temperature of ~50°C.

6. Denature the samples by heating to 85°C for 2 - 5 min, then load 3- 4µl of each into the sample wells. Run at 75W until the bromophenol blue in the sample buffer elutes from the bottom of the gel. If required, load a second set of the same reactions in four adjacent lanes. Run at 75 W again.

7. Dismantle the apparatus. Carefully take apart the glass plates. The gel remains attached to the plate that was not treated with siliconising fluid. Place a piece of Saran wrap on top of the gel and dry in an oven prewarmed at 80°C for 2 hr.

8. Autoradiography. Expose the gel to X-ray film for ~4 days.

2.2 Protein Methods

2.2.1 Determination of Protein Concentration

The concentrations of protein solutions were determined by measurement of the absorption at 280nm. Measurement of the absorption at 280nm is rapid and simple, but this method suffers from significant protein-to-protein variation, since the absorption at 280nm depends on the number of aromatic residues in the protein. Information about the pI, the aminoacid composition and the absorption at 280 nm can be obtained from Expasy-ProtParam Tool (www.expasy.ch). The absorption at 280 nm given by this program represents protein concentration of 1mg/ml. Using the required information about the protein, determination of the protein concentration by measuring the absorption at 280nm can be very accurate.

2.2.2 SDS-Polyacrylamide Gel Electrophoresis of Proteins

Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone - and SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length–i.e., the denatured polypeptides become "rods" of negative charge cloud with equal charge or charge densities per unit length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with 2- mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

There are two types of buffer systems in electrophoresis, continuous and discontinuous. A continuous system has only a single separating gel and uses the same buffer in the tanks and the gel. In a discontinuous system, a non-restrictive large pore gel, called a

stacking gel (4% polyacrylamide, pH 6.8), is layered on top of a separating gel called a resolving gel (7.5 - 15% polyacrylamide, pH 8.8). Each gel is made with a different buffer, and the tank buffers are different from the gel buffers. The resolution obtained in a discontinuous system is much greater than that obtained with a continuous system.

Electrophoretic mobility decreases with increasing polyacrylamide concentration, so high concentrations (12.5 or 15%) are appropriate for small polypeptides (12- 50 kDa) and lower concentrations (7.5 or 10%) are used for larger molecules (20 - 100 kDa).
The protocol presented below is adapted from Sambrook *et al.,* and from an equipment supplier's manual (Biorad).

1. Wash glass plates, plastic spacers and sample combs in dH_20 and air dry. Assemble in the gel casting chamber of a proprietary vertical electrophoresis apparatus.
2. Prepare resolving gel (section 2.4) and pour into the gap between the glass plates. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1cm). Carefully overlay the acrylamide solution with 0.1% SDS (for gels containing <8% acrylamide) or isobutanol (for gels containing>10% acrylamide).
3. After polymerisation is complete (30 minutes), pour off the overlay and wash the top of the gel several times with deionised water to remove any unpolymerised acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.
4. Pour the stacking gel solution (section 2.4) onto the surface of the polymerised resolving gel. Immediately insert a clean comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely.
5. While the stacking gel is polymerising prepare the samples by heating them to 100°C for 3 minutes in 4 x protein loading buffer (section 2.4.1) to denature the proteins.
6. After polymerisation is complete (30 minutes), remove the comb carefully. Using a squirt bottle, wash the wells with deionised water to remove any unpolymerised acrylamide. Place the gel in the electrophoresis apparatus. Fill the reservoir with Tris-glycine electrophoresis buffer.
7. Load the samples using a micropipette. Include a commercially prepared mixture of marker proteins (Biorad prestained SDS-PAGE standards, Broad range).
8. Attach a power supply so that the anode is at the bottom of the gel. Run the gel at 150 V for 45 min to 1 hr, or until the bromophenol blue in the sample buffer just elutes from the bottom of the gel. Dismantle the apparatus and Coomassie stain the gel as described below, or prepare a Western blot.

2.2.2.1 Coomassie staining

1. Dissolve 0.25 g of Coomassie Brilliant Blue r-250 in 100 ml of 10% acetic acid, 40% methanol and filter.
2. Immerse the gel in several volumes of staining solution and shake gently for 30-40 min at room temperature.
3. Decant the stain and save it for future use. Destain the gel by shaking in a solution containing 5% acetic acid and 10% methanol, for 5 - 20 hr. Change the destain 2 -3 times during this period.
4. Wash the gel in water and vacuum dry at 80°C for 2hrs.

2.2.2.2 Western Blotting

Freshly-electrophoresed SDS-polyacrylamide gels are dipped into transfer buffer (0.025M Tris-HCl, 20% (v/v) methanol, pH 8.3), then laid flat on pre- wetted nitrocellulose paper supported on three layers of transfer buffer- wetted filter paper resting on the anode (+ve electrode). The gel is overlaid with three wetted filter papers, and then either the cathode (-ve electrode) or another layer of nitrocellulose / gel / blotting paper. Care should be taken to exclude bubbles between gel and nitrocellulose, and between nitrocellulose and paper.

The assembly (apparatus purchased from BIORAD) is placed in a plastic tray resting on the anode. The anode (electrode on the nitrocellulose paper side of the assembly) is connected to the anode - and the gel side cathode to the cathode - of an appropriate powerpack. A current of 500mA is passed for 20-30 min to effect transfer.

Transfer is found to be essentially quantitative - for thin assemblies - after electrophoresis under these conditions. One hour should be sufficient for transfer of all but the most recalcitrant proteins. If in doubt, blot gels in duplicate, remove one and stain for protein after 1 hr and continue blotting other.

Disassemble assembly and rinse membrane in saline or other buffer before further treatment.

Indirect enzyme immunoassay

Blocking: nitrocellulose blots are briefly rinsed in transfer buffer, then soaked for 1 hr at 37°C, or 2 hr at room temperature in blocking buffer (PBS / 5% skimmed milk powder). This procedure allows saturation of all non-specific protein binding sites on the blots.

Binding of specific antibodies to proteins: this is achieved by incubation of blots in antisera (primary antibody) diluted 1/10- 1/1000 in incubation buffer, in sealed boxes for 1 hr on a shaking waterbath at room temperature (22°C).

Binding of secondary antibodies: this is achieved by incubation of blots in IgG raised against the animal used for the production of the primary antibody i.e., rat anti rabbit

IgG to detect anti C1q IgG raised in rabbit. Usually these are commercially available and the dilution is indicated in the manufacturer's instructions. These secondary antibodies are usually conjugated to a substrate (i.e., HRP).

Washing: Blots are washed by shaking in 100 ml/wash (PBS-0.1% tween 20), 3x10 min, at room temperature. Increase number and duration of washes if background is a problem.

Detection of antibody binding

Drain the filter and place it on a sheet of Saran Wrap, so that the face carrying the proteins is uppermost. Mix ECL detection reagents (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Allow 0.125ml/cm^2 membrane. Apply the reagents to the face of the membrane and incubate for 1 min at room temperature. Drain off excess reagent and wrap the membranes in Saran Wrap. Place protein side up in an autoradiography cassette. Expose to X-ray film for 15sec, aligning the top of the film with the top edge of the membrane, then develop the film immediately. Finally align the developed film with the membrane and mark the positions of the prestained molecular weight markers.

2.2.3 Protein Expression and Purification

2.2.3.1 The pET and pRSET Expression Systems

The pET and pRSET systems have been developed for the cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in pET and pRSET plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that almost all of the cell's resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein a few hours after induction. These systems have the ability to maintain target genes transcriptionally silent in the uninduced state. Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, thus eliminating plasmid instability due to the production of proteins potentially toxic to the host cell. Once established in a non-expression host, plasmids are then transferred into expression hosts containing a chromosomal copy of the T7 RNA polymerase gene under lacUV5 control, and expression is induced by the addition of IPTG.

pET and pRSET vectors also contain different sequences adjacent to the cloning sites that encode a number of peptide "tags", which perform various functions when fused with the target protein. Some of the fusion tags facilitate detection and purification of the target protein, whereas others increase the probability of biological activity by affecting solubility in

the cytoplasm or export to the periplasm. In this work, pET-15b and pRSET-C were used; both contain a His·Tag that facilitates the detection and purification of the target protein.

Preparative scale protein expression was carried out according the following protocol:

1. Pick a single colony of *E. coli* BL21(DE3)pLysS from a freshly streaked plate and inoculate 50 ml LB containing 34μg/ml chloramphenicol in a 250ml Erlenmeyer flask. Incubate with shaking at 37°C until the OD 600 reaches 0.6–1.0.
2. Collect the cells by centrifugation. Resuspend the cells in 10ml fresh medium plus antibiotic and use this to inoculate 500ml medium.
3. Incubate with shaking at 37°C until OD_{600} reaches 0.4–1 (0.6 recommended; about 3 h).
4. Remove 1ml sample for the uninduced control. To the remainder, add IPTG from a 100 mM stock to a final concentration of 1 mM (T7lac promoter) and continue the incubation for 2–3 h.
5. Place the flasks on ice for 5 min and then harvest the cells by centrifugation at 5000 x g for 5 min at 4°C. Save the supernatant for further analysis.
6. Resuspend the cells in 0.25 culture volume of cold 20 mM Tris-HCl pH 8.0, and centrifuge as above.
7. Remove the supernatant and completely resuspend the pellet in ~50 ml of cold 20 mM Tris-HCl, 150 mM NaCl, pH 8.0 to yield a concentration factor of 10X (500 ml culture to 50 ml buffer volume).
8. Completely lyse the cells by one of the following methods:

a) French Press. Perform two passes at 20,000 psi using a chilled pressure cell.

b) Lysozyme treatment plus sonication. Add lysozyme to a final concentration of 100 μg/ml from a freshly prepared 10mg/ml stock in water. Incubate at 30°C for 15 min. Mix by swirling and sonicate on ice using a microtip with the power level set between 4–5, at 40-50% duty for 15-20 bursts. It is important to keep the sample cold during sonication to avoid heat denaturation of proteins.

9. Take a 1.5ml sample of the lysate and centrifuge at 14,000 x g for 10 min to separate the soluble and insoluble fractions. Transfer 100μl of the soluble supernatant to a new tube. Remove and save the remaining supernatant for protein purification.
10. Add 4X protein loading buffer to the 100μl soluble fraction sample. Heat for 3 min at 70°C to denature proteins and then store at –20°C until SDS-PAGE analysis.

2.2.3.2 His-tag Protein Purification

Proteins were purified according to the following protocol:

1. Use a 1ml Ni column (HiTrap from Pharmacia) equilibrated with sonication buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM imidazole, pH 8.0).
2. Pass the lysate from step 9 (section 2.2.3.1) through the column. Save the flow-through. Wash the columns with approximately 30 ml of sonication buffer at pH 8.
3. Begin elution by increasing elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.5 M Imidazole, pH 8.0) proportion with gradual increase or isocratic step (step volume usually 10 ml). A gradient is better for a first run. Contaminants will elute first as imidazole concentration increases. Elute the protein by a 100% elution buffer.
5. Analyse fractions by a SDS-PAGE and Coomassie staining.

2.2.3.3 Purification using Ion Exchange Chromatography

Ion exchange resins contain charged groups. These may be acidic in nature (in which case the resin is a cation exchanger) or basic (in which case it is an anion exchanger).
Cation and anion exchangers may be broken down further into weak and strong exchangers (reflecting binding affinity). Usually, samples are loaded under low ionic strength conditions and bound material is eluted using either a step or gradient elution of buffer with higher ionic strength. Generally speaking, a protein will bind to a cation exchange resin if the buffer pH is lower than the isoelectric point (pI) of the protein, and will bind to an anion exchange resin if the pH is higher than the pI.

Elution of proteins from ion exchange resins:

In this work, the UNO Q Biochromatography column from BIORAD was used to purify the human calreticulin S-domain and the rat gC1qBP mature protein.
Proteins bound to ion exchange resins are bound via non-covalent ionic (salt-bridge) interactions. We can compete for these ionic binding sites on the resin with other ionic groups, namely, salts. There are two general types of methods when eluting with a salt solution: Gradient elution and Step elution. A gradient elution refers to a smooth transition of salt concentration (from low to high) in the elution buffer. Low elution buffer used for the elution of proteins: 20 mM Tris-Cl, 150 mM NaCl, and pH 8.0. High elution buffer: 20 mM Tris-Cl, 0.5 mM NaCl, pH 8.0.

Weakly binding proteins elute first, and stronger binding proteins elute last (i.e. they require higher salt concentrations in the buffer to compete them off the column). A gradient salt concentration can be made using a gradient maker. In its simplest form, this consists of two containers connected by a siphon (or tube at the bottom). One container contains the low salt buffer, and the other contains high salt buffer. The buffer is withdrawn from the low salt

container. This will produce a linear gradient from low to high salt concentrations over the total volume of the gradient (usually 20 ml).

If we know the concentration range of salt over which a protein of interest will elute we can simply elute with a buffer containing that concentration of salt. This is known as a step elution. Step elutions are generally faster to run, and elute the protein in a smaller overall volume than with gradient elutions. They generally work best when contaminants elute at a significantly different salt concentration than the protein of interest.

The above purification methods were programmed in the BioLogic HR Chromatography System from BIO-RAD.

Finally the fractions collected were analysed by SDS-PAGE and visualized by Coomassie staining, as described in sections 2.2.2 and 2.2.2.1

Thrombin digestion

Thrombin specifically cleaves target proteins containing the recognition sequence LeuValProArgGlySer and to release the fusion partner from the desired protein. Thrombin was purchased from Novagen (Cat. No. 69671), supplied with 10X thrombin cleavage buffer (20 mM Tris-HCl pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) and a cleavage control protein.

It is necessary to dialyse the fusion protein against thrombin buffer before digesting it with thrombin. Thrombin is inhibited by high ionic strength. 250 mM NaCl reduces thrombin activity to 75% of normal and 2 M NaCl almost completely inhibits enzyme activity. Also, Thrombin is known to be inhibited by > 2 M urea, > 20 mM β-mercaptoethanol (β-ME), >0.1% SDS, > 50 mM imidazole, and pH values below 6 and above 9.

Unit definition: One unit is defined as the amount of enzyme needed to cleave 1μg of fusion protein in 16 hours at 20°C in a 200 μl reaction containing 20 mM Tris-HCl pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$.

2.2.4 C4 purification from human plasma (Dodds, A.W., 1993).

The procedure described is for the purification of C4 from 10ml of human EDTA plasma, which should be fresh or stored at –70°C. However, it is suitable for volumes of plasma from 0.5 to 50 ml, with slight adjustment of the size of column and volume of the gradient. The following buffers are used and are mixed to give buffers of the required ionic strength.

Buffer A: 20 mM Tris-HCl, 50 mM e-aminocaproic acid, 5 mM EDTA, 0.02 % NaN_3, pH 7.5.

Buffer B: Buffer A with 1 M NaCl.

Buffer A/B: 75% buffer A and 25% buffer B. Just before use add 500μM serine protease inhibitor Pefabloc SC (Roche, Cat. No. 1 873 601).

Preparation of plasma and purification of C4

Take 30ml of fresh blood and centrifuge at 3000 x g for 10 min. Transfer the supernatant (10ml) into a clean tube and add 200μl of 0.1 M Pefabloc, 110μl 0.4 M EDTA pH 8.0 and 4.1 ml buffer A/B. Centrifuge at 10,000 g for 10 min and transfer the supernatant into a clean tube.

Load the supernatant at a flow rate of 1ml/min, onto a column (5ml, HiTrap, Q-Sepharose, Pharmacia), equilibrated with 30ml 95% buffer A, 5% buffer B (i.e., 50 mM NaCl). Wash the column with 25ml buffer A/B (i.e., 250mM NaCl) and elute with a 25ml linear gradient (starting with 100% buffer A/B and 0% buffer B to 100% buffer B). Collect 1ml fractions.

The column can be run at 4°C or at RT but in the later case fractions should be collected into tubes on ice. Analyse the fractions on SDS-PAGE to find those containing C4 and determine concentration by measuring the absorbance at 280nm. Prepare aliquots and store in -70°C until use.

2.3 Immunoassays

2.3.1 Separation of serum from blood samples and storage

Some complement components are extremely labile and blood samples should be processed as soon as possible after venepuncture. Serum should then be prepared as described in the following section:

Transfer the blood to a vessel and clot by incubating for 30-45 minutes at RT. Separate the serum from the clot by centrifuging at 2000 x g for 5 min at 2-4°C. Aspirate the serum and aliquot into suitable storage tubes. Store at –70°C.

2.3.2 C1q haemolytic complement assay

2.3.2.1 Preparation of erythrocytes (Antibody-sensitised sheep erythrocytes)

Sheep erythrocytes (E) sensitized with IgG or IgM antibody (A) will initiate the classical pathway of complement if Ca^{++} and Mg^{++} ions are present. Prepare antibody-sensitised sheep erythrocytes as described in the following protocol:

1.Centrifuge sufficient sheep blood at 2000 g for 10 min at 2°C and discard the plasma. Wash the red cells three times in ten times the cell volume with GVB/10 mM EDTA (section 2.4.1) and resuspend to 5% (v/v) in the same buffer.

2.Lyse a 0.1 ml aliquot with 2.9 ml water and measure the absorbance at 541 nm; calculate the concentration of cells as shown in table 2.1 and adjust the concentration to 1 x 10^9/ml in GVB/10 mM EDTA.

Table 2.1 Absorbance values and wavelengths used to calculate sheep erythrocyte concentrations

Sheep E concentration	Absorbance	Wavelength
1 x 10^9/ml	0.370	541
5 x 10^8/ml	0.185	541
2 x 10^8/ml	0.654	414
1 x 10^8/ml	0.327	414

The absorbance is measured on a lysate prepared by the dilution of 100µl of cell suspension in 2.9ml of water.

3. Titrate the anti-sheep E antibody (rabbit haemolytic serum- provided by Dr. R.B. Sim, University of Oxford) by serial double dilution in GVB/10 mM EDTA in a microtitre plate and mixing 50µl of diluted antibody with an equal volume of 1 x 10^9/ml E to find the minimum dilution which does not cause agglutination. This is the working dilution of the antibody.

4. Pre-warm equal volumes of the working dilution of antibody and sheep E (1 x 10^9/ml) in GVB/10 mM EDTA to 37°C. Whilst keeping the reagents at 37°C and continuously shaking mix antibody and cells and incubate for 30 min at 37°C.

5. Centrifuge the sensitised cells at 2000 g for 5 min at 2°C, wash once in GVB/10 mM EDTA, twice in GVB^{++} (section 2.4.1), and resuspend to the original volume in GVB^{++}. Sensitised cells can be stored at 4°C for up to one week.

2.3.2.2 Preparation of haemolytic intermediates (EAC1q)

Pre-coating of sensitised sheep erythrocytes (EA) with complement components from early in the activation pathways allows activation and/or binding of the component under test. Lysis is achieved by adding the remaining components, which will only bind if the assayed component is present. As all the components with the exception of the test component are present in excess, the degree of lysis is proportional to the concentration of the test component.

For the preparation of EAC1q cells, mix 2 ml of EA cells at 10^9/ml in $DGVB^{++}$ with 1ml of fresh human serum diluted in 20 ml PBS-5mM EDTA. Incubate on ice for 30 min, centrifuge, and wash twice in $DGVB^{++}$ and finally resuspend in 18 ml of $DGVB^{++}$.

2.3.2.3 Testing calreticulin S-domain for complement inhibition

On a 96-well plate mix 100µl of EAC1q cells at 10^8/ml in $DGVB^{++}$ with 100µl of 1/40 dilution of C1q-deficient serum in $DGVB^{++}$ (provided by Dr. R.B.Sim, University of Oxford). Add 50µl of purified calreticulin S-domain (see section 3.1) dilutions in $DGVB^{++}$ (start from 1mg/ml and dilute down to 0.05µg/ml). Incubate the plate for 60 min at 37°C. Centrifuge the plate for 10 min at 1200rpm, transfer the supernatant into a clean 96-well plate and read the absorbance at 405 nm.

2.3.3 Antibody Capture Assays and Competition ELISA

Antibody capture assays can be used to detect and quantitate antigens or antibodies and can be used to compare the epitopes recognized by the different antibodies. The general protocol is the following: an unlabelled antigen is immobilized on a solid phase, and the antibody is allowed to bind to the immobilized antigen. The antibody can be labelled directly or can be detected using a labelled secondary reagent that will specifically recognise the antibody. The amount of antibody that is bound determines the strength of the signal. This assay is most useful when both a specific antibody and milligram quantities of purified or semi-purified antigen are available.

In a competition ELISA, different concentrations of the antigen are peincubated with the antibody. This is then added to the plate coated with the antigen (immobilized antigen). Antibody binding is detected using a secondary labelled antibody and from the readings a standard curve is produced. The amount of the antibody that is bound determines the degree of inhibition. Antigen concentrations should span the dynamic range of inhibition, i.e., the range of inhibitor concentrations that produces detectable changes in the amount of inhibition. This must be determined empirically in an initial assay in which antigen concentration is varied from 10^{-6}M to 10^{-12}M. If possible, initial concentration should be ~100µg/ml, followed by nine 1:4 serial dilutions in blocking buffer. These are assayed for their ability to inhibit binding of conjugate to antigen-coated plates. From this initial assay, fifteen 1:2 antigen dilutions spanning the central segment of the dynamic range of inhibition are used as standard antigen-inhibitor dilutions. Inhibitor curves are most sensitive in the region of the curve where small changes in inhibitor concentrations produce maximal changes in the amount of inhibition (normally 15% to 85% inhibition). For the gC1qBP and CRT standard curves start

with 50µl of 20µg/ml of protein (recombinant gC1qBP or native human CRT) in blocking buffer (PBS-tw) and do serial 2-fold dilutions (20µg/ml , 10µg/ml , 5µg/ml, 2.5µg/ml , 1.25µg/ml , 625ng/ml, 312ng/ml , 156ng/ml, 78ng/ml , 39ng/ml , 20ng/ml , 10ng/ml , 5ng/ml , 2.5ng/ml and 1.25ng/ml).

NB: The amount of antigen that is bound per well is 0.1µg maximum. Need to test the antigen in solution as a competitor in the range of 0-1µg/well.

2.3.3.1 ELISA procedure for measuring antibody titre in antisera

In this type of ELISA (Enzyme-Linked Immunosorbent Assay), the antigen (peptide or protein) is bound to the microtiter plate first. The antiserum containing the anti-peptide antibody is then added to the well and allowed to bind. Finally, a second antibody, specific for the first antibody and labelled for detection, is added to the well and allowed to bind. The second antibody usually has an enzyme conjugated to it. This enzyme catalyses the formation of colored substance, e.g., *p*-nitrophenol, from a colourless substrate, *p*-nitrophenylphosphate. This colored substance is then quantified and the amount of antibody present can be calculated.

The protocol is described below:

1. Using a multichannel pipette, dispense 100µl antigen (1µg/ml in 0.1M Na_2CO_3, pH9) solution into each well of a microtitre plate (leave wells 11 and 12 empty) and tap or shake to evenly distribute antigen. Wrap coated plates in plastic wrap and incubate for 2 hr at room temperature.
2. Aspirate out the antigen and wash all the wells twice with 200µl PBS containing 0.1% tween-20. Fill all the wells with PBS-0.1% tween20 (PBS-tw) and leave for 1 hr at room temperature.
3. Add 100µl antibody samples [rabbit anti-calreticulin polyclonal (Stressgen, Cat. No. SPA-600), or rabbit antiserum against gC1qBP] diluted in PBS-tw to each coated well, and incubate the plate for 1 hr at room temperature.
4. Wash three times with 200µl of PBS-tw. After last wash, remove residual liquid by wrapping the plate in a large paper tissue and gently flicking it face down onto several paper towels.
5. Add 100µl (dilution 1:10000 in PBS-tw) of the anti rabbit IgG alkaline phosphatase conjugate (Sigma Immunochemicals) and incubate at room temperature for 1 hr.
6. Wash as in step 4
7. Add 100µl of pNPP (SIGMA FAST pNPP Substrate Tablet Set) substrate solution into each well and incubate at room temperature for 20 minutes. Monitor hydrolysis qualitatively

by visual inspection or quantitatively using a microtiter plate reader. Hydrolysis of NPP appears yellow. For spectophotometer measurement of NPP hydrolysis, use a 405-nm filter.

8. To calculate the antibody titer of the sera:

a. Plot absorbance vs. antiserum dilution using the mean and range or standard deviation for each duplicate or triplicate set.

b. Estimate the inflection point of the post-immune graph. This is a point before saturation occurs.

c. Interpolate the titer by drawing a line down to the x-axis.

2.3.3.2 Competition ELISA to detect soluble antigens (calreticulin and gC1qBP) in biological fluid samples.

For measuring the protein levels in serum/synovial fluid samples, do serial 2-fold dilutions of serum/ synovial fluid starting with 1:2 dilution in blocking buffer (1:2, 1:4, 1:8, 1:16).

Add 50µl of 1/150 dilution of the gC1qBP antiserum (mix A, B, D, see figure 4.4) to the rec gC1qBP (and serum) dilutions and/or 50µl of 1/800 dilution of the rabbit anti-calreticulin polyclonal antibody to the CRT (and serum) dilutions. Incubate the 96 well-plate at RT for 1 hr. Controls were wells containing buffer instead of gC1qBP or CRT i.e., 50µl of 1/150 dilution of the rabbit anti-gC1qBP polyclonal antibody and 50µl PBS-tw.

Transfer the above dilutions onto a gC1qBP and/or CRT coated plate and incubate at RT for 1 hr. Rinse three times with 200µl of PBS-tw. After last rinse, remove residual liquid and add 100µl (dilution 1:10000 in PBS-tw) of the anti rabbit IgG alkaline phosphatase conjugate (Sigma Immunochemicals) and incubate at room temperature for 1 hr.

Wash three times in PBS-tw and after last rinse add 100µl of pNPP (SIGMA FAST pNPP Substrate Tablet Set) substrate solution into each well and incubate at room temperature for 20 minutes. Monitor hydrolysis on a spectrophotometer using a 405-nm filter.

2.3.4 Detection of MASP-2 mediated C4 cleavage.

The following method describes a microtiter plate based assay that determines the amount of C4b deposited onto an activator of the MBL pathway without any interference from the classical pathway (Petersen *et al.*, 2000):

1. Coat microtiter wells (Maxisorb, Nunc, cat. no 442404, Fisher Scientific) with 10µg/ml mannan* (M7504 Sigma) in 100µl of 15mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6 (coating buffer). After incubation overnight at room temperature, block residual protein binding sites

by addition of 200µl 0.1% (w/v) HSA in 10mM Tris-HCL, 140mM NaCl, 1.5 mM NaN3, pH 7.4 (TBS) for 1 hour.

2. After wash in TBS with 0.05% Tween 20 and 5 mM $CaCl_2$ (TBS/tw/Ca^{++}) add dilutions of serum (starting from 1:20 and diluted 2-fold) made in 20 mM Tris-HCL, 10 mM $CaCl_2$, 1M NaCl, 0.05% (v/v) Triton X-100, 0.1% (w/v) HSA, pH 7.4 (MBL binding buffer). Wells receiving only buffer are used as negative controls. All dilutions should be added in duplicate.

3. Following incubation overnight at 4°C and wash in TBS/tw/Ca^{++}, add 100µl/well of 1µg/ml human C4 (prepared as described in section 2.2.4) in BBS (4mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1mM $MgCl_2$, pH 7.4, autoclaved). The wells are then incubated for 90 minutes at 37°C to allow deposition of C4b onto the surfaces.

4. Following another wash in TBS/tw/Ca^{++} deposited C4b is detected by adding 100µl of 1:1000 dilution in TBS/tw/Ca^{++} of chicken anti human C4c-AP (Immunsystem AB, Cat. 05-032). After incubation for 90 minutes at room temperature, the wells are washed in TBS/tw/Ca^{++}.

5. The presence of AP is determined by addition of 100µl substrate solution (Sigma Fast p-Nitrophenyl Phosphate tablet sets, Sigma) and incubation at room temperature for 20 minutes. Read the OD at 405nm using a microtiter plate reader.

*Different polyssacharides or proteins can be used in place of mannan depending on the experiment. These include (for this work), Pneumovax II™, calreticulin, soluble CR1, or intact cells e.g. *Acanthamoeba*. Concentration used for coating with the above proteins: 10µg/ml. Cells were coated at a concentration of 10^5/ml.

2.3.5 ELISA to detect C3b deposition (C3 cleavage assay)

Repeat steps 1-3 as described in section 2.3.4. Then continue as follows:

After C4b is deposited on the wells, wash three times with TBS/tw/Ca^{++}, add dilutions of serum made in BBS buffer and incubate for 3 hours at 37°C to allow C4b deposited on the wells to attach to the C2 contained in serum and form the C3 convertase C4b2b, which cleaves the C3 present in serum. Wash wells three times with TBS/tw/ Ca^{++}, add 100µl of peroxidase-conjugated goat anti human C3 (ICN Biochemicals, Cat. No. 55237) (1/2000 dilution in BBS) and incubate for 90 minutes at 37°C.

Following another three washes in TBS/tw/Ca^{++} add 100µl/well of TMB peroxidase EIA substrate (90% solution A and 10% solution B [Biorad]) and incubate at 37°C for 20-30

minutes. The reaction is stopped by adding 50µl of 1 M H_2SO_4 into each well and the absorbance is read at 450 nm.

2.3.6 ELISA to measure MBL levels in serum

This ELISA assay was developed to measure the levels of MBL in serum samples. The protocol is the following:

1. On a mannan coated microtiter plate (coated as described in section 2.3.4) add dilutions of serum with known MBL levels (control) and dilutions of the serum to be tested. Dilutions are made in MBL-binding buffer (see section 2.3.4).
2. Incubate the plate overnight at 4°C to allow binding of MBL to the substrate.
3. Wash three times in 200µl TBS/tw/Ca^{++} and add the monoclonal anti-MBL antibody (Hyb 131-01, Statens Serum Institut) at a concentration of 0.2-0.5µg/ml in TBS/tw/Ca^{++}. Incubate for 90 min at room temperature with gentle agitation.
4. Wash three times in 200µl TBS/tw/Ca^{++} and add goat anti-mouse IgG (whole molecule) conjugated to alkaline phosphatase (Sigma Immunochemicals) at 1:10,000 dilution in TBS/tw/Ca^{++}. Incubate for 60 min at room temperature with gentle agitation.
5. Following another three washes in TBS/tw/Ca^{++}, determine the presence of AP as described in section 2.3.4 step 5.
6. Make a standard curve using the control serum and from this calculate the MBL levels in the test serum.

2.3.7 Depletion of serum by immunoprecipitation

This procedure was used to produce C1q-depleted serum.

Dynabeads® Protein A

Dynabeads® Protein A (DYNAL Biotech. Cat. No. 100.01) are uniform, magnetizable polystyrene beads coated with recombinant Protein A. The 32,000 molecular weight protein is covalently coupled to the surface of 2.8µm Dynabeads. Protein A has a high specificity for immunoglobulins and is therefore suitable for the one-step capture of antibodies. The native bacterial cell wall protein is a single polypeptide chain of MW 42,000 with four Ig Fc binding sites, two of which are active. The protein A employed in this product is a 32,000 MW recombinant protein containing all four binding sites for the Fc region of immunoglobulins.

The beads can be coated with purified antibodies or IgG isolated directly from acites, serum, tissue culture supernatants or other samples. Dynabeads Protein A are added to the

immunoglobulin-containing sample. During a short incubation time, the immunoglobulins will bind to Dynabeads Protein A through the Fc region. The test tube is placed in a Dynal Magnetic Particle Concentrator (Dynal MPC) to collect the Dynabeads at the tube wall, and the supernatant is discarded. The beads are now coated with IgG and can be used to deplete serum from the relevant protein.

Dynabeads washing procedure

The amount of beads needed is 100µl Dynabeads suspension to an excess of 100µg antibody in sample (to which an estimated 25µg human IgG will bind). The washing procedure that will be described is facilitated by the use of a magnet (Dynal MPC).

1. Resuspend the Dynabeads® Protein A, thoroughly in the vial (e.g. by vortexing 1-2 minutes).
2. Transfer 100µl of the bead suspension to a polypropylene or polystyrene test tube.
3. Place the test tube in the Dynal MPC for one minute and pipette off the fluid.
4. Remove the test tube from the MPC and add 0.5ml 0.1 M Na-phosphate buffer pH 8.1
5. Repeat steps 3, 4 and 3 to complete washing procedure.

Dynabeads IgG capture procedure.

Dynabeads are resuspended in an adjusted volume so that sample and Dynabeads volumes together is the same as the bead-volume originally pipetted from the vial. The procedure is the following:

1. Resuspend the Dynabeads washed in the above section, in 87µl 0.1 M Na-phosphate buffer.
2. Add 13 µl (100µg) of rabbit anti-C1q polyclonal antibody (DAKO, Cat. No. A0136) to the washed Dynabeads
3. Incubate with slow tilt rotation mixing for 60 min.
4. Place the test tube in the Dynal MPC for 2 minutes and pipette off the fluid.
5. Remove the test tube from the Dynal MPC and add 0.5ml 0.1 M Na-phosphate buffer pH 8.1. (For downstream immunoprecipitation or storage of Dynabeads, 0.1% BSA can be added to the buffer for protein stability.)
6. Repeat steps 4, 5, 4, 5, 4.

The Ab-coated Dynabeads will be used for depletion of antigen from serum as will be described in the following section.

Depletion of antigen from serum using antibody coated Dynabeads

100µl of Dynabeads are coated with 25µg of IgG (as described in the above section), and to deplete serum of an antigen the concentration of the antigen should be less than 25µg/100µl serum. For the depletion the following protocol was used:

1. Resuspend the IgG coated Dynabeads in 90µl 0.1 M Na-phosphate buffer.
2. Add 50-100µl of serum to the washed IgG coated Dynabeads
3. Incubate with slow tilt rotation mixing for 60 min.
4. Place the test tube in the Dynal MPC for 2 minutes and transfer the depleted serum into a clean tube.
5. Prepare a Western blot with depleted serum and serum before depletion to confirm depletion.

The serum depleted of C1q was compared to C1q-depleted serum purchased from Quidel, Cat. No. A509. On western blot and ELISA assays detecting with rabbit anti-C1q polyclonal antibody, both C1q-depleted sera produced the same results.

2.4 Materials

2.4.1 Formulae (in alphabetical order)

6% Acrylamide, 7M Urea (for sequencing gels): 6% acrylamide, 7 M urea, 1 x TBE, pH 8.3. Store in a dark glass bottle at RT.

BBS:4mM barbital, 145mM NaCl, 2mM $CaCl_2$, pH 7.4, autoclave and keep at 4°C

Destaining solution (SDS-PAGE): 5% acetic acid and 10% methanol

$DGVB^{++}$: 71 mM NaCl, 2.5 mM sodium-diethylbarbiturate, 0.05% (v/v) gelatin, 3% (w/v) D-glucose, 1 mM $MgCl_2$ and 0.15 mM $CaCl_2$.

DNA Loading Buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol.

Electroblotting Buffer: 25 mM Tris-base, 192 mM glycine, 10% methanol, pH 8.3

GVB (isotonic veronal buffered saline with gelatine): melt 10 ml of 10% (w/v) gelatin by heating in boiling water and add to 200 ml of 5 X VB. Make up to 1 litre with water. Store to 2-4 °C. Store up to 48 hours.

GVB^{++}: mix 200 ml GVB with 1 ml of 30mM $CaCl_2$ and 2 ml of 100mM $MgCl_2$. Store for up to 48 hours.

LB (Luria-Bertani) Medium: Mix 10g of tryptone, 5g of yeast extract, 5g of NaCl and 1g of maltose in 1 litre of dH_2O. Autoclave and store at RT.

LB Agar: As LB Medium, plus 12g of agar per litre. Store at 4°C.

LSB: 10mM Tris, 10mM $CaCl_2$, 0.05% Triton X-100, 0.1% HSA, pH 7.4, containing increasing concentrations of NaCl: 10mM, 30mM, 50mM, 100mM, 200mM and 500mM. Store at 4°C

MBL binding buffer: 20mM Tris-HCl, 10mM $CaCl_2$, 1M NaCl, 0.05% (v/v) Triton X-100, 0.1% (w/v) HSA, pH 7.4

Mini-prep Solution I: 10 ml 40% glucose, 5 ml 1 M Tris HCl pH 8.0, 4 ml 0.5 M EDTA pH8.0, distilled water to 200 ml

Mini-prep Solution II: 20 ml 10% SDS, 8 ml 5 M NaOH, distilled water to 200 ml

Mini-prep Solution III: 23 ml glacial acetic acid, 120 ml 5M potassium acetate, distilled water to 200 ml

PBS (phosphate buffered saline): 150 mM NaCl, 140mM KCl, 10mM Na_2HPO_4, 5mM KH_2PO_4, pH 7.4. Autoclave and store at RT.

4 x Protein Loading Buffer: 0.2 M Tris-Cl, 40% Glycerol, 8% SDS, 8% beta-mercaptoethanol, 0.4% bromophenol blue. Aliquot and store at -20 °C

Resolving Gel:

10% Gel: 6.7 ml of 30% acrylamide mix, 5 ml of 1.5 M Tris (pH 8.8), 0.2 ml of 10% SDS, 0.2 ml of 10% ammonium persulfate, 0.008 ml of TEMED, distilled water up to 20 ml.

12% Gel: 8.0 ml of 30% acrylamide mix, 5.0 ml of 1.5 M Tris (pH 8.8), 0.2 ml of 10% SDS, 0.2 ml of 10% ammonium persulfate, 0.008 ml of TEMED, distilled water up to 20 ml.

SOB Medium: Mix 20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl and 10 ml of 250 mM KCl in 980 ml of distilled water. Autoclave, then add 10 ml of sterile 1 M $MgCl_2$. Store at 4 °C.

SOC Medium: SOC is identical to SOB, except that it contains 20mM glucose. Prepare immediately before used, by mixing 1 volume of filter-sterilised 2M glucose with 100 volumes of SOB.

Stacking Gel (4% acrylamide): 1.3 ml of 30% acrylamide mix, 2.5 ml of 0.5 M Tris-Cl (pH 6.8), 100 µl of 10% SDS and distiled water up to 10 ml. Immediately before use, add 10 µl of TEMED and 100 µl of 10% APS.

TAE Buffer: 40 mM Tris-acetate, 1mM EDTA, pH 8.0. Store at RT.

TBE Buffer: 0.1 M Tris-borate, 2mM EDTA, pH 8.3. Store at RT.

TBS:10mM Tris-HCl, 140mM NaCl, 1.5mM NaN_3. Store at RT.

TE: 10 mM Tris-Cl, 1 mM EDTA, pH 7.5. Store at RT.

Tfb I: 30 mM potassium acetate, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15% glycerol. Filter sterilise and store at -20°C

Tfb II: 10 mM Na-MOPS, 75 mM $CaCl_2$, 10 mM KCl, 15% glycerol. Filter-sterilise and store at -20°C.

Tris-Glycine Elctrophoresis Buffer: 25 mM Tris-base, 192 mM glycine, 0.1% SDS, pH8.3. Store at RT.

5 X VB (five-fold concentrated veronal buffered saline): dissolve 85 g NaCl and 3.75 g Na-barbitone in 1 l of water. Dissolve 5.75 g barbituric acid in 600 ml of hot water. Mix the two solutions and make up to 2 l with water, fix pH at 7.4-7.6. Store up to one month.

2.4.2 Vectors

The vectors that were used in this work were, the pET15-b (purchased from Novagen), the pRSET-C (purchased from Invitrogen), the pCR2.1 (purchased from Invitrogen) and the pGEM-T Easy (purchased from Promega). The following figures represent the vector maps where the multiple cloning sites are shown.

