ANTIBODIES TO C1Q AND C-REACTIVE PROTEIN IN AUTOIMMUNE INFLAMMATORY DISEASE

James McBain (B.Sc)

M.Sc

A thesis submitted for the degree of Master of Science to the Faculty of Medicine, University of Glasgow.

Department of Immunology

University of Glasgow

Western Infirmary

Glasgow, G11 6NT

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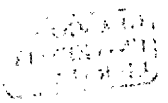

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Abstract

Background

Patients with increased levels of autoantibodies often have associated inflammatory disease. Immune complexes are often seen in inflammatory disease. They may be formed by antibodies produced against the C1q molecule which has been altered by the inflammatory process, and also by antibodies produced against C reactive protein (CRP) plasma levels of which become greatly increased during inflammation. These immune complexes can induce complement consumption, resulting in low serum levels of complement components, leading to pathogenesis of chronic inflammation and inflammatory disease. Raised levels of C3d are an indicator of increased complement consumption. It is not known whether antibodies to C1q and/or antibodies to CRP are responsible for autoimmune inflammatory disease or whether they are produced as a result of the disease.

Aims

The aim of this thesis is to establish a link between levels of antibodies to C1q, antibodies to CRP, and inflammatory disease and thus provide evidence that these antibodies may be involved in the pathogenesis of chronic inflammation leading to inflammatory disease. This thesis tries to determine if (a) patients with inflammatory disease have a significantly different than normal level of antibodies to C1q, and of antibodies to CRP, and whether this depends on immunoglobulin isotype and type of inflammatory disease. Also investigated was (b) possible associations of antibodies to C1q, and/or antibodies to CRP with age and biological markers of autoimmune inflammatory disease. Another aim was (c) to determine if there were increased levels

of C3d, an indicator of complement consumption, thus providing indirect evidence of the presence of immune complexes in patients with inflammatory disease.

Subjects and Methods

Patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and heterogenous autoimmune inflammatory disease (HAID) were tested for antibodies to C1q, antibodies to CRP, C1q levels and antibodies to double stranded DNA using enzyme-linked immunosorbent assay (ELISA) methods. Complement C3, C4 and C1 inhibitor levels were measured by nephelometry and antinuclear antibodies (ANA) by immunofluorence techniques. An ELISA method was developed to measure C3d levels in patients with SLE, renal disease and HAID.

Results

It was demonstrated that differences in antibody titres to C1q depend on the type of inflammatory disease and immunoglobulin isotype, and there is an association between antibody titres to C1q (of IgG, IgA and IgM isotype) and age and various biological markers of disease activity. Inflammatory disease appears to affect the complement levels of C3, C4 and C1 inhibitor. There is a direct link between increased antibody titres to CRP and inflammatory disease and IgG antibodies to CRP increase as C3 and C4 levels decrease. Also, patients with inflammatory disease have increased levels of C3d.

Conclusions

The results show there is strong evidence that antibodies to C1q and antibodies to CRP of certain immunoglobulin isotype (IgG, IgA and IgM) form immune complexes which induce complement consumption. The resulting complement deficiency would impair the ability of complement to clear immune complexes. An autoimmune response is then triggered and so the cycle goes on leading to chronic inflammation and inflammatory disease. Future studies should try to determine how and why this inflammatory cycle is initiated. Certainly, it appears that measurement of antibodies to CRP and possibly antibodies to C1q may prove to be a useful tool for diagnosing inflammatory disease.

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Author's Declaration

These studies represent original work made by the author, and have not been submitted in any form to any other University. Where use has been made of material provided by others, acknowledgement has been given to the person concerned.

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Abbreviations

CRP	C-reactive protein
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol-bis(α-aminoethyl ether) N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbant assay
GBM	glomerular basement membrane
HAID	heterogenous autoimmune inflammatory disease
HUVS	hypocomplementaemic urticarial vasculitis syndrome
MASP	MBP-associated serine protease
MBP	mannan-binding protein
MPGN	membranoproliferative glomerulonephritis
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus

Glossary

Antibody Protein with the moleclular properties of an immunoglobulin, produced by plasma cells, in response to stimulation by antigen and capable of specific combination with antigen

Antigen A substance that activates lymphocytes by interacting with the combining sites of T- or B-cell receptors

Autoantibody Antibody capable of specific reaction with an antigen that is a normal constituent of the body of the individual by which that antibody was formed

C1q A subcomponent of the first component (C1) of the classical pathway which, after binding to the Fc regions of immunoglobulins induces activation of the cascade of proteins composing the classical pathway

C3d A fragment produced following the breakdown of complement as a result of the activation of the classical and/or alternate pathways

Complement An enzymatic system of serum proteins that is activated by many antigen-antibody reactions and which is essential for

antibody mediated immune haemolysis and bacteriolysis and plays an important part in several other biological reactions

CRP — C Reactive protein : an acute phase beta-globulin which reacts with C-substance, a polysacharride derived from pneumococcus, in the presence of calcium ions

Dialysis — The use of semipermeable membranes to separate substances of differing molecular weights in solution

Fc fragment — Consists of the C terminal half of the two heavy chains linked by disulphide bonds. No activity as an antibody but carries sites for fixation of complement

Glomerulonephritis — A term applied to a group of kidney diseases, in all of which the major lesion is in the glomeruli

Heavy chain — A polypeptide chain present in all immunoglobulin molecules. Each chain is linked,usually by disulphide bonds to a light chain and to another identical heavy chain

Hypocomplementaemia — Any condition in which serum complement levels are low

IgG — The major immunoglobulin in the serum of humans, the molecular weight of which is 150 000 = 7S. It fixes complement and crosses human placenta

IgA — The major immuoglobulin of the external secretions, but also present in serum and has many antibody activities

IgM — High molecular weight (19S) immunoglobulin which is a cyclic pentamer of five basic units of heavy and light chains linked by disulphide bonds. There is also a monomeric form (7-8S). It has a high carbohydrate content and fixes complement

Immune complex — A macromolecular complex of antigen and antibody molecules bound specifically together. May be present in soluble form especially in antigen excess or as a precipitate

Immunodeficiency — Any condition in which a deficiency of humoral or cell-mediated immunity exists

Immunoglobulins — Member of a family of proteins each made up of light chains and heavy chains, linked together. All antibodies are immunoglobulins

Ochterlony test — Double diffusion precipitin test in which wells are cut in

an agar plate and filled with appropriate solutions of antigen and antibody which then diffuse through the agar gel to meet to form lines of precipitate

7S antibody Immunoglobulin molecules with sedimentation coefficient of about 7S used as a synonym for IgG

Sjogrens disease Chronic inflammatory disease of lacrimal and salivary glands often with conjunctivitis and rheumatoid arthritis

Systemic Lupus Erythematosus Disease causing widespread degeneration of connective tissue and lesions

Urticaria Skin rash characterised by localised, increased erythematosus, itchy weals due to histamine and vasoactive substances

Chapter 1

1 Introduction

1.1 The complement system

The complement system is an important humoral system which mediates many activities that contribute to inflammation and host defence (1). It consists of a group of individual proteins that are activated in the form of enzyme cascades. It non-specifically complements the specific effect of antibody in the opsonisation and lysis of micro-organisms. Four of the functions the complement system performs are :

1. cell activation : i.e. activation of neutrophils
2. opsonisation - rendering cells vulnerable to phagocytosis by the adherence of opsonins
3. cytolysis : i.e. the breakdown and destruction of foreign cells and bacteria (2)
4. prevention of immune precipitation : the conservation of immune complexes in soluble state to ensure their clearance from the circulation by the mononuclear phagocytic system.