SPECIAL NOTE

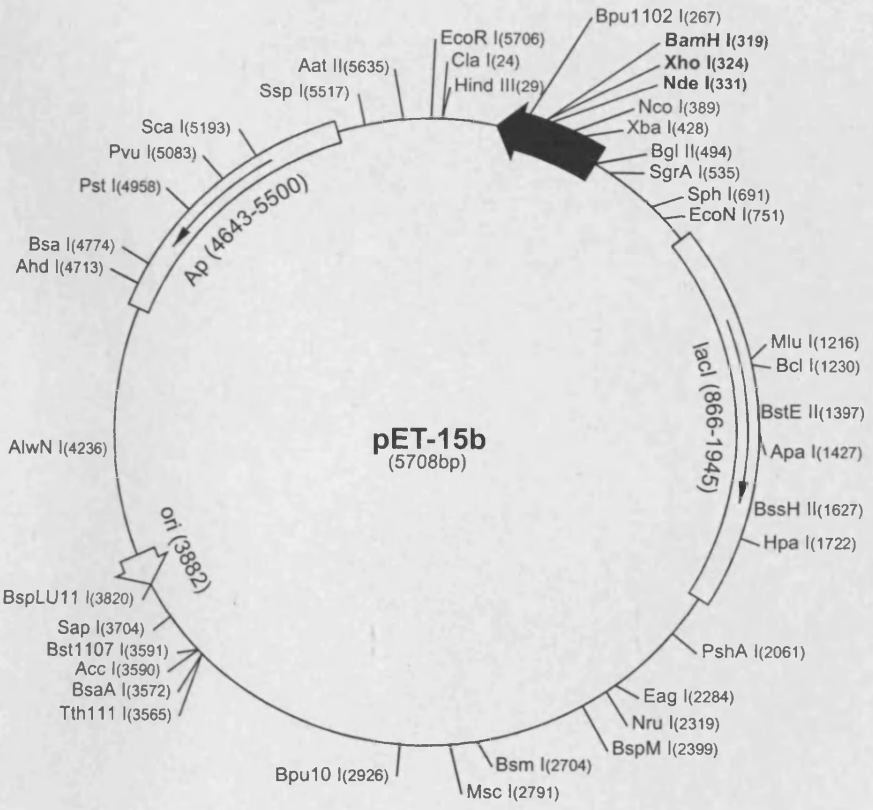
This item is tightly bound and while every effort has been made to reproduce the centres force would result in damage.

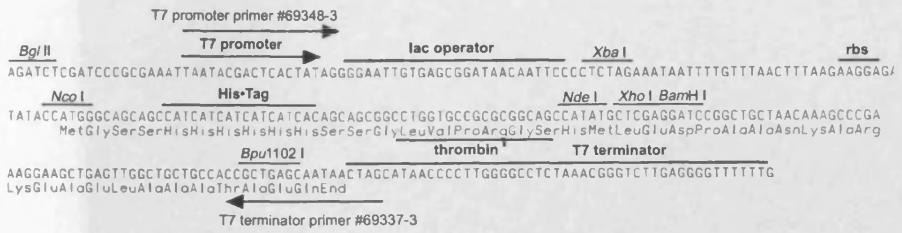
pET-15b Vector

TB045 5/99

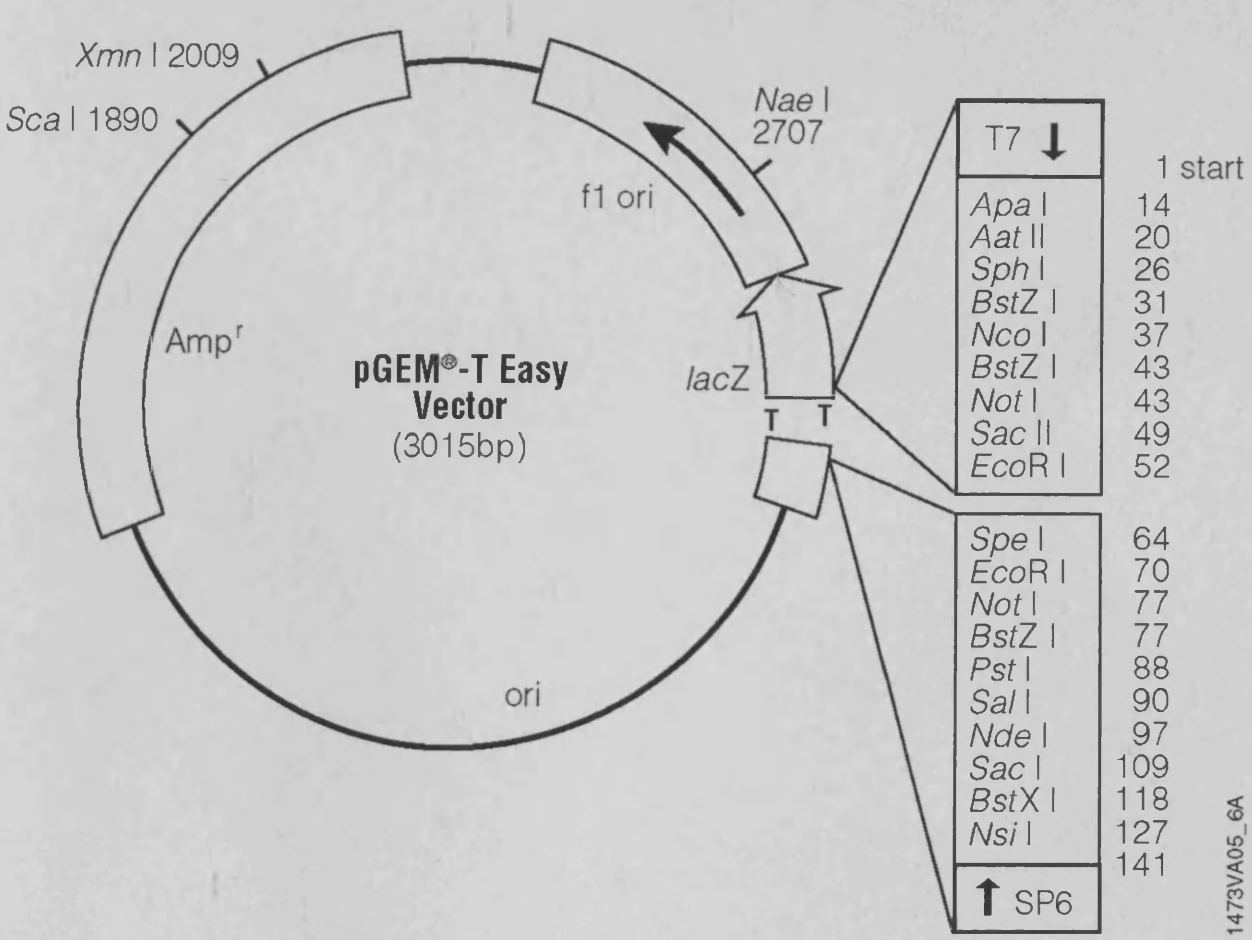
The pET-15b vector (Cat. No. 69661-3) carries an N-terminal His•Tag® sequence followed by a thrombin site and three cloning sites. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below.

pET-15b sequence landmarks	
T7 promoter	463-479
T7 transcription start	452
His•Tag coding sequence	362-380
Multiple cloning sites	
(*Nde* I - *Bam*H I)	319-335
T7 terminator	213-259
lacI coding sequence	(866-1945)
pBR322 origin	3882
bla coding sequence	4643-5500





pET-15b cloning/expression region



The pGEM®-T Easy Vector System is a convenient system for the cloning of PCR products. The multiple cloning site is flanked by restriction enzyme sites for *BstZ* I, *Not* I and *EcoR* I, allowing three options for removal of the insert with a single digest.
Rapid ligation: The 2X Rapid Ligation Buffer provided allows reactions to be completed in 1 hour at room temperature.
Blue/White Screening: T7 and SP6 RNA polymerase promoters flank a multiple cloning region within the α-peptide coding region for β-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by color screening on indicator plates.
F1 Origin of Replication: Allows the preparation of single-stranded DNA.

Comments for pCR®2.1
3929 nucleotides

LacZα gene: bases 1-545
M13 Reverse priming site: bases 205-221
Multiple Cloning Site: bases 234-355
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

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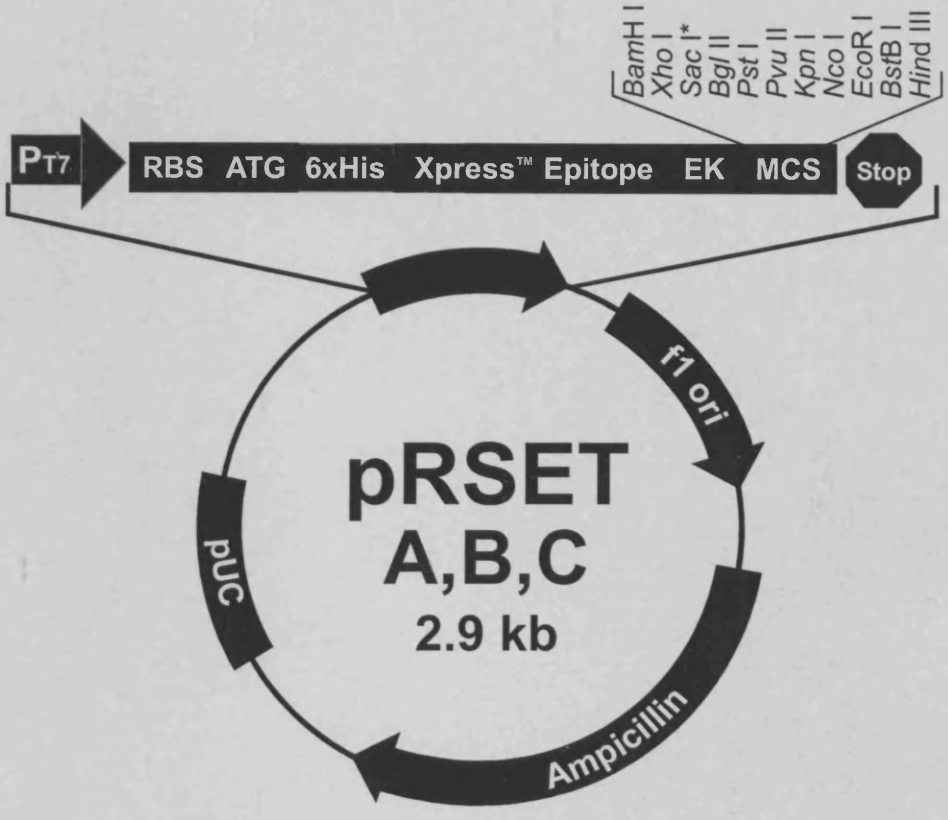

*Version C does not contain *Sac* I

Comments for pRSET A
2897 nucleotides

T7 promoter: bases 20-39
6xHis tag: bases 112-129
T7 gene 10 leader: bases 133-162
Anti-Xpress™ epitope: bases 169-192
Multiple cloning site: bases 202-248
pRSET reverse priming site: bases 295-314
T7 transcription terminator: bases 256-385
f1 origin: bases 456-911
bla promoter: bases 943-1047
Ampicillin (*bla*) resistance gene (ORF): bases 1042-1902
pUC origin: bases 916-2852 (C)

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2.5 Oligonucleotides

Primers gC1qBPsense, gC1qBPanti sense, hCALFWR and hCALREV were purchased from vhBio (vhBio Limited, Newcastle upon Tyne, UK). T7 promoter primer and T7 terminator primer were purchased from Novagen (CN BIOSCIENCES LTD. Nottingham, UK). The T7 gene 10 leader primer and the pRSET reverse primer were purchased from Invitrogen. In the following list of primers modifications used to introduce restriction sites, start codons, stop codons, are underlined.

Primer name	**Sequence (5'-3')**	**Position**	**Purpose and comments**
hCALFWR	ATAGAATTCCATATG **CGTTGCAAGGATGAT AGTTT**	484-503 in human CRT	*Nde*I modified for subcloning into pET15-b
hCALREV	TGAATTCAGATCTTTA **TGGGTTGTCGATCTGC CGGG**	938-957 in human CRT	*Bgl*II modified for subcloning into pET15-b Stop codon (TTA)
gC1qBPsense	GGATCC**ACCCCGCGA TGCTCCCTCTG**	38-57 in rat gC1qBP	*Bam*HI modified for subcloning into pRSET-C
gC1qBPantisense	**AGTCAGTAGAACTGTG AGAC**GAATTC	874-893 in rat gC1qBP	*Eco*RI modified for subcloning into pRSET-C
T7 promoter primer	TAATACGACTCACTAT AGGG	in pET15-b	Used for sequencing
T7 terminator	GCTAGTTATTGCTCAG CGG	in pET15-b	Used for sequencing
T7 gene 10 primer	TGAGGTTGTAGAAGT TCCG	in pRSET-C	Used for sequencing
pRSET reverse primer	TAGTTATTGCTCAGC GGTGG	in pRSET-C	Used for sequencing

PART I

Are levels of soluble C1q binding proteins in plasma/serum and synovial fluid indicative or prognostic markers in the course of Rheumatoid Arthritis and SLE?

CHAPTER 3

Results: Calreticulin Levels in Systemic Lupus Erythematosus And Rheumatoid Arthritis

Levels of calreticulin in rheumatoid arthritis and systemic lupus erythematosus

As elaborated in the introduction, calreticulin is a multifunctional protein, most abundant in the ER. The protein has well recognised physiological roles in the ER as a molecular chaperone and Ca^{++} -signalling molecule. It has also been found bound to membranes of organelles, at the cell surface and in the extracellular environment, where it has been shown to exert a number of physiological and pathological effects. Two forms of calreticulin have been described, ectoclareticulin and endocalreticulin. Ectocalreticulin was suggested to bind to the cell exterior via interaction with a KDEL receptor and α6 integrin. Endocalreticulin was described as being bound to the inside of the cell membrane via interaction with the integrin cytoplasmic domain (Zhu, Zelinka *et al.*, 1997).

Calreticulin has been characterized as an autoantigen in autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis and Sjogren's syndrome. The role of calreticulin in the pathophysiology of these inflammatory diseases is unclear. The hypothesis that calreticulin S-domain inhibits complement activation via the classical pathway was tested *in vitro*. Levels in serum samples from patients with SLE and RA and in synovial fluid samples from patients with RA were measured following the hypothesis that a pathogenic involvement of the potential autoantigen in these systemic diseases would correlate with altered levels at the local inflammatory site and/or systemically (serum).

This chapter first describes the expression and purification of the C1q-binding domain of calreticulin (S-domain). We tested its ability to inhibit complement activation through the classical pathway and finally we measured the levels of this protein in synovial fluid samples and serum samples of rheumatoid arthritis patients and serum samples of SLE patients. Recombinant human CRT S-domain was prepared for the following purposes: First, to produce anti-human antibodies with the aim of measuring the levels of the protein in the synovial fluids and sera of rheumatoid arthritis patients, and sera of SLE patients. Second, to investigate binding of the S-domain to C1q and determine the functional significance of such binding. Finally, we investigate binding of native calreticulin and recombinant calreticulin domains to MBL and determine the functional importance of such binding.

3.1 Cloning, expression and purification of the calreticulin S-domain.

Recombinant human CRT-S domain was expressed in *E. coli* using the vector pET-15b, which generates His tagged fusion proteins.

Oligonucleotide primers were generated for the recombinant CRT S-domain. The sense primer (hCALFWD) was modified to include a site for *Nde*I digest designed to allow in-frame subcloning into pET15-b. Antisense primer (hCALREV) was modified to include a site

for *Bgl*II digest and a stop codon (section 2.5). The S-domain nucleotide sequence and amino acid sequence are shown in figures 3.1.

Human calreticulin S-domain cDNA (cloned in pTrxfus by Dr. N.J. Lynch, University of Leicester, Leicester, UK) was used as a template for PCR. A PCR product of the expected size was obtained and ligated into pCR2.1. Plasmid DNA was prepared, digested with *Nde*I and *Bgl*II, then separated on an agarose gel. CRT S-domain was cut from the gel, extracted using the Ultra-Silica DNA Purification kit (section 2.1.8) and ligated into pET15-b that had been linearised with *Nde*I and *Bgl*II. The construct was transformed into *E.coli* strain TOP10F, plated on LA plates containing 50μg/ml ampicillin and incubated overnight at 37°C. Plasmid DNA was isolated from a single recombinant colony and the DNA was transformed into *E.coli* expression strain BL21 (DE3) pLysS, plated on LA plates containing 50μg/ml ampicillin and 35μg/ml chloramphenicol and incubated overnight at 37°C. Plasmid was isolated from a single colony and sequenced (manually sequenced, see section 2.1.11) with primers "T7 promoter primer" and "T7 terminator primer" (purchased from Novagen) to confirm that the subcloning had been successful, i.e., the insert was cloned in frame.

A single colony of *E. coli* BL21 (DE3) pLysS harbouring the appropriate pET-15b construct was inoculated into LB medium containing 50μg/ml ampicillin and 35μg/ml chloramphenicol and grown overnight at 37°C (overnight culture). 2L Erlenmeyer flasks each containing 500ml of LB without antibiotics were inoculated with 8 ml of the overnight culture. Protein expression was induced in late-log phase by addition of 0.4 mM IPTG for 3 hours at 37°C. The cells were pelleted by centrifugation at 5000 x g for 10 min at 4°C and stored at –20°C for protein purification.

The cell pellet was resuspended in ice-cold buffer containing 20mM Tris, pH 8.0, 0.5M NaCl, 5mM Imidazole, 0.1% Triton X-100 and sonicated at 4°C. The sonicate was centrifuged for 10 min, at 10000 x g, at 4°C and the supernatant was transferred into a clean tube. The supernatant was applied on a Ni^{++} charged iminodiacetic acid-Sepharose column (5 ml Hi-Trap Chelating; Amersham Pharmacia Biotech) and eluted with an Imidazole gradient (figure 3.2). The binding buffer contained 50mM Tris-HCl, pH 8.0, 0.5M NaCl, 5mM Imidazole and the elution buffer contained 50mM Tris-HCl, pH 8.0, 0.5M NaCl, 0.5M Imidazole. The fractions were analysed by SDS-PAGE and those containing the protein were dialysed against 20mM Tris pH8.0, 200mM NaCl, 2mM $CaCl_2$ and digested with 3.5 units of thrombin for two hours at RT to remove the fusion partner (figure 3.3). The digested material was loaded into the Ni^{++} column to remove the histidine tag using the buffers described above. The flow through containing the protein was collected and dialysed against 20mM Tris pH 8.0, 50mM NaCl. After dialysis, the CRT-S domain was purified on a UNO-Q

column (Biorad) using a NaCl gradient with a binding buffer containing 20mM Tris pH 8.0, 50 mM NaCl and the elution buffer contained 20mM Tris pH 8.0, 1M NaCl.

Approximately 200mg of CRT- S domain were expressed in a soluble state from a 2.5L induced culture. 125 mg were purified in the purification trials and the protein preparation was 95% pure as estimated from SDS-PAGE analysis (figure 3.3). It can be seen from figure 3.3 that the S-domain appears to be in the form of a dimer (homodimer due to the presence of a cysteine residue) even under reducing conditions. This is because the S-domain has a very rigid structure due to the presence of a large number of proline residues (Elgaard, Riek *et al.*, 2001) and a low isoelectric point (pI=4.09).

Samples of the recombinant CRT-S domain were sent for mass spectroscopy analysis and protein sequencing (Protein and Nucleic Acid Chemistry Laboratory, Centre for Mechanisms of Human Toxicity, University of Leicester).

The mass spectroscopy results indicated a MW of 29,984 ±8.56Da; this corresponds to the homodimer linked by the disulfide bond. Protein sequencing identified the first 5 amino acids of the CRT-S domain R C K D D.

```
   1 - ATGCTGCTATCCGTGCCGTTGCTGCTCGGCCTCCTCGGCCTGGCCGTCGCCGAGCCTGCC - 60
     -  M  L  L  S  V  P  L  L  L  G  L  L  G  L  A  V  A  E  P  A
  61 - GTCTACTTCAAGGAGCAGTTTCTGGACGGAGACGGGTGGACTTCCCGCTGGATCGAATCC - 120
     -  V  Y  F  K  E  Q  F  L  D  G  D  G  W  T  S  R  W  I  E  S
 121 - AAACACAAGTCAGATTTTGGCAAATTCGTTCTCAGTTCCGGCAAGTTCTACGGTGACGAG - 180
     -  K  H  K  S  D  F  G  K  F  V  L  S  S  G  K  F  Y  G  D  E
 181 - GAGAAAGATAAAGGTTTGCAGACAAGCCAGGATGCACGCTTTTATGCTCTGTCGGCCAGT - 240
     -  E  K  D  K  G  L  Q  T  S  Q  D  A  R  F  Y  A  L  S  A  S
 241 - TTCGAGCCTTTCAGCAACAAAGGCCAGACGCTGGTGGTGCAGTTCACGGTGAAACATGAG - 300
     -  F  E  P  F  S  N  K  G  Q  T  L  V  V  Q  F  T  V  K  H  E
 301 - CAGAACATCGACTGTGGGGGCGGCTATGTGAAGCTGTTTCCTAATAGTTTGGACCAGACA - 360
     -  Q  N  I  D  C  G  G  G  Y  V  K  L  F  P  N  S  L  D  Q  T
 361 - GACATGCACGGAGACTCAGAATACAACATCATGTTTGGTCCCGACATCTGTGGCCCTGGC - 420
     -  D  M  H  G  D  S  E  Y  N  I  M  F  G  P  D  I  C  G  P  G
 421 - ACCAAGAAGGTTCATGTCATCTTCAACTACAAGGGCAAGAACGTGCTGATCAACAAGGAC - 480
     -  T  K  K  V  H  V  I  F  N  Y  K  G  K  N  V  L  I  N  K  D
 481 - ATCCGTTGCAAGGATGATGAGTTTACACACCTGTACACACTGATTGTGCGGCCAGACAAC - 540
     -  I  R  C  K  D  D  E  F  T  H  L  Y  T  L  I  V  R  P  D  N
 541 - ACCTATGAGGTGAAGATTGACAACAGCCAGGTGGAGTCCGGCTCCTTGGAAGACGATTGG - 600
     -  T  Y  E  V  K  I  D  N  S  Q  V  E  S  G  S  L  E  D  D  W
 601 - GACTTCCTGCCACCCAAGAAGATAAAGGATCCTGATGCTTCAAAACCGGAAGACTGGGAT - 660
     -  D  F  L  P  P  K  K  I  K  D  P  D  A  S  K  P  E  D  W  D
 661 - GAGCGGGCCAAGATCGATGATCCCACAGACTCCAAGCCTGAGGACTGGGACAAGCCCGAG - 720
     -  E  R  A  K  I  D  D  P  T  D  S  K  P  E  D  W  D  K  P  E
 721 - CATATCCCTGACCCTGATGCTAAGAAGCCCGAGGACTGGGATGAAGAGATGGACGGAGAG - 780
     -  H  I  P  D  P  D  A  K  K  P  E  D  W  D  E  E  M  D  G  E
 781 - TGGGAACCCCCAGTGATTCAGAACCCTGAGTACAAGGGTGAGTGGAAGCCCCGGCAGATC - 840
     -  W  E  P  P  V  I  Q  N  P  E  Y  K  G  E  W  K  P  R  Q  I
 841 - GACAACCCAGATTACAAGGGCACTTGGATCCACCCAGAAATTGACAACCCCGAGTATTCT - 900
     -  D  N  P  D  Y  K  G  T  W  I  H  P  E  I  D  N  P  E  Y  S
 901 - CCCGATCCCAGTATCTATGCCTATGATAACTTTGGCGTGCTGGGCCTGGACCTCTGGCAG - 960
     -  P  D  P  S  I  Y  A  Y  D  N  F  G  V  L  G  L  D  L  W  Q
 961 - GTCAAGTCTGGCACCATCTTTGACAACTTCCTCATCACCAACGATGAGGCATACGCTGAG - 1020
     -  V  K  S  G  T  I  F  D  N  F  L  I  T  N  D  E  A  Y  A  E
1021 - GAGTTTGGCAACGAGACGTGGGGCGTAACAAAGGCAGCAGAGAAACAAATGAAGGACAAA - 1080
     -  E  F  G  N  E  T  W  G  V  T  K  A  A  E  K  Q  M  K  D  K
1081 - CAGGACGAGGAGCAGAGGCTTAAGGAGGAGGAAGAAGACAAGAAACGCAAAGAGGAGGAG - 1140
     -  Q  D  E  E  Q  R  L  K  E  E  E  E  D  K  K  R  K  E  E  E
1141 - GAGGCAGAGGACAAGGAGGATGATGAGGACAAAGATGAGGATGAGGAGGATGAGGAGGAC - 1200
     -  E  A  E  D  K  E  D  D  E  D  K  D  E  D  E  E  D  E  E  D
1201 - AAGGAGGAAGATGAGGAGGAAGATGTCCCCGGCCAGGCCAAGGACGAGCTGTAG - 1254
     -  K  E  E  D  E  E  E  D  V  P  G  Q  A  K  D  E  L  *
```

Figure 3.1 The nucleotide sequence and the predicted amino acid sequence of human calreticulin. The highlighted section corresponds to the S-domain. The underlined section corresponds to the P-domain of the calreticulin molecule.

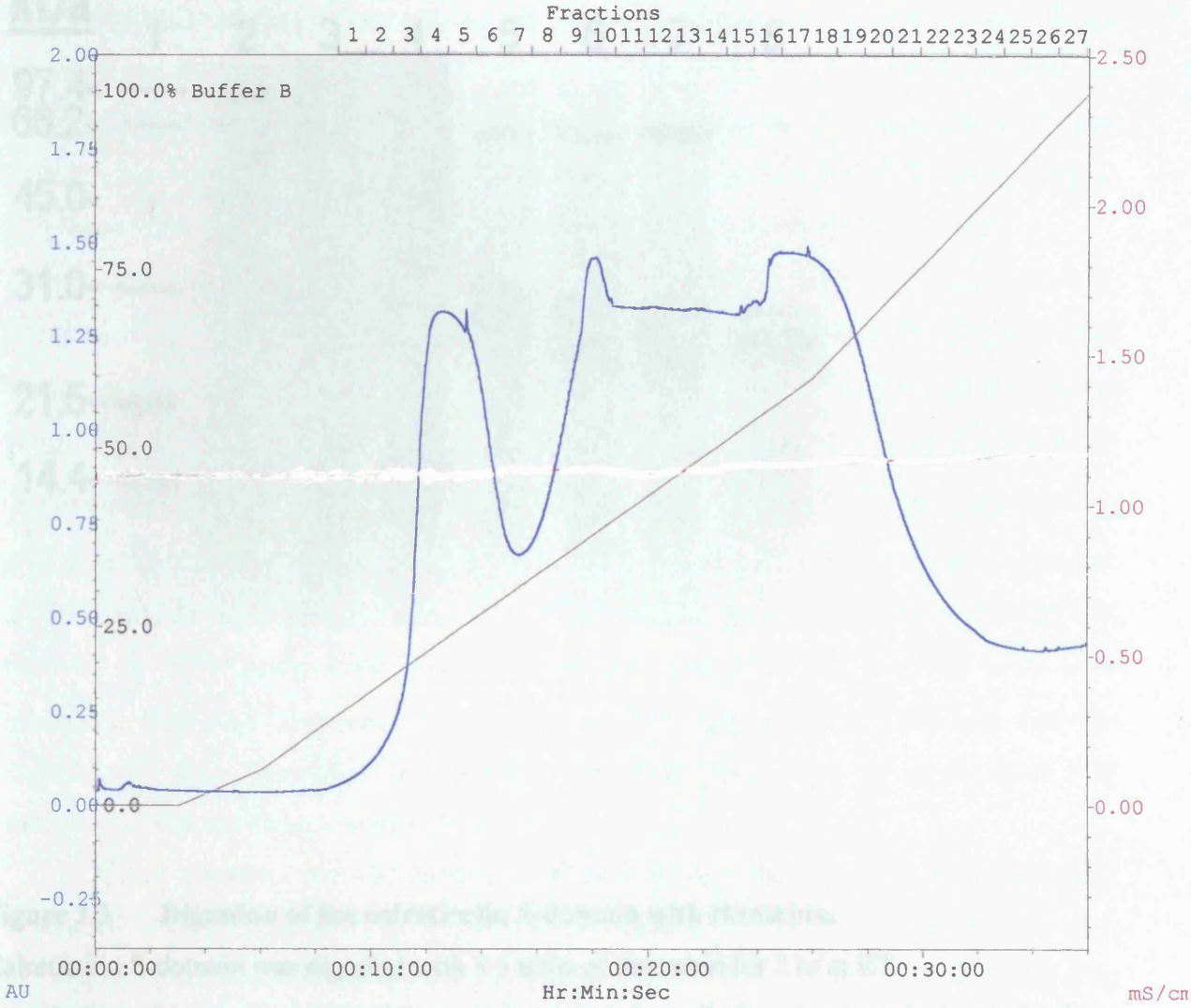


Figure 3.2 Elution profile of the calreticulin S-domain purification on a Ni^{++} charged HiTrap Chelating column.

The protein (calreticulin S-domain) is contained in fractions 9-18. The solid blue line indicates the absorbance at 280 nm. The protein was eluted with a linear gradient from 5mM to 500mM Imidazole as indicated by the black line.

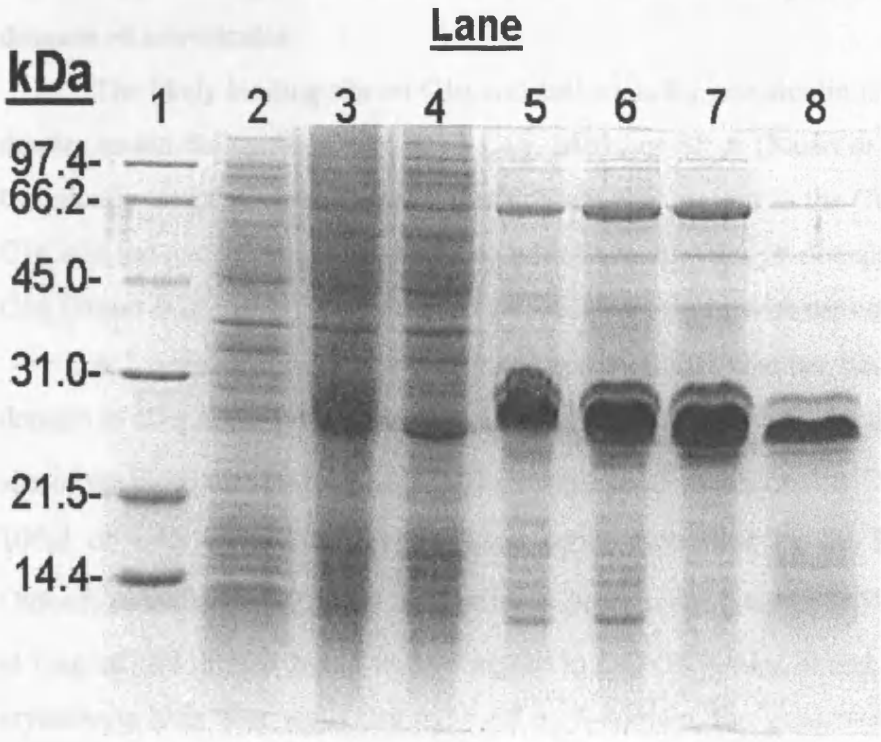


Figure 3.3 Digestion of the calreticulin S-domain with thrombin.

Calreticulin S-domain was digested with 3.5 units of thrombin for 2 hr at RT.

Lane 1: Biorad low molecular weight protein marker, Lane 2: *E. coli* culture before induction of S-domain expression, Lane 3: *E. coli* culture 3 hrs after induction, Lane 4: Flow through of Ni^{++} column, Lane 5: S-domain before digestion with thrombin, Lane 6: S-domain after 1hr digestion, Lane 7: S-domain after 2hr digestion, Lane 8: digested S-domain after UNO-Q purification.

3.2 C1q binding site of calreticulin. Inhibition of complement activation by the S-domain of calreticulin

The likely binding site on C1q and collectins for calreticulin is a region of high charge density in the collagenous portion of C1q, MBL, or SP-A (Stuart *et al.,* 1996). A region of calreticulin contains short sequences, which are also present in the CUB domains of C1r and C1s, and has overall sequence similarity with CUB domains (S-domain). This region, binds to C1q (Stuart *et al.,* 1996) and inhibits re-association of C1q with the complex $C1r_2s_2$.

A haemolytic assay was performed to determine whether binding of recombinant S-domain to C1q could prevent complement activation possibly by blocking the $C1r_2s_2$ binding site of the molecule. EAC1q cells (100 µl/well) at 10^8/ml in $DGVB^{++}$ buffer were mixed with 100µl of 1/40 dilution of C1q-deficient serum (provided by Dr. R.B.Sim, University of Oxford) in $DGVB^{++}$. 50 µl per well of the S-domain (dialysed in $DGVB^{++}$) dilutions (starting at 1mg/ml and diluted 2-fold to 0.05 mg/ml in $DGVB^{++}$) were added. Controls for maximum erythrocyte lysis were wells that received no S-domain, for spontaneous lysis were wells that received no serum and controls for complete lysis were wells that received water. The microtiter plate was incubated for 60 min at 37°C centrifuged at 1200 rpm and the supernatants were transferred into a clean microtiter plate. Lysis of erythrocytes was measured at 405 nm using a spectrophotometer.

It was observed that the S-domain of CRT inhibits haemolysis in a concentration dependent manner (figure 3.4). At low concentrations of S-domain (<100ng/ml) high percentage of erythrocyte lysis is observed due to classical pathway activation from the C1q contained in the erythrocytes. At higher concentrations of S-domain, a low percentage of lysis is observed, because the S-domain binds C1q and possibly blocks binding of the molecule $C1r_2s_2$, thereby preventing complement activation. When there is no S-domain added (buffer only), there is 90% of erythrocyte lysis because the C1q presented on the erythrocytes activates the classical pathway in the C1q deficient serum. At 10µg/ml S-domain approximately 10% inhibition of erythrocyte lysis is observed. At 200µg/ml S-domain, 45-50% inhibition in erythrocyte lysis is observed. As shown in figure 3.4 addition of 1mg/ml S-domain causes maximum inhibition of cell lysis (50% lysis). Inhibition of cell lysis by the S-domain could be enhanced by incubating the EAC1q cells with the purified S-domain prior to addition of C1q-deficient serum.

3.3 Binding of native human calreticulin and recombinant human calreticulin domains to MBL.

Native calreticulin and recombinant human calreticulin domains were tested for binding to purified native MBL (obtained from J. Jensenius, Aarhus University, Denmark). The recombinant human calreticulin domains tested were the P-domain (A. Gingras, University of Leicester, UK), the S-P domains (these two domains were expressed and purified together by A. Gingras, University of Leicester, UK) and the S-domain (prepared as described in section 3.1).

A 96 well plate was coated with CRT and rec CRT domains at 10μg/ml in 15mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6. Dilutions (in CRT buffer-10mM Tris, 10mM $CaCl_2$, 0.05% Triton X100, 0.1% HSA, pH 7.4, containing increasing concentrations of NaCl – 10mM, 30mM, 50mM, 100mM, 200mM and 500mM) of purified native MBL (2μg/ml, 1μg/ml, 0.5μg/ml and 0μg/ml) were added to the wells and the plate was incubated at 4°C, overnight. MBL binding to CRT and CRT domains was detected using mouse monoclonal anti-MBL antibody. Figure 3.5 shows binding of recombinant S-domain (A), recombinant P domain (B) and native calreticulin (C) to MBL. It can be seen from the figure that as the concentration of NaCl increases, the binding to MBL decreases.

A C4 cleavage assay was performed in order to determine functional activation of the lectin pathway by native calreticulin and recombinant domains of calreticulin. The assay is similar to the one described in Chapter 2 (section 2.3.4) exept that in this case the concentration of NaCl in the MBL binding buffer is not more than 10 mM because CRT does not bind at high salt concentrations. Briefly, 96 well plates were coated with 10μg/ml antigen (S-domain, P-domain, native calreticulin), blocked and washed as described previously. Wells received dilutions of C1q-depleted serum (prepared as described in section 2.3.7) and dilutions of C1q-depleted serum preincubated with the antigen. Following overnight incubation at 4°C, purified C4 was added and the plate was incubated for 90 min at 37°C. C4b deposition was detected as described in section 2.3.4 (Figure 3.6). It was shown that preincubation of C1q-depleted serum with the antigen (P, P-S, and native CRT) inhibited MBL binding to CRT. This demonstrates that the MBL interaction is specific.

We also tested the effect that $CaCl_2$ concentration has on MBL binding to native CRT and recombinant CRT S-domain. For this, microtiter plates were coated with the antigens as described previously. Dilutions of purified MBL (containing MASPs) (Statens Serum Institut) in 10mM Tris, 0.05% Triton X100, 0.1% HSA, pH 7.4 containing different concentrations of $CaCl_2$ (0mM, 2mM, 10mM) were added to the wells. Following o/n incubation at 4°C the first microtiter plate was incubated with mouse monoclonal anti human MBL (131-01) for 90min at RT. The second microtiter plate was incubated with purified C4

for 90 min at 37°C. MBL binding was measured using anti mouse IgG-AP antibody (first microtiter plate) and C4b deposition was measured using anti human C4-AP antibody (second microtiter plate). The results are shown in figures 3.7 and 3.8 respectively. It can be seen from the figures that binding of native CRT and recombinant S-domain to MBL does not appear to be Ca^{++} dependent. At higher concentrations of $CaCl_2$ (10mM), MBL binding to CRT and CRT S-domain decreases because CRT binding to MBL is inhibited at high salt concentrations as shown in figure 3.5.

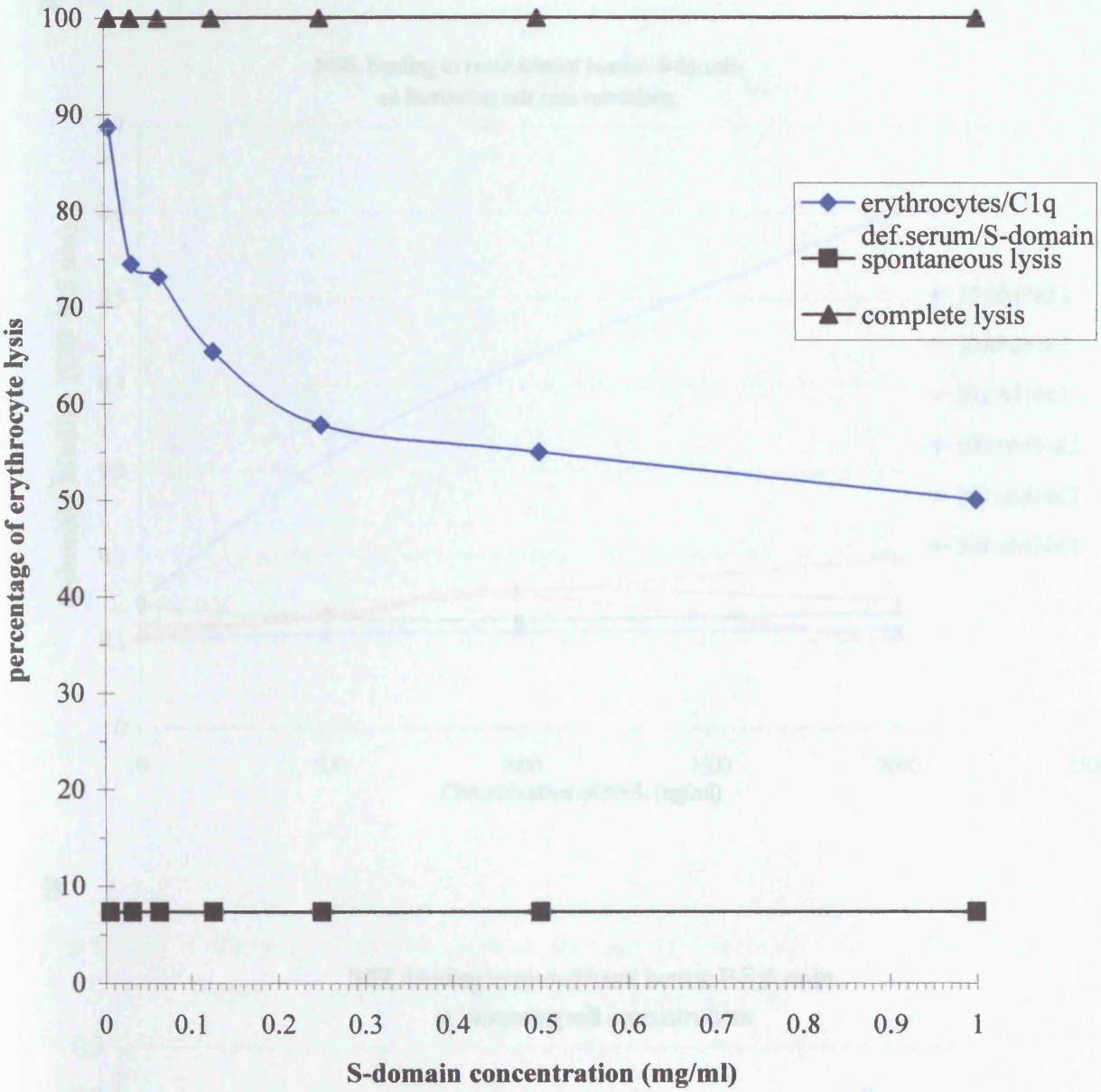
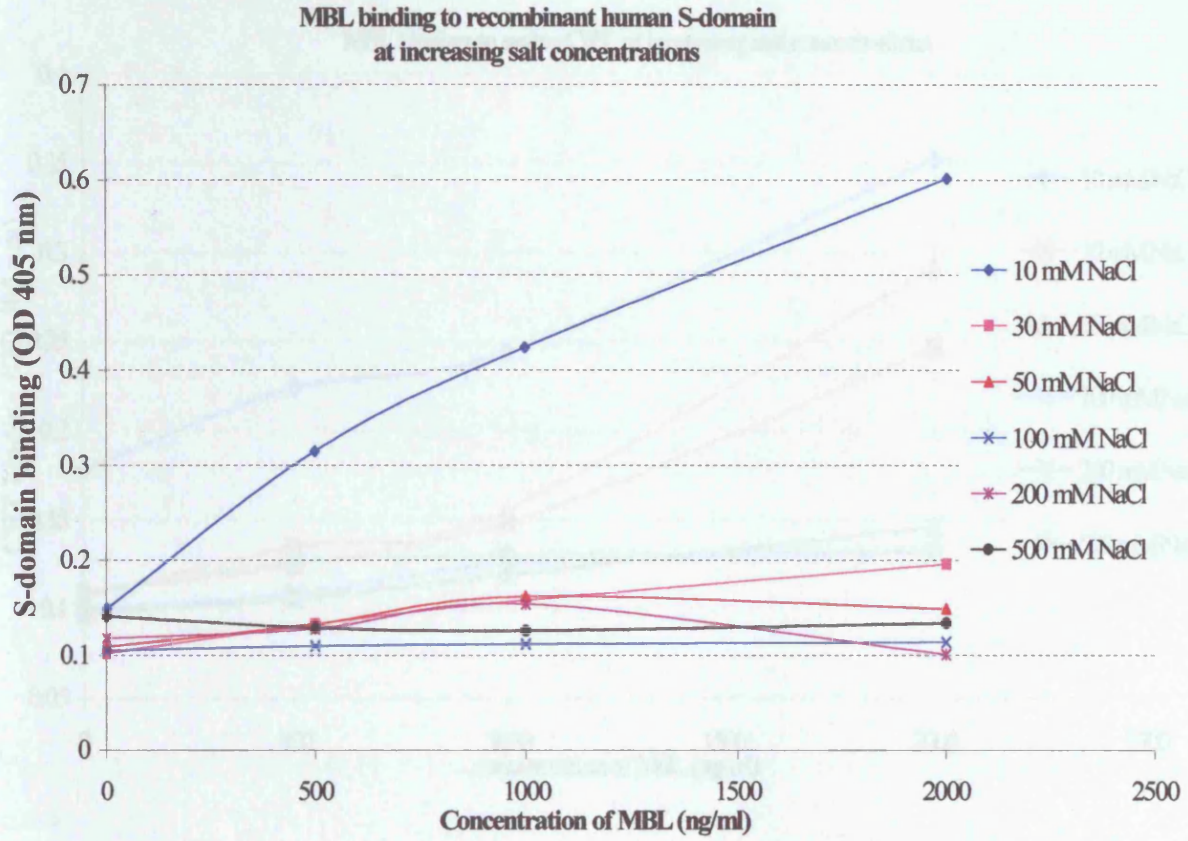
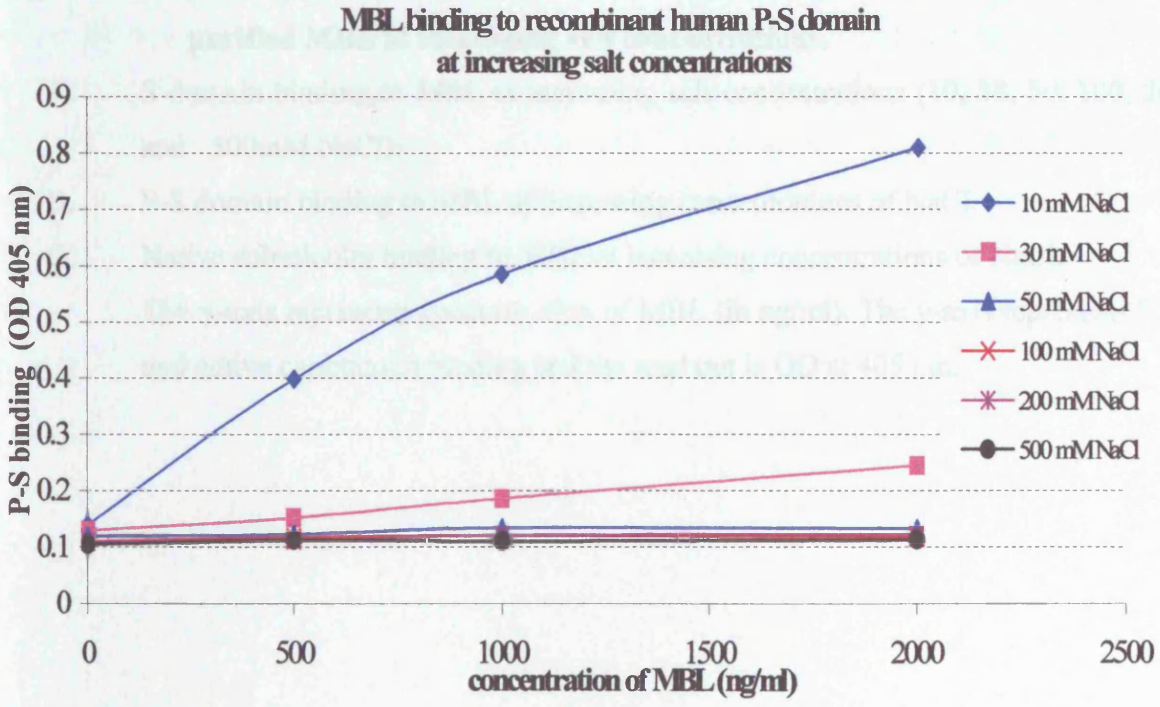


Figure 3.4 Inhibition of complement dependent lysis by the calreticulin S-domain. Erythrocytes (EAC1q) were incubated with S-domain and C1q deficient serum, and compared against complete (EAC1q incubated with water) and spontaneous lysis (EAC1q incubated with $DGVB^{++}$). The S-domain inhibits haemolysis of the sheep erythrocytes in a concentration dependant manner. At low concentrations of S-domain (<100ng/ml) high percentage of erythrocyte lysis is observed. As the concentration of the S-domain increases there is a decrease in the percentage of erythrocyte lysis.

A


MBL binding to recombinant human S-domain
at increasing salt concentrations
S-domain binding (OD 405 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
500
1000
1500
2000
2500
Concentration of MBL (ng/ml)
10 mM NaCl
30 mM NaCl
50 mM NaCl
100 mM NaCl
200 mM NaCl
500 mM NaCl

B


MBL binding to recombinant human P-S domain
at increasing salt concentrations
P-S binding (OD 405 nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
500
1000
1500
2000
2500
concentration of MBL (ng/ml)
10 mM NaCl
30 mM NaCl
50 mM NaCl
100 mM NaCl
200 mM NaCl
500 mM NaCl

C

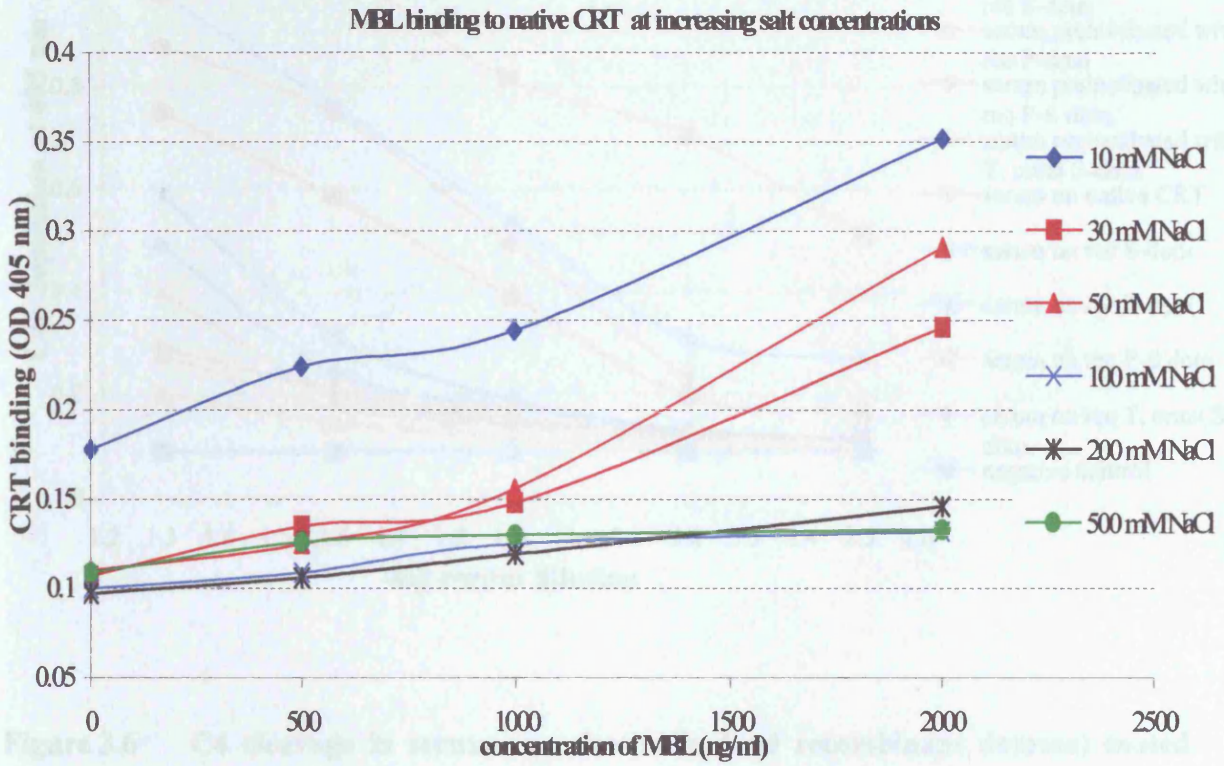


Figure 3.5 Binding of native calreticulin and recombinant calreticulin domains to purified MBL at increasing salt concentrations.

A. S domain binding to MBL at increasing salt concentrations (10, 30, 50, 100, 200 and 500mM NaCl).

B. P-S domain binding to MBL at increasing concentrations of NaCl.

C. Native calreticulin binding to MBL at increasing concentrations of NaCl.

The x-axis represents concentration of MBL (in ng/ml). The y-axis represents S, P and native calreticulin binding and the read out is OD at 405 nm.

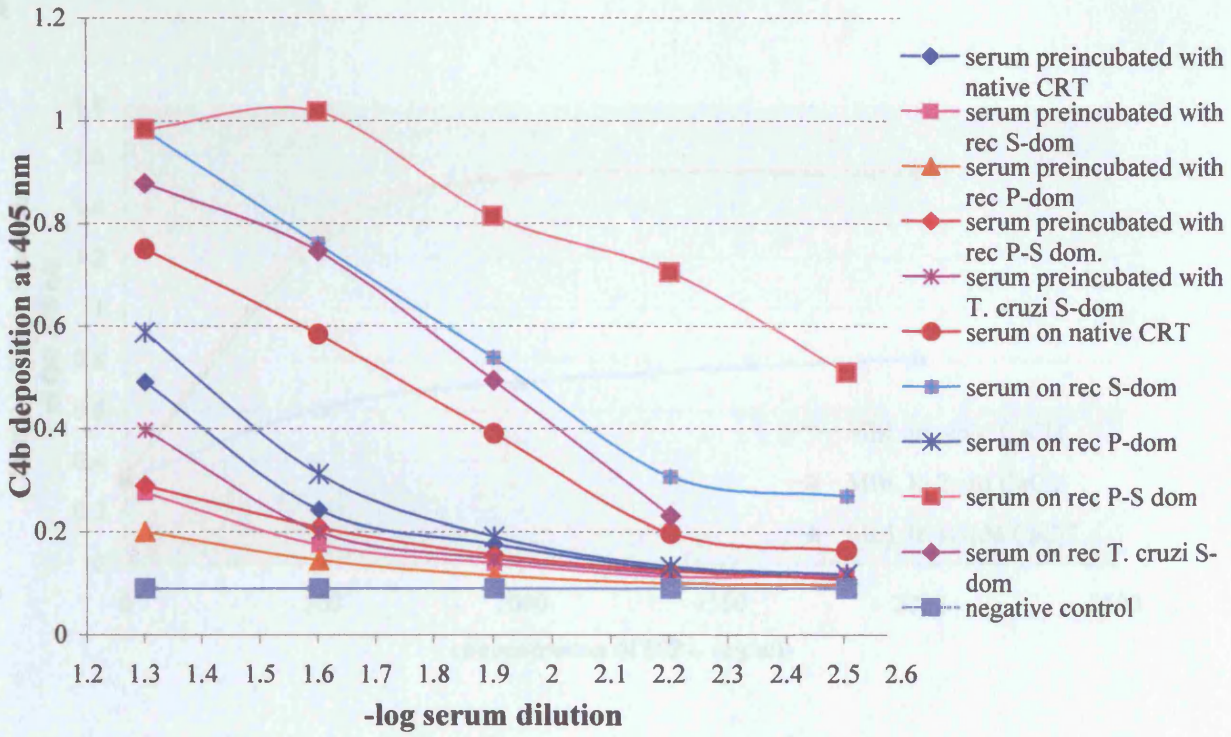
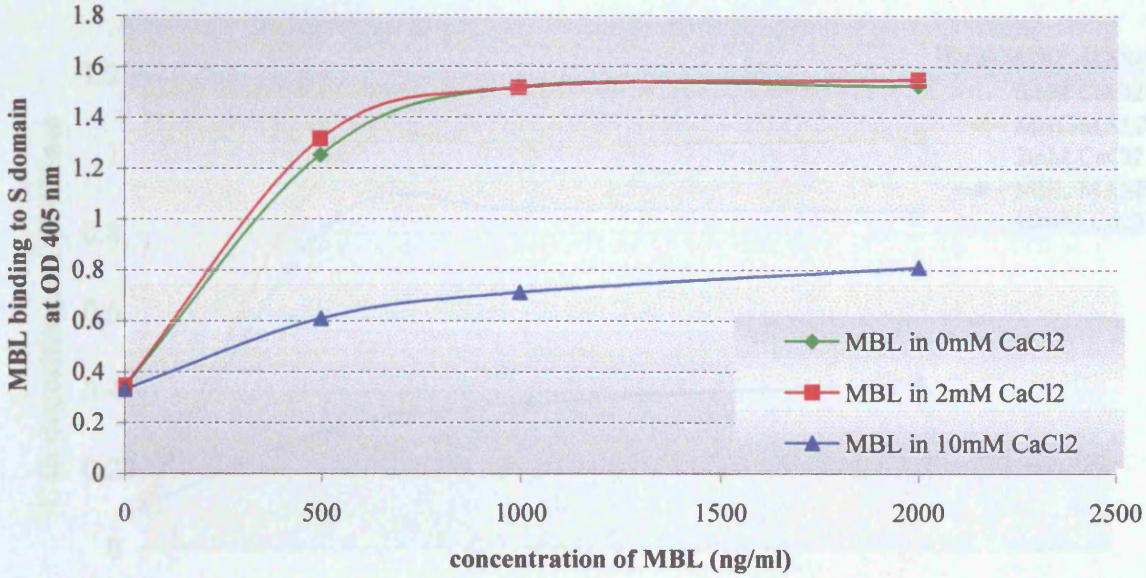


Figure 3.6 C4 cleavage in serum on calreticulin (and recombinant domain) coated plate.

In this assay, C1q-depleted serum (starting dilution 1/30 in 10mM NaCl, 10mM Tris, 10 mM $CaCl_2$, 0.05% triton, 0.1%HSA, pH7.4) is preincubated with CRT and recombinant CRT domain (1µg/ml). This is added to a 96-well plate coated with 10µg/ml CRT or recombinant CRT domain. For comparisson, C1q-depleted serum dilutions not incubated with CRT (or CRT recombinant domains) are added to the wells. Following overnight incubation at 4°C, purified C4 is added and the plate is incubated at 37°C for 90 min. C4b deposition is detected using chicken anti-C4 antibody conjugated to alkaline phosphatase. As shown in the figure, C4 cleavage is inhibited by preincubation of C1q-depleted serum with calreticulin (and recombinant domains). The X-axis represents serum dilutions (-log serum dilutions) and the y-axis represents C4b deposition at OD_{405} nm.

A



B

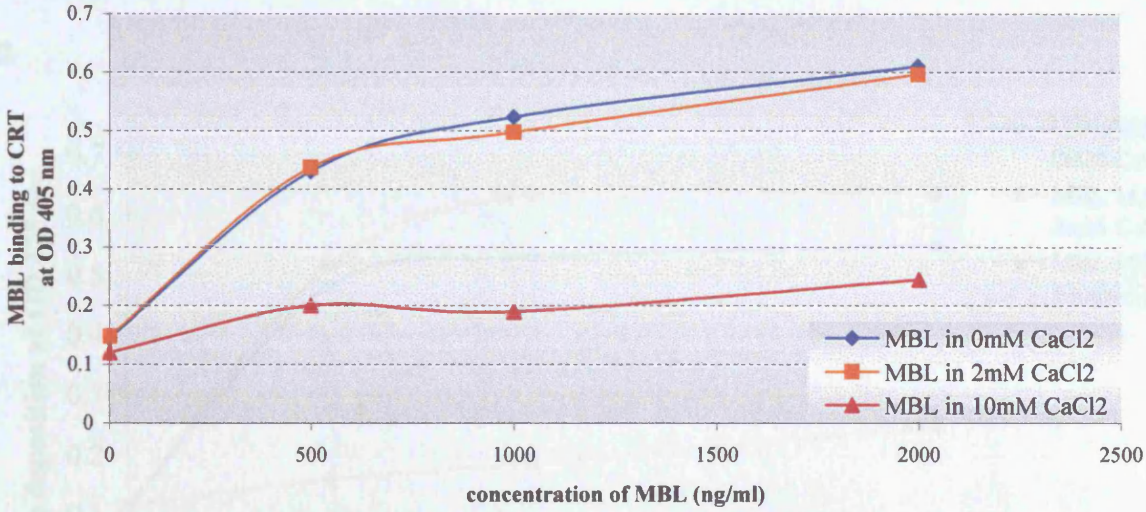
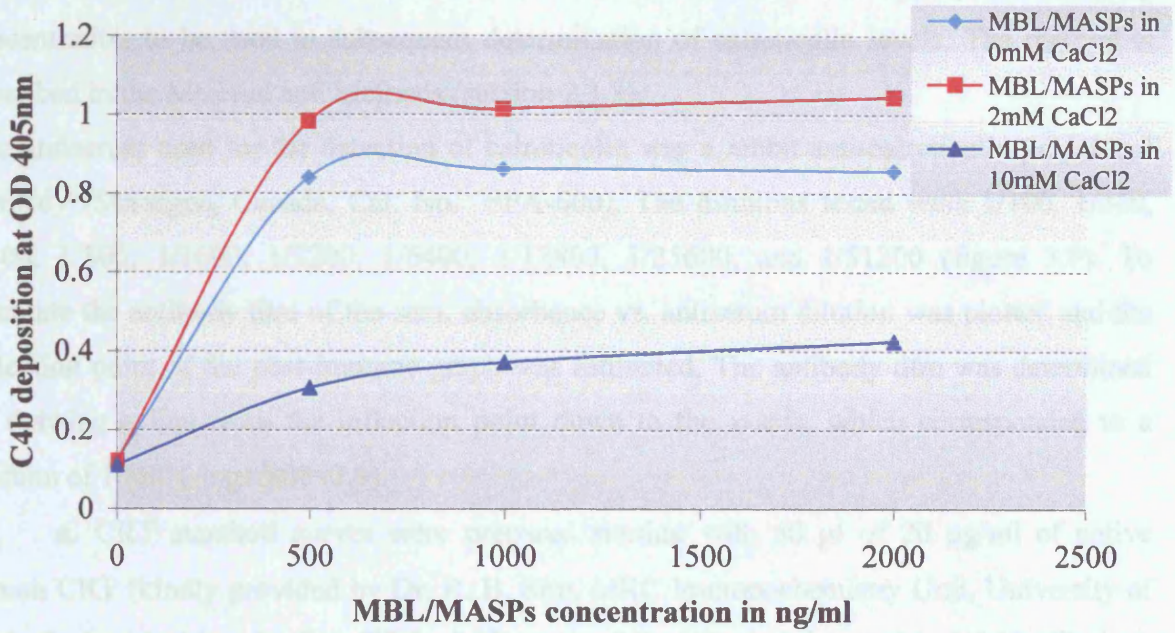


Figure 3.7 Effect of $CaCl_2$ on MBL binding to recombinant calreticulin S-domain (A) and native calreticulin (B).

Purified MBL (in 10mM NaCl, 10mM Tris, 0.05% triton, 0.1%HSA, pH7.4) was added onto S domain (A) and native CRT (B) coated plates under different $CaCl_2$ concentrations (0mM, 2mM and 10mM). The x-axis represents the concentration of MBL in ng/ml; the y-axis represents MBL binding detected using the monoclonal anti human MBL antibody (131-01). The read-out is OD_{405}.

A



B

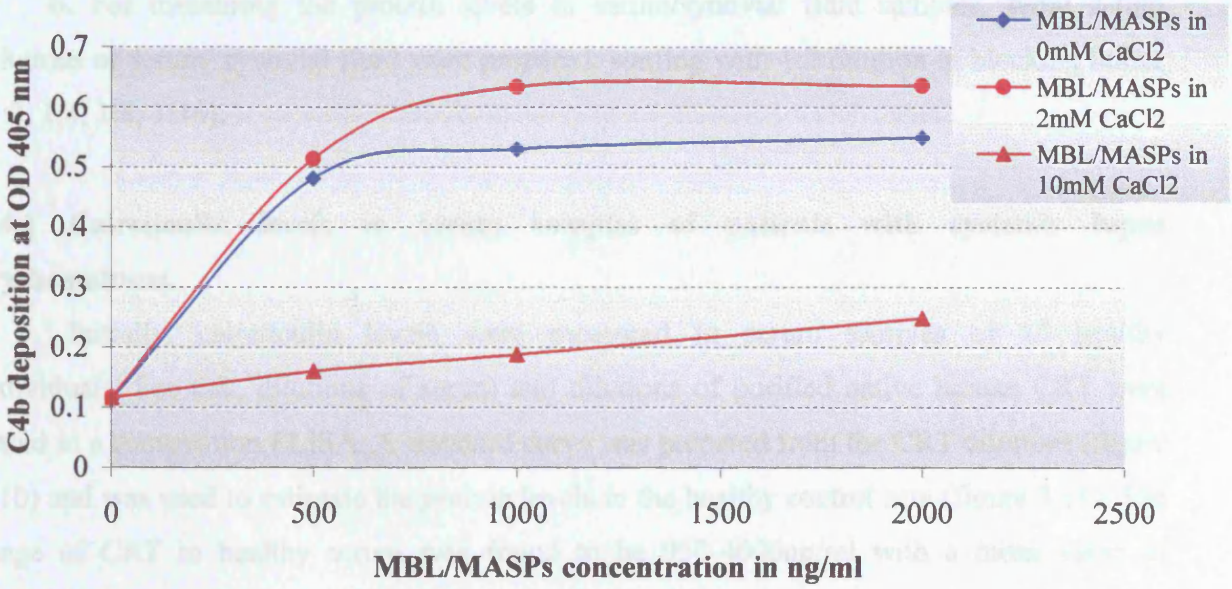


Figure 3.8 C4b deposition on recombinant calreticulin S-domain (A) and native calreticulin (B) coated plates.

Purified MBL (containing MASPs) was incubated on calreticulin S-domain (A) and native calreticulin (B) coated plates. Purified C4 was added and incubated for 90min at 37°C. C4b deposition was detected using chicken anti C4 antibody. The x-axis represents MBL (MASPs) concentration, the y-axis represents C4b deposition and the read-out is OD_{405}.

3.4 Calreticulin levels in synovial fluid aspirates/sera of patients with rheumatoid arthritis and in sera of patients with systemic lupus erythematosus.

An inhibition ELISA was set up in order to determine the appropriate antibody concentration to be used in subsequent determination of calreticulin levels. The method is described in the Material and Methods (section 2.3.3).

The antiserum used for the detection of calreticulin was a rabbit anti-calreticulin polyclonal antibody (Stressgen, Canada, Cat. No. SPA-600). The dilutions tested were 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400, 1/12800, 1/25600, and 1/51200 (figure 3.9). To calculate the antibody titre of the sera, absorbance vs. antiserum dilution was plotted and the inflection point of the post-immune graph was estimated. The antibody titre was determined by drawing a line from the inflection point down to the x-axis, which corresponded to a dilution of 1/800 (-log1/800=2.9).

a. CRT standard curves were prepared starting with 50 µl of 20 µg/ml of native human CRT (kindly provided by Dr. R. B. Sim, MRC Immunochemistry Unit, University of Oxford) in blocking buffer (PBS, 0.1%tween 20) followed by serial 2-fold dilutions (20µg/ml, 10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml, 625ng/ml, 312ng/ml, 156ng/ml, 78ng/ml, 39ng/ml, 20ng/ml, 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml).

b. For measuring the protein levels in serum/synovial fluid samples, serial 2-fold dilutions of serum/ synovial fluid were prepared, starting with 1:2 dilution in blocking buffer (1:2, 1:4, 1:8, 1:16).

3.4.1 Calreticulin levels in serum samples of patients with systemic lupus erythematosus.

Initially, calreticulin levels were measured in serum samples of 15 healthy individuals. For this, dilutions of serum and dilutions of purified native human CRT were tested in a competition ELISA. A standard curve was prepared from the CRT dilutions (figure 3.10) and was used to estimate the protein levels in the healthy control sera (figure 3.11). The range of CRT in healthy serum was found to be 950-4000ng/ml with a mean value of 2074ng/ml.

The calreticulin inhibitor curve was also used to measure the levels of protein in the sera of 54 patients diagnosed with systemic lupus erythematosus (Table 3.1, Figure 3.11). The range of CRT in sera of patients diagnosed with SLE was found to be 700ng/ml to 5120ng/ml with a mean value of 1761ng/ml and for the majority of the samples the concentration of CRT ranges from 750-2250ng/ml (see Table 3.1). Figure 3.11 shows a comparison between the levels of CRT in healthy individuals and the levels of CRT in patients diagnosed with SLE.

Table 3.1 The following table shows the values for the CRT levels in sera of 54 patients diagnosed with systemic lupus erythematosus.

SLE sample No.	[CRT] in ng/ml
1	4352
2	1600
3	4224
4	1600
5	4224
6	1600
7	3680
8	3200
9	2880
11	2200
13	1700
14	1400
15	1050
16	1120
17	1840
18	1040
19	3520
21	1300
23	4480
24	4608
25	4160
26	1600
27	3360
28	1600
29	800
30	900
31	3000
32	1000
33	1360

34	700
35	2800
36	1600
37	800
38	1760
40	1950
41	960
42	800
43	1760
44	5120
45	2240
46	640
47	960
48	1600
49	2500
50	1440
51	960
52	1680
53	880
54	1920
55	2080
56	960
57	2000
58	1440

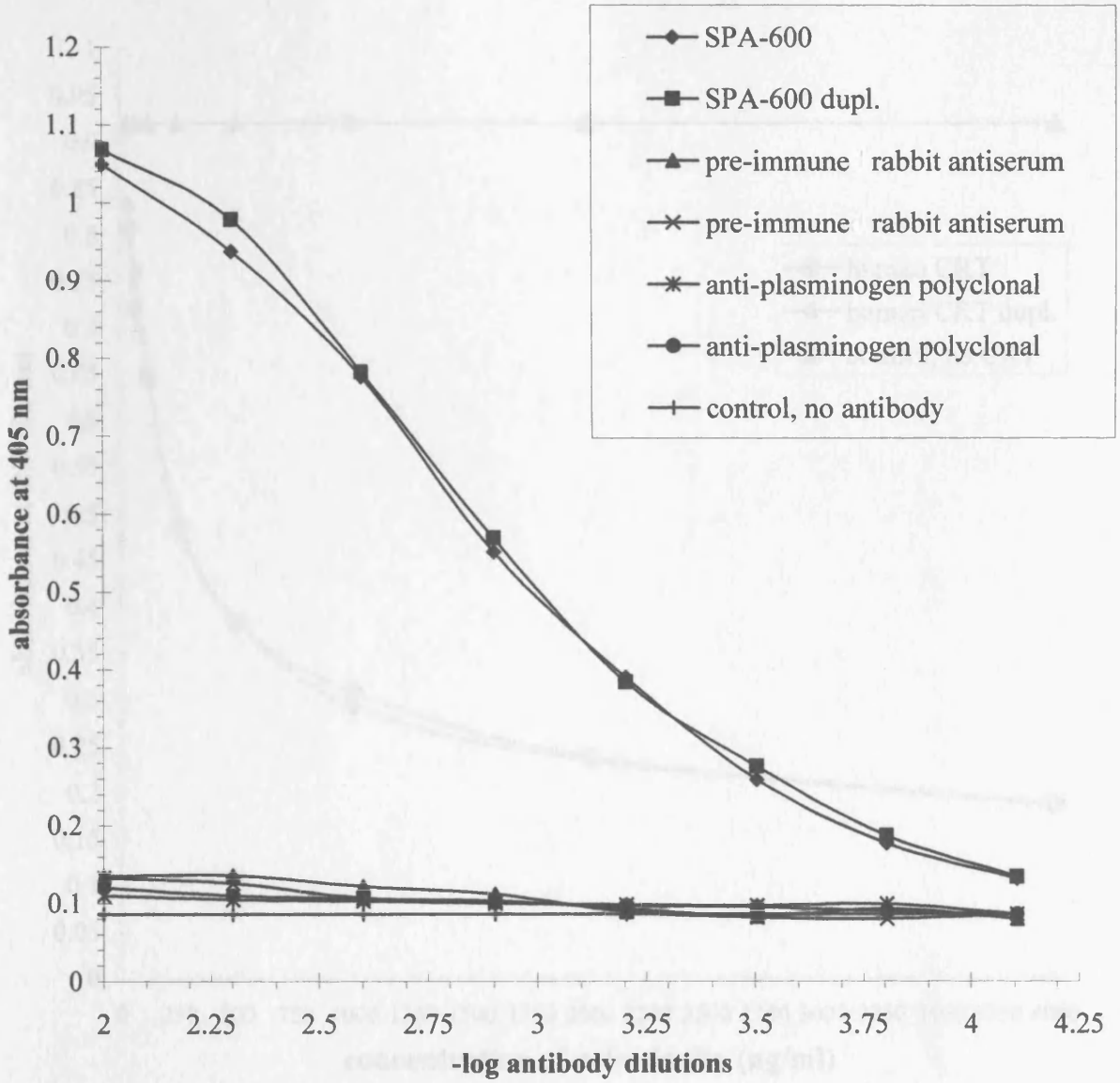


Figure 3.9 Measurement of the titer of the polyclonal antibody against human calreticulin (SPA-600).

The antigen (human calreticulin) is bound to the polystyrene microtiter plate. The antiserum containing the polyclonal antibody SPA-600 is added to the well and allowed to bind. A second antibody, anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase, is then added. For control, preimmune rabbit antiserum and an irrelevant anti-plasminogen rabbit antiserum are used. The titre of the antiserum is that dilution which corresponds to the inflection point of the absorbance vs. dilution curve, in this case 1/800.

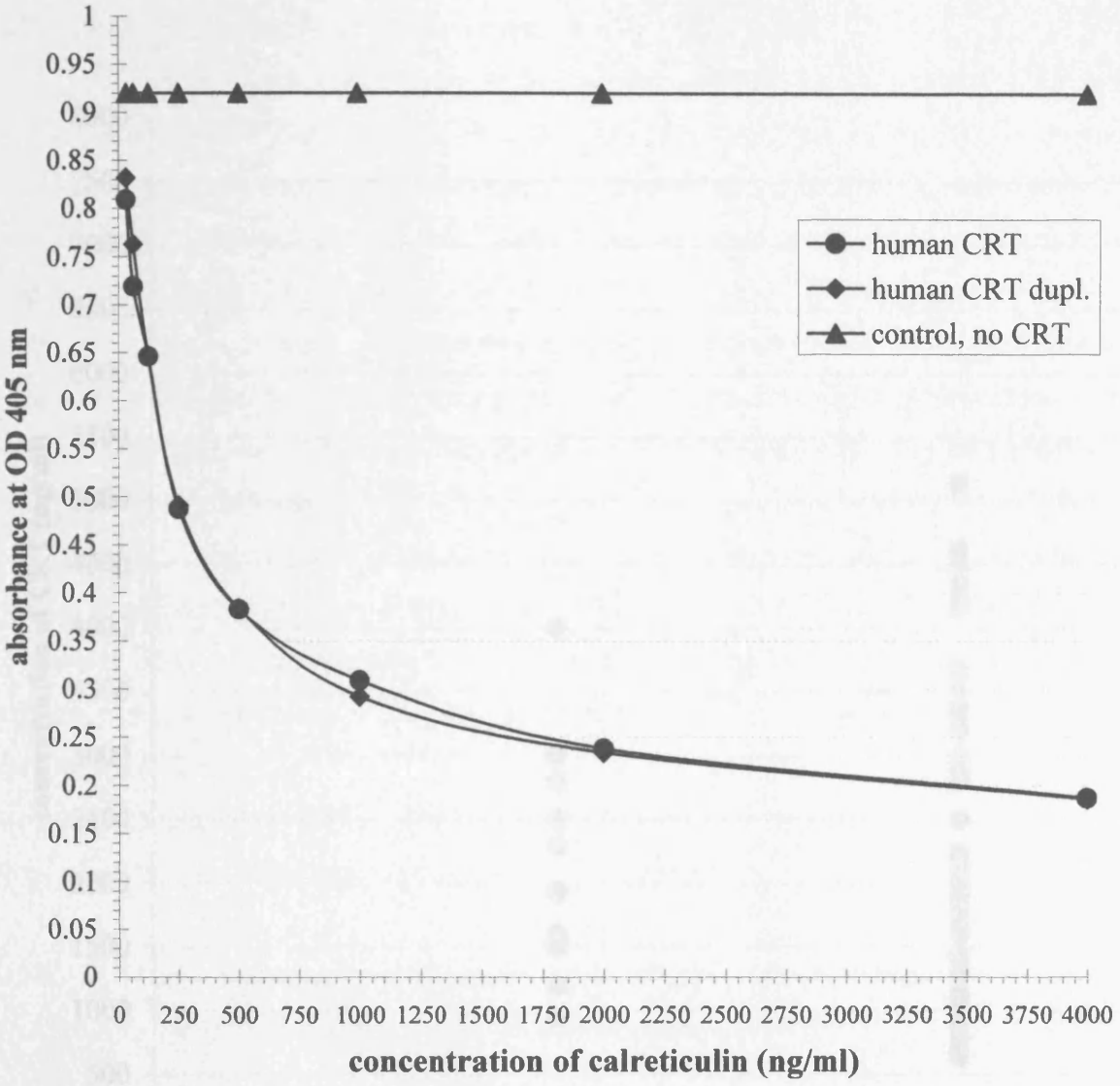


Figure 3.10 Calreticulin inhibitor curve.

50 µl of 4000ng/ml of protein (native human CRT) in blocking buffer (PBS/0.1%tween 20) was diluted 2-fold (4000ng/ml, 2000ng/ml, 100ng/ml, 500ng/ml, 250ng/ml, 125ng/ml, 62.5ng/ml, 31.25ng/ml). The assay is most accurate in the region of the curve where small changes in inhibitor concentrations produce maximal changes in the amount of inhibition (100-1000ng/ml).

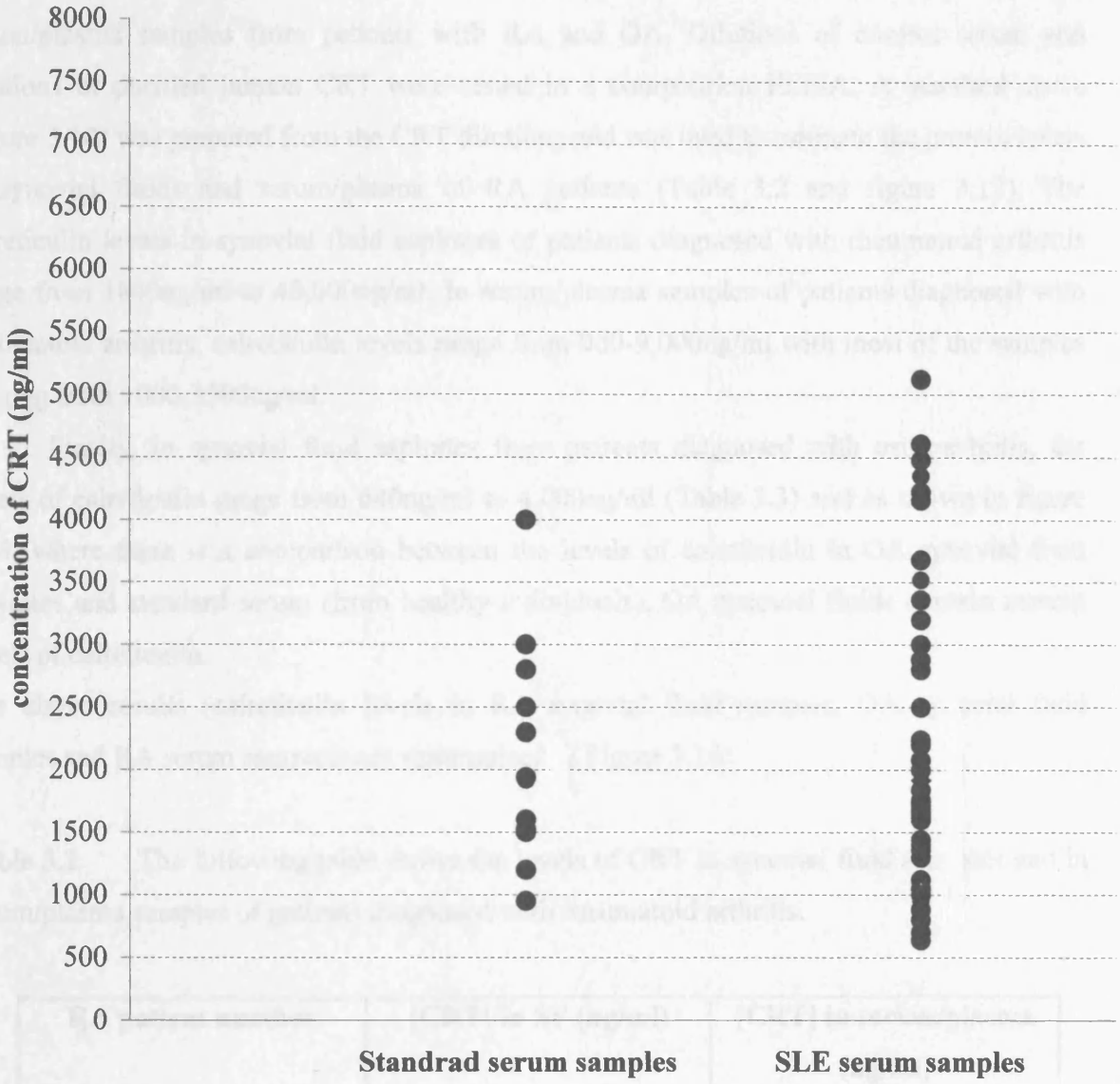


Figure 3.11 Calreticulin levels in serum samples from healthy individuals and serum samples from patients diagnosed with SLE.