1.1.1 Complement pathways

The complement system can be activated by one of three enzyme cascade pathways. The most studied are the classical pathway and the alternative pathway, but all end in the cleavage of C3, the central event in the

complement system (3). These pathways can play an important role in the pre-immune phase as well as amplifying the humoral and cellular immune response. The pathway components are shown in Table 1.1.1.

Most components are ß globulins and consist mainly of one or two peptide chains joined by disulphide bonds. The main exceptions are C1q with its unique structure and C4 with three peptide chains and the collectin MBP (1).

1.1.2 The Classical pathway

To initiate classical pathway activation the antigen bound antibody on the cell surface or in an immune complex interacts with the first component of the classical pathway, C1. As the components were numbered in the order of their discovery, the activation sequence is C1→ C4 → C2 → C3 → C5 through to C9 (Figure 1.1.2). The classical pathway evolved to take advantage of the specificity of antibodies in identifying targets for activation, to provide an effector mechanism for antibody - mediated functions. Antigen -antibody complexes are the most common classical pathway activators, but there are others such as the MBP pathway (described later in 1.1.5).

The C1component is a multimeric complex which consists of the C1q protein and $C1r_2C1s_2$ tetramer, the integrity of which is Ca^{2+} dependant. The C1q protein is the recognition subunit that can interact with the immunoglobulin molecule. The intact C1 complex is required for activation, so the process can prevented by dissociation of C1qrs when the calcium is removed. Thus, the anticoagulant compounds EDTA, EGTA, citrate, and oxalate that work by chelating calcium are also anti-complementary compounds. EDTA also prevents activation of the alternative pathway because

it chelates magnesium, required for both the classical pathway and alternative pathway C3 convertases (2,3).

There are several steps involved in the activation of C1 leading to the formation of the C1 esterase C1s. Binding of C1 to immune complexes via C1q, the recognition unit of C1, induces a conformational change that occurs within the C1q molecule. This change favours spontaneous activation of C1r which, once activated, enzymatically cleaves a peptide bond in the second C1r monomer. The two enzymatically active monomers then cleave the two C1s molecules which activates them. This process is called "autocatalytic activation" of C1r and can occur in serum even in the absence of activators of C1 (1). The sequential binding of C1r and C1s generates C1 esterase enzyme activity for the cleavage and activation of C4 and C2. Both molecules are cleaved to produce a and b binding fragments. The complex C4bC2aC2b is termed the classical pathway C3 convertase which cleaves C3 into two fragments, C3a which possesses anaphylatoxins and chemotaxins activity and C3b which binds to the initiating complex and promotes many of the biological properties of complement. The C5 convertase generated initiates the final lytic pathway.

1.1.3 The Alternative activation pathway

The alternative pathway is relatively inefficient in the tissues in comparison to the classical pathway as high concentrations of the various components are needed. However, C3 convertase is generated without the need for antibody, C1, C4 or C2 because the activators are bacterial cell walls and endotoxin. The initial cleavage of C3 in the alternative pathway happens

continuously and independently generating a low level of C3b. C3b is an unstable substance, which becomes inactive if a suitable acceptor surface is not found. When a suitable acceptor surface is found, C3b is able to use factors D and B of the alternative pathway to produce the active enzyme C3bBb which can break down more C3, providing still more C3b. This is known as the "positive feedback loop" of the alternative pathway. In the presence of properdin C3bBb is stabilised in the presence of properdin to form the C5 convertase of the alternative pathway (2,4).

1.1.4 The Lytic pathway

The final lytic pathway of complement in both the classical pathway and alternative pathway involves the sequential attachment of the components C5, C6, C7, C8 and C9 and results in lysis of the target cell.

The component C5 is cleaved into C5a and C5b fragments. The C5b fragment remains bound to the C5 convertase and acts as an acceptor for the C6 component, which in turn binds C7. This binding causes a conformational change in C7 which allows it to attach to the lipid bilayer of the plasma membrane through which holes are punctured. The C5b67 complex then in turn causes a change in C8 also allowing it to attach to the lipid bilayer. Multiple units of the C9 component and the assembly of the membrane attack complex then enlarge the punctures. Proteins, nucleic acids and high molecular-mass substances leave through these punctures and the cell becomes lysed and dies (5).

1.1.5 The MBP pathway – the Collectins

Lectins are carbohydrate-binding proteins other than antibodies and enzymes (11). Collectins are a family of C-type (Ca^{2+} dependant) lectins with collagenous regions. There are five such lectins: lung surfactant protein A and D (SP-A and SP-D), conglutinin, mannan-binding protein (MBP), and CL-43. They are composed of trimeric subunits containing a collagenous section and a C-terminal globular carbohydrate-recognising domain containing the 14 invariant amino acids characteristic of the C-type lectins. Complete molecules MBP and SP-A molecules are composed of up to six such subunits, while conglutinin, SP-D and CL-43 contain up to four subunits.

The collectins bind to carbohydrates on yeast, bacteria and viruses. To be of biological use, humoral lectin should also possess structures with biological effector functions. MBP has been demonstrated to be capable of complement-mediated lysis of mannan-coated erythrocytes, which is C4 dependant (7), and killing bacteria (8). Also, when MBP binds to zymosan, a cell wall preparation of baker's yeast, it activates the $C1r_2C1s_2$ complex without the involvement of C1q (9) and MBP activates C4 and C2 through the action of a new C1s-related serine protease called MASP (MBP-associated serine protease) (10). As in the C1q molecule, the MBP molecule has tetramers of subunits which contain N-terminal collagen-like helixes and a C-terminal globular recognition domain. The MASP molecule dimerizes like C1r and C1s and also shares 40% of their amino acid residues. The structures of

these two proteins show MBP to be homologous to C1q and MASP to $C1r_2C1s_2$.

Obsevations on the opsonising capacity of human serum indicate that the lectin pathway is of major importance and was directly correlated to the concentration of MBP in indivdual sera. (11).

Another likely biological effector function of the collectins is associated with the observation that these molecules interact the C1q receptor (now called the collectin receptor)(12). MBP, SP-A and conglutinin inhibit the binding of radiodinated C1q to purified, solid-phase collectin receptor and to myeloid cell line U937, and radioiodinated collectin receptor binds to the above collectins coated onto plastic. The collagenous section reacts with the collectin receptor found on many cells including phagocytes.

The available structural and functional information on the collectins indicate that this family of lectins plays a significant role in the innate antimicrobial defence (13).

1.1.6 Clinical relevence associated with the complement system

The inherited deficiency of C1 esterase inhibitor can produce an uncontrolled activation of the early components of the complement system which causes angioedema in the skin, the gastrointestinal and genitourinary tracts and the larynx (14). C2 deficiency is the commonest one associated with lupus. Lupus–like syndromes of malar flush, arthralgia, glomerulonephritis, fever or chronic vasculitis have been associated with patients who have inherited C1, C3 or C4 deficiency. This deficiency usually compromises the ability to combat viral antigens and leads to immune complex diseases.

Individuals with C3 deficiency are more likely to suffer from life threatening infections such as pneumonia, septicaemia and meningitis while those with deficiencies of C5, C6, C7 or C8 have presented with gonococcal infection (15).

Diseases such as systemic lupus erythematosus, which consume the complement system via the classical or alternate pathways, can impair complement activity, which increases susceptibility to infection. The consumption of the early classical pathway components reduces their capacity to neutralise or lyse certain viruses.