Calreticulin levels in healthy individuals range from 950ng/ml to 4000ng/ml with a mean value of 2074ng/ml, in serum samples of patients diagnosed with SLE from 700ng/ml to 5120 ng/ml and for most of the SLE samples the concentration of calreticulin is 750-2250 ng/ml (Table 3.1).

Points represent means of duplicates from one of several experiments.

3.4.2 Calreticulin levels in synovial fluid aspirates and serum/plasma samples of patients with rheumatoid arthritis/osteoarthritis.

CRT levels were measured in control serum (section 3.4.1), synovial fluid and serum/plasma samples from patients with RA and OA. Dilutions of control serum and dilutions of purified human CRT were tested in a competition ELISA. A standard curve (figure 3.10) was prepared from the CRT dilutions and was used to estimate the protein levels in synovial fluids and serum/plasma of RA patients (Table 3.2 and figure 3.12). The calreticulin levels in synovial fluid aspirates of patients diagnosed with rheumatoid arthritis range from 1840ng/ml to 40,000ng/ml. In serum/plasma samples of patients diagnosed with rheumatoid arthritis, calreticulin levels range from 960-9,000ng/ml with most of the samples ranging from 1000-3500ng/ml.

Finally, in synovial fluid aspirates from patients diagnosed with osteoarthritis, the levels of calreticulin range from 640ng/ml to 4,000ng/ml (Table 3.3) and as shown in figure 3.13, where there is a comparison between the levels of calreticulin in OA synovial fluid aspirates and standard serum (from healthy individuals), OA synovial fluids contain normal levels of calreticulin.

The above results (calreticulin levels in RA synovial fluid samples, OA synovial fluid samples and RA serum samples) are summarised in Figure 3.14

Table 3.2 The following table shows the levels of CRT in synovial fluid aspirates and in serum/plasma samples of patients diagnosed with rheumatoid arthritis.

RA patient number	[CRT] in SF (ng/ml)	[CRT] in serum/plasma (ng/ml)
1	6100	6400
2	17600	8480
3	20800	8000
4	16000	3500
5	22400	3250
6	20800	3800
7	6400	2880
8	3200	1920
9	1840	1560
10	17920	3400
11	16160	1840

12	2400	960
13	12800	2200
14	4000	5760
15	7000	3360
16	5760	1980
17	40000	6080
18	6400	-
19	4960	-
20	3000	-
21	6720	-
22	16000	-
23	3360	-
24	15880	-
25	16320	-
26	7520	-
27	18800	-
28	16640	-
29	16480	-
30	4000	-
31	20800	-
32	>20000	-
33	-	1760
34	-	1440
35	-	960
36	-	2080
37	-	1920
38	-	1920
39	-	1600
40	-	1360
41	-	1440
42	-	1360

Table 3.3 The following table shows CRT levels in synovial fluid samples of patients diagnosed with osteoarthritis (2-7, 9-15) and patients diagnosed with other types of arthritis (16, 1 and 8).

Patient number	[CRT] in SF (ng/ml)
1 (psoriatic arthritis)	3000
2	1840
3	4000
4	2400
5	2800
6	3200
7	640
8 (spondiloarthritis)	14400
9	1280
10	1920
11	1900
12	1900
13	2000
14	2080
15	3360
16 (arthritis pseudogout)	2800

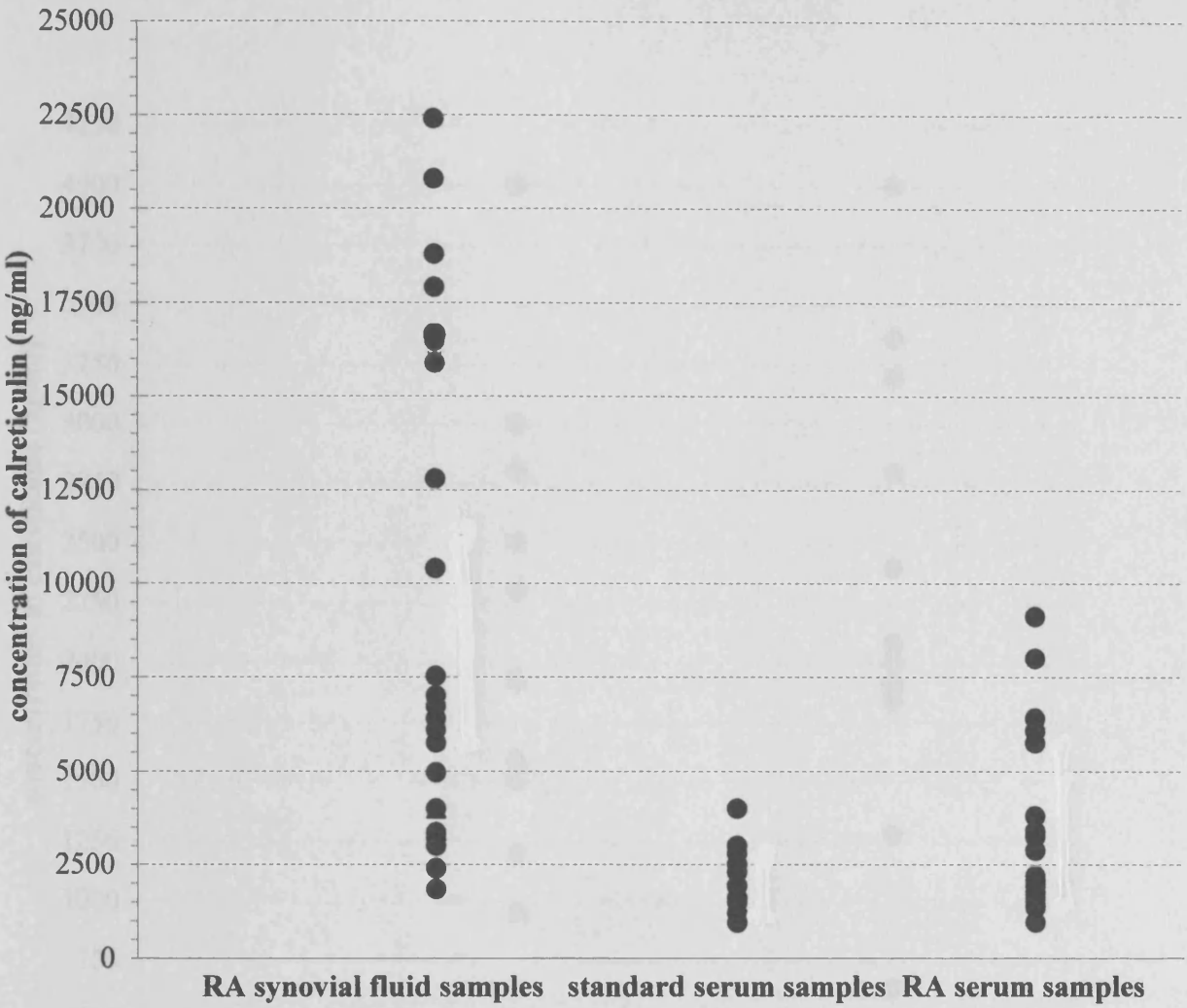


Figure 3.12 Calreticulin levels in serum samples of healthy individuals and serum samples/synovial fluid aspirates of patients diagnosed with rheumatoid arthritis (RA).
Calreticulin levels in serum samples of healthy individuals (standard serum samples) range from 950ng/ml to 4000ng/ml. The levels in serum samples of patients diagnosed with RA range from 960 ng/ml to 9000 ng/ml (Table 3.2). The levels in synovial fluid aspirates of RA patients range from 1840 ng/ml to 40,000 ng/ml (the sample containing 40,000ng/ml calreticulin is not shown in this figure-Table 3.2).
Points represent means of duplicates from one of several experiments.

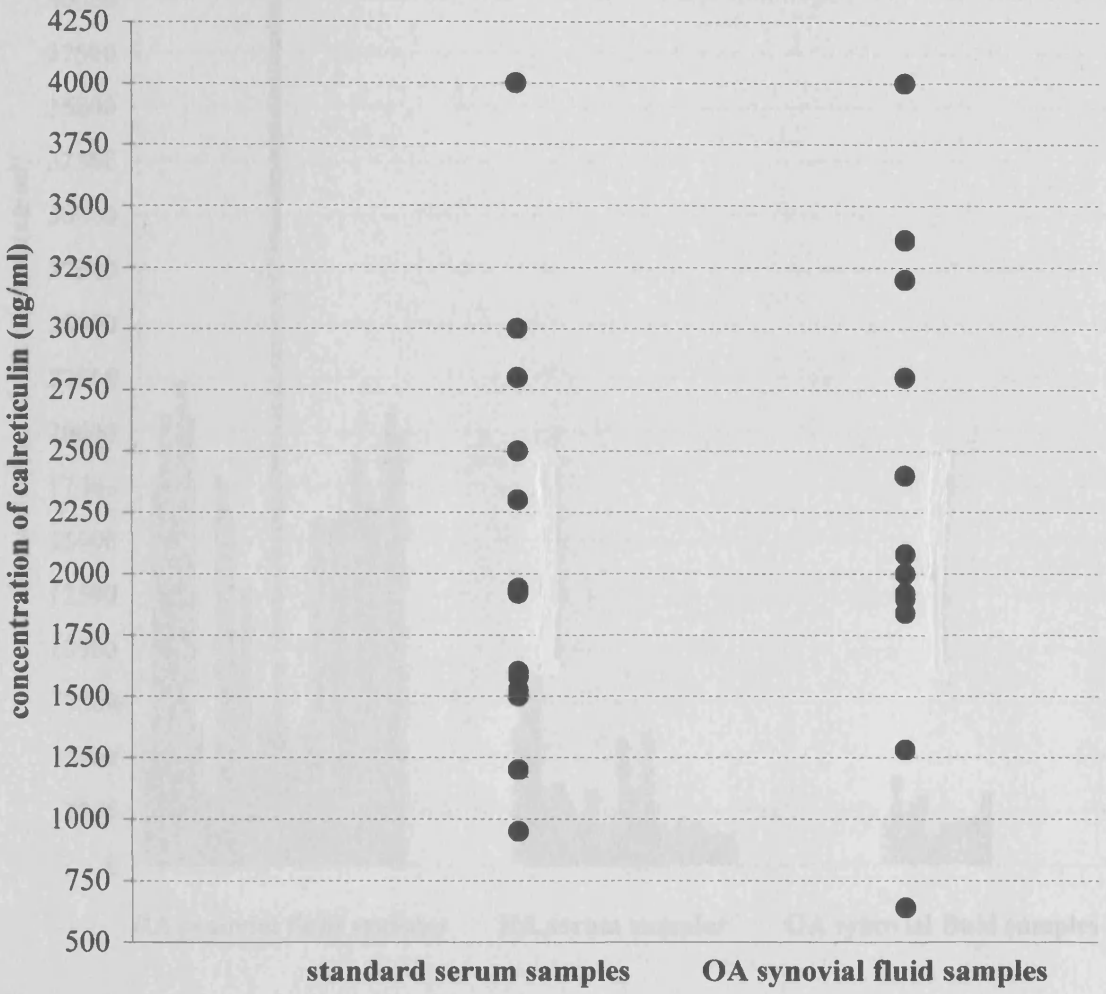


Figure 3.13 Calreticulin levels in healthy individuals (standard serum samples) and in synovial fluids of patients diagnosed with osteoarthritis (OA).

Calreticulin levels in serum samples of healthy individuals (standard serum samples) range from 950ng/ml to 4000ng/ml. Calreticulin levels in OA synovial fluid aspirates range from 640ng/ml to 4,000ng/ml.

Points represent means of duplicates from one of several experiments.

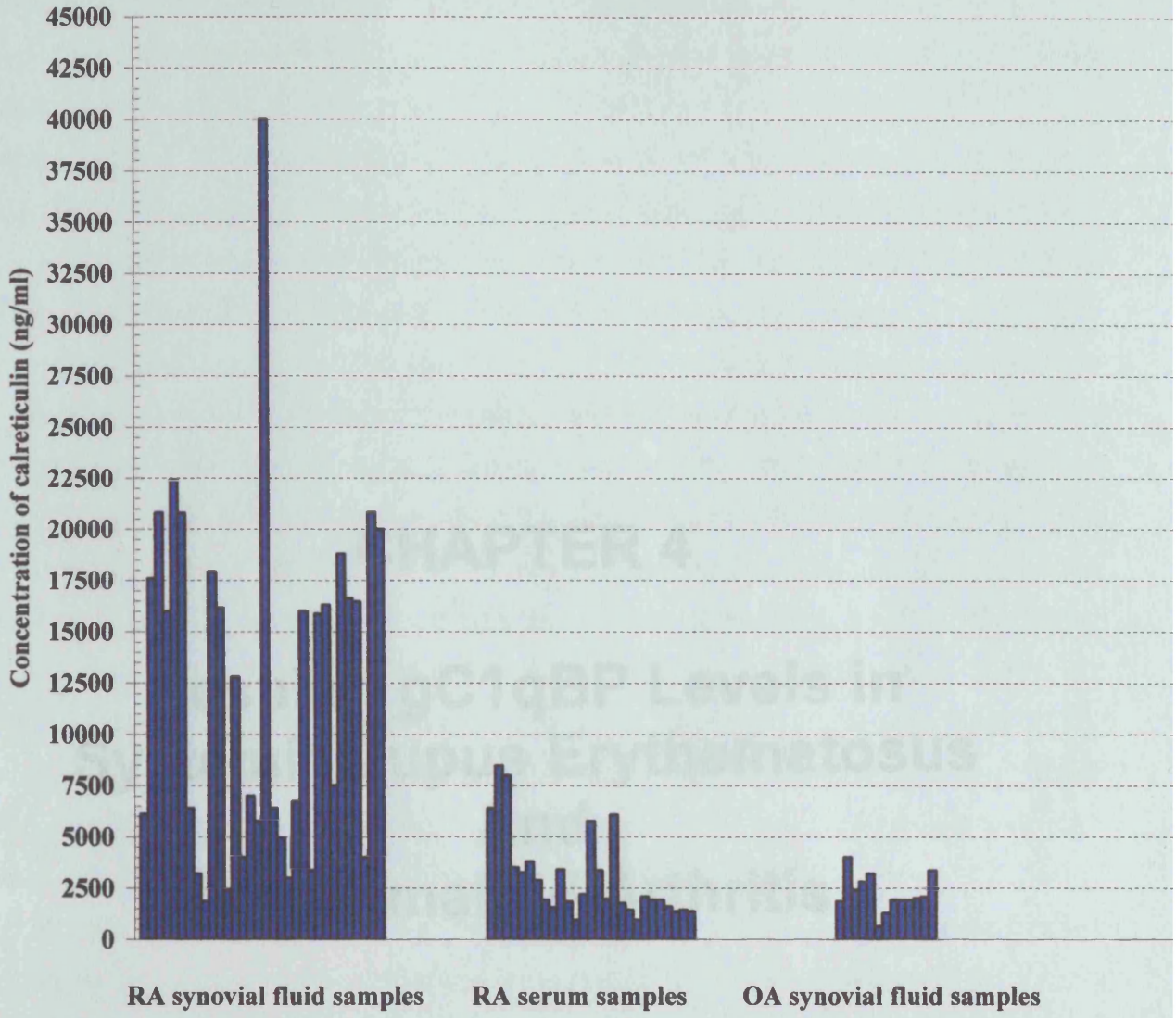


Figure 3.14 Calreticulin levels in synovial fluid aspirates and serum/plasma of patients diagnosed with rheumatoid arthritis (RA) and synovial fluid aspirates of patients diagnosed with osteoarthritis (OA).

RA serum/plasma samples and OA synovial fluid aspirates have physiological levels of calreticulin (1000-5000ng/ml). RA synovial fluid aspirates have increased levels of calreticulin starting from 2,500 ng/ml to 40,000 ng/ml.

CHAPTER 4

Results: gC1qBP Levels in Systemic Lupus Erythematosus And Rheumatoid Arthritis

Levels of gC1qBP in rheumatoid arthritis and systemic lupus erythematosus

Even from experiments that date back almost 30 years when the first indication of the presence of C1q binding sites/receptors on lymphocytes was first demonstrated (Dickler and Kunkel 1972) it was apparent that the role of C1q is not limited to the recognition and triggering of the classical pathway, but that C1q itself may directly mediate immune effector functions and thus play a significant role in inflammation (Ghebrehiwet and Muller-Eberhard 1978; Ghebrehiwet and Peerschke 1998). Indeed, subsequent reports from several laboratories have shown that the interaction of C1q with cell surface structure(s) or receptors induces a wide range of biological functions, including phagocytosis, chemotaxis, and the generation of pro-coagulant activity (Ghebrehiwet and Peerschke 1998; Peerschke and Ghebrehiwet 1998; Sim, Moestrup *et al.,* 1998). The interaction of C1q with cells induces a wide range of biologic responses, which include inositol-1,4,5-trisphosphate (IP3) production and generation of pro-coagulant activity on platelets (Peerschke, Reid *et al.,* 1993); chemotaxis on mast cells (Ghebrehiwet, Kew *et al.,* 1995), eosinophils (Kuna, Iyer *et al.,* 1996), neutrophils (Eggleton, Reid *et al.,* 1998) and fibroblasts (Oiki and Okada 1988); generation of antiproliferative response on B and T cells (Ghebrehiwet, Habicht *et al.,* 1990; Chen, Gaddipati *et al.,* 1994); and enhancement of CR1-mediated phagocytosis and superoxide production by neutrophils (Guan, Burgess *et al.,* 1991; Goodman and Tenner 1992). Although the specific role of gC1qBP in many of these responses is still being worked out, its ubiquitous expression, including expression in breast cancer cell lines MCF7 and SKBr3 and in glial cells (Ghebrehiwet, Lim *et al.,* 2001), predicts that gC1qBP may play a significant role in specific ligand-mediated responses.

gC1qBP is predominantly found associated intracellularly with the mitochondria and the nucleus. In addition to C1q, gC1qBP also binds key proteins of the blood coagulation system such as thrombin (Ghebrehiwet and Peerschke 1998), fibrinogen (Lu, Galanakis *et al.,* 1999), vitronectin (Lim, Reid *et al.,* 1996), and high molecular weight kininogen (Joseph *et al.,*, 1996). Recent evidence also suggests that gC1qBP binds to proteins on the surface of pathogenic microorganisms such as protein A of *Staphylococcus aureus* (Nguyen, Ghebrehiwet *et al.,* 2000) and internalin B (InlB) of *Listeria monocytogenes* (Braun, Ghebrehiwet *et al.,* 2000). Conversely, intracellular gC1qBP has been shown to bind the cytoplasmic tail of the [alpha]1B-adrenergic receptor (Xu, Hirasawa *et al.,* 1999) and to PKCμ.

In this work, we cloned and purified the rat gC1qBP and then using this recombinant protein as a standard we determined the levels of gC1qBP in serum samples from healthy individuals and compared them with synovial fluid samples/sera from patients diagnosed with

RA, synovial fluid samples from patients diagnosed with OA and serum samples from patients diagnosed with SLE.

4.1 Cloning, expression and purification of rat gC1qBP protein

Recombinant rat gC1qBP was prepared in order to produce anti-rat antibodies with the aim to measure the levels of the protein in sera of patients diagnosed with systemic lupus erythematosus and in the synovial fluid aspirates/sera of patients diagnosed with rheumatoid arthritis.

Clone RB7.4 [lambda ZAP II rat brain cortex library- (Stratagene); Lynch, Reid *et al.*, 1997], which contained the ORF of rat gC1qBP, was used as a template for PCR with primers gC1qBPsense and gC1qBPantisense (materials and methods section 2.5) (Figure 4.1). The sense primer (gC1qBPsense) includes a *Bam*HI restriction site designed to allow in-frame subcloning into the expression vector pRSET-C. The antisense primer (gC1qBPantisense) includes an *Eco*RI restriction site for subcloning into the expression vector.

The expected 859bp long PCR product was obtained, run on an agarose gel, the DNA band was extracted using the Ultra-Silica DNA Purification kit (section 2.1.8), ligated into pGEMT-Easy and transformed into chemically competent *E. coli* TOP10F'. Plasmid DNA minipreps were prepared, digested with *Bam*HI and *Eco*RI and separated on an agarose gel. After electrophoresis the 859bp fragment was cut from the gel and extracted using the Ultra-Silica DNA Purification kit (section 2.1.8). Plasmid pRSET-C was digested with *BamH*I and *Eco*RI. The linearised vector and the gC1qBP fragment were ligated and transformed into chemically competent *E. coli* strain TOP10F', plated on LA plates containing 50μg/ml ampicillin and incubated overnight at 37°C. Plasmid DNA was isolated from a single recombinant colony and the DNA was transformed into *E.coli* expression strain BL21(DE3)pLysS, plated on LA plates containing 50μg/ml ampicillin and 35μg/ml chloramphenicol and incubated overnight at 37°C. Plasmid was isolated from a single colony and sequenced (section 2.1.11) with primers T7 and pRSET reverse (section 2.5), to confirm that the insert was cloned in frame.

A single colony of *E. coli* BL21(DE3)pLysS harbouring the appropriate pRSET-C construct was inoculated into LB medium containing 50μg/ml ampicillin and 35μg/ml chloramphenicol and grown overnight at 37°C (overnight culture). 2L Erlenmeyer flasks each containing 500ml of LB without antibiotics were inoculated with 8 ml of the overnight culture. Protein expression was induced in late-log phase by addition of 1 mM IPTG for 3 hours at 37°C. The cells were pelleted by centrifugation at 5000 x g for 10 min at 4°C and stored at –20°C for protein purification.

The cell pellet was resuspended in ice-cold buffer containing 20mM Tris, pH 8.0, 0.15M NaCl, 5mM Imidazole, 0.1% Triton X-100 and sonicated at 4°C. The sonicate was centrifuged for 10 min, at 10,000 x g, at 4°C. The cell pellet was resuspended in 6ml buffer containing 20mM Tris pH 8.0, 0.15M NaCl, 5mM Imidazole and 0.2% SDS. As the protein was in the form of inclusion bodies, we chose to use 0.2% SDS to solubilise it. After incubation for 30 min at RT-shaker the sample was centrifuged and the supernatant was applied on a Ni^{++} charged iminodiacetic acid-Sepharose column (5 ml Hi-Trap Chelating; Amersham Pharmacia Biotech) previously equilibrated with 20mM Tris pH 8.0, 0.15M NaCl, 5mM Imidazole, 0.2% SDS and eluted with an imidazole gradient (figure 4.2). The elution buffer contained 50mM Tris-HCl pH 8.0, 0.5M NaCl, 0.5M Imidazole and 0.2% SDS. The fractions were analysed by SDS-PAGE and those containing the protein were dialysed in two steps against a buffer containing decreasing amounts of SDS to a final buffer containing no SDS. The sample was run on a SDS-PAGE gel (Figure 4.3) and stained as described in the materials and methods (section 2.2.2). The concentration of the recombinant gC1qBP was estimated to be 0.5mg/ml and the preparation was 95% pure.

```
   1 - CGGCACGAGCCGGTCGTCCTTCCCTTTGCACGTGTCCACCCCGCGATGCTCCCTCTGCTG - 60
     - R  H  E  P  V  V  L  P  F  A  R  V  H  P  A  M  L  P  L  L
  61 - CGCTGCGTGCCCCGCGCCCTGGGTGCCGCCGCCACGGGCCTCCGAGCCTCCATCCCGGCC - 120
     - R  C  V  P  R  A  L  G  A  A  A  T  G  L  R  A  S  I  P  A
 121 - CCGCCGCTTCGGCATTTACTGCAGCCCGCGCCCCGGCCATGCCTCCGGCCCTTCGGTTTG - 180
     - P  P  L  R  H  L  L  Q  P  A  P  R  P  C  L  R  P  F  G  L
 181 - CTCAGCGTACGAGCCGGCTCCGCTCGGCGCTCTGGCCTCCTGCAGCCCCCAGGTTCCTGC - 240
     - L  S  V  R  A  G  S  A  R  R  S  G  L  L  Q  P  P  G  S  C
 241 - GCGTGCGCTGCGGCTCTGCACACGGAAGGAGACAAGGCCTTTGTTGAATTTTTGACTGAT - 300
     - A  C  A  A  A  L  H  T  E  G  D  K  A  F  V  E  F  L  T  D
 301 - GAAATTAAGGAAGAAAAGAAGATCCAGAAGCATAAGTCCCTTCCCAAAATGTCTGGAGAT - 360
     - E  I  K  E  E  K  K  I  Q  K  H  K  S  L  P  K  M  S  G  D
 361 - GGGGAGCTGGAAGTGAACGGCACGGAGGCTAAATTATTGCGCAAAGTTGCCGGAGAAAAG - 420
     - G  E  L  E  V  N  G  T  E  A  K  L  L  R  K  V  A  G  E  K
 421 - ATCACTGTCACTTTCAACATTAACAATAGCATCCCTCCAACCTTTGATGGTGAAGAGGAG - 480
     - I  T  V  T  F  N  I  N  N  S  I  P  P  T  F  D  G  E  E  E
 481 - CCCTCACAGGGGCAGAAGGCCGAAGAGCAGGAGCCAGAACTGACATCAACTCCCAATTTT - 540
     - P  S  Q  G  Q  K  A  E  E  Q  E  P  E  L  T  S  T  P  N  F
 541 - GTGGTGGAAGTGACAAAGACTGATGGCAAGAAGACCCTTGTACTGGACTGCCACTATCCT - 600
     - V  V  E  V  T  K  T  D  G  K  K  T  L  V  L  D  C  H  Y  P
 601 - GAGGACGAGATCGGACACCAAGATGAGGCCGAGAGTGACATTTTCTCTATTAAGGAAGTG - 660
     - E  D  E  I  G  H  Q  D  E  A  E  S  D  I  F  S  I  K  E  V
 661 - AGCTTTCAGACCACTGGTGACTCTGAGTGGAGGGATACAAACTACACACTCAACACAGAC - 720
     - S  F  Q  T  T  G  D  S  E  W  R  D  T  N  Y  T  L  N  T  D
 721 - TCCCTGGACTGGGCCTTGTATGACCACCTAATGGATTTCCTTGCGGACCGAGGGGTGGAT - 780
     - S  L  D  W  A  L  Y  D  H  L  M  D  F  L  A  D  R  G  V  D
 781 - AACACTTTTGCAGATGAGTTGGTGGAGCTCAGCACAGCCCTGGAGCACCAGGAATATATC - 840
     - N  T  F  A  D  E  L  V  E  L  S  T  A  L  E  H  Q  E  Y  I
 841 - ACCTTTCTTGAGGACCTCAAAAGCTTTGTCAAGAGTCAGTAGAACTGTGAGACTGAGGGC - 900
     - T  F  L  E  D  L  K  S  F  V  K  S  Q  *  N  C  E  T  E  G
 901 - TCTAATATAAACGGCCAGCTCTCCTGGGCGAGCAAAAGCTGCCTTGACCTCACAACTATG - 960
     - S  N  I  N  G  Q  L  S  W  A  S  K  S  C  L  D  L  T  T  M
 961 - CTTTGCCCTGGCTGTCATCCTAATATATGGGGGAAAGCAAGTTTAAATTATCGCCGTTAC - 1020
     - L  C  P  G  C  H  P  N  I  W  G  K  A  S  L  N  Y  R  R  Y
1021 - ACCTCCATTTACGATTCCTTTGGGCTCTTTTCCTGTACACATCTATTATTTGTAGATTTT - 1080
     - T  S  I  Y  D  S  F  G  L  F  S  C  T  H  L  L  F  V  D  F
1081 - TGTAGTACATGATGATGAACAATAAACTGACTTCCTCGTGCCGA - 1124
     - C  S  T  *  *  *  T  I  N  *  L  P  R  A
```

Figure 4.1 cDNA sequence and predicted amino acid sequence of rat gC1qBP.

The sequence shown in blue corresponds to the mature protein. The sequences shown in red correspond to the annealing sites of the primers (gC1qBPsense and gC1qBPantisense) used for cloning into pRSETC.

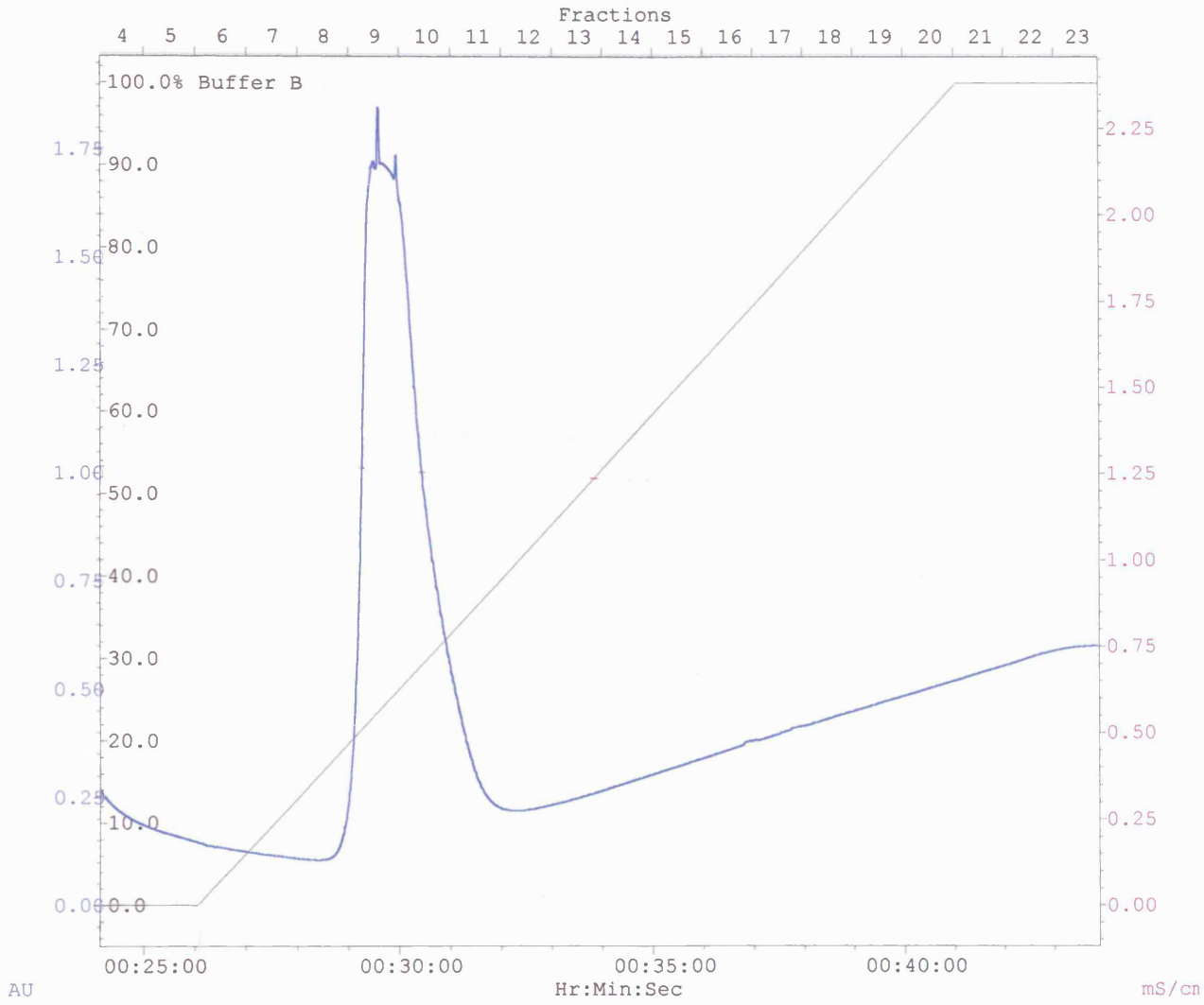


Figure 4.2 Elution profile of gC1qBP purification on a Ni^{++} charged HiTrap Chelating column.

Protein is contained in fractions 8, 9 and 10. The solid blue line indicates the absorbance at 280 nm. The protein was eluted with a linear gradient from 5mM to 500mM Imidazole as indicated by the black line.

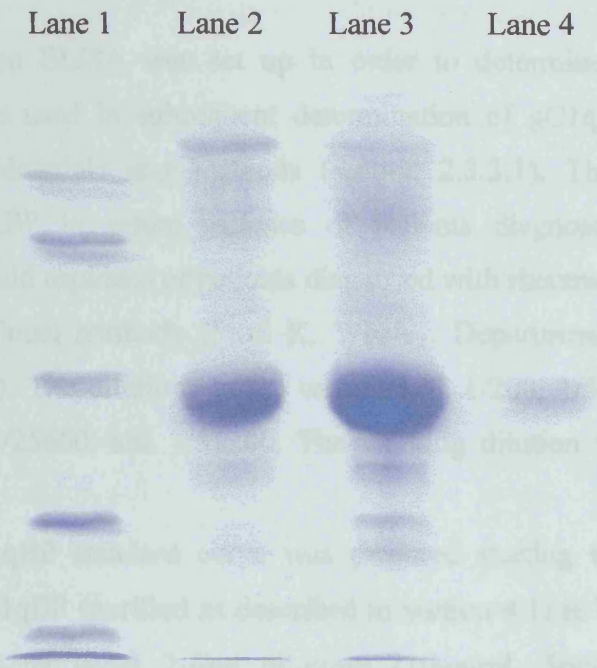


Figure 4.3 gC1qBP fractions after purification using HiTrap chelating column

Lane 1: Biorad low molecular weight protein marker, Lane 2: elution fraction 8, Lane 3: elution fraction 9, Lane 4: elution fraction 10.

4.2 gC1qBP levels in synovial fluid aspirates/sera of patients diagnosed with rheumatoid arthritis and in sera of patients diagnosed with systemic lupus erythematosus.

An inhibition ELISA was set up in order to determine the appropriate antibody concentration to be used in subsequent determination of gC1qBP levels. The method is described in the Materials and Methods (section 2.3.3.1). The antiserum used for the detection of gC1qBP in serum samples of patients diagnosed with SLE, and serum samples/synovial fluid aspirates of patients diagnosed with rheumatoid arthritis was the rabbit anti-gC1qBP polyclonal antibody (Prof. K. Whaley, Department of Microbiology, Kuwait University, Kuwait). The dilutions tested were 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200, 1/6400, 1/12800, 1/25600, and 1/51200. The working dilution was estimated to be 1/300 (Figure 4.4).

a. The gC1qBP standard curve was prepared starting with 50µl of 10 µg/ml of recombinant rat gC1qBP (purified as described in section 4.1) in blocking buffer (PBS/0.1% tween20) followed by serial 2-fold dilutions (10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml, 625ng/ml, 312.5ng/ml, 157ng/ml, 78ng/ml, 39ng/ml, 19.5ng/ml).

b. For measuring the protein levels in serum, serial 2-fold dilutions of serum were prepared, starting with 1:2 dilutions in blocking buffer (1:2, 1:4, 1:8, 1:16, 1:32). The levels of gC1qBP in healthy serum were expressed in ng/ml as well as in OD_{405} units and the latter was used to compare with the levels of gC1qBP in serum samples of patients diagnosed with SLE and serum samples/synovial fluids of patients diagnosed with rheumatoid arthritis.

4.2.1 gC1qBP levels in serum samples of patients diagnosed with systemic lupus erythematosus.

Initially gC1qBP levels were measured in sera from healthy controls. For this, dilutions of serum and dilutions of purified protein (recombinant rat gC1qBP) were tested with a competition ELISA. A standard curve was prepared from the rec gC1qBP dilutions (Figure 4.5) and was used to estimate the protein levels in the healthy control serum. 15 samples of serum from different healthy individuals were used and the range of gC1qBP in serum was found to be 1680-3000 ng/ml, a normal distribution around the mean of 1920ng/ml. Sera from patients diagnosed with SLE were compared with a standard serum (containing 1920ng/ml gC1qBP) by measuring the OD at 405 nm. Any decrease observed in the OD_{405} of the SLE sera when compared to control sera (Table 4.1), corresponds to increase in gC1qBP levels. This is shown in Figure 4.6.

Table 4.1 The following table shows the OD_{405} units for the gC1qBP levels in sera of patients diagnosed with systemic lupus erythematosus, the OD_{405} units in control serum and the relative concentration of gC1qBP in the SLE samples (normalised to the standard serum, SLE OD_{405} / Control OD_{405}).

SLE sample No.	SLE OD_{405} (1/32 dilution)	Control OD_{405} (1/32 dilution)	Relative concentration
1	0.50	0.548	0.91240876
2	0.458	0.548	0.83576642
3	0.44	0.548	0.80291971
4	0.513	0.548	0.93613139
5	0.487	0.548	0.88868613
6	0.446	0.548	0.81386861
7	0.419	0.548	0.76459854
8	0.482	0.548	0.87956204
9	0.485	0.548	0.8850365
11	0.605	0.6055	0.99917424
13	0.404	0.6055	0.66721718
14	0.515	0.6055	0.85053675
15	0.5825	0.6055	0.96201486
16	0.602	0.6055	0.99421965
17	0.536	0.6055	0.88521883
18	0.511	0.6055	0.84393064
19	0.506	0.6055	0.835673
21	0.4	0.485	0.82474227
23	0.4135	0.485	0.85257732
24	0.422	0.485	0.87010309
26	0.5235	0.485	1.07938144
27	0.396	0.485	0.81649485
28	0.446	0.485	0.91958763
29	0.449	0.485	0.9257732
25	0.4175	0.491	0.8503055
30	0.437	0.491	0.89002037
31	0.49	0.491	0.99796334
32	0.420	0.491	0.85539715

33	0.46	0.491	0.93686354
36	0.4245	0.491	0.86456212
37	0.508	0.491	1.03462322
38	0.397	0.491	0.80855397
34	0.38	0.4075	0.93251534
35	0.46	0.4075	1.12883436
40	0.47	0.4075	1.15337423
41	0.367	0.509	0.72102161
42	0.409	0.509	0.80353635
43	0.4055	0.509	0.79666012
44	0.342	0.509	0.6719057
45	0.375	0.509	0.7367387
46	0.41	0.509	0.80550098
47	0.523	0.509	1.02750491
48	0.465	0.509	0.91355599
49	0.427	0.509	0.8388998
50	0.33	0.38	0.86842105
51	0.343	0.38	0.90263158
52	0. 335	0.38	0.881578
53	0.4	0.38	1.052
54	0.34	0.38	0.89473684
55	0.351	0.38	0.92368421
56	0.486	0.38	1.27894737
57	0.43	0.38	1.13157895
58	0.41	0.38	1.07894737
59	0.46	0.59	0.77966102
60	0.34	0.59	0.57627119
61	0.343	0.59	0.58135593
62	0.364	0.59	0.61694915
63	0.489	0.59	0.82881356
64	0.43	0.59	0.72881356
65	0.442	0.59	0.74915254
66	0.406	0.59	0.68813559

Statistical analysis: t-test for independent samples

In one-sample t-test, the observed mean (from a single sample) is compared to an expected (or reference) mean of the population (e.g., some theoretical mean), and the variation in the population is estimated based on the variation in the observed sample (T-value).

The test assumes that the data in the two groups (control serum and SLE serum samples) are normally distributed. If the resultant *t* value is statistically significant, then one can conclude that the means in the two groups are different (that is, in the two populations from which the observations where sampled). The *p*-level reported with a *t*-test represents the probability of error involved in accepting our research hypothesis about the existence of a difference. This is the probability of error associated with rejecting the hypothesis of no difference between the two categories of observations (corresponding to the groups) in the population when, in fact, the hypothesis is true. The higher the p-value, the less we can believe that the observed relation between variables in the sample is a reliable indicator of the relation between the respective variables in the population. Results that yield $p \leq 0.05$ are considered borderline statistically significant but this level of significance still involves a high probability of error (5%). Results that are significant at the $p \leq 0.01$ level are commonly considered statistically significant, and $p \leq 0.005$ or $p \leq 0.001$ levels are often called "highly" significant. (http://www.statsoftinc.com/textbook/stathome.html).

Statistical analysis: One-way *t* test

H_0: the mean of SLE serum samples=1(standard serum)

H_A: the mean of SLE serum samples≠1(standard serum)

Variable	N	Mean	StDev	SE Mean	T	P	95.0% CI
SLE	61	0.8350	0.1007	0.0129	-12.79	**<0.0001**	(0.8092, 0.8609)

The above results are summarized in Figure 4.6 where there is a comparison between levels of gC1qBP in the healthy controls (standard serum) and levels of gC1qBP in the serum samples of patients diagnosed with SLE. As shown in the graph, the levels of gC1qBP in serum samples of patients diagnosed with SLE are increased by approximately 15% compared to the levels of gC1qBP in serum samples of healthy individuals. This increase is significant with a p-value < 0.0001 and a standard deviation of 0.1007.

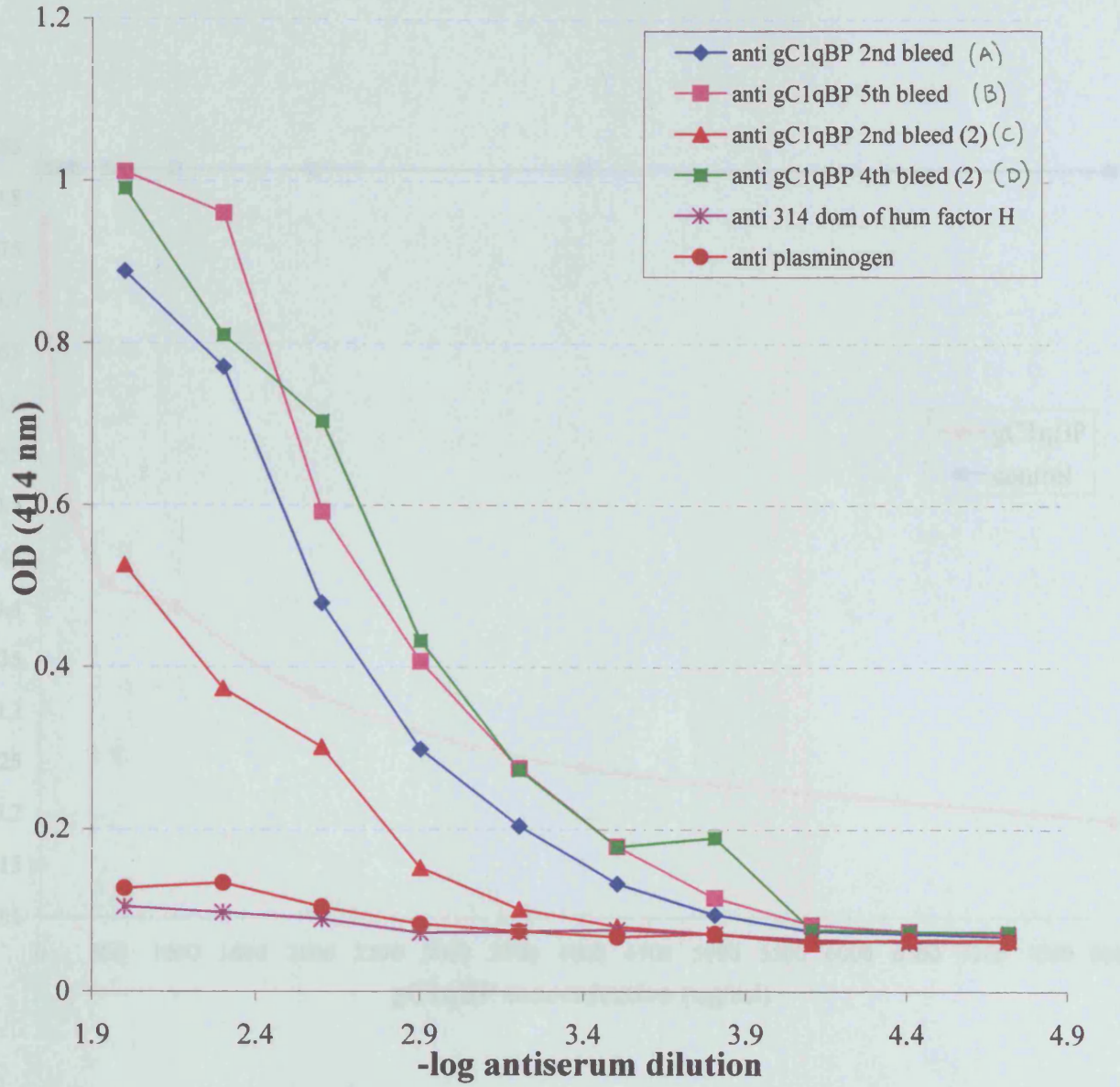


Figure 4.4 Measurement of the titer of the polyclonal antisera raised against rat gC1qBP.

The antigen (rat gC1qBP) was bound to the polystyrene microtiter plate. The antisera raised against gC1qBP (A, B, C, and D) were added to the wells and allowed to bind. To detect binding of the polyclonal antisera to the antigen, a second antibody, anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase, was added. For control, an irrelevant polyclonal antibody raised against human factor H and an irrelevant anti-plasminogen rabbit antiserum were used. The antibody titer of the sera was calculated by plotting absorbance vs. antiserum dilution and was determined by drawing a line down to the x-axis, which corresponded to a dilution of 1/300 (-log1/300=2.477).

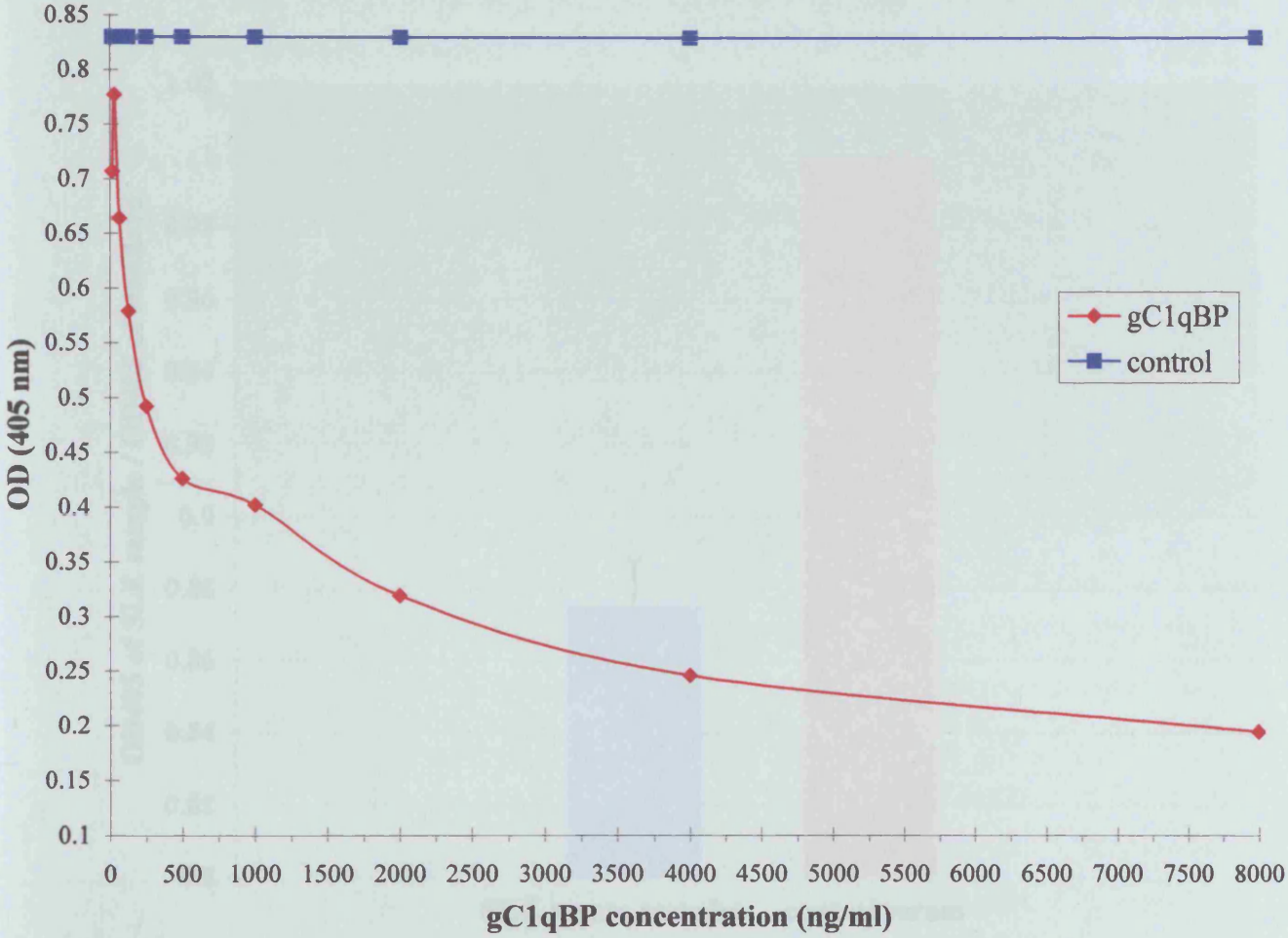


Figure 4.5 gC1qBP inhibitor curve.

50 µl of 8000ng/ml of protein (rat gC1qBP) in blocking buffer (PBS/0.1%tween 20) were serially diluted 2-fold (8000ng/ml, 4000ng/ml, 2000ng/ml, 100ng/ml, 500ng/ml, 250ng/ml, 125ng/ml, 62.5ng/ml, 31.25ng/ml), preincubated with polyclonal anti-gC1qBP and transferred to a gC1qBP-coated plate. The assay is most sensitive in the region of the curve where small changes in inhibitor concentrations produce maximal changes in the amount of inhibition (100-1000ng/ml).

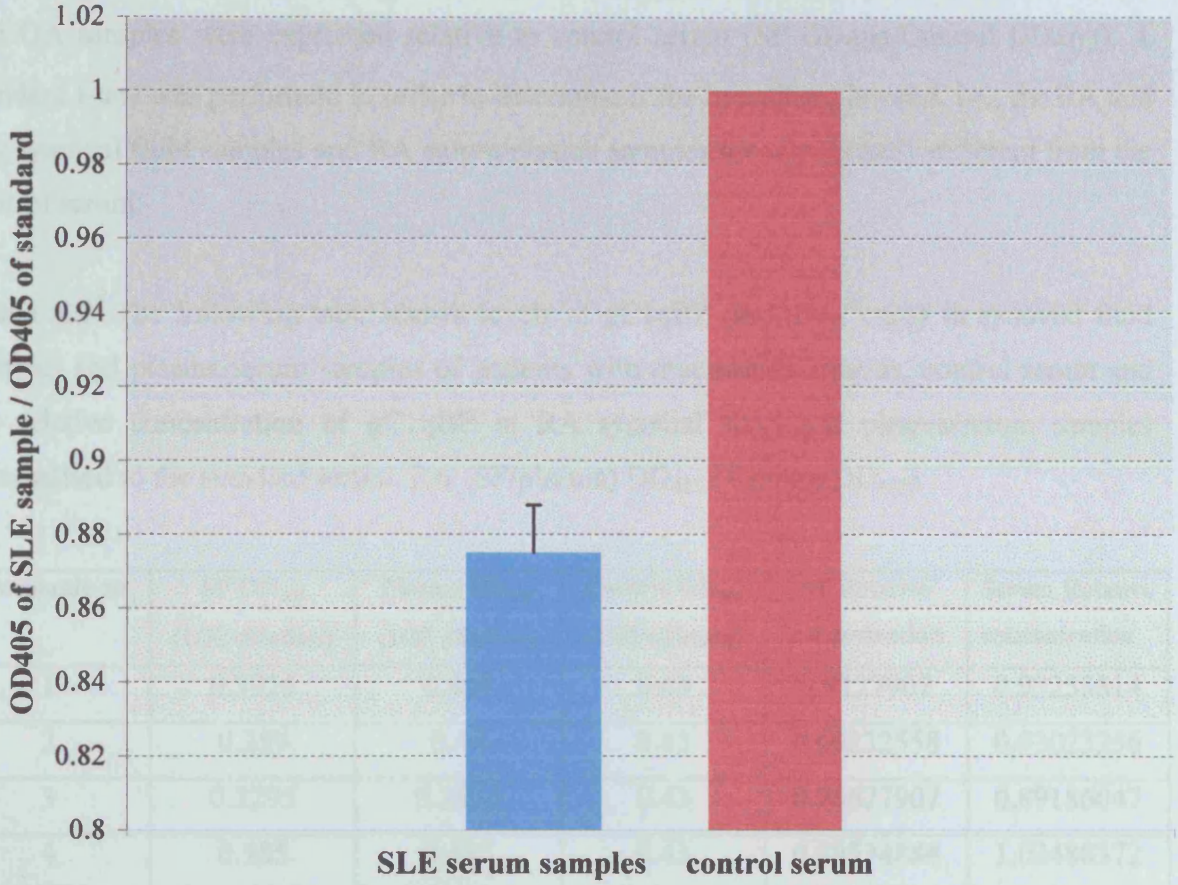


Figure 4.6 Competition ELISA: Detection of gC1qBP levels in sera of patients diagnosed with SLE.

Sera from patients diagnosed with SLE (n=61) compared with a standard serum (the gC1qBP concentration of which corresponds to the mean concentration observed in 15 control sera). The levels of gC1qBP in SLE are expressed as OD_{405} of SLE sample / OD_{405} of standard. Any decrease in OD_{405} of SLE samples compared to control sample, represents an increase in the level of gC1qBP contained in the SLE samples.

4.2.2 gC1qBP levels in synovial fluid aspirates/sera of patients diagnosed with rheumatoid arthritis.

Control serum with known gC1qBP levels (as determined in section 4.2.1) was used to determine gC1qBP levels in synovial fluid and serum/plasma samples from patients with RA and synovial fluid samples from patients with OA. The patient samples were compared to the control serum by measuring the OD_{405} nm. (Table 4.2-4.3 and figures 4.7-4.8). The RA and OA samples were expressed relative to control serum (SF OD_{405}/Control OD_{405}). A standard t-test was performed in order to determine if the hypothesis is valid, i.e., the RA and OA synovial fluid samples and RA serum/plasma samples are significantly different from the control serum.

Table 4.2 The following table shows levels of gC1qBP (in OD_{405} units) in synovial fluid samples and plasma/serum samples of patients with rheumatoid arthritis, control serum and the relative concentration of gC1qBP in RA synovial fluid and plasma/serum samples [normalised to the standard serum, RA (SF/plasma) OD_{405} / Control OD_{405}].

RA sample no	SF OD_{405} (1/32 dilution)	Plasma OD_{405} (1/32 dilution)	Control OD_{405} (1/32 dilution)	SF Relative concentration	Serum Relative concentration
1	0.3925	0.401	0.43	0.9127907	0.93255814
2	0.259	0.40	0.43	0.60232558	0.93023256
3	0.3295	0.3835	0.43	0.76627907	0.89186047
4	0.385	0.445	0.43	0.89534884	1.03488372
5	0.4925	0.4155	0.43	1.14534884	0.96627907
6	0.694	0.6145	0.669	1.03736921	0.91853513
7	0.715	0.63	0.669	1.06875934	0.94170404
8	0.6695	0.6385	0.669	1.00074738	0.95440957
9	0.70	0.609	0.669	1.04633782	0.9103139
10	0.7095	0.7115	0.669	1.06053812	1.06352765
11	0.3915	0.3695	0.354	1.1059322	1.04378531
12	0.45	0.45	0.354	1.27118644	1.27118644
13	0.44	0.42	0.354	1.24293785	1.18644068
14	0.415	0.45	0.354	1.17231638	1.27118644
15	0.47	0.46	0.354	1.32768362	1.29943503
16	0.48	-	0.4	1.2	-
17	0.554	-	0.4	1.385	-

18	0.54	-	0.4	1.35	-
19	0.52	-	0.4	1.3	-
20	0.455	-	0.4	1.1375	-
21	0.45	-	0.3945	1.14068441	-
22	0.44	-	0.3945	1.11533587	-
23	0.386	-	0.3945	0.97845374	-
24	0.48	-	0.3945	1.21673004	-
25	0.53	-	0.3945	1.34347275	-
26	0.56	-	0.3945	1.41951838	-
27	0.623	-	0.5	1.246	-
28	0.583	-	0.5	1.166	-
29	0.557	-	0.5	1.114	-
30	0.282	-	0.409	0.68948655	-
31	0.392	-	0.409	0.95843521	-
32	0.4	-	0.409	0.97799511	-
33	-	0.2778	0.3257	-	0.85293215
34	-	0.3502	0.3257	-	1.0752226
35	-	0.3761	0.3257	-	1.15474363
36	-	0.3072	0.3257	-	0.94319926
37	-	0.3656	0.3257	-	1.12250537
38	-	0.3128	0.3257	-	0.960393
39	-	0.3935	0.3257	-	1.20816702
40	-	0.2899	0.3257	-	0.8900829
41	-	0.3632	0.3257	-	1.11513663
42	-	0.3281	0.3257	-	1.00736874

Statistical analysis: One-way *t* test

One-way *t* test was performed to compare the difference between the control serum and the RA synovial fluids, and between the control serum and the RA serum samples.

H_0: the mean of RA synovial fluids=1(standard serum)

H_A: the mean of RA synovial fluids≠1(standard serum)

Variable	N	Mean	StDev	SE Mean	T	P	95.0% CI
RA-SF	32	1.1056	0.1950	0.0345	3.06	**0.0045**	(1.0352,1.1759)

H_0: the mean of RA serum samples=1(standard serum)

H_A: the mean of RA serum samples≠1(standard serum)

Variable	N	Mean	StDev	SE Mean	T	P	95.0% CI
RA-sera	25	1.0376	0.1331	0.0266	1.41	**0.17**	(0.9826, 1.0925)

It can be seen from the statistical analysis that the RA synovial fluids are different from the control serum and the difference is significant with p=0.0045. The RA serum samples are not statistically different from the control serum with p =0.17

Table 4.3 The following table shows levels of gC1qBP (in OD_{405} units) in synovial fluid samples of patients with osteoarthritis (2-15) and other types of arthritis (1, 8 and 16), in control serum and the relative concentration of gC1qBP in OA synovial fluid samples [normalised to the standard serum, OA (SF/plasma) OD_{405} / Control OD_{405}].

patient number	**SF OD_{405} (1/32 dilution)**	**Control OD_{405} (1/32 dilution)**	**OA SF Relative concentration**
2	0.34	0.398	0.854271
3	0.26	0.398	0.653266
4	0.33	0.398	0.829146
5	0.34	0.398	0.854271
6	0.307	0.398	0.771357
7	0.405	0.398	1.017588
9	0.385	0.409	0.94132
10	0.49	0.409	1.198044
11	0.33	0.409	0.806846
12	0.38	0.409	0.929095
13	0.35	0.409	0.855746
14	0.43	0.409	1.051345
15	0.395	0.409	0.96577
16 (arthritis pseudogout)	0.361	0.409	0.882641
1 (psoriatic arthritis)	0.225	0.398	0.565327
8 (spondiloarthritis)	0.495	0.409	1.210269

Statistical analysis: One-way *t* test

One-way *t* test was performed to compare the difference between the control serum and the OA synovial fluids, and a two-way t test was performed to compare the difference between the OA synovial fluid and the RA synovial fluid samples.

One-way t test:

H_0: the mean of OA synovial fluids=1(standard serum)

H_A: the mean of OA synovial fluids≠1(standard serum)

Variable	N	Mean	StDev	SE Mean	T	**P**	95.0% CI
OA-SF	13	0.9021	0.1383	0.0384	-2.55	**0.025**	(0.8185, 0.9857)

Two-way t test:

H_0: the mean of RA synovial fluid samples is greater than the mean of OA synovial fluid samples.

Variable	N	Mean	StDev	SE Mean
C2	32	1.106	0.195	0.034
C4	13	0.902	0.138	0.038

T	**P**	95.0% CI	Degrees of Freedom	polled StDev
3.42	**0.0007**	(0.083, 0.323)	43	0.181

It can be seen from the results that the levels of gC1qBP in the OA synovial fluids are significantly different (borderline statistically significant difference) from the control serum (p=0.025). As shown by the two-way *t* test, there is a highly significant difference between the levels of gC1qBP in the RA synovial fluid samples and the OA synovial fluid samples (p=0.0007).

The above results for rheumatoid arthritis and osteoarthritis synovial fluids and serum/plasma samples are summarized in Figures 4.7 and 4.8.

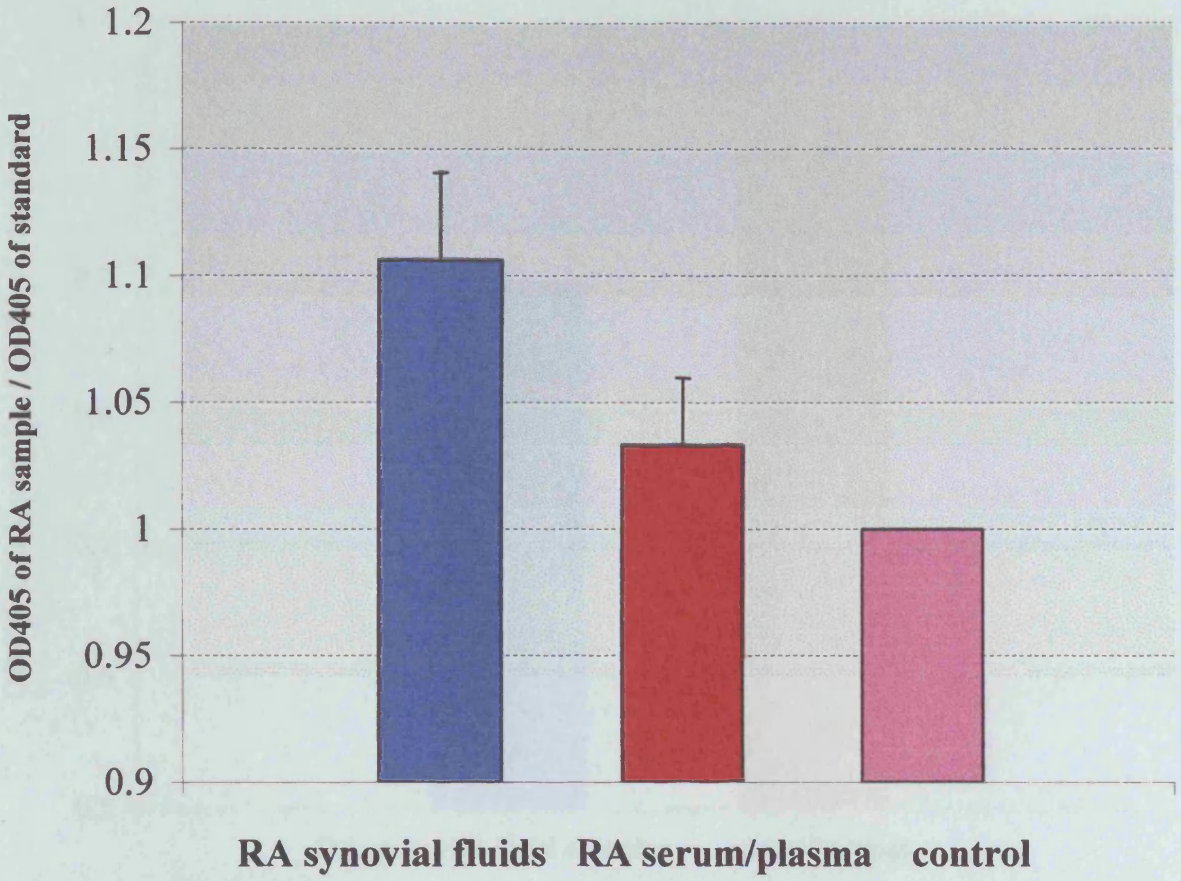


Figure 4.7 Competition ELISA: Detection of gC1qBP levels in synovial fluid samples and serum samples from patients diagnosed with rheumatoid arthritis.

Sera (n=25) and synovial fluid (n=32) samples from patients diagnosed with RA are being compared with a standard serum. The levels of gC1qBP are expressed in OD_{405} of RA sample / OD_{405} of standard. Any increase in OD405 of RA samples compared to control sample (of the same group), represents a decrease in the level of gC1qBP contained in the RA samples of the chosen group. Error bars represent SEM.

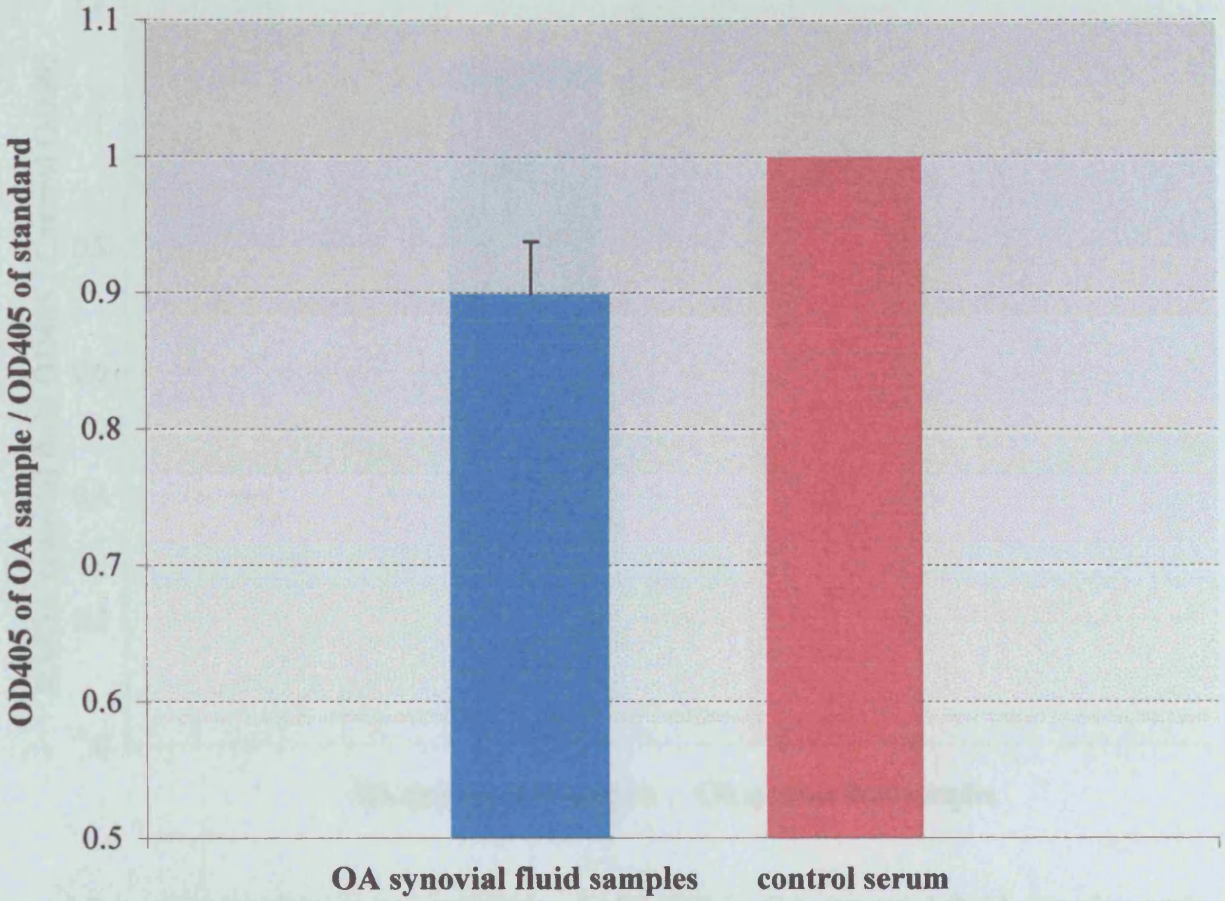


Figure 4.8 Competition ELISA: Detection of gC1qBP levels in synovial fluid from patients diagnosed with osteoarthritis (OA).

Sera from patients diagnosed with OA (n=13) compared to a standard serum (that has a gC1qBP concentration which corresponds to the mean concentration observed in 15 control sera). The levels of gC1qBP are expressed in OD_{405} of OA sample / OD_{405} of standard. Any decrease in OD_{405} of OA samples compared to the standard sample, represents an increase in the level of gC1qBP contained in the OA samples. The x-axis represents OA synovial fluid samples and control serum, the y-axis represents the concentration of gC1qBP expressed relative to control serum. Error bars represent SEM.

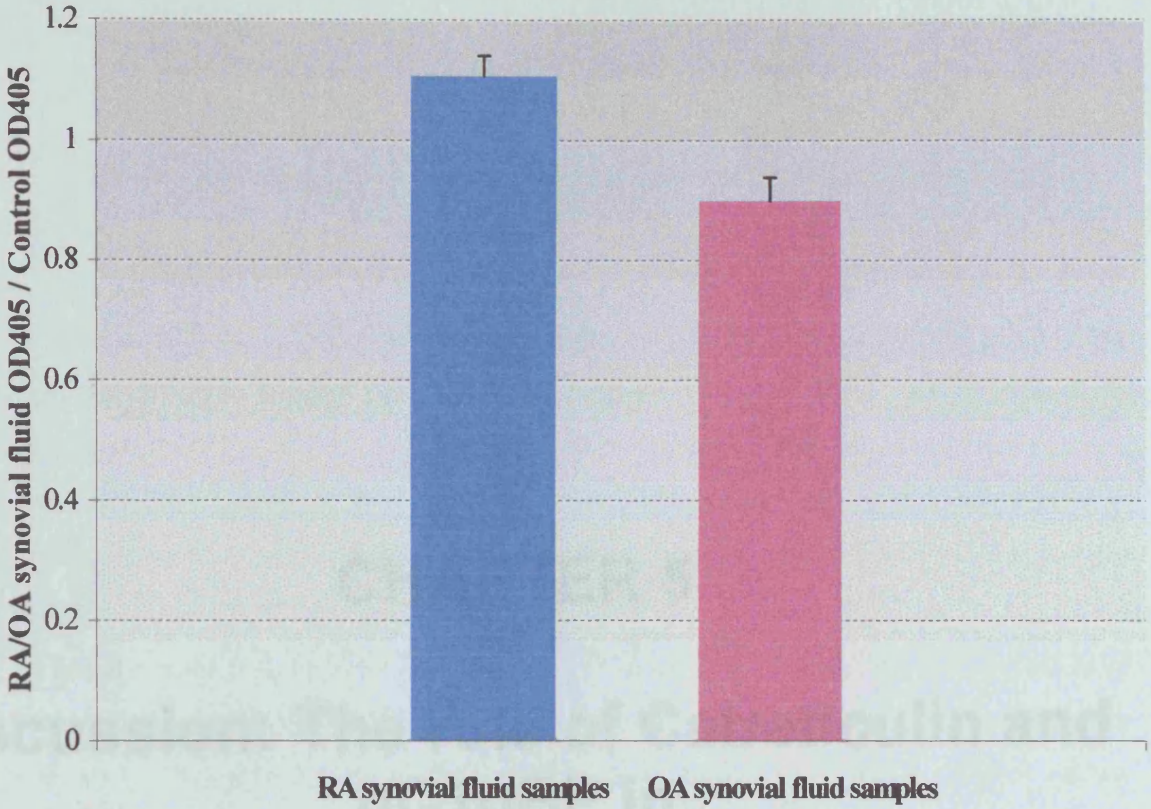


Figure 4.9 Comparison between levels of gC1qBP in OA synovial fluid samples and RA synovial fluid samples.

Synovial fluid (n=32) samples from patients diagnosed with RA are being compared with synovial fluid samples from patients diagnosed with OA (n=13). The x-axis represents the relative concentration of gC1qBP in RA and OA synovial fluid samples (normalised to the standard serum, RA/OA synovial fluid OD_{405} / Control OD_{405}). Error bars represent SEM.

CHAPTER 5

Discussion: The role of Calreticulin and gC1qBP in Systemic Lupus Erythematosus and Rheumatoid Arthritis.

Chapter 5a: Discussion

A possible role for calreticulin in SLE and RA

5.1 Possible involvement of calreticulin in the control of CD8 mediated cytotoxicity.

Naive T cells (mature recirculating T cells that have not yet encountered their antigens) are activated to produce armed effector cells. These are induced to proliferate and differentiate into effector cells of the immune response in an MHC class I-dependent fashion. The activation of naive antigen specific T cells by APC occurs in the lymphoid tissues and organs through which naive T cells (Th0) are constantly passing. The three cell types that can serve, as APCs are dendritic cells, macrophages and B cells. The most distinctive feature of APCs is the expression of co-stimulatory molecules, of which the B7.1 and B7.2 molecules are the best characterised (Janeway, Walport *et al.,* 2001).

The activation of T cells by APCs leads to their proliferation and the differentiation of their progeny into armed effector T cells. This depends on the production of cytokines, in particular the T cell growth factor IL-2, which binds to a high affinity receptor on the activated T cell. Effector T cells can mediate a variety of functions. Their most important functions are the killing of infected cells by CD8 cytotoxic T cells and the activation of macrophages by Th1 cells, which together make up cell-mediated immunity, and the activation of B cells by both Th2 and Th1 cells to produce different classes of antibody, thus driving the humoral immune response (Spellberg and Edwards 2001).

The effector molecules produced by armed T cells fall into two broad classes: cytotoxins, which are stored in specialised lytic granules and released by cytotoxic CD8 T cells, and cytokines and related membrane-associated proteins, which are synthesised *de novo* by all effector T cells (Spellberg and Edwards 2001).

The lytic granules are modified lysosomes that contain at least two distinct classes of cytotoxic effector proteins that are expressed selectively in cytotoxic T cells. Such proteins are stored in the lytic granules in an active form, but conditions within the granules prevent them from functioning until after their release (Spellberg and Edwards 2001). One of these cytotoxic proteins, known as perforin, polymerises to form transmembrane pores in target cell membranes. The other class of cytotoxic proteins comprises at least three proteases called granzymes, which belong to the same family of enzymes-the serine proteases- as the digestive enzymes trypsin and chymotrypsin (Janeway, Walport *et al.,* 2001). Granules that store perforin and granzymes can be seen in armed CD8 cytotoxic effector cells in tissue lesions (Janeway, Walport *et al.,* 2001). When purified granules from cytotoxic T cells are added to target cells *in vitro*, they lyse the cells by creating pores in the lipid bilayer. The pores consist of polymers of perforin, which is a major constituent of these granules. The pore that is

formed allows water and salt to pass rapidly into the cell. With the integrity of the cell membrane destroyed the cells die rapidly (Janeway, Walport *et al.*, 2001).

It has been suggested that an alternative function of cell-surface calreticulin might be implicated in the upregulation of the immune system. Previous research has shown that in the activated CD8 T cells, calreticulin is targeted to the cytotoxic granules (Burns, Littlefield *et al.*, 1992), where it has been proposed to prevent perforin from forming pores in the granule membrane, either by Ca^{++} chelation (Dupuis, Schaerer *et al.*, 1993) or direct interactions with perforin (Andrin, Pinkoski *et al.*, 1998). Recent work has suggested that calreticulin has a more active role in preventing autolysis of the lymphocyte by binding directly to the cell surface (Fraser, Karimi *et al.*, 2000). Experiments performed on erythrocytes showed that calreticulin bound to the membrane, where it prevented the insertion of perforin and hence prevented cell lysis. The ability of CRT to bind directly to membranes has also been demonstrated in endothelial cells where thrombosis was inhibited by the stimulation of nitric oxide production (Kuwabara, Pinsky *et al.*, 1995).

In the case of rheumatoid arthritis various subsets of T cells including CD4 (+), CD8 (+), CD45RO (+), and CD45RA (+) were shown to have comparable ability to induce synovial fibroblast activation (Yamamura, Gupta *et al.*, 2001). Synovial fibroblasts can also function as accessory cells for T cell activation by superantigens and other stimuli. Highly purified resting T cells, even in the absence of T cell mitogens, can induce activation of synovial fibroblasts when co-cultured for 6-24 h (Yamamura, Gupta *et al.*, 2001).

These results establish an Ag-independent effector function for resting T cells that is likely to be important in inflammatory compartments in which large numbers of T lymphocytes and fibroblasts can come into direct contact with each other. In such cases, the presence of calreticulin in the surface of synovial fibroblasts could prevent their lysis by the CD8 cells as described previously, resulting in control of inflammation.

5.2 Possible involvement of calreticulin in antigen presentation, phagocytosis and complement activation.

5.2.1 Calreticulin in antigen presentation and phagocytosis.

There are two distinct subpopulations of CD4 T cells, these are known as Th1 and Th2 lymphocytes. Th1 cells are the principal mediators of delayed type hypersensitivity. The cytokine chiefly responsible for their proinflammatory effect is IFN-γ. IFN-γ stimulates phagocytosis (Livingston, Appel *et al.*, 1989), oxidative burst (Johnston and Kitagawa 1985), and intracellular killing of microbes (Diamond, Lyman *et al.*, 1991). IFN-γ also upregulates expression of class I and class II major histocompatibility complex (MHC) molecules (Volk,

Gruner *et al.,* 1986; Male, Pryce *et al.,* 1987) on a variety of cells, thereby stimulating antigen presentation to T cells.

Calreticulin has demonstrated the ability to enhance MHC class I presentation (Sadasivan, Lehner *et al.,* 1996) and exhibit an antiangiogenic effect (Cheng, Hung *et al.,* 2001). Newly synthesised MHC class I α chains assemble in the endoplasmic reticulum with a membrane bound protein, calnexin. When this complex binds α-2-macroglobulin receptor (CD91) it dissociates from calnexin, and the partially folded MHC class I molecule then binds to the peptide transporter TAP by interacting with one molecule of the TAP-associated protein tapasin (Figure 5.1). Calreticulin and Erp57 also bind to form part of this complex. The MHC class I molecule is retained within the endoplasmic reticulum until released by the binding of a peptide, which completes the folding of the MHC molecule. Peptides generated by the degradation of proteins in the cytoplasm are transported into the lumen of the endoplasmic reticulum by TAP. Once peptide has bound to the MHC molecule, the petide: MHC complex leaves the endoplasmic reticulum and is transported through the Golgi apparatus to the cell surface.