1.2 The Complement Component C1q

1.2.1 Structure of complement component C1q

C1q is a serum protein of molecular weight 410 kDa (16). It is a large, highly cationic glycoprotein. Electron microscopy shows the C1q molecule is a large complex hexametric structure (fig. 1.2.1) which has been shown to consist of 18 polypeptide chains of three kinds, six A chains, six B chains and six C chains (17,18). The three types differ in amino acid sequence but are similar in overall organisation : a short, N - terminal segment containing a cysteine residue followed by a collagen-like, Gly-Xaa-Yaa repeating sequence and then by a C-terminal, non-collagen-like sequence. Disulphide bond linkages, between A- B and C-C chains, in the N-terminal segments of the chains and the formation of triple helices between the collagen-like regions of each of the A-, B- and C chains, results in the assembly of the eighteen

polypeptide chains into an overall ' bunch of tulip-like' structure. The insertion of a threonine residue interupts the middle of the collagen-like region of the A-chain between the 10th and the 11th Gly-Xaa-Yaa repeating triplets. The C-chain is interupted by the insertion of three residues , Ala-Ile-Pro, between the 11th and 12th Gly-Xaa-Yaa repeats. These interruptions in the A- and C- chains disrupt the triple-helix at its mid-point causing the C-terminal portions of the collagen-like stalks of C1q to diverge away from the axis of the rest of the molecule i.e. 'bending the stalks' (19,20,21).

The hexameric globular region represents the recognition site of C1q to acceptor molecules : a variety of substances have binding site(s) for C1q or interact directly with the cationic C1q molecule resulting in activation of C1 and the classical pathway of complement.

Substances and cells that which interact directly with C1q include :

- Immunoglobulins e.g. Fc portion IgM, IgG (IgG3, IgG1, IgG2)
- Serum proteins e.g. C-reactive protein (CRP), $C1r_2$ - $C1s_2$ complex, fibronectin
- Polyanions e.g. PO_4 ,SO_4
- Viruses e.g. envelope of certain RNA viruses, Moloney viruses, HIV-1
- C1q receptors on : B-cells, PMNs, monocytes, fibroblasts, endothelial cells, platelets

1.2.2 Genomic structure of C1q

The A-, B- and C chains of human C1q have been characterised at the cDNA and genomic levels with the genes being approximately 2.6 kb, 2.5 kb and 3.2 kb long respectively each containing a single intron of approximately 1.2 kb(22).The intron is located within the collagen-like region coding sequence in the A- and C- chain genes and immediately precedes the sections coding for the interruption in the repetitive nature of the Gly-Xaa-Yaa repeating sequences in these chains. The A-, B- and C- chain genes are arranged 5' to 3' in twos ordered at A-B-C within the region 1p34. 1p36.3 on a 24 kb DNA stretch on chromosome 1.

1.2.3 Binding of C1q to immunoglobulins

The interaction between C1 and immunoglobulins takes place between the C1q globular domains and the Cγ2 domain of IgG or the Cμ3 domain of IgM. This was shown by experiments in which the globular domains were shown to inhibit the reaction between immune complex and C1 after the stalks were removed by collagenase digestion (23). Studies of the effect of ionic strength on thermodynamics of the interaction suggest a stronger bond is formed between C1q and IgG than C1q and IgM due to three salt bridges

being formed as each C1q globular domain is bound in the case of IgG whereas only two are formed in the case of IgM (24,25).

1.2.4 Interaction of C1q with IgG (Figure 1.2.4)

In order to activate the complement process, the shape of the C1q molecule must be distorted in a certain way in a process that depends on the attachment of the C1q heads to at least two Fc sites. The C1q molecule is not flexible enough to bind to the two sites on an IgG molecule simultaneously, therefore bivalent binding is only achieved when C1q spans two IgG molecules. The two IgG molecules must be within 40nm for a single C1q molecule to bridge them because this is the maximal armspan of C1q. The distances between neighbouring IgG molecules depends on the density of the bound immunoglobulins, on an antibody- coated cell . For example, on an erythrocyte with 1000 molecules of bound IgG, only 1% of the molecules are close enough to be spanned by C1q with 20 000 molecules, 20% of the molecules can be spanned (3).

1.2.5 Interaction of C1q with IgM

A pentameric IgM molecule has at least three binding sites for C1q and their spatial distribution allows a single C1q molecule to bind to a single IgM molecule firmly enough to be activated. However, the IgM binding sites are normally hidden and only become exposed when the IgM molecule binds to antigen and adopts a staple-shaped configuration (126).

1.2.6 Immune complex clearance

Clearance of immune complexes occurs by activation of the complement system. The classical pathway of the complement system may be activated by IgM and IgG containing immune complexes after binding to the globular head portions of C1q (26). Binding of Fc regions of immunoglobulins to the globular head portions of C1q induces conformational changes of the the collagen-like region, which alters the dynamic equilibrium between C1q and the other subcomponents of C1 (4,27). This results in the activation of the classical pathway which then results in the conservation of immune complexes in soluble state and ensures their clearance from the circulation by the mononuclear phagocytic system (4).

1.2.7 Clinical relevence associated with C1 complex

The C1 macromolecule is derived from five independently regulated genes and the catabolism of C1 is complex. The C1q component is generated from 18 polypeptides, each of which must undergo extensive postsynthetic modification to permit assembly. The association of C1s and C1r subcomponents with C1q is constrained by primary and higher order structure (the stringency of which is not yet known). Considering these complicated mechanisms involved in normal C1 metabolism, the relative infrequency of genetic deficiencies of C1 is surprising (28).

There are four C1 deficiency syndromes subgroups:

Type I : no C1q is detected in serum using conventional methods for protein

Type II : a dysfunctional C1q protein is present in normal or reduced amounts

Type III : an inherited deficiency of C1r (with associated decrease in steady state C1s serum levels).

Type IV : a hypercatabolic form of C1 deficiency

The hypercatabolic form of C1 deficiency was the first to be noted in which studies showed that decreased C1q was associated with several immunoglobulin deficiencies but that the levels were lowest in patients with severe combined immunodeficiency (SCID) (29,30), and that bone marrow transplantation restored C1q levels to normal (31). The basis of these observations was revealed in a study which demonstrated hyper catabolism of C1q in association with hypogammaglobulinemia (32). However, the C1q deficiency in SCID is of minor importance because the extreme deficit in cellular and humoral immunity accounts for the devastating clinical manifestations of the disorder.

Although most C1q deficient patients develop symptoms in early childhood some do not develop until adulthood. The symptoms can be prominent :

- cutaneous manfestations of systemic and discoid lupus erythromatosis
- renal disease
- arthritis and arthralgias
- cold intolerance or Raynaud phenomenon
- mucosa lesions (often stomatitis) (28)

Antinuclear antibodies, anti-Sm and circulating immune complexes are frequently found in patients serum and more rarely, anti-Ro antibodies. Skin and renal biopsies in several patients may also have symptoms fairly typical

for the renal and/or systemic collagen vascular disease manifested. C1inhibitor and C1s serum levels are elevated which emphasises the independent regulation of C1q, C1r and C1s synthesis and catabolism.

It has been suggested that C1q deficiency is also associated with increased susceptibility to infections (34) though it is difficult to be certain because some SLE patients, without primary complement deficiency, have increased susceptibility. Also, some C1q deficients have no symptoms, reflecting the importance of other genetic or environmental factors necessary for expression of disease.

1.2.8 Function and disease characteristics of antibodies to C1q

After the binding to an immune complex, the physiological role of C1q may be influenced by antibodies to C1q. Decreased C1 activation by binding of antibodies to C1q to the collagen-like region of C1q could functionally resemble a C1q deficient state and result in immune complex precipitation in the tissues. Alternatively, antibodies to C1q may also stimulate C1 activation after binding to C1q. The association between antibody titres to C1q and hypocomplementaemia may result from such stimulation (35) although studies on activation of the classical pathway in the presence of antibodies to C1q could not demonstrate any effect so, at present, there is no evidence to support the possibility that antibodies to C1q directly influence C1 activation (36).