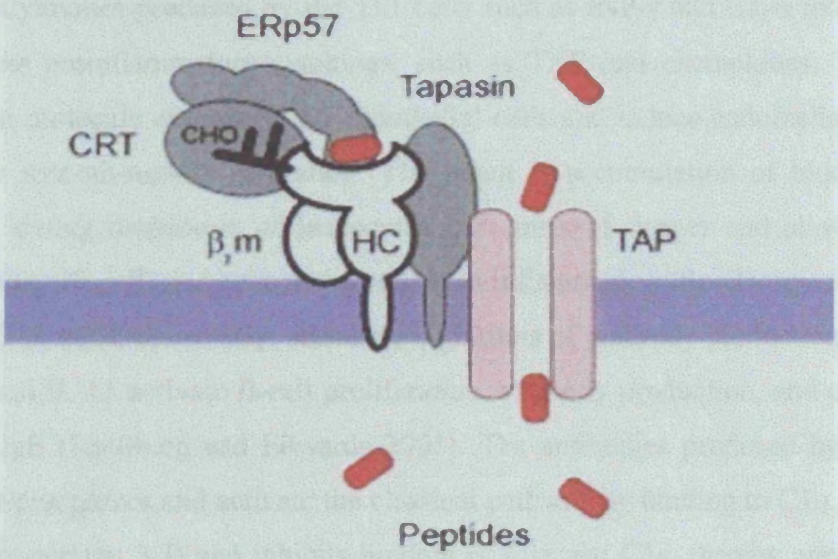


Figure 5.1 A schematic view of the MHC class I peptide loading complex is shown with suggested modes of interaction of the various components indicated. (Adapetd from Immunol Rev (1999), Volume 172: 21-28)

It has been suggested that calreticulin present on macrophages might play a role in the removal of apoptotic cells by macrophages (Ogden, deCathelineau *et al.*, 2001). The hypothesis is that CRT acts to bind the collagenous tails of C1q and MBL and then signals for uptake due to its interaction with CD91 (Ogden, deCathelineau *et al.*, 2001). This is suggested to initiate engulfment of the apoptotic cell by a process leading to concurrent uptake of extracellular fluid and the formation of spacious phagosomes. It can also stimulate engulfment of attached cells. Accordingly, uptake of apoptotic cells by these processes is suggested to occur by macropinocytosis. This mechanism which involves pattern recognition molecules of the innate immune system, a multifunctional cellular protein and an evolutionary conserved clearance receptor, may be an ancient method the body has evolved for ridding itself of a potentially harmful self-antigen and could be of significant importance in the clearance of apoptotic cells in SLE.

5.2.2 Involvement of calreticulin in inflammation through Th1 and Th2 CD4-T cells.

Inhibition of complement activation through the classical pathway

Cytokines produced by the Th1 cells such as INF-γ and LT-α induce other cell types to secrete proinflammatory cytokines, such as TNF and chemokines. They also stimulate adhesion molecule expression on endothelial cells and induce endothelial cell retraction and vascular smooth-muscle relaxation. The result is accumulation of blood in dilated, leaky vessels, easing diapedesis of leukocytes into areas of danger and allowing recruitment of innate immune cells and opsonins resulting in inflammation (Spellberg and Edwards 2001).

Th2 cells, conversely, stimulate high titers of antibody production. In particular, IL-4, IL-10, and IL-13 activate B-cell proliferation, antibody production, and class switching from IgG to IgE (Spellberg and Edwards 2001). The antibodies produced by the Th2 cells bind immune complexes and activate the classical pathway by binding to C1q. CRT binds C1q (as shown in section 3.2) and inhibits binding of C1r and C1s, thereby preventing complement activation and inflammation. In rheumatoid arthritis, activated CD4 T cells stimulate B cells through cell surface contact and through the binding of αLβ2 integrin, CD154, and CD28, to produce rheumatoid factors. The precise role of rheumatoid factors is not known but they are thought to be involved in the activation of complement through the formation of immune complexes (Choy and Panayi 2001). These complexes bind to C1q and activate complement through the classical pathway. Binding of calreticulin to C1q prevents association with C1r and C1s and inhibits complement activation through the classical pathway.

Involvement of intracellular calreticulin in antigen presentation and possible activation of regulatory T cells.

In addition to their stimulatory effects, Th1 and Th2 cells cross-regulate one another. The IFN-γ secreted by Th1 cells directly suppresses IL-4 secretion and thus inhibits differentiation of naive Th0 cells into Th2 cells. Conversely, IL-4 and IL-10 inhibit the secretion of IL-2 and INF-γ, blocking the ability of Th0 cells to polarise into Th1 cells. IL-10 is perhaps the most anti-inflammatory cytokine known. It inhibits the secretion of proinflammatory cytokines, suppresses phagocytosis, the oxidative burst and the intracellular killing, and inhibits antigen presentation to T cells, causing T cell anergy. Like IL-10, IL-4 and IL-13 also inhibit phagocytosis and intracellular killing, suppresses inflammatory cytokine production and may induce T cell anergy (Spellberg and Edwards 2001).

An imbalance between T helper cell (Th) 1 and Th2-like cytokines has been described in several autoimmune diseases. Organ specific autoimmune diseases such as multiple sclerosis (MS) and inflammatory bowel diseases (IBD) are caused by Th1 dominant immune responses (van Roon, van Eden, *et al.,* 1997). On the contrary, systemic autoimmune diseases such as systemic lupus erythematosus (SLE) and Sjogren's syndrome (SS) are characterized by Th2 dominant imbalance of cytokine production (van Roon, van Eden, *et al.,* 1997).

In patients with rheumatoid arthritis, there is a predominance of activated mononuclear cells producing IL-1, TNF-α, and IL-6, cytokines, which are major contributors to inflammatory responses and joint destruction (Nouri, Panayi *et al.,* 1984). Type 1 (Th1) cell activity, which has been shown to predominate in the joints of patients with RA (Schulze-Koops, Lipsky *et al.,* 1995), is known to stimulate macrophages towards production of IL-1 and TNF-α (Romagnani 1994). Regulatory type 2 (Th2) cell cytokines, in particular IL-4 and IL-10, have been shown to suppress T1 responses, IL-1, and TNF-α production and cartilage degradation by RA inflammatory cells from synovial fluid and tissue (van Roon, van Roy *et al.,* 1995). The low level of T2 cell cytokine production in RA has suggested a lack of suppressive T cells in these patients (Schulze-Koops, Lipsky *et al.,* 1995). Therefore, patients with RA may benefit from the activation of regulatory T cells that provide active cellular suppression through production of suppressive cytokines, such as IL-4 and IL-10.

With respect to the induction of suppressive T cells, it has been suggested that heat-shock proteins (hsps) may provide antigenic stimuli leading to the immunomodulation of rheumatoid arthritis (van Roon, van Eden *et al.,* 1997). In an experimental model of arthritis, administration of (human) hsp60 before or during induction of arthritis prevented the induction or enhanced remission of adjuvant arthritis (Lopez-Guerrero, Ortiz *et al.,* 1994). In line with this are the observations on patients with juvenile chronic arthritis in which T cell reactivity to human (self) hsp60 preceded remission of the disease (De Graeff-Meeder, van

der Zee *et al.*, 1991; de Graeff-Meeder, van Eden *et al.*, 1995). The downregulation of experimental and human juvenile chronic arthritis is likely due to recognition of self-hsp60 as (over) expressed in the arthritic (stressed) joints of these subjects (Kleinau, Soderstrom *et al.*, 1991). Expression of self-hsp60 molecules may activate regulatory T cells, which can cause suppression of T cells that induce arthritis (van Roon, van Eden *et al.*, 1997).

As previously discussed, calreticulin has the ability to enhance MHC class I presentation of antigenic peptides (Sadasivan, Lehner *et al.*, 1996). These are recognised by CD4 or CD8 T cells and together with other regulatory molecules such as hsp60, activate regulatory Th2 cells to produce IL-4 and IL-10 that have been shown to suppress Th1 response, IL-1 and TNF-α production and cartilage degradation by RA inflammatory cells from synovial fluid and tissue (van Roon, van Roy *et al.*, 1995).

5.3 Calreticulin levels in healthy serum and in serum/synovial fluid samples from patients with autoimmune diseases (SLE and RA).

In this work we measured levels of soluble calreticulin in healthy serum, in sera from patients diagnosed with systemic lupus erythematosus and in sera/synovial fluid aspirates from patients diagnosed with rheumatoid arthritis. We found that the levels of soluble calreticulin in serum from healthy individuals range from 950ng/ml to 4000ng/ml with a mean value of 2074ng/ml. It was observed that the accuracy of this measurement depended on the condition of the sera with haemolysed sera containing high levels of soluble calreticulin (4000-9000ng/ml) most likely due to externalisation of intracellular calreticulin during cell lysis. In this study, 15 non-haemolysed sera from different healthy controls were used.

The levels of calreticulin in sera from patients diagnosed with SLE range from 700ng/ml to 5120ng/ml with a mean value of 1750ng/ml (figure 3.11). Calreticulin levels in sera from patients with rheumatoid arthritis range from 900ng/ml to 9000ng/ml with a mean value of 3,031ng/ml (figure 3.12). Interestingly, calreticulin levels in synovial fluid samples from patients diagnosed with RA range from 1840 ng/ml to 40,000 ng/ml and for the majority of the samples the levels of calreticulin were found to be more than 6000ng/ml (figure 3.12). Calreticulin levels in synovial fluid samples from patients diagnosed with osteoarthritis (13 samples) were within the normal range, 600ng/ml-4000ng/ml (figure 3.13). Three more synovial fluid samples were included in this study; these were from patients diagnosed with psoriatic arthritis, arthritis pseudogout and spondiloarthritis. The levels of calreticulin in the above samples were 3000ng/ml, 2800ng/ml and 14400ng/ml, respectively.

It can be seen from the above results that calreticulin levels are particularly elevated in the synovial fluid of patients diagnosed with rheumatoid arthritis. In the inflamed synovium

there is hyperproliferation of synovial tissue and infiltration of blood-derived cells. Autoantibodies present in the RA synovium mediate cells lysis, which results in the externalisation of intracellular proteins such as calreticulin into the inflamed synovium. Calreticulin might also be released from these cells as a result of an increased apoptotic event during inflammation. Osteoarthritis is a condition, which is readily distinguished from rheumatoid arthritis. Osteoarthritis is not an autoimmune disease, it's a complex response of joint to aging and to genetic and environmental factors, characterised by degeneration of cartilage, bone remodelling and overgrowth of bone. In osteoarthritis, the levels of calreticulin were found to be within the normal range. It seems that elevated levels of calreticulin is a characteristic feature of RA and is linked to autoimmune conditions.

The levels of calreticulin in the serum samples from SLE patients were found to be within the normal range. As mentioned in the introduction, SLE is an autoimmune disease, which is characterised by autoantibody production and immune complex formation. Calreticulin that is being externalised during apoptosis could be consumed in the removal of apoptotic cells and immune complexes through phagocytosis and complement activation as described in section 5.2. Consumption of calreticulin in complement activation and phagocytosis, explains the normal levels of this protein in serum of patients diagnosed with SLE.

5.4 The role of gC1qBp in clearance of apoptotic cells; Significance of apoptotic cell clearance in inflammation.

Systemic lupus erythematosus (SLE) is arguably *the* prototypic multi-systemic autoimmune disease. The skin, joints, kidneys and the central nervous system are frequent targets of autoimmunity in SLE, although the disease can affect virtually any organ system. While the occurrence of B cell and T cell dysfunction is now established in SLE, the precise pathogenesis of the disease remains unknown. Several alternate hypotheses for the pathogenesis of SLE have been proposed. It is possible, and perhaps quite probable, that several of these proposed mechanisms might be operative simultaneously. These mechanisms include: loss of antigen-specific tolerance with antigen-driven immune response against self-antigens, enhanced B cell responsiveness, enhanced T cell responsiveness, immunity or tolerance to cryptic self-antigens or self-antigens modified by apoptosis associated proteolysis, and/or abnormal clearance of apoptotic antigens and molecular mimicry against self-antigens triggered by microbial agents.

The phagocytosis of apoptotic cells is thought to be important in the resolution of inflammation (Savill 1997). Complement has also been implicated in the phagocytosis of apoptotic cells by the observation that C1q binds specifically to the surface blebs of apoptotic cells (Korb and Ahearn 1997) and that C3 depletion of human serum impairs uptake of apoptotic cells by human monocyte-derived macrophages *in vitro* (Mevorach, Mascarenhas *et al.,* 1998). Binding of C1q to apoptotic cells is mediated via the C1q receptors present on the surface of the cells such as gC1qBP and cC1qR (calreticulin). Apoptotic cells express on their surface blebs many of the autoantigens of SLE (Casciola-Rosen, Anhalt *et al.,* 1994) and they also expose on their cellular membranes the negatively charged phospholipids that are the ligands of antiphospholipid autoantibodies (Fadok, Savill *et al.,* 1992; Casciola-Rosen, Rosen *et al.,* 1996). In addition, there is evidence that the enzymes specific to the cell death program may cleave and modify many autoantigens of SLE, which may reveal cryptic epitopes (Casciola-Rosen, Andrade *et al.,* 1999). These findings suggest that apoptotic cells are a major source of autoantigens in SLE and that an impairment of their physiological clearance may promote the development of autoimmunity. Indeed, macrophages from humans with SLE have been shown to exhibit a reduction in the phagocytic uptake of apoptotic cells *in vitro* (Herrmann, Voll *et al.,* 1998).

One of the characteristics in SLE is the antigen-driven immune response against self-antigens. This results in inflammation due to formation of immune complexes and

complement activation through the classical pathway. C1q receptors (gC1qBP and cC1qR) are able to bind to C1q and inhibit serum haemolytic activity, either in the case of cC1qR by binding to the collagen-like stalks and preventing the assembly of C1 (Ghebrehiwet, Randazzo *et al.,* 1984), or in the case of gC1qBP, by competing for the same binding site on the globular heads of C1q as immune complexes.

5.5 Role of gC1qBP in platelet activation and inflammation

Platelets are formed in the bone marrow from giant polyploid cells called megakaryotes. In hemostasis platelets release potent vasoconstrictors (thromboxane A2 and serotonin) from their intracellular granules, aggregate and form a plug at the site of vessel injury, and provide a surface for the activation of soluble coagulation factors (Wyngaarden, Smith *et al.,* 1992). In addition to their role in primary hemostasis, they participate in inflammatory processes that may contribute to the development of thrombosis, atherosclerosis, and vasculitis (Wyngaarden, Smith *et al.,* 1992).

C1q has been shown to modulate platelet interactions with collagen and immune complexes, and has been identified at sites of vascular injury and inflammation, as well as in atherosclerotic lesions. Platelets express a variety of C1q binding sites, including gC1qBP. A recent paper suggests that platelet adhesion to collagen or other adhesive/matrix protein coated surfaces may be caused by gC1qBP expression, and that aggregated platelets and platelet microparticles shed gC1qBP from platelets following aggregation (Peerschke and Ghebrehiwet 2001).

Platelet microparticles contain the major platelet integrin $alpha_{IIb}/beta_3$ (GPIIb-IIIa) and express other glycoproteins associated with platelet activation and secretion, such as P-selectin and thrombospondin. In addition, microparticles derived from activated platelets function as an important surface for coagulation factors and their regulators and bind annexin V. *In vitro* evidence suggests that both fluid phase and immobilised C1q impact platelet function. Platelets adhesion to C1q-coated surfaces is accompanied by platelet activation (Cazenave, Assimeh *et al.,* 1976) as reflected by induction of the $alpha_{IIb}/beta_3$ integrin, which functions as the platelet fibrinogen receptor (Ghebrehiwet, Lu *et al.,* 1996). Moreover C1q multimers have been found to aggregate platelets (Cazenave, Assimeh *et al.,* 1976). This aggregation is inhibited by antibodies against either the cC1qR (Ghebrehiwet, Lu *et al.,* 1996) or, gC1qBP (Peerschke and Ghebrehiwet 1998). Moreover, platelets aggregation in response to aggregated C1q can be inhibited by both the collagen tail of C1q and the purified globular fraction. In addition, an 18 amino acid sequence representing the amino-terminal of gC1qBP has been shown to inhibit aggregated C1q-induced platelet aggregation and to prevent platelet adhesion to immobilised C1q (Peerschke and Ghebrehiwet 1998). Platelets appear to regulate

gC1qBP expression by limiting expression to activated platelets that are adhering to sites of vascular damage and to microparticles shed from platelets following aggregation in response to strong agonists. On platelets, the gC1qBP may contribute to platelet interactions at sites of vascular injury by recognition of C1q in inflammatory lesions and C1q multimers as part of immune complexes. If gC1qBP was expressed on activated platelets, it could bind C1q and inhibit complement activation by competing for the same binding site on the globular heads of C1q as immune complexes. It has been suggested (but not convincingly shown) by Ghebrehiwet's group that such a mechanism might play a significant role in the control of inflammation at sites of tissue injury as observed in a numerous autoimmune diseases including SLE.

5.6 gC1qBP binds to vitronectin and high molecular weight kininogen.

gC1qBP has been found to bind to the human serum protein vitronectin- a potent inhibitor of complement activation by formation of an inactive terminal complement complex. The binding site lies in the acidic stretch of the N-terminal region of gC1qBP. Including gC1qBP as a vitronectin-binding protein, at least four different types of cellular binding proteins for the adhesion factor have been described; integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ expressed on endothelial and other cells have been implicated in cell adhesion and migration on vitronectin substrate (Preissner 1991; Felding-Habermann and Cheresh 1993) or in the endocytosis of denatured vitronectin, respectively (Panetti and McKeown-Longo 1993). Heparan sulfate proteoglycans on endothelial cells are able to mediate cell adhesion and spreading on immobilized vitronectin (de Boer, de Groot *et al.,* 1993) and possibly mediate uptake, whereas recent data indicate that glycolipid-anchored urokinase receptor serves as an additional high affinity receptor for soluble multimeric forms of vitronectin (Wei, Waltz *et al.,* 1994). As heparin competes for the binding of vitronectin to proteoglycans and $\alpha v\beta 5$ has been described (de Boer, Preissner *et al.,* 1992; Vogel, Lee *et al.,* 1993), possible interference with the gC1qBP interaction may be expected as well. Together, the functional properties of vitronectin as an indirect opsonin (Parker, Frame *et al.,* 1988), a participating component in apoptosis (Savill, Dransfield *et al.,* 1990) and a major complement inhibitor are likely to be expressed in concert with gC1qBP. The distribution pattern of vitronectin and its novel binding protein in the vascular system may very well indicate a functional linkage of gC1qBP expressing cells and vitronectin-rich extracellular matrix. Both components can thereby act in a cooperative fashion in processes of wound healing and immune defence.

gC1qBP has been reported to bind to high molecular weight kininogen (HK) (Chavakis, Kanse *et al.,* 2001). Proteolytic cleavage of single-chain, (HK) by kallikrein releases the short lived vasodilator bradykinin and leaves behind a two-chain, high molecular

weight kininogen (Hka) reported to bind to the β2-integrin Mac-1 on neutrophils and exert anti-adhesive properties by binding to the urokinase receptor and vitronectin. One interaction defining the anti-adhesive properties of kininogen is the binding to the extracellular matrix protein vitronectin (VN) (Chavakis, Kanse *et al.,* 2000). When leukocytes migrate from the bloodstream into sites of inflammation or injury, they undergo a complex sequence of adhesion and locomotion steps (Chavakis, Kanse *et al.,* 2001). For this, expression and up-regulation of various adhesion receptors on the surface of leukocytes and vascular cells are required. Leukocyte rolling depends on selectins, while firm adhesion to and transmigration through the endothelium are mediated by the b2-integrins Mac-1 and LFA-1, which interact with their counter-receptor ICAM-1 on the endothelial cells.

HK seems to bind to the aminoterminus of vitronectin, where gC1qBP binds. So it is possible that HKa and gC1qBP compete with each other for binding to vitronectin.
HK also functions as a coagulation cofactor and has been shown to bind to vascular endothelial cells, in zinc-dependent reaction. The initiating protein of the clotting cascade, factor XII can also bind to the endothelial cells and HK and factor XII compete for binding, which suggest interaction with a common receptor. Joseph *et al.,* (Joseph, Ghebrehiwet *et al.,* 1996) described binding of gC1qBP to HK and factor XII. It is not clear whether gC1qBP binding to vitronectin and HK has any significant role in inflammation but it could be involved in wound healing and immune defence.

5.7 gC1qBP levels in healthy serum and in serum/synovial fluid samples from patients with autoimmune diseases (SLE and RA).

Levels of soluble gC1qBP in sera from healthy individuals, sera from patients diagnosed with systemic lupus erythematosus, sera/synovial fluid samples from patients diagnosed with rheumatoid arthritis and synovial fluid samples from patients diagnosed with osteoarthritis, were measured. In healthy sera the levels of soluble gC1qBP range from 1680 ng/ml to 3000 ng/ml, with a mean value of 1920ng/ml. The levels of gC1qBP in serum samples were expressed in OD_{405} units because recombinant rat gC1qBP was used in the assay. A standard curve was made using a standard serum containing average levels of gC1qBP and the serum/synovial fluid samples were compared to that standard serum using OD_{405} units.

As shown in table 4.1 and in figure 4.6, gC1qBP levels are significantly increased in serum samples from patients with systemic lupus erythematosus compared to standard serum. This increase is significant as shown by the statistical analysis (t-test for independent samples) where the P value is 0.0000. Increased concentration of gC1qBP is probably one of the results of cell apoptosis, which leads to externalisation of the cell proteins, including

gC1qBP. gC1qBP is thought to be involved in the control of inflammation by binding to C1q and preventing binding of immune complexes and therefore preventing complement activation. Also, if gC1qBP is expressed on activated platelets, as suggested by Ghebrehiwet's group, could prevent aggregation of platelets in response to aggregated C1q at sites of vascular injury and therefore prevent complement activation resulting in the control of inflammation. As described in section 5.4, C1q binding to apoptotic cells through gC1qBP, results in clearance of apoptotic cells through Phagocytosis - a process important in the resolution of inflammation.

Tables 4.2 and 4.3 and figures 4.7, 4.8 and 4.9 show levels of gC1qBP in serum/synovial fluid samples from patients diagnosed with rheumatoid arthritis and synovial fluid samples from patients diagnosed with osteoarthritis. It can be seen that the levels of gC1qBP in RA serum samples are not significantly different from the control serum ($p=0.17$), whereas there is a highly significant difference in the levels of gC1qBP between the RA synovial fluid samples and the control serum ($p=0.045$) and between OA synovial fluid samples and control serum ($p=0.025$). When RA synovial fluid samples were compared with OA synovial fluid samples, it was found that RA synovial fluid samples were significantly different ($p=0.0007$) from OA synovial fluid samples and this difference represents decreased levels of gC1qBP.

As discussed earlier for calreticulin, there is increased apoptosis and inflammation in RA joints. Increased apoptosis results in increased consumption of gC1qBP by C1q in the clearance of immune complexes. This accounts for the reduced concentration of gC1qBP detected in RA joints. OA synovial fluid samples have increased concentration of gC1qBP; this probably represents more physiological levels, osteoarthritis is a non-autoimmune disease and the gC1qBP present is most probably externalised during cell lysis. Usually in osteoarthritis there is no inflammation (except in advanced stages of the condition) and therefore gC1qBP in the OA joint is not consumed as much as in the RA joint.

5.8 Conclusions and future work.

We conclude that CRT levels are normal in serum samples from patients diagnosed with SLE and increased in the synovial fluids of patients diagnosed with RA. Serum samples from RA patients and synovial fluid samples from OA patients did not have significantly altered levels of CRT. gC1qBP levels are within the physiological range in serum samples from patients with rheumatoid arthritis, decreased in their synovial fluids, increased in synovial fluids of patients with osteoarthritis and in serum samples from patients diagnosed with systemic lupus erythematosus.

This is the first study to report measurement of levels of CRT and gC1qBP in SLE and RA.

Unfortunately, as can be seen from the appendices I and II, incomplete clinical and laboratory information about the majority of the patients is a limiting factor in the establishment of a definite role of gC1qBP and CRT in SLE and RA and for this reason, only hypotheses can be made. Medication taken by the patients could also have a significant effect in the levels of the measured proteins. Long-term suppressive drug therapy with side effects such as thrombocytopenia, proteinuria and neutropenia could alter the levels of gC1qBP and CRT. Therefore, in order to establish the role of CRT and gC1qBP in SLE and RA, it would be necessary to investigate a more controlled group of patients with samples taken at different stages of the desease.

Appendices

Appendix 1. Clinical and laboratory information of patients diagnosed with systemic lupus erythematosus.

Patient No.	Age	diagnosis	Age of onset (years)	Duration of disease (years)	Clinical symptoms	Lab results
1 (T.A) Female	61	SLE	33	27	Arthralgia, anaemia	ANA=1:160, C3=0.75g/l C4<0.1g/l, IgG=28.9 g/l, IgM=2.68 g/l, IgA=4.92 g/l
2 (T.P) Female (22/12/00)	24	SLE	17	7	Arthralgia, photosensitivity Raynaud's phenomenon Exanthema.	ANA=1:160, C3=0.97 g/l C4=0.09 g/l, DNA:(-), ENA: (-)
3 (G.E) Female (12/12/00)	21	SLE	21	0.5	exanthema, serositis (pleuritis, pericarditis, peritonitis), fever, arthritis haematuria, leukocyteuria	ANA=1:2560, C3=1.08 g/l C4=0.148 g/l, dsDNA:(-), αRo(SSA)(+) ANA of pleural effusion: (+)
4 (K.M) Female (30/10/00)	37	SLE	17	20	Arthralgia, serositis, fever Raynaud's phenomenon, anaemia, kidney involvement, exanthema	ANA=1:10240 C3=1.06 g/l, C4=0.18 g/l αCL:1gG=1.92 g/l, IgM=0.30 g/l dsDNA=71%, WBC:11,000, PLT:350,000 cryoglobulins:0.07g/l, ESR:44 in 1st hr
5 (G.E) same patient as in 3 (11/10/00)						WBC= 6500, PLT= 268000 C3=1.08 g/l, C4=0.15 g/l, ANA=1:640 αRo(SSA) (+), dsDNA(-), urine analysis:normal
6 (D.E) Female	33	SLE	31	2	Arthralgia, pericarditis, anaemia, leucopaenia.	ANA=1:640, dsDNA (-), C3=1.11 g/l, C4=0.24 g/l ENA: αRo(SSA) (+), αLa(SSB) (+)
7 (X.L) Female	32	SLE	30	2	Arthralgia, arthritis Raynaud's phenomenon	RF: (+), ANA=1:80 C4=0.24 g/l, C3=0.88 g/l dsDNA= 1:160, PLT=520000, αCl (-), WBC: 11200, ESR=17/1st hour

8 (X.D) Female (13/11/00)	39	SLE	21	18	Arthralgia, arthritis Raynaud's phenomenon, fever Alopecia, photosensitivity exanthema, serositis	RF=1:640, ANA=1:20480 C3=0.90 g/l,C4=0.38 g/l ENA:αnRNP(+), α-Ro(SSA) (+), αCL:IgG=1.34 g/l, IgM=1.41 g/l, dsDNA=33%
9 (M.M) Female (10/12/00)	63	**subacute** SLE	39	24	Raynaud's phenomenon photosensitivity, exanthema sicca manifestations Livedo Reticularis	ANA=1:320, C3=0.94 g/l, aCL(-) C4=0.04 g/l, hypergammaglobulemia ENA: αRo(SSA) (+), IgG/IgA normal dsDNA (-).
10 (M.M) Female	65	Sjogren	55	10	arthralgia, tiredness, sicca manifestations enlarged parotids	ANA (-), ENA (-), dsDNA (-) C3=1.45 g/l, C4=0.27 g/l
11 (S.M) Female	40	SLE	34	6	Alopecia, exanthema, anaemia photosensitivity, leucopaenia Raynaud's phenomenon, Lemphadenopathy.	ANA=1:2560, C3=0.77 g/l, WBC:2700 ENA: αRo(SSA)(+), dsDNA (+), PLT normal, C4=0.18 g/l
12 (P.S) Female	46	MCTD	37	9	arthralgia, arthritis, fever photosensitivity, exanthema Raynaud's phenomenon	ANA=1:10240 dsDNA=13% (normal=10%) C3=0.63 g/l, C4=0.19 g/l
13 (X.P) Female	48	SLE	51	3	Raynaud's phenomenon arthralgia, exanthema	diffused hypergammaglobulinaemia C3=1.45 g/l, C4=0.28 g/l, ANA=1:80 αRo(SSA)(+) weak
14 (K.A) Female	32	SLE	20	12	arthralgia, arthritis, Raynaud's phenomenon Lymphadenopathy, exanthema (porphyria) thrombosis/vein	IgG=32.20 g/l, IgM/IgA normal C3=0.50 g/l, C4=0.10 g/l RF(-), ANA=1:10240, dsDNA=1:1280 αRo(SSA)(+), αLa(SSA)(+), αnRNP(+)
15 (G.L) Female	35	SLE/RA	26	11	arthralgia, arthritis, fever	C3=1.03 g/l, C4=0.20 g/l ANA=1:1280, dsDNA(+),αSm(+)

16	(K.D) Female	64	SLE	46	18	arthralgia, arthritis, dyspnoea Raynaud's phenomenon, fever photosensitivity, pericarditis lung fibrosis, secondary Sjogren	ANA=1:2560, dsDNA(+)21%, ENA: αRo(SSA)(+), αnRNP(+). α-CL(-), cryoglobulins (-) C3=0.98 g/l, C4=0.26 g/l
17	(M.E) Female	22	SLE	9	13	arthralgia, fever, splenomegaly Raynaud's phenomenon, Livedo Reticularis, pericarditis leucopaenia	ANA=1:1280, dsDNA(+) 27% C3=0.46 g/l, C4=0.07 g/l αRo(SSA)(+), ESR:110mm/1st hr
18	(K.A) Female	27	SLE	21	6	arthralgia, arthritis, fever Livedo Reticularis	ANA=1:5120, dsDNA: 49% C3=0.16 g/l, C4=0.06 g/l
19	(T.A) Female (same patient as in 1)	61	SLE	33	27	arthralgia, anaemia	ANA=1:160, ESR: 125mm C3=0.75 g/l, C4<0.10 g/l IgG=28.90 g/l, IgM=2.68 g/l, IgA=4.92 g/l
20	(P.A) Female	57	Sjogren	50	7	Raynaud's phenomenon, photosensitivity, leucopaenia sicca manifestations, anaemia	ANA=1:1280, dsDNA(-), ENA(-) leucocytes:3000
21	(K.B) Female	37	SLE	33	4	arthralgia, arthritis, exanthema photosensitivity	ANA=1:160, dsDNA=200 (co<100) C3=0.59 g/l, C4=0.10 g/l, RF=82 (normal<20)
22	(G.K) Male	54	anti-PL syndrome	43	11	arthralgia, fever, Raynaud's phenomenon Thrombosis of the veins	ANA(-), dsDNA(-), ENA(-) C3=1.11 g/l, C4=0.32 g/l α-CL= IgG: 0.70 g/l, IgM: 0.12 g/l
23	(G.E) same patient as in 3 (5/2/00)						WBC= 11200, PLT= 110000, RF(-), C3=1.08 g/l, C4=0.17 g/l, dsDNA (-), ANA=1:640, urine analysis=normal.
24	(X.L) same patient as in 7 (19/12/00)						DNA=1:320, C3=0.68 g/l, C4=0.156 g/l PLT=490000, WBC=14300
25	(M.M) same patient as in 9 (2/2/01)						
26	(X.D) same patient as in 8 (18/01/01)						

27	(K.M) same patient as in 4 (15/12/00)						WBC=11000, PLT=356000, BSR=48/1st hour.
28	(T.P) same patient as in 2 (11/6/00)						
29	(B.P) Female	19	SLE	17.5	1.5	thrombopenia, exanthema	ANA=1:80, dsDNA(-), PLT:12000 C3=1.03 g/l, C4=0.144 g/l
30	(K.A) Female	22	SLE	19	3	arthralgia, arthritis, photosensitivity Raynaud's phenomenon	ANA=1:320 dsDNA=1:834 1st time dsDNA=1:160 2nd time. C3=0.76 g/l C4=0.11 g/l
31	(P.E) Female	65 dead	SLE	60	5	arthritis, fever, exanthema hematuria, leucopaenia, vasculitis renal impairment	ANA(+), DNA(+), C3-C4=low
32	(A.A) Female	34	SLE	19	15	arthralgia, arthritis,exanthema Raynaud's phenomenon Photosensitivity	ANA=1:1280, dsDNA(+) C3=0.79 g/l, C4=0.13 g/l ENA: αRo(SSA)(+), cryoglobulins (+)
33	(P.G) Male	41	antiPL/ SLE	38	3	Raynaud's phenomenon, arterial/vein thrombosis Hepatic impairment	α-CL: IgG=1.82 g/l, IgM=3.48 g/l ANA=1:320, C3=0.83 g/l, C4=0.17 g/l
34	(D.E) same as in 4						
35	(T.M) Female	34	SLE	30	4	arthralgia, fever, anaemia leucopaenia, thrombopaenia, splenomegaly, lemphadenopathy	WBC=3000, RF=138, C3=0.72 g/l, C4=0.05 g/l dsDNA=1:40 increased, ENA: αRo(SSA)(+), α-La(SSB)(+), α-Sm(+),α-nRNP(+).
36	(X.S) Female	56	SLE	39	17	exanthema, ulcers	ANA=1:80, αRo(SSA)(+).

37 (D.E) Female	65	SLE/RA overlap	51	14	arthralgia, arthritis, exanthema Photosensitivity, sicca manifestations	RF(+), ANA=1:640, dsDNA(-) C3=1.07 g/l, C4=0.19 g/l
38 (O.S) Female	35	SLE	21	14	arthralgia, arthritis, alopecia butterfly rash, exanthema, renal impairment, lymphadenopathy	ANA=1:5120, dsDNA=27%(normal 5%) C3=0.39 g/l, C4=0.06 g/l, RF=1:640 ENA(-), α-CL(-), cryoglobulins (-).
39 (S.A) Male	74	vasculitis	73.5	0.5	fever, weight loss, cough lung impairment	PLT=442000, ANA(-), ANCA(-), ESR=75mm
40 (T.S) Female	68	SLE	52	16	Photosensitivity, exanthema leucopaenia, anaemia, thrombopaenia haematuria	ANA=1:1280, dsDNA=1:20 (normal=1:10) ENA: αRo(SSA)(+), α-nRNP(+),α-Sm(+) RF(-)
41 (P.K) Female	60	SLE/ anti-PL syndrome	40	20	20 years ago: arthritis, pleuritis 30/04/01: no symptoms on medication	PLT=442000, WBC=12100 ESR=53mm 1st hr, PTT(LA)=54.7 sec C3=0.98 g/l, C4=0.11 g/l, dsDNA=139 IU/ml, α-CL=105 GPL and 10 MPL
42 (T.T.F) Female	30	SLE	2	28	arthralgia, arthritis, fever, Raynaud's phenomenon, exanthema, haemolytic anaemia, lymphadenopathy	PLT=140000, WBC=8100, ANA 1:160 fine specled, dsDNA (-), hyperglobulinaemia C3=0.88 g/l, C4=0.11 g/l, RF(-)
43 (T.T.F) Female	30	SLE	2	28	no symptoms	
44 (T.E) Female	53	SLE	24	29	arthralgia, CVA (tromboembolic) anaemia	ANA=1:1280 fine specled, αRo(SSA)(+), αLa(SSB) (+), dsDNA=304 IU/ml, aCL= 341 GPL and 16.5 MPL, C3=1.04 g/l, C4=0.13 g/l, Hb= 0.111 g/l, WBC= 13600, PLT=256000, ESR=55mm 1st hr.
45 (B.A) (3/2/01) Female						

46 (K.O) Female	51	SLE	16	35	Previous symptoms: arthralgia, leucopaenia, photosensitivity anaemia Raynaud's phenomenon Present symptoms: none, medication: none	WBC=3900, PLT=209000, RF(-) C3=1.42 g/l, C4=0.17 g/l, dsDNA (-), ANA=1:1280 fine specled
47 (Z.A.M) Female (30/3/01)	30	SLE	15	15	Raynaud's phenomenon Medication: Prednisolone (5mg daily) Azathioprine (150mg daily)	WBC=2900, PLT=244000, ESR 11mm 1st hr, Hb=13.4 g/dl, C3= 0.468 g/l, C4=0.0371 g/l αCL=15.2 GPL, 17.2 MPL, α-β2GPI= 2.5 GPL and 36.0 MPL, ANA= 1:2560 diffuse, dsDNA=232 IU/ml
48 (Z.A.M) Female (10/5/01)						
49 (B.A) Female (17/4/01)						
50 (E.J) Female	36	SLE			cerebral lupus	DNA=52, C3=1.25g/l, C4=0.2g/l
51 (K.J) Female	50	SLE			Medication: Hydroxychloroquine Azathioprine	C3=1.22 g/l, C4=0.16 g/l, DNA=26
52 (H.K) Female	41	arthritis				DNA=255, C3=0.85 g/l, C4=0.23 g/l
53 (J.T) Female	31	SLE			skin lesions Medication: Hydroxychloroquine Azathioprine	DNA=26, C3= 1.22 g/l, C4=0.16 g/l
54 (C.B) Female	30	SLE			retinal vasculitis Medication: Azathioprine	ANA=1:1000, DNA=70 IU/ml, C3=1.18 g/l, C4=0.22 g/l
55 (R.N) Male	18	SLE			presented as juvenile chronic arthritis, anaemia	DNA=214, C3=0.65 g/l, C4=0.07 g/l

56 (B.E) Female	51	SLE			Medication: Azathioprine	DNA>300 IU/ml, C3=0.98 g/l, C4=0.17g/l
57 (H.B) Female	33	SLE			Haemolysis, hypertension, anaemia renal impairment, Medication: Azathioprine	DNA>300, C3=0.88 g/l, C4=0.30 g/l
58 (G.J) Female	45	SLE			Medication: Hydroxychloroquine Azathioprine	ANA:1:256, C3=0.95 g/l, C4=0.14 g/l, DNA=14 IU/ml
59 Female	31	SLE	12	19	Pulmonary fibrosis Medication: Hydroxychloroquine	ACA+
60 Female	36	SLE	32	4	arthritis, lymphadenopathy Medication: Hydroxychloroquine	ANA=640, dsDNA=3.8, ESR=42
61 Female	24	SLE/ myositis	19	5	arthritis, renal impairment pulmonary fibrosis. Medication: Azathioprine, warfarin, cyprofloxacin, nifedipine	ANA=320, dsDNA=31, ESR=38
62 Female		SLE	4		arthritis Medication: Hydroxychloroquine	
63 Male	34	SLE/RA	31	3	arthritis, skin rash Medication: Hydroxychloroquine, voltarol	
64 Female	70	SLE	36	34	arthritis, skin rash. Medication: Hydroxychloroquine	ANA=80, dsDNA=17, ESR=49, a-CL(+)
65 Male	55	SLE	51	4	arthritis, renal impairment, skin rash photosensitivity Medication: Hydroxychloroquine, nabumetone	ANA=40, ESR=8, a-CL(+)
66 Female	38	SLE	19	19	skin rash, photosensitivity Medication: Hydroxychloroquine	ESR=5

***Patient samples and clinical data provided by Dr. Galanopoulou Vasiliki and Dr. Galanopoulou Dimitra, Papageorgiou Hospital, Thessaloniki, Greece.**

Appendix II: Clinical and laboratory information of patients diagnosed with rheumatoid arthritis

Patients 1-17: No clinical information available

Patient No.	Age (yrs)	Diagnosis (yrs)	Age of Onset (yrs)	Duration (yrs)	Clinical/Lab information	Medication
18	62	seropositive erosive RA	44	18	PV=1.77	Methotrexate (10mg weekly) Hydrochloroquine (400mg daily) Diclofenac (50mg 3 times daily), HRT, Paracetamol
19	66	Erosive RA	61	5	PV=1.73, RF status:unknown splenectomy	Methotrexate (15mg weekly) Arthrotec (150mg once daily) Penicillin
20	76	seropositve erosive RA	66	10	PV= 1.91	Methotrexate (20mg weekly) Prednisolone (10mg daily) Sulphasalazine (2g daily) Nabumetone (1g nocte)
21	36	seropositive erosive RA	32	4		Hydrochloroquine (200mg daily) Azeclofenac (100mg twice daily)
22	55	seronegative erosive RA	41	14	PV=1.74	Methotrexate (12.5mg weekly) Sulphasalazine (2g daily) Budesonide (9mg monthly)
23	61	seronegative erosive RA	57	4	PV=1.72	Sulphasalazine (2g daily)

24	77	seropositive RA	76	1	PV=1.96	Prednisolone (5mg daily) Colchicine (500 mg once daily)
25	78	seropositive RA	70	8	PV=1.88	Prednisolone (5mg daily) Sulphasalazine (2g daily)
26	76	seropositive	75	1	PV=1.89	Nabumetone (1g nocte)
27	58	seropositive erosive RA	42	16	PV=1.58	Methotrexate, Arthrotec, Prednisolone, Sulphasalazine
28						
29	75	seropositive non erosive RA	51	24		Surgam (600mg daily)
30	28	RA	18	10		Sandimmum, Methotrexate Presolon, anti-inflammatory.
31	51	RA	46	5	synovial membrane inflammation/right knee	Methotrexate,Sandimmum Cortisone, Gold.
32	68	RA	65	3	synovial membrane Inflammation/both knees	Methotrexate,Sandimmum Cortisone, Gold.

Patients 33-42: No clinical information available

Patient samples and clinical data provided by Dr. Nikolaos Afantenos, Rheumatology,Clinic Rhodes, Greece and Dr. Peter Sheldon, University of Leicester, Leicester, UK.

Physiological values

[A. Ward, P. Riches, R. Fifield and A. Smith (1996) PRU "Handbook of clinical immunology", PRU Publications]

IgG= 5.2-18g/l
IgM= 0.02-0.2g/l
IgA= <0.02g/l
RF < 15 I.U/ml
C3= 0.75-1.65g/l
C4= 0.14-0.54g/l
ANA <1:80
α-CL IgG<0.2g/l, IgM<0.2g/l
White blood cells (WBC): 4000-10000/μl
Platelets (PLT): 150000-400000/μl
Erythrocyte Sedimentation Rate (ESR): for females 1-20 mm/1st hour, for males 1-13 mm/1st hour.
Extactable Nuclear Antigen (ENA): αRo(SSA), dsDNA, αLa(SSB), αnRNP, αSm

Systemic diseases

Antibody	RA	SS	DM-PM	MCTD	SLE
RF	80%	90%	30	Rare	20
ANA	50%	75%	20	99	99
Anti-ss-A(Ro)	20%	60%	10	20	35
Anti-ss-B(La)	-	50%	-	<10	15
Anti-ds-DNA	-	<10%	-	-	55
Anti-ss-DNA	-	<10%	-	-	55
Anti-Sm	-	-	-	<10	35
Anti-Histon	25%	-	-	-	30
Anticentromer	<10%	-	-	-	-
Anti-SCL-70	-	-	-	-	-
Anti-UI-RNP	<10%	<10%	15	100	35
Anti-hnRNP/RNA	-	-	-	100	-
FANA	45%	-	-	-	-

Schaffler, A. Braun, J. Renz, U. (1992) "Klinikleitefaden" Jungjohann Verlagsgesellschaft, mbH, Stuttgart.

Abbreviations:

RA: Rheumatoid Arthritis
SS: Sjogren's Syndrome
DM-PM: Dermatomyositis-Polymyositis
MCTD: Mixed-Connective-Tissue-Disease
SLE: Systemic Lupus Erythematosus

PART II
Development of pathway specific assays, to monitor activity of complement activation complexes in human and murine serum/plasma samples.

CHAPTER 6

Results: Functional Characterisation of Lectin Pathway Activity

Functional characterisation of lectin pathway activity

Mannose-binding lectin (MBL) is a calcium dependent collagenous serum lectin involved in innate immunity. It is a pattern recognition molecule, which binds to mannose and N-acetylglucosamine found on microbial surfaces. Upon binding, the protein activates the lectin pathway of the complement system independently of antibody, via MBL associated serine proteases (MASPs) of which MASP-2 cleaves C4 and C2. MBL also interacts directly with phagocytic cells and initiates phagocytic uptake (Ogden, deCathelineau *et al.*, 2001). It has been suggested that the interaction of MBL with phagocytic cells is mediated via C1q receptors and that the recognition that CR1 could act as a receptor for C1q suggested that CR1 might also be a receptor for MBL (Ghiran, Barbashov *et al.*, 2000).

The objective of the present work is to characterise the functional activity of the lectin pathway and determine biologically relevant binding surfaces. We find that MBL binds to the pneumococcal polysaccharide vaccine Pneumovax II™ and to the surface of pathogenic or non-pathogenic strains of *Acanthamoeba*.

We also investigated the role of alternative pathway on lectin pathway activation using mice deficient in Factor B and C2 (*H2 Bf/C2*$^{-/-}$) and Factor B (*H2 Bf*$^{-/-}$) and C1q (*C1q a*$^{-/-}$). Finally we investigated C1q and MBL binding to soluble CR1 receptor (sCR1) using *in vitro* assays with purified proteins.

6.1 Activation of the lectin pathway by Pneumococcal vaccine.

The pneumococcal polysaccharide vaccine (commercial name: Pneumovax II™, Merck Sharp & Dohme B.V., Netherlands) used in this study, consists of 23 types of pneumococcal polysaccharide. These are: type 1, 2, 3, 4, 5, 6B, 7F, 8, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. The vaccine contains 12.5 μg/ml of each of the polysaccharides.

In this study we have used the capacity of the MBL-MASPs complex to cleave C4 and deposit the cleavage product C4b onto the activator to determine the ability of Pneumovax II™ (P/VAX II) to activate the lectin pathway. 96 well plates (Maxisorp, Nunc, Cat. No. 442404) were coated with 10μg/ml of the mixture of P/VAX II polysaccharides (0.434 μg/ml of each polysaccharide) in coating buffer, overnight at RT. The plates were then blocked in 200 μl of TBS-0.1% (w/v) HSA for 1 hour at RT and washed in TBS-0.1% tween-5 mM $CaCl_2$. Human sera with known concentrations of MBL were diluted in MBL binding buffer (section 2.3.4), added to the wells and incubated overnight at 4°C. The wells were washed in TBS-0.1% tween-5 mM $CaCl_2$ and purified human complement component C4

was added at concentration of 1μg/ml in BBS buffer. The C4b deposition was detected as described in section 2.3.4

Figure 6.1 shows C4b deposition from samples with high (1200ng/ml), medium (750ng/ml) and low (<100ng/ml) levels of MBL. Lectin pathway dependent C4b deposition was inhibited by preincubation with mannan. For this, mouse C1q $\alpha^{-/-}$ plasma was preincubated with 0.2mg/ml mannan for 2 hr at RT and dilutions in MBL binding buffer were added to the P/VAX II coated wells. The plate was incubated o/n at 4°C and purified human C4 was added (C4b deposition assay, section 2.3.4). C4b deposition was observed in the C1q $\alpha^{-/-}$ mouse plasma. No C4b deposition was observed in the C1q $\alpha^{-/-}$ mouse plasma that was preincubated with mannan (Figure 6.2).

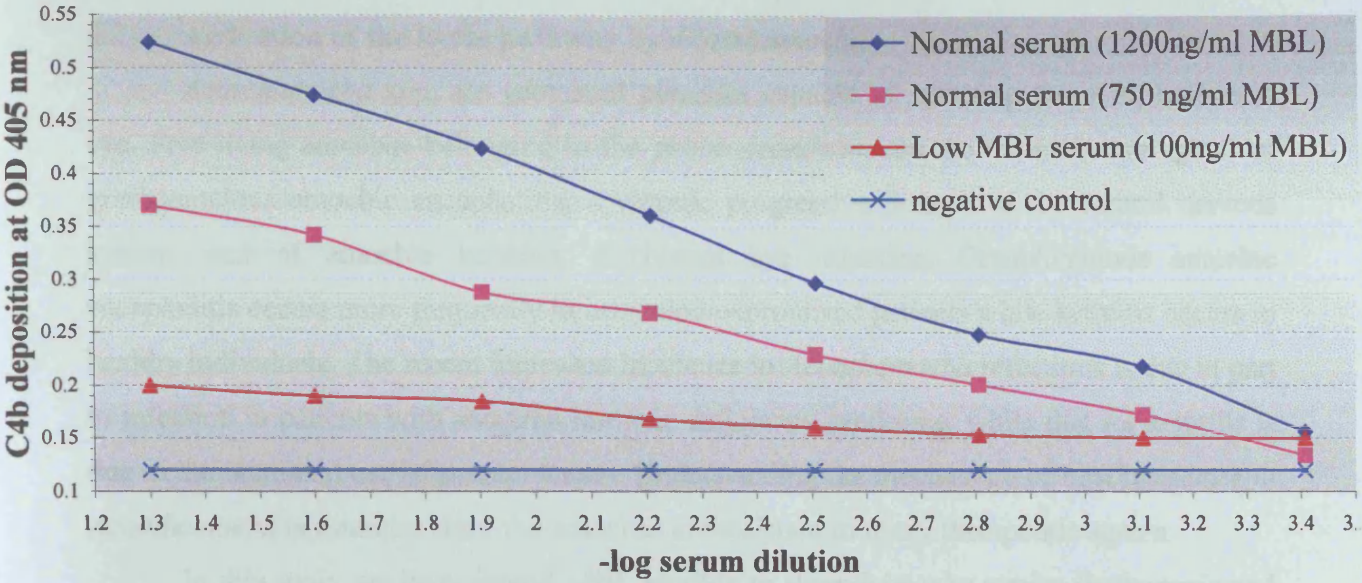


Figure 6.1 Lectin pathway activation by Pneumovax II.

Serum samples were diluted in MBL binding buffer and incubated in P/VAX II coated microtiter wells o/n at 4°C. C4b deposition was dependent on the concentration of MBL contained in the sera. Maximum C4b deposition was observed with the high MBL containing serum and minimum C4b deposition was observed with the low MBL-containing serum.

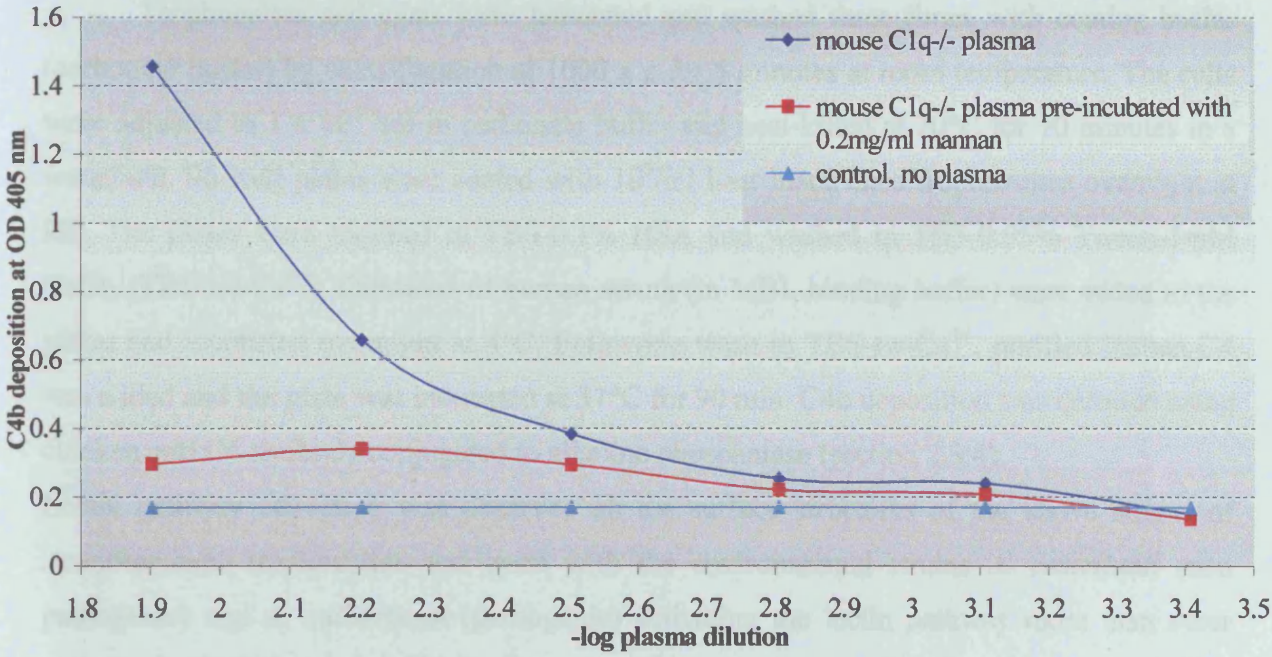


Figure 6.2 Inhibition of lectin pathway activity by incubation with mannan.

C1q $\alpha^{-/-}$ plasma dilutions in MBL binding buffer and C1q $\alpha^{-/-}$ plasma preincubated for 2 hr at RT with 0.2mg/ml mannan in MBL-binding buffer, were added to the P/VAX II coated wells o/n at 4°C. Purified human C4 was then added and the deposition of C4b was measured. Lectin pathway-dependent C4 cleavage was inhibited by preincubation of the C1q $\alpha^{-/-}$ plasma with mannan.

6.2 Activation of the lectin pathway by *Acanthamoeba*

Acanthamoeba spp. are protozoal parasites capable of infecting the skin, brain and eye. Free-living amoebae belonging to the genus *Acanthamoeba* are the causative agents of granulomatous amoebic encephalitis, a chronic progressive disease of the central nervous system, and of amoebic keratitis, a chronic eye infection. Granulomatous amoebic encephalitis occurs more frequently in immunocompromised patients while keratitis occurs in healthy individuals. The recent increased incidence in *Acanthamoeba* infections is due in part to infection in patients with acquired immune deficiency syndrome, while that for keratitis is due to the increased use of contact lenses. Understanding the mechanism of host resistance to *Acanthamoeba* is essential since the amoebae are resistant to many therapeutic agents.

In this study we investigated MBL binding to *Acanthamoeba* strains (pathogenic and non-pathogenic) and their ability to activate complement through the lectin pathway.
Acanthamoeba species and strains used in this study are given in Table 6.1. Trophozoites were maintained in a semi-defined axenic broth medium as described previously (Hughes and Kilvington, 2001). Cysts were prepared from the trophozoite cultures using Neff's chemically defined encystment medium (Hughes and Kilvington, 2001; Neff *et al.*, 1964).

Trophozoites and cysts were harvested and washed three times with coating buffer (carbonate buffer) by centrifugation at 1000 x g for 5 minutes at room temperature. The cells were adjusted to 1 x 10^5 /ml in carbonate buffer and heat-killed at 70°C for 10 minutes in a waterbath. 96 well plates were coated with 10^5/ml heat inactivated trophozoites overnight at RT. The plates were blocked in TBS-0.1% HSA and washed in TBS-0.05% Tween-5mM $CaCl_2$ (TBS-tw-Ca^{++}). Dilutions of human serum (in MBL binding buffer) were added to the plates and incubated overnight at 4°C. Following wash in TBS-tw-Ca^{++}, purified human C4 was added and the plate was incubated at 37°C for 90 min. C4b deposition was detected using chicken anti C4 antibody conjugated to alkaline phosphatase (section 2.3.4).
Lectin pathway activation was observed on the surface structures of the above strains of *Acanthamoeba* trophozoites and cysts with the environmental strains *A. castellanii* (non pathogenic) and *A. culbertsoni* (pathogenic) activating the lectin pathway more than other pathogenic clinical isolates. This is shown in Figure 6.3

To test MBL binding to *Acanthamoeba*, plates were coated with the environmental *Acanthamoeba* strain 150/1A and blocked as described above. Serum dilutions and serum dilutions pre-incubated with 0.2mg/ml and 2μg/ml mannan in MBL binding buffer were added to the wells and the plate was incubated overnight at 4°C. Following wash in TBS/tw/Ca, 0.2μg/ml of monoclonal antibody raised in mouse against human MBL (131-01) was added and the plate was incubated at RT for 90 min. Binding of MBL to *Acanthamoeba* was detected using goat anti-mouse IgG conjugated to alkaline phosphatase (section 2.3.6).

The results are shown in Figure 6.4. We find that no binding of MBL to *Acanthamoeba* is observed from serum preincubated with mannan.

The ability of serum preincubated with mannan to activate the lectin pathway was tested on *Acanthamoeba* coated plates. Plates were coated with *A. castellanii* and *A. culbertsoni* and blocked as described above. Serum (containing 1300ng/ml MBL) dilutions and serum dilutions preincubated with 3μg/ml mannan were added to the wells and the plate was incubated overnight at 4°C. C4b deposition was detected as previously described. We observed 50% decrease in lectin pathway activation when using serum preincubated with 3μg/ml mannan as compared to the lectin pathway activation when using MBL containing serum (Figure 6.5).

Table 6.1 *Acanthamoeba* species and strains studied

(Dr. Simon Kilvington, Department of Microbiology and Immunology, University of Leicester provided the strains and the information on the source and pathogenicity)

Species	Strain	Source	Pathogenicity
A. polyphaga	Ros	Keratitis	+
A. quina	L1A	Environmental	-
A. culbertsoni	ATCC 30171 (A-1)	Environmental	+
A. castellanii	CCAP 1501/1a	Environmental	-
Acanthamoeba sp	Ac-Char	Keratitis	+
Acanthamoeba sp	Ac-Cole	Keratitis	+

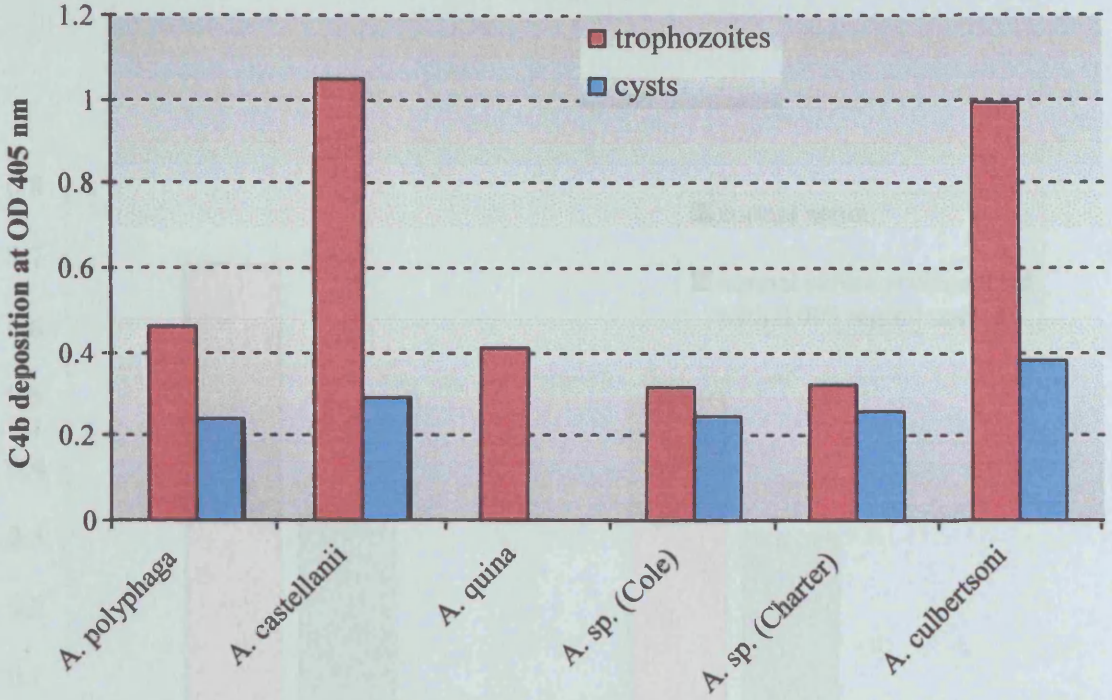


Figure 6.3 Lectin pathway activation by *Acanthamoeba* strains.

The plates were coated with 10^5/ml of heat inactivated *Acanthamoeba* strains (trophozoites and cysts). Human serum dilutions were added and complement activation through the lectin pathway was detected by C4b deposition.

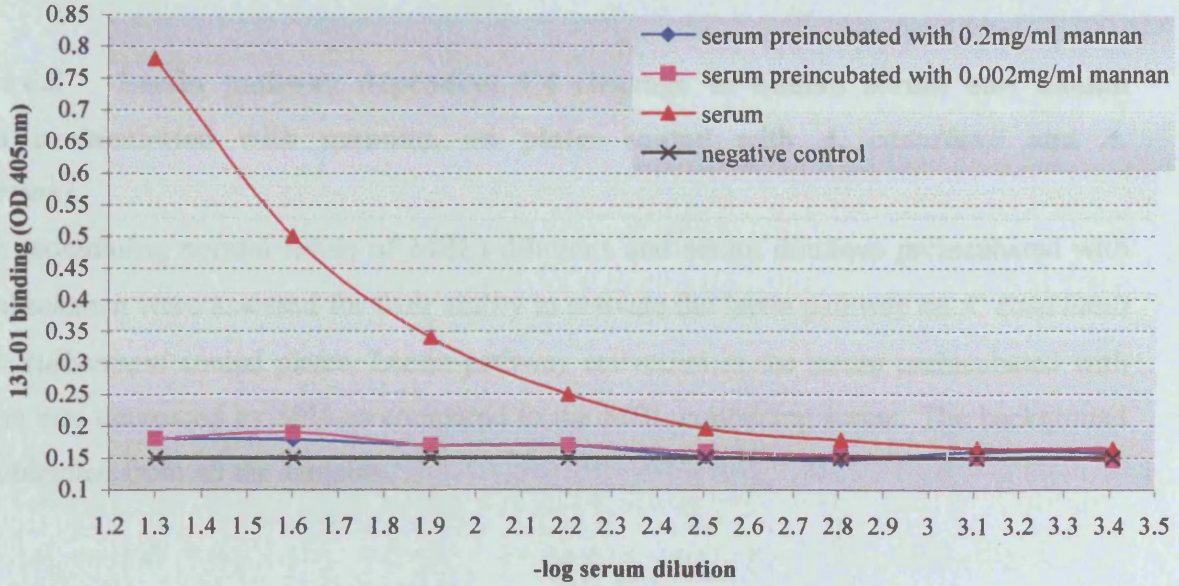


Figure 6.4 Inhibition of MBL (contained in serum) binding to *Acanthamoeba* strains by incubation with mannan.

Serum was preincubated with different concentrations of mannan (0.2mg/ml and 0.002mg/ml) and added to *Acanthamoeba*-coated plates. To assess MBL binding to *Acanthamoeba*, 131-01 was added. No binding was observed from serum preincubated with mannan.

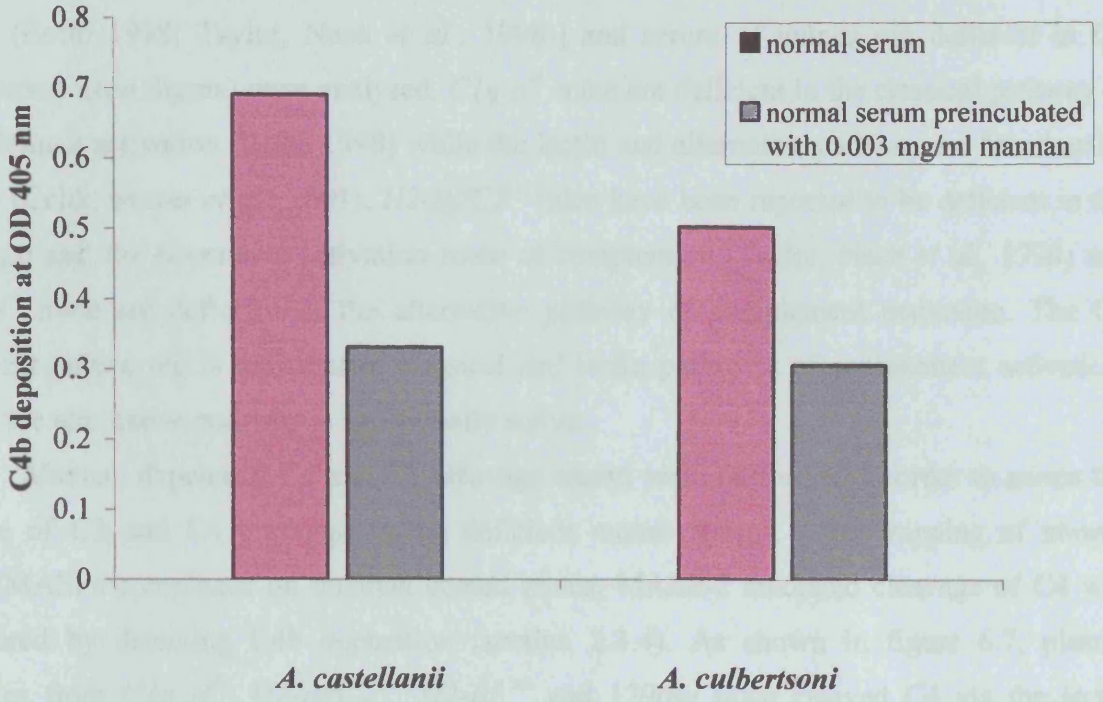


Figure 6.5 Lectin pathway dependent C4 cleavage in human serum and human serum preincubated with mannan, on plates coated with *A. castellanii* and *A. culbertsoni.*

Serum (containing normal levels of MBL) dilutions and serum dilutions preincubated with 3μg/ml mannan were assessed for their ability to activate the lectin pathway on *A. castellanii* and *A. culbertsoni* coated plates. Lectin pathway activation in the serum preincubated with mannan was decreased by 50% as compared to the MBL containing serum. The background was subtracted from all the samples.

6.3 Contribution of the alternative pathway to lectin pathway activation

In order to study the interaction of the alternative pathway amplification loop of complement activation with the lectin pathway activation route, plasma of mouse strains deficient in the components C1q, Factor B, Factor B and C2 [kindly provided by Dr. Marina Botto (Botto 1998; Taylor, Nash *et al.,* 1998)] and serum of guinea pig deficient in C4 (purchased from Sigma) were analysed. *C1q a*$^{-/-}$ mice are deficient in the classical pathway of complement activation (Botto 1998) while the lectin and alternative pathway are functionally active (Celik, Stover *et al.,* 2001). *H2-Bf/C2*$^{-/-}$ mice have been reported to be deficient in the classical and the alternative activation route of complement (Taylor, Nash *et al.,* 1998) and *H2-Bf*$^{-/-}$ mice are deficient of the alternative pathway of complement activation. The C4 deficient guinea pig is deficient in classical and lectin pathways of complement activation, while the alternative pathway is functionally active.

Mannan dependent C4 and C3 cleavage assays were performed in order to assess the degree of C3 and C4 cleavage in the deficient mouse strains. After trapping of plasma MBL/MASPs complexes on mannan coated plates, MASP-2 mediated cleavage of C4 was measured by detecting C4b deposition (section 2.3.4). As shown in figure 6.7, plasma samples from *C1q a*$^{-/-}$, *H2-Bf/C2*$^{-/-}$, *H2-Bf* $^{-/-}$ and 129/Sv mice cleaved C4 via the lectin pathway route and thereby completed the first step to generate the lectin pathway C3 convertase. As *H2-Bf/C2*$^{-/-}$ lack C2 required to form the lectin pathway C3 convertase, we assessed and compared the ability of the sera with selective complement deficiencies to cleave C3 in this lectin specific activation assay (section 2.3.5). Lectin pathway dependent C3 cleavage was detected, in mice deficient in C1q, which could be inhibited by pre-incubating the plasma with mannan (Figure 6.8) whereas no C3 cleavage was seen in *H2-Bf/C2* $^{-/-}$ and *H2-Bf* $^{-/-}$ mice (Figure 6.9). These data demonstrate that *C1q a*$^{-/-}$ mice have a fully functional lectin pathway, whereas *H2-Bf/C2*$^{-/-}$ mice present as deficient in all complement activation routes. *H2-Bf* $^{-/-}$ mice have functional classical and lectin pathways as shown by the C4b deposition assay but they do not have a functional alternative pathway.

C4 deficient guinea pig serum has functional alternative pathway but it does not have functional classical and lectin pathways because it cannot form the classical/lectin C3 convertase. Activation of the alternative pathway in C4 deficient guinea pig serum (if any) can be attributed to spontaneous hydrolysis of C3 or to a serine protease, which has the ability to directly cleave C3. It has been suggested that MASP-1 has the ability to directly cleave C3 (Matsushita, Endo *et al.,* 2000) and we tested this hypothesis on C4 deficient guinea pig serum using a C3 cleavage assay. The results of the C3 cleavage assay are shown in figure 6.10. The data demonstrate that C4 deficient guinea pig serum does not cleave C3, although it

has functional alternative pathway. C4b deposition by the C4 deficient guinea pig serum was also undetectable (data not shown).

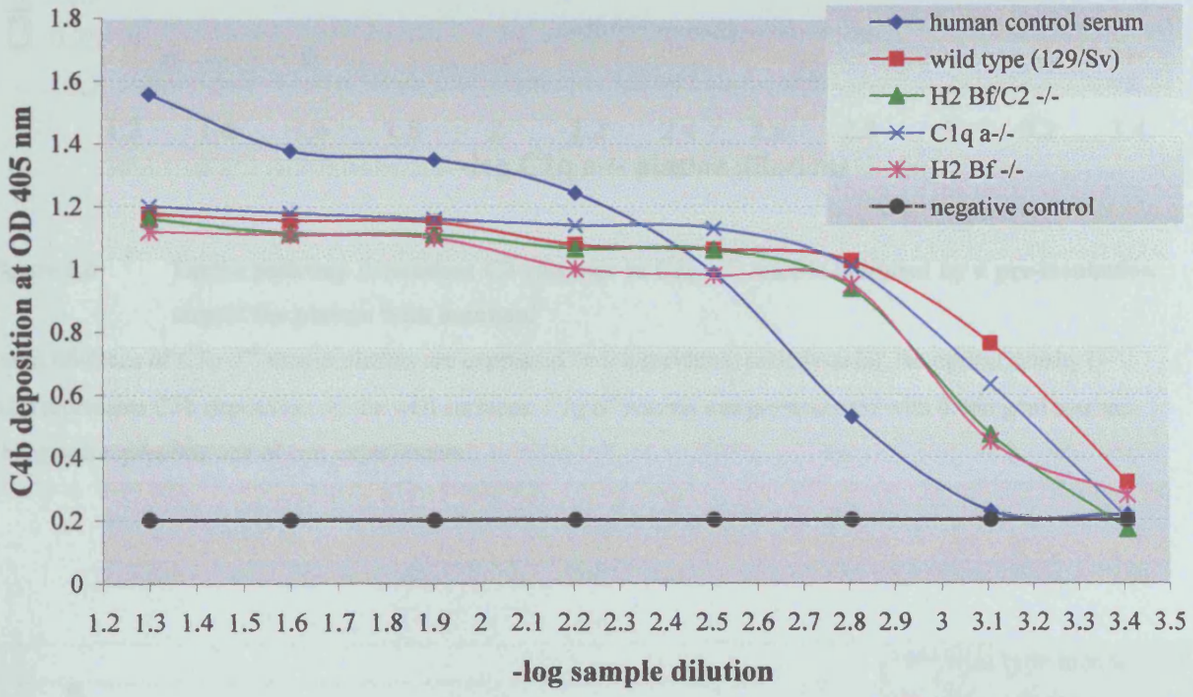


Figure 6.7 **Lectin pathway dependent C4 cleavage in plasma samples of *H2-Bf/C2 -/-*, *C1q a -/-*, *H2-Bf$^{-/-}$* and 129/Sv.**

A human control serum (MBL level 3.6 μg/ml) was assayed in parallel as a positive control. Serial dilutions are expressed on a logarithmic scale (x-axis), the optical density (y-axis) is the read-out of C4b deposition on the well surfaces. Each point is the mean of a duplicate experiment. For the negative control, plasma was substituted by buffer only. This graph represents one of two experiments.

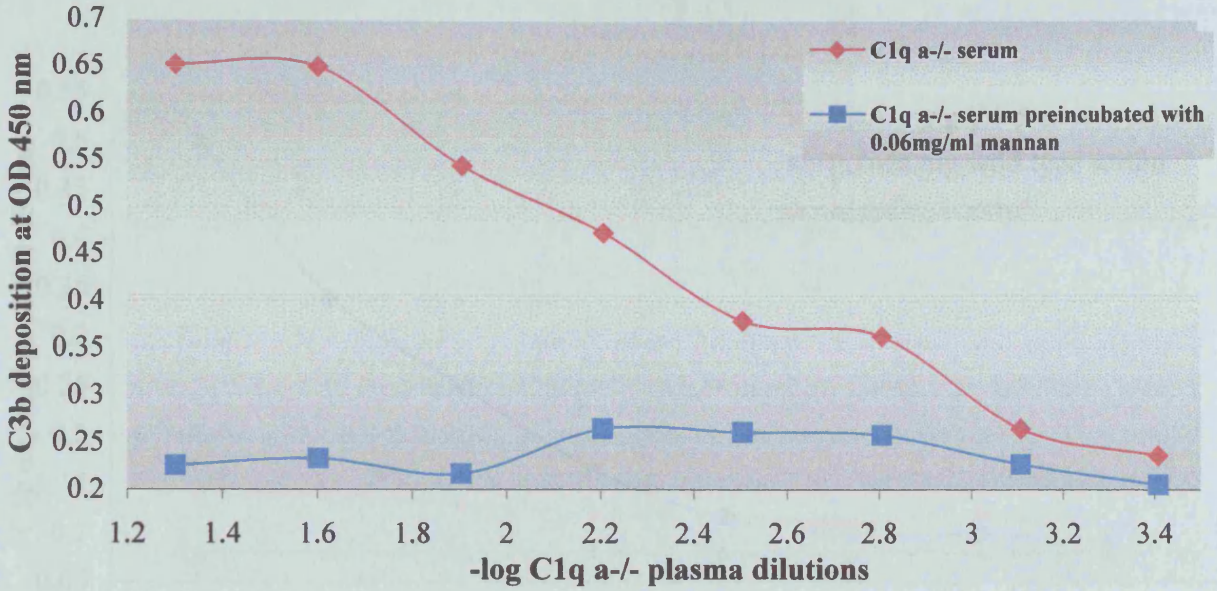


Figure 6.8 **Lectin pathway dependent C3 cleavage in *C1q a* $^{-/-}$ mice is inhibited by a pre-incubation step of the plasma with mannan.**

Serial dilutions of *C1q* $\alpha^{-/-}$ mouse plasma are expressed on a logarithmic scale (x-axis); the optical density (y-axis) represents C3b deposition on the well surfaces. *C1q* $\alpha^{-/-}$plasma was preincubated with 0.06mg/ml mannan. This graph represents one of two experiments.

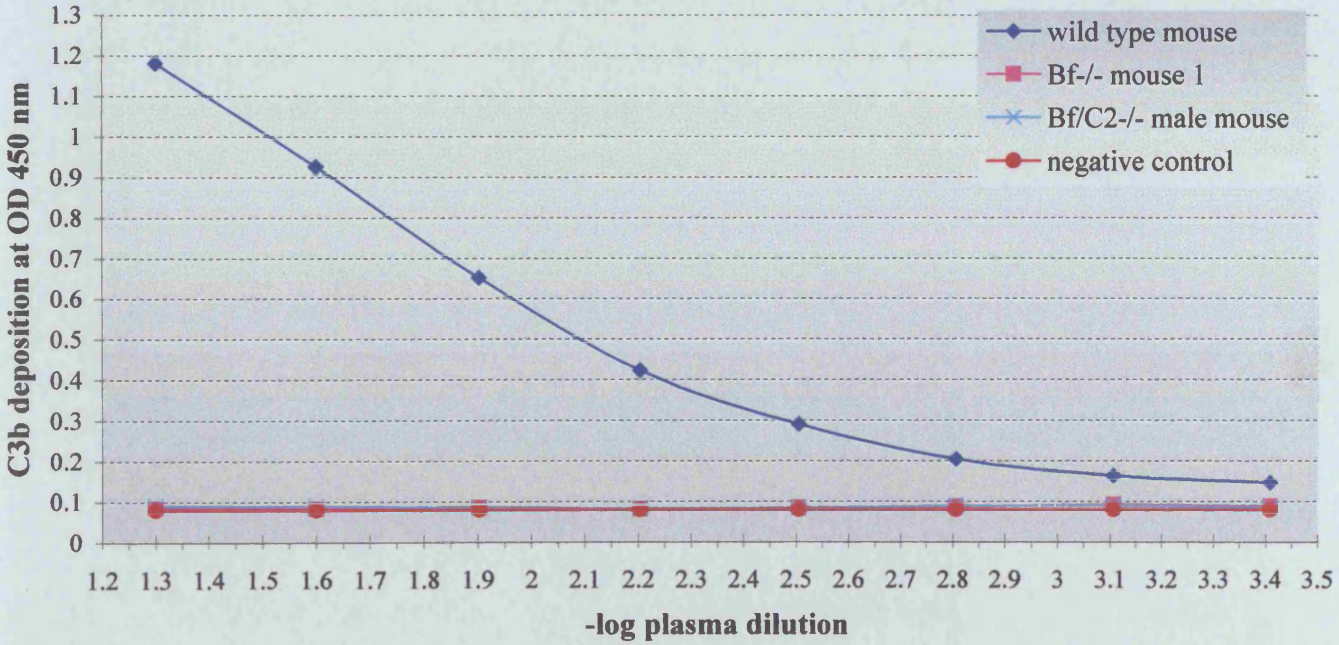


Figure 6.9 **C3 cleavage in plasma samples of *H2-Bf/C2* $^{-/-}$, *H2-Bf* $^{-/-}$ and wild type (129/Sv).**

Serial dilutions are expressed on a logarithmic scale (x-axis); the optical density (y-axis) is the read-out of C3b deposition on the well surfaces. Each point is the mean of a duplicate experiment. For the negative control, serum or plasma was substituted by buffer only. This graph represents one of two experiments.

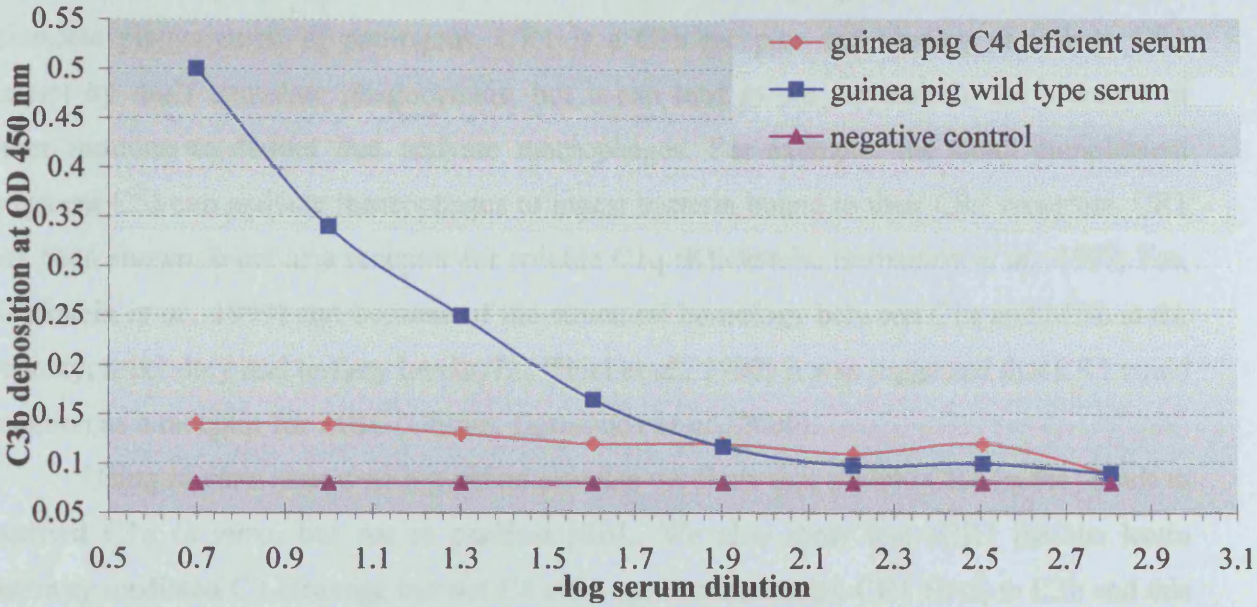


Figure 6.10 C3b deposition in serum samples from wild type guinea pig and C4 deficient guinea pig. Serum samples from wild type and C4 deficient guinea pig are expressed on a logarithmic scale (x-axis). C3b deposition is measured at OD_{450} nm (y-axis). For negative control, wells received buffer instead of serum. Points represent duplicates of one of several experiments.