Combined data suggests that antibodies to C1q may exert a pathogenetic role by contributing to immune complex deposition or formation in glomeruli which results in inflammation (37).

Various studies have suggested that the occurrence of antibodies to C1q may have no pathological or diagnostic significance (38,39). However, the occurrence of antibodies to C1q may be associated with specific diseases and the serial measurement of antibody titres to C1q may provide an objective tool for patient management. The first cross- sectional study on associations between antibody titres to C1q and clinical variables of disease activity in SLE patients demonstrated that the highest antibody titres to C1q were found in patients with active lupus nephritis (35). Subsequent studies demonstrated that rises in antibody titres to C1q have predictive value for ensuing relapses of lupus nephritis, and a comparison between titres of anti-dsDNA antibodies and antibodies to C1q suggested both equally effective in predicting renal relapses of SLE (41,42).

Immune complexes may be formed either in the circulation or locally in tissues. C1q molecules which are part of an immune complex, are potential antigens for antibodies to C1q. Thus, the formation and size of immune complexes may be increased by antibodies to C1q. The induction of the lattice formation of immune complexes can lead to inflammatory reactions such as the development of glomerulonephritis (43).

Antibodies to C1q may also bind to C1q that has already deposited in tissues leading to *in situ* immune complex formation which is considered to play an important role in the pathogenesis of glomerulonephritides, The preferential binding of antigens on the glomerular basement membrane (GBM) may play a role in this type of immune complex formation (44).

Evidence for the involvement of antibodies to C1q in disease include the findings that large C1q containing circulating immune complexes have

been associated with lupus nephritis (44,45) and with rheumatoid vasculitis (46). Studies of renal biopsies of lupus patients invariably demonstrate the presence of immunoglobulins and C1q in (40) glomeruli and significant increases of antibody titres to C1q preceding the development of lupus nephritis lends further support to a pathogenetic role of antibodies to C1q in the development of nephritis. Antibodies against the collagen-like region of C1q were recovered from glomeruli of 80% of SLE patients suffering from diffuse proliferative lupus nephritis.

Systemic lupus erythematosus (SLE) is considered the prototype of an immune complex disease in humans. Immune complexes that can be detected in both the tissues and circulation increase in numbers as autoantibody production increases. Each step in the clearance of immune complexes, including activation of the complement system, binding to complement receptors for transportation and the degradation of immune complexes, may be disturbed in SLE patients.

Because the classical pathway of the complement system prevents precipitation of immune complexes, patients with deficiencies of components of the classical pathway of the system may develop SLE-like syndromes suggesting that changes in the interaction between immune complexes and complement lead to SLE development (47,48,49). Activation of the classical pathway by binding of C1q to an immune complex serves to maintain immune complex solubility by preventing aggregation. Therefore activation of the classical pathway by immune complexes depends on their interaction with C1q and is a prerequisite for the prevention of immune complex diseases.

When antibodies to C1q were reported to be present in the serum of SLE patients in 1984 (50), it was considered that antibodies to C1q may not only participate in immune complex formation, but may also interfere with the physiological immune complex clearance by interaction of antibodies to C1q with C1q. Thus, antibodies to C1q may play a pathogenetic role in immune complex diseases such as SLE.

A number of assays have been employed to measure circulating immune complexes in order to relate titres of circulating immune complexes to the presence of disease manifestation (51). The most widely used is the solid phase C1q binding assay, a radioimmunoassay based on the interaction between C1q and immune complexes, where immune complexes bind to C1q which is fixed to polystyrene tubes (52). From this assay it was found that as well as immune complexes there were low molecular weight (7S) C1q precipitins which were present in high titres in SLE patients' serum, especially patients with lupus nephritis (53,54,55,56,57).

Proof for the antibody nature of "7S C1q precipitins" was supplied in 1984 (50). Monomeric IgG was demonstrated to bind to C1q through its antigen binding rather than its complement binding region. Procedures known to destroy the ability of IgG to bind to C1q like exposure to low pH, reduction and alkalation, did not influence the binding of IgG to C1q in the serum of SLE patients (50). Therefore CH2 binding of IgG to C1q was not responsible for the binding of 7S IgG to C1q . The epitope against which antibodies to C1q were directed was found to be located on the collagen like region of C1q. (58,59). Also the binding of IgG antibodies to C1q was of high affinity

directed against C1q bound to a solid phase and not affected by the fluid phase C1q (60).

In recent years evidence was provided for the presence of IgG antibodies to C1q in a number of autoimmune and renal diseases (Table 1.2.8). As well as SLE, IgG antibodies to C1q were found to be present in patients with hypocomplementaemic urticarial vasculitis syndrome (HUVS), membranoproliferative glomerulonephritis (MPGN), rheumatoid arthritis and systemic sclerosis (61,62,63). The occurrence of antibodies to C1q as a natural antibody in the general population was also investigated and it was found that the occurrence of IgG antibodies to C1q increased at an older age (64).

1.3 C-Reactive Protein and antibodies to C-Reactive Protein

The serum concentration of a number of proteins, such as an increase in the alpha and beta globulins, change rapidly during infection and illness. These are called acute phase proteins and their concentration can increase up to 1000 times their normal levels and they remain elevated throughout the infection (65,66). An example of an acute phase beta-globulin is C Reactive Protein, so-called because in the presence of Ca^{2+} ions. It reacts and precipitates with C-substance, a polysaccharide derived from pneumococcus, which is how it was first discovered (67).

The origin and function of CRP are obscure, but its increased presence in blood serum indicates that an inflammatory reaction is going on somewhere in the body and its estimation is particularly valuable as an objective and quantitative measure of illness in e.g. clinical trials.

CRP is a molecule of distinctive pentameric structure (Figure1.3) which binds avidly to DNA immune complexes from dead or damaged cells, after which it then activates the classical pathway via C1 as efficiently as IgG antibodies. Therefore CRP can induce all the known inflammatory responses of the complement system. When CRP binds to bacteria it promotes complement binding which facilitates the uptake of bacteria by phagocytes, known as opsonisation. Acute phase proteins are therefore important in the innate immunity to infection and CRP is likely to offer the most useful screen for inflammation in samples obtained from a wide range of patients (68).

Antibodies to CRP have been used to show the presence of CRP in the brains of those suffering from Alzheimers disease (69) and have been reported in patients suffering from Toxic Shock Syndrome (TOS) (70). TOS resembles autoimmune diseases such as SLE or systemic scleroderma. Consuming adulterated olive oil caused the affected patients to develop symptoms resembling the above diseases therefore this project will try to ascertain if there is a correlation between levels of antibodies to CRP and inflammation.

1.4 The Complement Component C3d

The complement system plays an essential role in the body's defence system against pathogenic organisms. The C3 component is the key component common to both the classical pathway and the alternate pathway. When activated via bacterial invasion, the C3 molecules break down into different fragments including C3d, the size of which is small enough to pass through the glomerular basement membrane and which can be detected in urine samples.

Activation of the complement cascade is of importance in the pathogenesis of immune damage in such disorders as rheumatoid arthritis and SLE. Measurement of intact components such as C3 and C4 provides only limited information concerning complement activation. In rheumatoid arthritis, for example, levels of C3 may be normal or raised in the presence of complement consumption since C3 behaves as an acute-phase reactant (71). In certain diseases, such as SLE and childhood autoimmune chronic active hepatitis, low levels of C4 are observed and are often genetically determined by the possession of C4 null allelic variants (72,73). Therefore interest has focused on measurement of the products of complement breakdown in an attempt to investigate in vivo activation of both classical and common pathways. The classical pathway is activated by immune complexes and the alternate pathway by bacterial lipopolysaccharides. Both result in the enzymic cleavage of C3, generating C3d. Fixed C3 is the major mediator of the adherence functions of the complement system involving polymorphs, lymphocytes and macrophages (74). Bound C3b is also necessary for the terminal complement sequence to proceed. This generates C5a, an important chemotactic attractant, and leads to stable fixation of the lytic C5b-9 complex (75).

Following its initial activation to C3b, metabolism of the C3 molecule proceeds, via the mediation of the C3b inactivator (I) and ß1Hglobulin (H), to cleave into the C3c and C3dg fragments (76). C3c is rapidly catabolised and cleared. In contrast, the turnover of C3d is relatively slow and as a result of its small molecular size (35 000-43 000 mol.wt), it can reach the plasma by diffusion from extravascular sites of complement activation (77). C3dg

provides a binding site for complement receptor type 2 (CR2), which is a plasma-membrane structure involved in B-cell activation and proliferation.

Therefore measurement of the plasma concentration of C3d is a more useful index of in vivo complement activation in humans and also the ratio of its concentration to that of intact C3 reflects the relative kinetics of C3 synthesis and catabolism. Renal disease in SLE is of variable severity with protean manifestations, ranging from asymptomatic proteinuria and haematuria to active nephritis to acute or chronic renal failure. The clinical management is complicated by the extreme clinical and pathological diversity in that patients with SLE can have a broad range of glomerular lesions including glomerulonephritis and intestinal nephritis (78). Many patients with lupus nephritis have very severe extrarenal systemic disease and may be acutely ill with high fevers and increased anti nDNA antibodies and low complement levels (79).

1.5 Aims of this project

Immune complexes can induce complement consumption resulting in low serum levels of complement components (80). Raised levels of C3d are an indicator of increased complement consumption. Immune complexes, which are often seen in inflammatory disease, may be formed by antibodies generated by the C1q molecule, altered by the inflammatory process and, by antibodies generated by CRP levels, which become greatly increased by inflammation.

The aim of this thesis is to establish a link between levels of antibodies to C1q, antibodies to CRP, and inflammatory disease and thus provide

evidence that these antibodies may be involved in the pathogenesis of chronic inflammation leading to inflammatory disease.

The following was carried out to try to achieve this aim :

- the development of two quantitative methods, one to measure titres of antibodies to C1q, the other to measure titres of antibodies to CRP, of isotypes IgG, IgA and IgM in both cases
- determining if patients with inflammatory disease have a significantly different than normal level of antibodies to C1q, and of antibodies to CRP, and whether this depends on immunoglobulin isotype and type of inflammatory disease
- the investigation of whether there are associations of antibodies to C1q, and of antibodies to CRP with age and biological markers of autoimmune inflammatory disease
- the development of a quantitative method to measure complement component C3d
- determining if there were increased levels of C3d in patients with inflammatory disease compared to normal

Chapter 2

2 Materials and methods

Complement C3, C4 and C1 inhibitor were all measured using nephelometry techniques. A Behring BNA II Nephelometer was employed.

The Double Diffusion technique was used to confirm the purity of C1q and a spectrophotometer was employed to assess the concentration of purified C1q.

ELISA methods were used to quantitatively measure antibodies to C1q, C1q levels, antibodies to CRP, anti ds-DNA antibodies and C3d levels. Antinuclear antibody titres were determined using immunofluorescence techniques.

2.1 The Patients

Blood sera from 97 patients from various hospitals in the West of Scotland area were involved in the experiments which comprised this thesis. The samples of blood sera were sent to the Department of Immunology, Western Infirmary, Glasgow for various immunological tests. A group of 64 patients with inflammatory diseases, were subdivided into groups of disease categories ; 27 patients with systemic lupus erythematosus (SLE), 23 with rheumatoid arthritis (RA) and 14 with symptoms of heterogenous autoimmune inflammatory diseases (HAID) which included urticaria, hypocomplementaemic urticarial vasculitis syndrome (HUVS), joint pain, Sjogrens, Raynaulds, scleroderma, mixed connective tissue disease (MCTD)

and arthralgia. 76 of the patients were also divided into a group of 38 positive for antibodies to dsDNA and a group of 38 negative for antibodies to dsDNA. The sera from a total of 30 healthy laboratory personnel (normal healthy donors) were used as controls.

The EDTA-plasma of 45 patients from various hospitals in the west of Scotland area, 15 SLE, 15 renal disease and 15 HAID, were used in the C3d experiments. The EDTA-plasma of 11 normal healthy donors were used as controls.

2.2 Definition and Clinical Criteria of SLE

SLE is defined as an inflammatory multisystem disease of unknown etiology with diverse clinical and laboratory manifestations and a variable course and prognosis. Immunologic aberations give rise to excessive autoantibody production, some of which cause cytotoxic damage while others participate in immune complex formation resulting in inflammation.

The clinical manifestations may be constitutional or result from inflammation in various organ systems, i.e. skin and mucous membranes, joints, kidney, brain, serous membranes, lung and heart. These organ systems may be involved individually or in any combination. Vital organs such as kidneys and the brain affected by SLE account for significant mortality. Mortality can result from tissue damage due to the disease process or its therapy.

Because of the various manifestations and variable courses of SLE, there is a need for classification criteria. The 1982 ACR criteria for the classification of SLE (127) helps distinguish patients with SLE from patients with other connective tissue diseases.

These criteria are :

- malar rash
- discoid rash
- photosensitvity
- oral ulcers
- haematologic disorder
- arthritis
- serositis
- renal disorder
- neurologic disorder
- immunologic disorder e.g. anti ds DNA antibody positive
- antinuclear antibody positive

Patients who satisfy at least four of these criteria are said to have SLE.

2.3 Definition and Clinical Criteria of Rheumatoid Arthritis

RA is a common severe inflammatory disorder, which affects people of all ages, but especially young adults and premenopausal woman, and is characterised by immune driven, chronic inflammation. Patients follow a variable course, involving many exacerbations and remissions of disease activity and many cases are chronic and progressive, resulting in severe disability and sometimes death.

Clinical features are that rheumatoid factors are detectable in the serum in most cases and the main target organ is the synovial lining of joints, bursae and tendon sheaths. Synovitis results in erosion of articular cartilage and marginal bone with subsequent joint destruction. Extra-articular features are common, numerous and sometimes serious. Most are due to serositis, nodule formation or vasculitis.

The Revised Diagnostic Criteria for the Classification of Rheumatoid Arthritis was set up in 1987 (128) and is listed as follows :

- morning stiffness for at least 1 hour every day for at least 6 weeks
- arthritis in at least three joints with swelling or fluid
- arthritis of hand joints
- symmetric joint swelling and involvement
- subcutaneous nodules
- radiographic changes typical of RA
- positive for rheumatoid factor test

Patients who satisfy at least four of these criteria are said to have RA.