6.4 Soluble CR1 inhibits lectin pathway mediated C3 cleavage

Complement receptor 1 (CR1)/ CD35 is present on phagocytes and it is known to stimulate phagocytosis of pathogens. CR1 is a C3b receptor and binding of C3b to CR1 cannot by itself stimulate phagocytosis, but it can lead to phagocytosis in the presence of other immune mediators that activate macrophages. For example, the small complement fragment C5a can activate macrophages to ingest bacteria bound to their CR1 receptors. CR1 has been shown to act as a receptor for soluble C1q (Klickstein, Barbashov *et al.*, 1997; Tas, Klickstein *et al.*, 1999) and because of the structural homology between C1q and MBL at the primary, secondary and tertiary levels (Lu, Thiel *et al.*, 1990) it was suggested that CR1 could function as a receptor for MBL (Ghiran, Barbashov *et al.*, 2000).

Using *in vitro* assays with purified proteins we show that soluble CR1 (sCR1) binds to purified C1q *in vitro*, but not to purified MBL. We also show that sCR1 inhibits lectin pathway mediated C3 cleavage but not C4 cleavage. On host cells, CR1 binds to C3b and this is then cleaved by factor I to yield inactive C3b, which results in inhibition of complement activation on host cell surfaces.

6.4.1 Binding of sCR1 to purified C1q and MBL.

Microtiter wells were coated with 10μg/ml sCR1 (Avant Immunotherapeutics, Needham, MA), blocked and washed as described in section 2.3.4. Dilutions of purified MBL (Statens Serum Institut) (starting with 1000ng/ml) and C1q (Quidel, Cat. No. A400) (starting with 1000 ng/ml) were added to the wells. MBL was diluted in TBS-0.05% tween containing increasing concentrations of $CaCl_2$ (0mM, 0.3mM and 5mM $CaCl_2$ and these buffers were used in all subsequent washes). C1q was diluted in TBS-0.05% Tween-5mM $CaCl_2$. The plate was incubated overnight at 4°C to allow binding of MBL and C1q to the sCR1-coated wells.

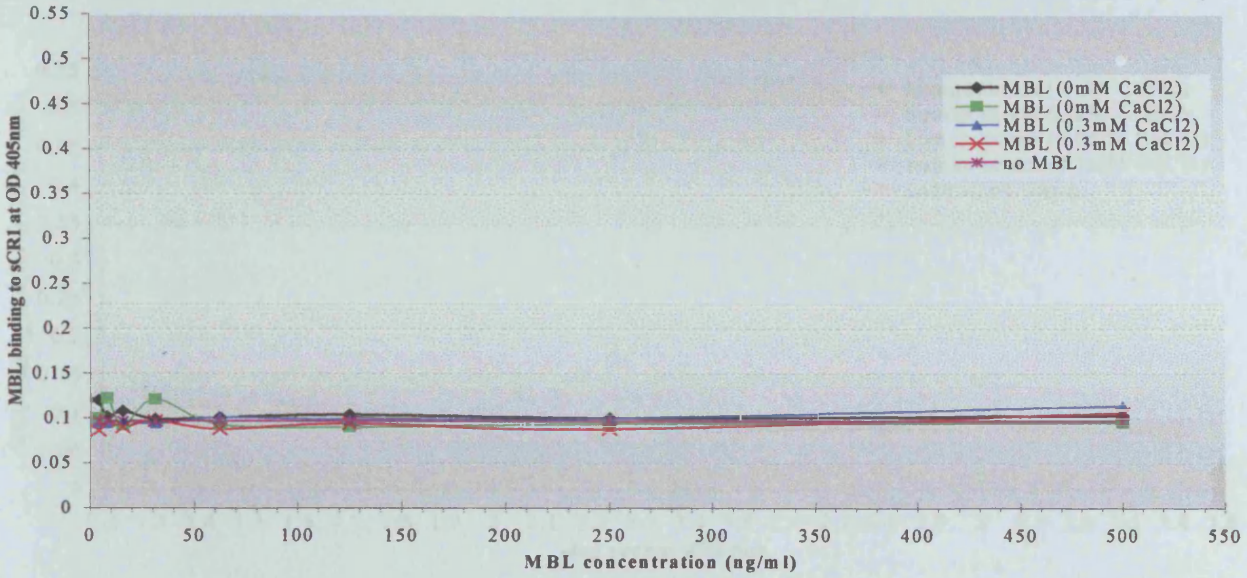
- Wells incubated with purified MBL (in TBS/tw and TBS/tw/0.3mM $CaCl_2$) were washed in TBS/tw and TBS/tw/0.3mM $CaCl_2$ and the monoclonal anti human MBL antibody 131-01 was added at a concentration of 0.2-0.5 μg/ml in TBS/tw and TBS/tw/0.3mM $CaCl_2$, respectively. Following incubation for 90 min at room temperature with gentle agitation the plate was washed in TBS/tw and TBS/tw/0.3mM $CaCl_2$ and goat anti-mouse IgG (whole molecule) conjugated to alkaline phosphatase was added at 1:10,000 dilution in TBS/tw and TBS/tw/0.3mM $CaCl_2$. The presence of AP was determined by the addition of pNPP.

- Wells incubated with C1q were washed in TBS/tw/5mM $CaCl_2$ and rabbit anti-human C1q polyclonal antibody (DAKO, Cat. No. A0136) diluted 1:2000 in TBS-tw-5mM

$CaCl_2$ was added. Following incubation for 90 min at room temperature with shaking, the plate was washed in TBS-tw-5mM $CaCl_2$ and goat anti-rabbit IgG (whole molecule) conjugated to alkaline phosphatase was added at 1:20,000 dilution in TBS/tw/5mM $CaCl_2$. The presence of AP was determined by the addition of pNPP.

The results are shown in Figure 6.11. We observed C1q binding to sCR1 in the presence of 5mM $CaCl_2$, but no MBL binding. Based on previous studies by Ghiran *et al.*, (Ghiran, Barbashov *et al.*, 2000) the optimal $CaCl_2$ concentration for MBL binding to sCR1 is 0.3mM. The experiment was repeated using human serum as a source of MBL and different concentrations of $CaCl_2$ and NaCl. The results are shown in Figure 6.12 where no MBL binding to sCR1 was observed under the chosen conditions.

A



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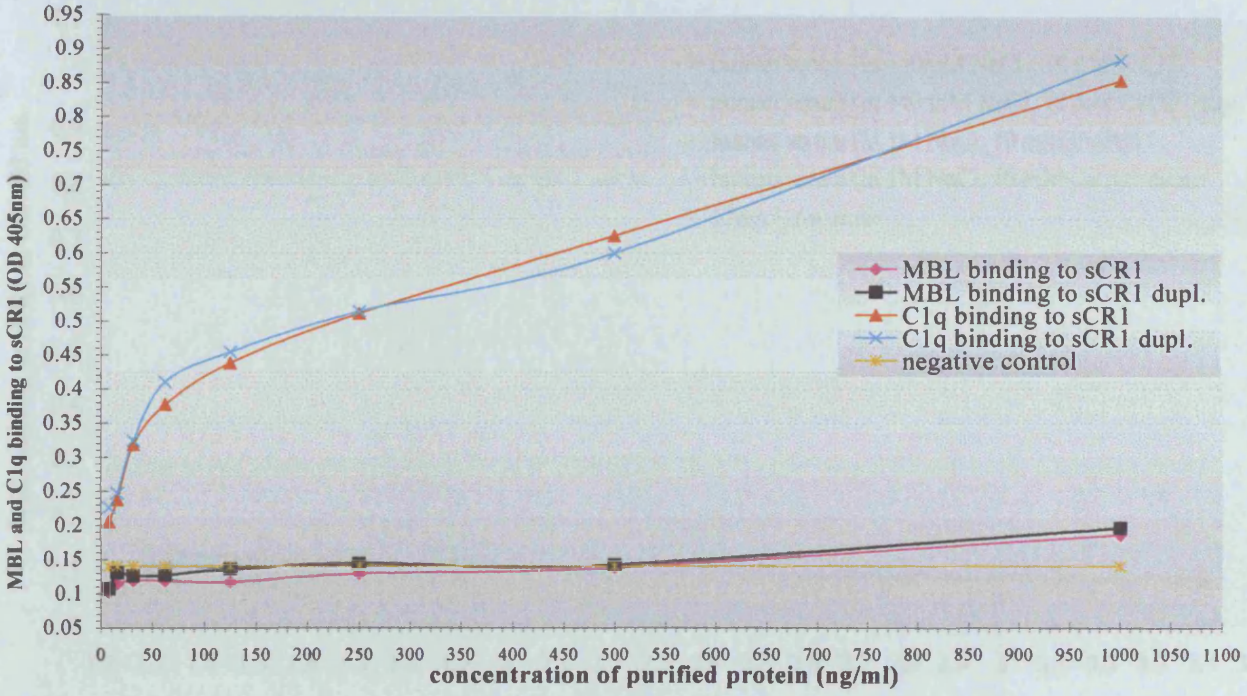
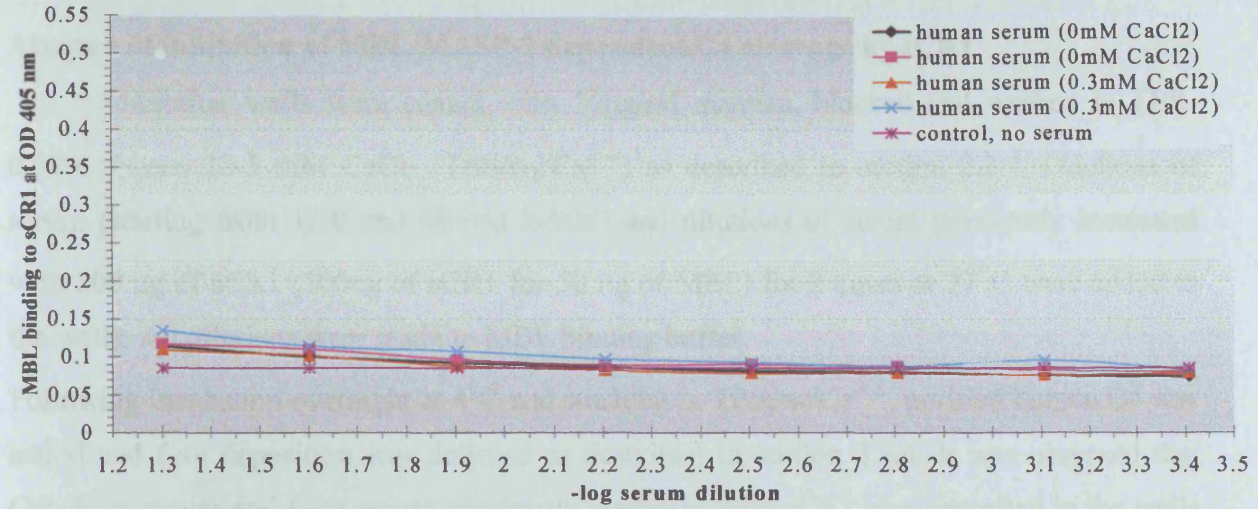


Figure 6.11 Binding of purified MBL and C1q to sCR1

A. The assay was performed in buffer containing 5mM $CaCl_2$. Purified C1q and MBL were allowed to bind to sCR1-coated plates o/n at 4C. Binding of C1q and MBL to sCR1 was detected as described in section 6.4.1 The y axis represents OD at 405 nm, the x axis represents concentration of MBL and C1q in ng/ml

B. MBL binding to sCR1 was tested under different $CaCl_2$ concentrations. Purified MBL was diluted in buffer containing no $CaCl_2$ and buffer containing 0.3mM $CaCl_2$ and was allowed to bind to sCR1-coated wells, o/n at 4°C.

A



B

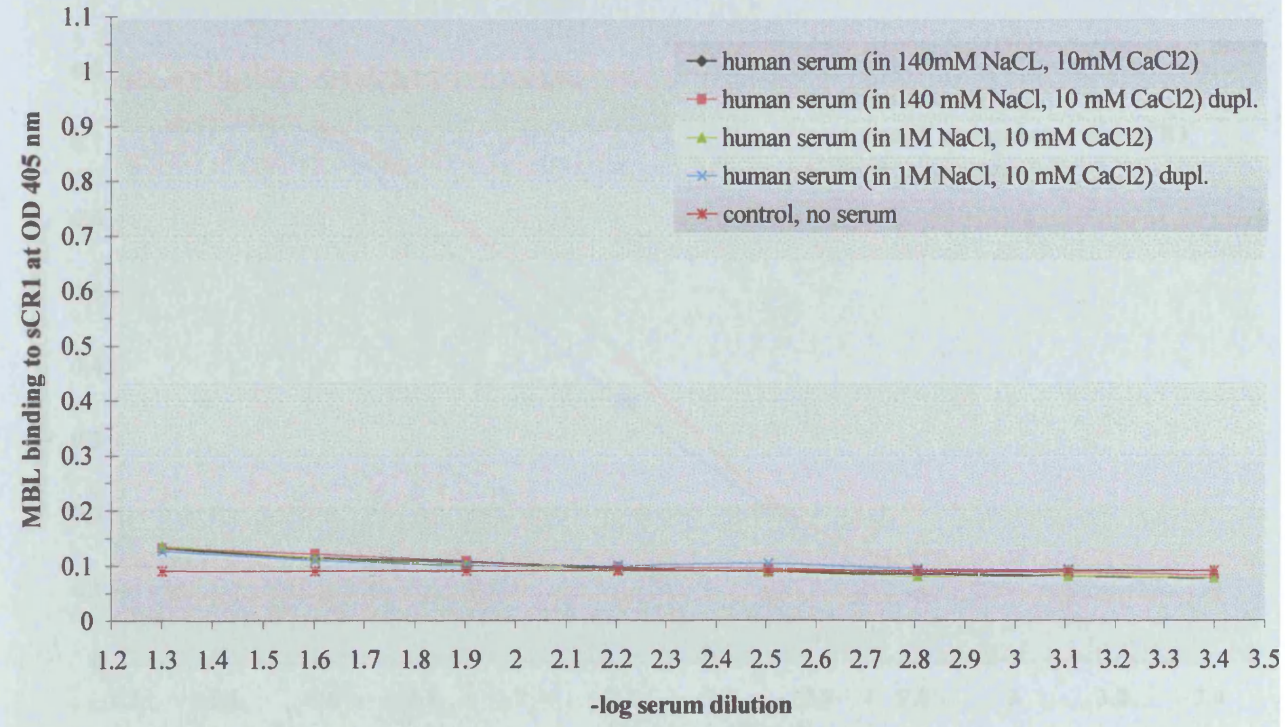


Figure 6.12 Binding of MBL (contained in serum) to sCR1 at increasing salt or Ca^{++} concentration

A. MBL binding to sCR1 was tested under different $CaCl_2$ concentrations. Human serum (containing known concentration of MBL) was diluted in buffer containing no $CaCl_2$ and buffer containing 0.3mM $CaCl_2$ and was allowed to bind to sCR1-coated wells, o/n at 4°C. Binding of MBL was detected as previously described. The y-axis represents OD at 405 nm, the x-axis represents –log serum dilution.

B. MBL (contained in serum) binding to sCR1 was tested under increasing NaCl concentration (140 mM and 1M NaCl). Binding was detected as previously described. The y-axis represents OD at 405 nm, the x-axis represents –log serum dilution.

6.4.2 Absence of inhibition of lectin pathway/alternative pathway dependent C3 cleavage

Absence of inhibition of MBL/MASP-2 dependent C4 cleavage by sCR1

Microtiter wells were coated with 10μg/ml mannan, blocked and washed in TBS-0.05% Tween 20-5 mM $CaCl_2$ (TBS/tw/Ca^{++}) as described in section 2.3.4. Dilutions of serum (starting from 1:20 and diluted 2-fold) and dilutions of serum previously incubated with 300 ng of sCR1 (300ng of sCR1 for 50 ng of MBL) for 2 hours at 37°C were added to the wells. All dilutions were made in MBL binding buffer.

Following incubation overnight at 4°C and washing in TBS/tw/Ca^{++}, purified human C4 was added and C4b deposition was detected as described in section 2.3.4. It was observed that C4b from serum and from serum previously incubated with sCR1 was deposited in the wells (Figure 6.13)

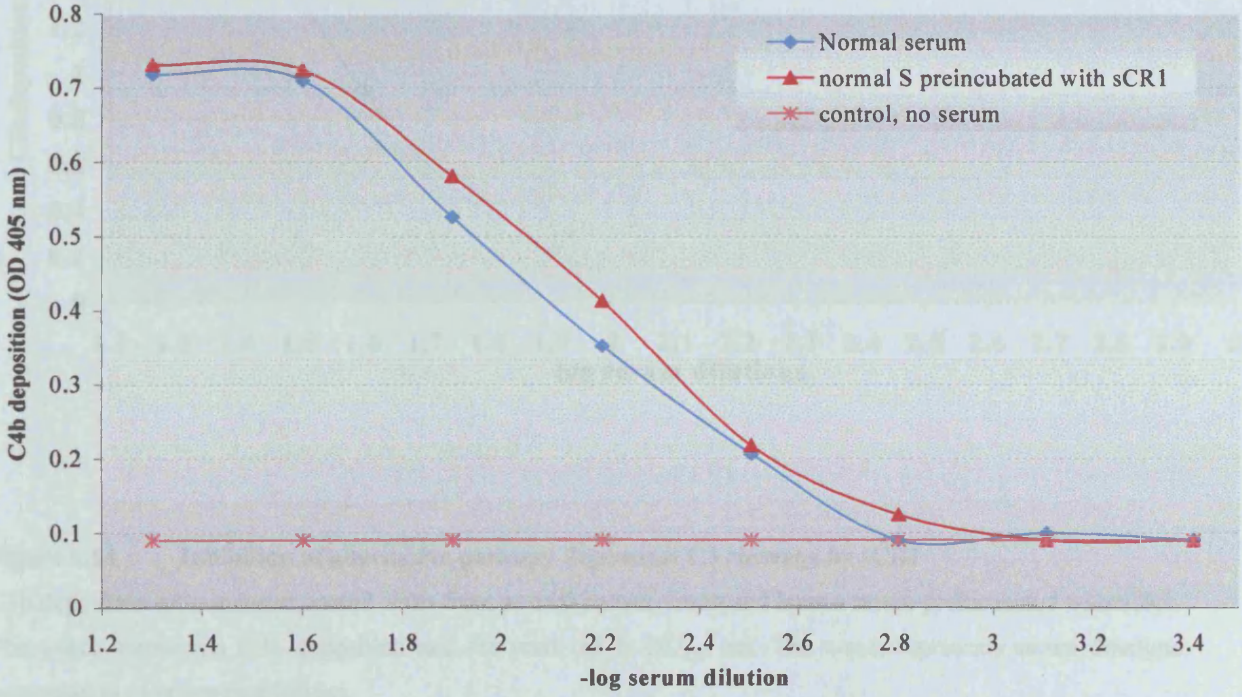


Figure 6.13 Absence of inhibition of MBL/MASP-2 dependent C4 cleavage by sCR1 under high salt conditions (1M NaCl).

C4b deposition onto mannan coated wells from normal human serum and human serum preincubated with sCR1. The y-axis represents C4b deposition and the read out is OD_{405} nm. The x-axis represents serum dilutions expressed as –log.

Competitive inhibition of alternative pathway dependent C3 cleavage by sCR1

In this study the role of sCR1 on the alternative pathway was investigated. It has been suggested that the role of CR1 in alternative pathway regulation is to displace Bb from C3b and to catalyse the cleavage of bound C3b by the plasma protease factor I to produce inactive C3b (known as iC3b). In order to test this hypothesis, dilutions in TBS-tw-Ca^{++} of (human) serum and serum pre-incubated with sCR1 were added onto mannan-coated wells. After o/n incubation at 4°C, C3b deposition was measured as described in section 2.3.4

The data demonstrate that soluble CR1, when present in serum, inhibits C3b deposition (Figure 6.14)

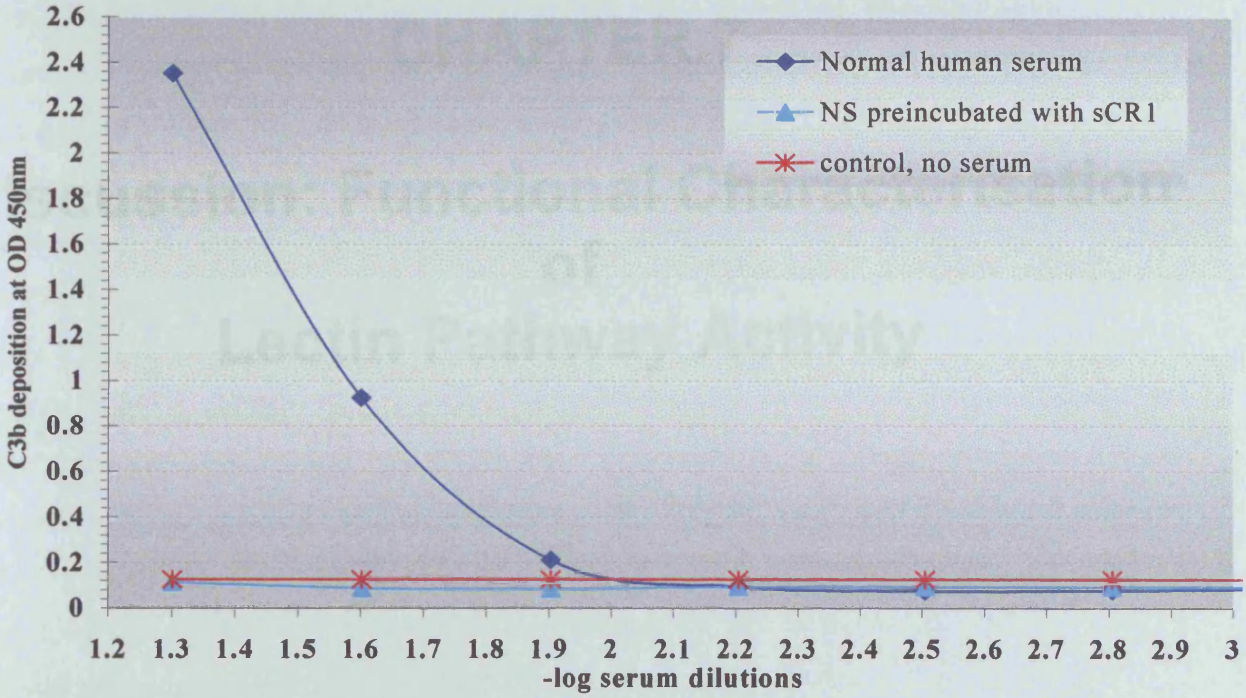


Figure 6.14 Inhibition of alternative pathway dependent C3 cleavage by sCR1

C3b deposition onto mannan coated wells from normal human serum and human serum preincubated with sCR1. The y-axis represents C3b deposition and the read out is OD_{450} nm. The x-axis represents serum dilutions expressed as –log serum dilutions.

CHAPTER 7

Discussion: Functional Characterisation of Lectin Pathway Activity

7.1 Pneumovax II mediated lectin pathway activation and clinical importance.

The different strains of *Streptococcus pneumoniae* have antigenically distinct capsular polysaccharides. The capsule prevents effective phagocytosis until the bacterium is opsonised by specific antibody and complement, allowing phagocytes to destroy it. Antibody to one type of *S. pneumoniae* does not cross-react with the other types, so an individual immune to one type does not have protective immunity to a subsequent infection with a different type. There are 84 known types of *S. pneumoniae*. These types are distinguished by serological tests and so are often known as serotypes.

Pneumovax consists of polysaccharides from 23 different serotypes. These serve as TI-2 antigens (thymus independent antigens; these are bacterial polysaccharides, polymeric proteins and lipopolysaccharides that stimulate naïve B cells in the absence of peptide-specific T-cell help) and contain no intrinsic B-cell stimulating activity. Whereas TI-1 antigens can activate both immature and mature B cells, TI-2 antigens can activate only mature B cells. Upon vaccination with the pneumococcal polysaccharides contained in pneumovax II, B cells will be stimulated to produce antibodies, initially IgM. There is evidence that helper T cells become activated and lead to class switching and production of IgG. It is not clear how T cells become activated because polysaccharide fragments such as those present in pneumovax II cannot produce peptide fragments that might be recognised by T cells on the B-cell surface. One possibility is that a component of the antigen binds to a cell surface molecule common to T cells of all specificities. IgG confers protection against some serotypes of *S. pneumoniae* because IgG binds to *S. pneumoniae* and activates the classical/lectin pathways of complement resulting in clearance of the pathogen, or by opsonisation and phagocytosis of the pathogen.

In the present study we have used the C4b deposition assay to test binding of MBL and lectin pathway activation mediated by the pneumococcal polysaccharides present in Pneumovax II. We find that MBL binds to the above polysaccharides and promotes complement activation (chapter 6, figures 6.1 and 6.2). We observed binding of MBL to pneumovax II in a dose dependant manner (high levels of C4b deposition correlated with high concentration of MBL), and preincubation of serum (containing MBL) with mannan resulted in inhibition of C4 cleavage.

These findings could be of significant clinical importance in patients that might fail to respond to the vaccine and have undetectable MBL levels. It is possible that such patients could be susceptible to infection by *S. pneumoniae* as a result of absence of the critical routes of elimination i.e. IgG mediated complement activation and opsonisation and lectin pathway mediated complement activation and opsonisation. Furthermore, it has been suggested that

MBL may inhibit the spread of microorganisms by blocking access to appropriate receptors (Neth, Jack *et al.*, 2000).

The above aspects should be the target of further research and will help to identify the clinical situations in which MBL replacement or adjunctive therapy (Valdimarsson, Stefansson *et al.*, 1998) might play a positive role.

7.2 Lectin pathway activation by *Acanthamoeba* strains.

Members of the genus *Acanthamoeba* are ubiquitous in natural environment and make up the majority of free-living amoebae found commonly in soil and fresh water. *Acanthamoeba* have a relatively simple life cycle, alternating between a trophozoite phase, which is capable of feeding and division, and a dormant stage represented by a thick-walled cyst. The cyst forms a means of survival during periods of unfavourable conditions. Cysts of *Acanthamoeba* have a double wall. The inner wall (endocyst wall) is mainly composed of cellulose, and the outer wall (ectocyst wall) of proteoglycan.

Pathogenic strains have been associated with cases of primary amoebic meningoencephalitis, and granulomatous amoebic encephalitis (Rodriguez-Zaragoza 1994). *Acanthamoeba* species can also harbour intracellular bacteria e.g. *Legionella pneumophila*, in a symbiotic relationship (Rodriguez-Zaragoza 1994). *Acanthamoeba* parasites also cause the vision-threatening corneal inflammatory disease *Acanthamoeba* keratitis (Visvesvara and Stehr-Green, 1990). Pathogenesis probably results from contaminated contact lenses, which bring the parasite in close proximity with the cornea. The infectious process involves attachment of parasites to the surface of the epithelial cell (Preston and King 1984). In the case of granulomatous amoebic encephalitis (GAE), the route of invasion seems to be the skin, lower respiratory tract or nasopharynx, with dissemination to the central nervous system via the hematogenous route. Central nervous system invasion can occur also through the neuroepithelium. GAE occurs more often in debilitated or immunocompromised patients. The majority of patients with GAE have been reported to have skin lesions, cirrhosis, neoplasia, systemic lupus erythematosus, bone grafts, diabetes, pneumonitis, or AIDS (Marciano-Cabral, Puffenbarger *et al.*, 2000).

The precise mechanism by which *Acanthamoeba* adheres to the surface of the cornea has not been elucidated. The carbohydrate chains of plasma membrane glycoconjugates of host cells are primary collision partners for many bacterial, fungal, and protozoal pathogens. In this respect, it has been reported that the adhesion of *Acanthamoeba polyphaga* to corneal epithelial cells in culture (Morton, McLaughlin *et al.*, 1991) and to extracellular matrix proteins (Gordon, Asem *et al.*, 1993) can be inhibited by methyl-α-D-mannopyranoside (α-

Man). Other studies have shown that phagocytosis of yeast (Allen and Dawidowicz 1990) and erythrocytes (Brown, Bass *et al.,* 1975) by *A. castellanii* could be inhibited by D-mannose.

It has been demonstrated that a clinical isolate of *A. castellanii* binds to primary cultures of rabbit corneal epithelium and that α-Man inhibited the binding (Yang, Cao *et al.,* 1997). This inhibition was specific; four other sugars did not inhibit the binding. It was also shown that *A. castellanii* binds most, if not all, of the mannose-containing glycoproteins (mannose-GPs) of corneal epithelium. The parasite does not bind to numerous other proteins lacking mannose residues (Yang, Cao *et al.,* 1997). *A. castellanii* organisms were found to bind to mannose-GPs of corneal epithelium regardless of whether the GPs were isolated by using agarose-bound s-ConA, LcH, or GNA. Although s-ConA, LcH, and GNA are all mannose-specific lectins, their requirements for binding with the sugar are distinct from one another. At least two interacting α-mannopyranosyl units (e.g., Mana1,2Man, Mana1,3Man, and Mana1,6Man) in the oligosaccharides are essential for binding to immobilized s-ConA (Narasimhan, Wilson *et al.,* 1979). For binding to LcH, in addition to two α-mannosyl end groups, the presence of a polylactosamine-type oligosaccharide containing an a-fucosyl residue attached to the asparagine-linked N-acetylglucosamine residue is required (Kornfeld *et al.,* 1981; Yamamoto *et al.* 1982), and for binding to GNA with high affinity, non-reducing terminal Mana1,3Man disaccharide units must be present (Shibuya *et al.,* 1988; Van Damme *et al.,* 1987). Thus, it appears that the nature of mannose linkage and of saccharides present in the vicinity of mannose in the oligosaccharide chains had little effect on the adhesion of the amoebae to GPs. It was further demonstrated that *A. castellanii* binds to a neoglycoprotein, Man-BSA.

Based on the finding that binding and internalization of yeast particles and erythrocytes by soil *A. castellanii* can be inhibited by D-mannose, Brown *et al.,* (Brown, Bass *et al.,* 1975) and Allen and Dawidowicz (Allen and Dawidowicz 1990) have suggested that a mannose receptor is responsible for phagocytosis in amoebae. From *Acanthamoeba* extracts, a 136-kDa mannose-binding protein was isolated (Yang, Cao *et al.,* 1997) with the ability to bind to Man-BSA. Moreover, a biotinylated protein of the same molecular mass (136 kDa) could be isolated by chromatography of the extracts of cell surface-labeled amoebae on a mannose-affinity column. This suggested that the mannose-binding protein of the amoeba is membrane associated (Yang, Cao *et al.,* 1997). It was proposed that one mechanism explaining *Acanthamoeba* adhesion to the corneal surface might involve interactions between the mannose-binding protein of the amoeba and mannose-GPs of corneal epithelium. It was also shown that subtle corneal injury exposes mannose-GPs on the surface of injured corneas. The newly exposed GPs may serve to provide additional attachment sites for the amoebae. This, in turn, could render the cornea susceptible to the infection (Jaison, Cao *et al.,* 1998).

Adhesion and invasion may be mediated by distinct molecules for example, varicella zoster virus, a virus that expresses GPs containing mannose 6-phosphate (Man6P) residues, first attaches to lung fibroblasts by binding to a heparin sulfate proteoglycan (Zhu, Gershon *et al.,* 1995). This binding subsequently promotes a low-affinity interaction of the virus with a Man6P receptor, the interaction necessary for viral entry into the host cells. It is of interest that *A. castellanii* produces unusually large amounts of N-acetylglucosamine (GlcNAc)-1-phosphotransferase, the enzyme that transfers GlcNAc-1-phosphate residues from UDP-GlcNAc to the 6-position of terminal mannose residues (Ketcham and Kornfeld 1992). It has been speculated that the first step in the pathogenesis of *Acanthamoeba* infection involves attachment of the parasite to the mannose residues of the plasma membrane GPs of host cells. This binding, in turn, may promote phosphorylation of selective mannose residues by the amoeba enzyme, and it may be the presence of the Man6P residues on the surface of host cells that is required for infection to occur.

In this study we show that environmental and pathogenic strains of *Acanthamoeba* trophozoites bind to MBL and activate complement through the lectin pathway. Preincubation of serum with mannan (serum depleted of MBL) reduced by 40% lectin pathway activation on plates coated with trophozoites. On the contrary, *Acanthamoeba* cysts did not activate lectin pathway significantly. Serum that contained no MBL due to preincubation with mannan, also activated the lectin pathway. This activation could be attributed to the amoebic lectin. The role of lectin pathway in the pathogenesis of *Acanthamoeba* infection has not been previously investigated and the role of the immune system in preventing infection with Acanthamoeba is unknown. *Acanthamoeba* are known to be resistant to several components of the immune system and repeated ocular infections do not induce protective immunity (Alizadeh *et al.,* 1995). Acanthamoeba activate the complement system but are resistant to complement mediated lysis (Toney and Marciano-Cabral, 1998).

MBL might have a protective role in Acanthamoeba infections. In the development of keratitis through its potential binding to mannose-GPs present in the corneal epithelium and inhibition of adherence of *Acanthamoeba,* especially in the surface of injured corneas where there is high exposure of mannose-GPs. Systemic infection via hematogenous spread, may also be prevented by *Acanthamoeba*-mediated complement activation and generation of C3b that results in the recruitment of phagocytic cells such as neutrophils and macrophages, which exert amoebicidal activity. These aspects could be of significant importance in *Acanthamoeba*-infected immunocompromised individuals and it remains to be tested if susceptibility to *Acanthamoeba* infections correlates with low MBL levels. The above aspects should be the targets of further research in order to determine a possible therapeutic role of purified MBL in fighting Acanthamoeba infections.

7.3 Contribution of the alternative pathway to lectin pathway activation

C3 is abundant in plasma and C3b is produced at a significant rate by spontaneous cleavage (C3 tick over). This occurs through the spontaneous hydrolysis of the thioester bond in C3 to form C3 (H_2O), which then binds plasma factor B. This allows factor D to cleave factor B to Ba and Bb and Bb remains associated with C3 (H_2O) to form the C3 (H_2O) Bb complex. This complex is the fluid phase C3 convertase and is thought to cleave many C3 molecules to C3a and C3b. This C3b attaches through its reactive thiolester group to the surfaces of host cells or to pathogens. C3b bound in this way is able to bind to factor B allowing its cleavage from factor D to yield a small fragment Ba and the active protease Bb. Properdin stabilises the C3bBb complex, which is the alternative pathway C3 convertase.

C3 is also cleaved by the classical/lectin pathway convertases C4b2b, into C3a and C3b. C3b is then deposited on the surface of the pathogen and the alternative pathway follows as described above. The same amplification loop enables the alternative pathway to contribute to complement activation initially triggered through the classical or lectin pathways.

In this study we investigated the role of the alternative pathway amplification loop on lectin pathway. We performed C4b and C3b deposition ELISA with three gene targeted mouse strains with deficiencies of the classical activation pathway only (*C1q* $a^{-/-}$), all of the three routes of complement activation, i.e. the classical, the lectin, and the alternative pathway (*H2-Bf/C2*$^{-/-}$) and of the alternative pathway only (*H2 Bf* $^{-/-}$). We observed that all three gene targeted mouse strains showed normal C4b deposition and that *H2 Bf/C2*$^{-/-}$ and *H2* $Bf^{-/-}$ showed no C3b deposition. This indicates that the alternative pathway amplification loop, which is not functional in the *H2 Bf* $^{-/-}$ mouse strain, is vital in the activation of complement because many C3b molecules are deposited in this way, resulting in complement activation.

Absence of C3b deposition by the guinea pig serum shows that MASP-1 does not directly cleave C3 as suggested by other research groups. As indicated in the results chapter, C4 deficient serum does not have functional classical and lectin pathways and therefore the C3 convertase cannot be formed. The alternative pathway is functional but as shown during this work (figure 6.14 and figure 6.8) its not activated unless there is initial C3b deposition from the classical/lectin pathway This C3b initiates the amplification loop and this results in the production of many C3b molecules. Taken the above into consideration, in the C4 deficient serum, any complement activation (if any) cannot be attributed to classical/lectin pathway activation but to direct cleavage of C3 by the lectin pathway serine protease MASP-1. We demonstrate that there is no detectable C3b deposition from the C4 deficient serum (figure 6.10) in our study and therefore we question the physiological role of the suggested MASP-1 function in C3 cleavage.

7.4 The role of CR1 in the regulation of complement activation. Binding of soluble CR1 to C1q and MBL.

Stringent regulation of the alternative complement pathway C3 convertase is essential for the prevention of C3 hypercatabolism and for the protection of host tissue from the deleterious effects of alternative pathway propagation. In primates this regulation involves primarily the soluble serum proteins factor H and factor I, as well as the membrane-resident proteins decay-accelerating factor (CD55), membrane cofactor protein (MCP, CD46), and complement receptor 1 (CR1, CD35) (Oran and Isenman 1999). CR1 inactivates the alternative pathway C3 convertase C3bBb, because CR1 (and other regulatory proteins) binds to C3b and displaces Bb. C3b bound to CR1 is cleaved by factor I to yield inactive C3b (iC3b) which can no longer form the C3-convertase with factor B [individuals with a genetic deficiency of factor I were first diagnosed as being deficient in C3 since their blood C3 level was only about 10% that of normal due to lack of down regulation of C3 convertases and the consequent high level of conversion of C3 to C3b (Law and Reid, 1995)]. In a mouse model of mesenteric ischemia/reperfusion, treatment with soluble Crry-Ig (mouse membrane complement inhibitor –mouse CR1- with decay-accelerating activity for classical/alternative pathways) inhibited complement activation (Rehrig, Fleming *et al.*, 2001). When Crry-Ig was given 30 min after the beginning of the reperfusion phase a decrease in ischemia/reperfusion-induced intestinal mucosal injury was observed and it was comparable to that occurring when it was given 5 min before the initiation of the reperfusion phase (Rehrig, Fleming *et al.*, 2001). This suggests that inhibitors of complement activation can be used into the reperfusion phase with significant capability to attenuate tissue damage.

CR1 displaces C2b from the classical C3 convertase (C4b2b) (Kim, Kinoshita *et al.*, 1995). C4b bound to CR1 is cleaved by soluble Factor I to inactive forms, C4d and C4c.

CR1 has been shown to act as a receptor for C1q (Klickstein, Barbashov *et al.*, 1997) and for MBL (Ghiran, Barbashov *et al.*, 2000), the primary opsonins of complement. In this study we show that CR1 binds C1q but it does not bind MBL under the conditions chosen (Chapter 6.4.1). We also show that CR1 is important in the regulation of the alternative pathway (figure 6.14). Serum preincubated with sCR1 does not cleave C3 as compared to control serum. On the contrary, C4b deposition is the same in serum preincubated with sCR1 as in control serum (Figure 6.13).

In conclusion, we suggest that sCR1 is an important protein involved in the regulation of complement activation by mediating conversion of C3b to inactive C3b (iC3b) and that it is not a relevant receptor for MBL.

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