2.4 Definition and Clinical Criteria of Renal Disease

Renal disease is mostly defined by the laboratory assessment of renal function and structure (81). The clinical features are :

- Renal Insufficiency and Renal Failure which is defined as a decrease in glomerular filtration rate and can be acute and chronic
- Nephritic and Nephrotic Syndrome : also known as nephritis, is defined as the total urinary protein excretion in excess of 3.5grams per day, reduced

serum albumen concentration, elevated serum lipid concentration and oedema

- Renal Tubular Syndromes, defined as disorders resulting from abnormal tubule handling of water and solutes, without renal deficiency
- Renal Disease with Urinary Tract Syndromes which arises from infection, obstruction and kidney stones
- Asymptomatic Urinary Abnormalities : defined as abnormalities in urinary protein excretion or in the urinary sediment that originate from the kidney, without renal insufficiency or urinary tract symptoms
- Hypertension of Renal Origin : defined as sustained elevation of arterial blood pressure as the result of diseases of the renal parenchyma or major blood vessels
- Asymptomatic Radiologic Abnormalities : defined as renal structural abnormalities observed on radiologic studies, without renal insufficiency, urinary tract symptoms, urinary abnormalities or hypertension

2.5 The ELISA Method

The antigen specific ELISA is an immunological configuration used to measure antibody activity in serum, secretions or culture fluids to a particular antigen (82). The concept is combining either antibody or antigen to spontaneously adsorb on polystyrene, polypropylene or polyvinyl permitted plastic reaction vessels to serve as solid phases for either antigen or antibody assays (83).

After incubation of the antigen or antibody with the microtitre plate,

excess antigen was washed off and the prospective antibody or antigen containing fluid was added. Although microplates allow simple adsorption and are convenient, there can be undesirable effects. Where the antigen coated solid phase was incubated with the potential antibody-containing specimen, efforts were made to prevent antibodies and particularly other immunoglobulins, from adsorbing directly onto the solid phase while permitting antibodies to bind the antigens. This was accomplished by adding the non-ionic detergent Tween 20 and/or the abundant protein gelatin to reaction buffer phosphate buffered saline.

After a suitable incubation time, the plate was again emptied, washed and the solid phase complex was incubated with the antibody–enzyme detection system, HRP. Finally, an appropriate substrate for the enzyme used was added and the extent of reaction, which was stopped by sulphuric acid, was measured after some fixed time period by determining the absorbency of the coloured product.

Sandwich ELISAs, which measure antigen quantity, are performed by immobilising capture antibodies on a microtitre plate, then adding the prospective antigen containing fluid. They are 10 to 100 times more sensitive than competitive assays (84).

2.6 Purification of complement component C1q

Purified complement components are plasma (serum) fractions

containing a high activity of the required component and low content of other proteins. On dilution these fractions behave as single components.

The preparation of purified C1q involved re-calcifying one unit of plasma (obtained from the blood transfusion service at Law Hospital) by adding 4ml of 1M $CaCl_2$ and stirring this overnight at 4° C. This was centrifuged at 4° C at 2000 rpm the next day for 15 minutes after which the supernatant was taken off and the pellet was discarded. The supernatant was now equivalent to serum.

125 ml of the serum was cooled in an ice bath (brought to 0° C by the addition of solid NaCl). One part volume of the serum was diluted with pH adjusted to 7.5 with either 1M hydrochloric acid or 1M NaOH. The serum was then dialysed for 4 hours at 2° C against 1 litre EGTA (26mM/l, pH 7.5), the conductivity being equivalent to NaCl (30mM/l). The dialysis sac containing the serum was then dialysed against 1 litre of the same buffer for a further 11 hours.

The precipitate was harvested by centrifugation at 10 000g for 30 minutes at 2° C and washed once with EGTA (26mM/l, pH 7.5) then dissolved in 32 ml of acetate buffer (20mM/l, pH 5) containing 0.75M/l NaCl and 10mM/l EDTA. Insoluable aggregates were removed by centrifugation at 10 000g for 30 minutes at 2° C.

The centrifuged supernatant was dialysed for 4 hours at 2° C against 4litres of EDTA (60mM/l, pH5, RSC 65mM/l). The precipitate was harvested by centrifugation at 10 000g for 30minutes at 2° C, washed once in EDTA(60mM/l, pH5) and dissolved in 32 ml PBS (5mM/l, pH7.5)

containing NaCl (750mM/l) and EDTA (10mM/l). Insoluble aggregates were removed by centrifugation (10 000g for 30minutes at 2°C).

Finally, the re-dissolved precipitate was dialysed at 2°C for 5 hours against 4 litres of EDTA.

2.6.1 Measurement of C1q protein concentration

The protein concentration of the purified C1q was determined by measuring absorbency using the extinction coefficient $\varepsilon^{1\%}_{cm} = 6.8$ at 280nm for C1q (85). This was performed using a spectrophotometer.

2.6.2 Double-diffusion technique to determine quality of purified C1q

The double- diffusion technique relies on the fact that when antiserum and antigen are placed in adjacent wells in agarose gel they each diffuse out from the wells. At the point where antigen and antibody meet in optimal proportions (equivalence) a line of precipitate develops.

A glass plate (80 mm x 80 mm) was cleaned by boiling for 10 minutes, then washed in de-ionised water, rinsed in alcohol and allowed to dry. A similar sized piece of gel bond was applied to the plate. The plate was then covered with 10ml of molten 1.5 % agarose in veronal buffer and allowed to solidify.

Six wells were then arranged around a central well, the wells being 3 mm in diameter and the distance between the centre of the central well and the centre of the peripheral wells being 10 mm. The central well was filled with purified C1q and the rest of the wells were filled with C1q antiserum (Diasorin Code 80274) at various dilutions and anti-whole human serum (SAPU 5044).

The gel was then placed in a glass humidity chamber on a horizontal surface at room temperature for 3 days. The gel was viewed using incident light against a dark background.

2.6.3 Electrophoresis method to determine the quality of purified C1q

Agarose gel electrophoresis was used to separate any proteins present in the purified C1q into distinct, well-defined zones. The proteins in the gel, when placed in an electrical field, will migrate towards one of the electrical probes.

The purified C1q was then electrophoretically separated six times on the same gel. A different antiserum was overlaid directly onto the gel surface along the axis of each electrophoretic migration except the first one, which was overlaid with a protein fixative. The antisera applied were antisera to C1q, C3, C4, kappa and lambda. The resulting antigen-antibody complexes became trapped in the pore structure of the gel. The gel was then dried and stained to reveal protein precipitin bands and protein reference electrophoretic pattern. Interpretation was made by visually comparing the specific precipitin bands with the reference protein electrophoretic pattern.

2.7 Measurement of C1q levels

A direct sandwich ELISA (Enzyme-Linked Immunosorbent Assay) system was used for the detection of C1q in human serum. Specific IgG antiserum to

C1q was coated onto high binding plates (Immunol 4), the sample applied and detected using the same specific antiserum conjugated to Biotin-avidin-HRP. Biotin-avidin system was utilised to increase sensitivity and also because of the lack of commercially available HRP conjugated antibodies to C1q.

In order to obtain biotin conjugate of antiserum to C1q, the following procedure was carried out. 1ml of antiserum was dialysed against 2 litres of PBS overnight. The protein concentration was then measured to ensure it was not greater than 5 mg/ml.

2.3mg Biotinamidocaproate dissolved in 100µl of dimethylformamide was added to the dialysed antiserum and left to stand at room temperature for 2 hours with occasional shaking. It was then dialysed against 4 litres of PBS overnight and stored at 4°C.

The measurement of C1q levels using the ELISA system was carried out as follows :

an ELISA plate was coated, 100µl/well, with C1q antiserum diluted to a concentration of 5µg/ml in 0.1M Na_2CO_3 ,0.1M $NaHCO_3$, pH 9.6 buffer and left to incubate overnight at 4°C. The plate was then washed 3 times in 0.05% Tween in PBS and then blocked by adding 200µl/well of 0.1% gelatin in PBS containing 0.05% tween for 1 hour at room temperature.

8 standard dilutions were made by making an initial dilution of normal human serum (SAPU, Law Hospital, Carluke) of 1/10 000 and doubly diluting until reaching 1/1 280 000 from which a standard curve is finally drawn. Test samples and a normal control were diluted at 1/10 000, 1/20 000 and 1/40 000. All dilutions were made in blocking buffer/ 0.086M EDTA. The plates were then washed 3 times in PBS/Tween. Standard curve samples were applied in

duplicate while test samples were applied in triplicate at 100μl/well and left to incubate at room temperature for 2 hours. The plate was then washed and 100μl/well of 5μg/ml C1q antiserum conjugated to Biotin diluted in blocking buffer to 1/5000 was added and incubated for 1 hour at room temperature. After washing, 100μl/well of avidin HRP diluted to 1/5000 in blocking buffer was added and incubated at room temperature for a further hour. After a final wash, 100μl/well of developing buffer (0.4mg/ml OPD in citrate-phosphate buffer, pH5.0, containing 4μl/ml of 3% hydrogen peroxide) was added and the reaction was stopped by 4N H_2SO_4.

The plate was then read at 490nm on a Dynatech MXR ELISA plate reader.

2.8 ELISA method to measure antibodies to C1q

Most studies have modified the solid phase C1q binding assay for the measurement of antibodies to C1q into an enzyme linked immunosorbent assay (ELISA) for the measurement of antibodies to C1q in which either whole, or the collagen-like region of C1q was employed (58,62,63). One pitfall in the measurement of antibodies to C1q is possible interference by binding of immune complexes in assays using whole C1q. This may be inhibited by using high ionic strength conditions in the incubation media. In 1990, complete inhibition of binding of immune complexes was demonstrated when the sodium chloride concentration used exceeded 0.15 M (46).

A second pitfall in IgG antibodies to C1q measurement is the binding of immune complexes containing small native ds-DNA fragments to the

collagen-like region of C1q which could give false positive results by IgG anti-dsDNA bound to C1q (86,87). This was also shown to be prevented by high ionic strength incubation medium (88,89). Notably binding of antibodies to C1q to C1q was shown not to be affected by high ionic incubation medium, therefore antibodies to C1q measurement should always be performed under high ionic strength conditions (36,86,50,90,91).

A third pitfall in IgG antibodies to C1q measurement may be cross reactivity with C1q by anti-collagen antibodies. Proteolysis by collagenase or exposure to oxydation of C1q may reveal epitopes on the collagen-like region of C1q that may also occur on collagen type II (92,93). IgG autoantibodies to collagen are present in the serum of SLE patients (94). This issue is addressed in 3.1.2. An ELISA plate was coated 100μl/well with purified C1q diluted to a concentration of 11μg/ml in 0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH 9.6 and incubated overnight at 4°C. The plate was then washed 3 times in 0.05% Tween in PBS and blocked by adding 200μl/well of 1% complement depleted foetal bovine serum in PBS containing 0.05% tween and 1M NaCl for 1 hour at 37°C.

8 standard dilutions were made by making an initial dilution of 1/200 and doubly diluting until reaching 1/12800. Test samples and a control were diluted at 1/200. All dilutions were made in the above blocking buffer, again containing 1M NaCl (high ionic strength to prevent IgG anti-dsDNA bound to C1q).

The plate was then washed 3 times. All samples were applied at 100μl/well and left to incubate for 2 hours at 37°C. The plate was then washed 3 times and 100μl/well of rabbit anti-human Ig/HRP conjugate at a

dilution of 1/1000 in blocking buffer was added and incubated at 37°C for 1 hour. After a final wash, 100μl/well of reaction substrate was added comprising of 0.4 mg/ml OPD in citrate-phosphate buffer, pH 5.0, containing 4μl/ml of 3% hydrogen peroxide. The reaction was stopped with 50μl/well 4Nsulphuric acid and the optical density was read on Dynatech MXR at 490nm.

2.9 ELISA method to measure anti DNA antibodies

Autoantibodies associated with the disease SLE mostly target intranuclear nucleic acids, proteins and RNP complexes and are called antinuclear antibodies (ANA). ANA is a test for any autoantibody that binds to non-specific antigens within a cell. One of the specific antigens, however, is double stranded DNA and a more specific test is needed to determine the presence of antibodies to dsDNA.

The genetic information of all living organism is contained in deoxyribonucleic acid (DNA). DNA forms a double stranded helix when intact because it pairs complementary bases on each strand and is carried on chromosomes. There are 3 forms of DNA which are antigenic in SLE : single stranded (ssDNA), double stranded (dsDNA) and left handed (zDNA).

Antibodies to dsDNA are detected in approximately 60% of patients with SLE. In general, antibodies to dsDNA levels reflect disease activity, but a lot of exceptions make antibody levels of limited value in many patients. For example, the strongest clinical association of antibodies to dsDNA has been with nephritis, although patients may have high levels of antibodies to dsDNA without renal disease.

A commercial kit (Cambridge Life Sciences Code M4196) was used to measure antibodies to double stranded DNA titres. Test samples, a reference standard and controls were diluted 1:100 in sample diluent, prediluted standards (0-800 IU/ml in increasing concentration) were all added 100µl/well to a U-shaped well microplate. An antigen coated micropin lid was then placed on top of the microplate, with the tips of the pins immersed in the samples and incubated for 15 minutes. 100 µl/ well of conjugate was added to a second U-shaped well microplate. The micropin lid was then washed and the pins immersed in the conjugate filled plate for 15 minutes. 200 µl/ well of substrate was then added to a flat-bottomed microplate, the micopin plate was washed and added to this plate. Finally, the micropin plate was discarded and 50 µl / well of 4M sulphuric acid was added to the flat-bottomed plate. The optical density was measured at 490nm on a Dynatech MRX microplate reader.

2.10 ELISA method to measure antibodies to C Reactive Protein

An ELISA plate was coated 100µl/well with C Reactive Protein (Sigma Cat No.9007-41-4) diluted to a concentration of 2.5 µg/ml in 0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH 9.6 and incubated overnight at 4°C. The plate was then washed 3 times in 0.05% Tween in PBS and blocked by adding 200µl/well of in PBS containing 0.05% Tween and incubated for 1 hour at 37°C.

8 standard dilutions were made by making an initial dilution of 1/200 and doubly diluting until reaching 1/25600. Test samples and a control were diluted at 1/200. All dilutions were made in the above blocking buffer.

The plate was then washed 3 times. All samples were applied 100μl/well and left to incubate for 1 hour at 37°C. The plate was then washed 3 times and 100μl/well of rabbit anti-human Ig/HRP conjugate at a dilution of 1/1000 in blocking buffer was added and incubated at 37°C for 1 hour. After a final wash, 100μl/well of reaction substrate was added comprising of 0.4 mg/ml OPD in citrate-phosphate buffer, pH 5.0, containing 4μl/ml of 3% hydrogen peroxide. The reaction was stopped with 50μl/well 4N sulphuric acid and the optical density was read on Dynatech MXR at 490nm.

2.11 Measurement of C3d levels

The C3d-ELISA provides a very sensitive technique for the evaluation of complement activation in biological fluids.

2.11.1 Preparation of C3d standard

A C3d standard was derived from SAPU normal human serum by incubation with 3mg/ml of inulin at 37°C for 1 hour, producing exhaustive activation of the alternative pathway C3 convertase. This was centrifuged at 1500xg for 30 minutes and then the supernatant was aliquoted and used as a C3d standard.

2.11.2 C3d measurement by Double Decker Rocket Immunoelecrophoresis (DDRIE)

For C3d analysis the blood to be tested was collected in sample bottles containing EDTA to prevent falsely elevated results due to breakdown products created by the coagulation process. EDTA also inhibits complement activation by chelating Ca and Mg ions which are essential components of the complement cascade. The samples were stored at -70°C to avoid "in vitro" complement activation during storage.

The DDRIE method involves antiserum being incorporated into agarose and wells being cut into the agarose to hold the antigen. When an electric current was applied the antigen migrates towards the anode. At first, soluble complexes are formed in antigen excess, but as the antigen migrates further it becomes more dilute. Eventually, equivalence is reached and an insoluble precipitate is formed. The precipitate redissolves and moves forward as the antigen reaches it and finally, when no more antigen remains to enter the precipitate, a stable arc is formed which becomes stationary. The area under the rocket is proportional to the concentration of the antigen.

Activation of the classical pathway, or alternate pathway, results in breakdown of C3 with the formation of C3d. In order to separate any C3d from native C3, electrophoresis is carried out in a two tier agarose gel. The first tier contains anti-C3c to remove native C3 and breakdown fragments except C3d. Free C3d then enters the anti-C3d gel, where the size of the rocket formed is proportional to the concentration of the C3d.

A glass plate was covered with a sheet of gel bond and had molten neutral 1% agarose, molten 1% agarose mixed with C3c antiserum and molten 2% agarose mixed with C3d antiserum and 8% polyethylene glycol(PEG)

poured onto it. When the gel had solidified a row of 3mm holes were cut using a gel punch.

A C3d standard was derived from normal human serum (obtained from SAPU, Law Hospital, Carluke) by incubation with 3mg/ml of inulin at 37°C for 1 hour, producing exhaustive activation of the alternate pathway C3 convertase. Following centrifugation at 1500xg for 30 minutes, the supernatant was removed and used as a standard. This standard was given an arbitrary value of 250 units/ml. A standard curve was derived by diluting the standard in PBS to give arbitrary values of 50, 25, 18.2, 12.5 and 5 U/ml.

The plate was placed in an electrophoresis tank and two Cambrelle wicks were moistened with the PBS in the tank and placed in contact with the edges. Then a low current 15-24Vwas passed through the plate to help minimise lateral diffusion. 5μl of test and standard serum were put into the wells, after which the voltage was increased to 40V for 18 hours.

The plate was then removed from the electrophoresis chamber, covered with blotting paper and dried. The dried film was placed in Coomassie Blue stain and then placed in alcohol to remove excess stain. The film was allowed to air dry.

2.11.3 C3d-ELISA method

Microtitre plates were coated with 100μl/well of rabbit anti-human C3d diluted to a concentration of 1μl/ml in PBS. Plates were then stored at 4°C for 24 hours, followed by incubation at 37°C for 1 hour with 100μl/well of 1% foetal bovine serum in PBS to eliminate non-specific binding to the plastic wells.

100μl/well of the standard and each of the test samples were added to an equal volume of 22% polyethylene glycol (PEG)2000 in PBS. Following incubation for 1 hour at 4°C, the mixture was centrifuged at 1500xg for 30 minutes at 4°C. This manoeuvre precipitates native C3 and related molecules, leaving C3d in the supernatant. The supernatant of the standard was diluted in PBS containing 0.01% foetal bovine serum to a concentration of 1/500 and then doubly-diluted to a final concentration of 1/64 0000 to obtain a standard curve. The supernatant of the plasma of the test samples were diluted in PBS and tested at a concentration of 1/500. All were added at 100μl/well and incubated at 37°C for 1 hour.

Plates were then washed 3 times in PBS and 100μl/well of horse-radish peroxidase-conjugated rabbit anti-human C3d (Dako P0387) diluted 1/2000 in PBS added to each well for 45 minutes at 37°C. After 3 more washes in PBS, 100μl/well of reaction substrate was added comprising of 0.4mg/ml OPD in citrate-phosphate buffer, pH5.0, containing 4μl/ml of 3% hydrogen peroxide. The reaction was stopped by 50μl/well 4N sulphuric acid and the optical density was read at 490nm.

Chapter 3

3 Results

3.1 The role of antibodies to C1q in inflammatory disease

The C1q molecule, altered by the inflammatory process, may generate antibodies capable of forming immune complexes. These immune complexes are often seen in inflammatory disease.

An investigation was carried out to determine if antibodies to C1q of various Ig isotype are associated with inflammatory disease. Initially, C1q, to be used as an antigen, had to be isolated and purified. Then a method, using ELISA to measure antibodies to C1q, had to be developed.

3.1.1 Purification of C1q

Using the extinction coefficient $\varepsilon^{1\%}_{cm} = 6.8$ at 280nm for C1q, the final volume obtained from plasma using the purification of C1q method described in 2.6.1 was diluted to a concentration of 1mg/ml, employing a spectrophotometer.

3.1.1.1 Double - diffusion method to determine the quality and provide evidence of purified C1q

Precipitins were formed between the purified C1q and the C1q antiserum at all dilutions, the strongest at 1:1, the next strongest at 1:2, and the weakest at 1:4 (Figure 3.1.1.1).

No precipitins were formed between the anti-whole human serum and the purified C1q.

3.1.1.2 Electrophoresis method to determine the quality of the purified C1q

The electrophoresis gel (figure 3.1.1.2) showed a single protein band in the protein-fixed migration. Protein analysis by immunofixation with C1q antiserum and C3, C4, kappa and lambda antisera identified this band as C1q. Immunofixation with C3, C4, kappa and lambda showed no discernible bands.

In conclusion, the purified C1q was free from other major serum proteins.

3.1.2 Determination of cross-reactivity with C1q by anti-collagen antibodies

A cross-reacting antibody is capable of combining with an antigen which did not specifically stimulate its production. The antigen for GMB antibodies is part of the glomerular basement membrane mainly consisting of different collagen chains (95). C1q has a collagen-like region. Because glomerular basement membrane (GBM) contains collagen, it was determined whether two commercially available blood serum samples containing antibodies to GBM (collagen) were positive for antibodies to C1q. If so, then cross-reactivity exists. The results are shown in Table 3.1.2.

The samples containing anti-collagen antibodies were negative for antibodies to C1q so there is no cross reactivity between antibodies to GBM and C1q.

3.1.3 Measurement of antibodies to C1q

3.1.3.1 Determination of standards and normal ranges

A serum of known arbitrary value for IgG antibodies to C1q, i.e. 1241 U/ml (from Professor Daha, Department of Nephrology, University Hospital,

Leiden, The Netherlands calculated using the method described in (40)) was used as a known value standard. A checkerboard technique, where the sample was tested at different sample, plate coating and conjugate dilutions, was employed to determine the optimum conditions for the standard curve. The values of IgG antibodies to C1q of serum from 27 healthy donors were obtained. In order to establish a normal range for IgG antibodies to C1q, the mean titre + 2 standard deviations of the serum samples of these healthy donors was regarded as the upper limit of normal levels. The levels are listed in Table 3.1.3.1.

The mean was found to be 71 U/ml, the standard deviation 42 U/ml, therefore the upper limit of normal was calculated to be 155 U/ml.

There are no agreed standard sera for IgA and IgM antibodies to C1q, therefore the normal ranges for these antibody isotypes was obtained by testing high titre IgG antibodies to C1q positive sera for corresponding IgA and IgM activity. This was done by an ELISA "checkerboard" technique. The optical densities of serum from 5 normal controls and 5 patients with high titre IgG antibodies to C1q were measured. In both cases the serum with the highest optical density was chosen for use as a standard for each isotype. Each of these standards was given an arbitrary value of 100 U/ml. The IgA antibody titres to C1q and IgM antibody titres to C1q of 30 healthy donors were measured using ELISA technique. The mean titre + 2 standard deviations was considered the upper limit of normal. The levels are listed in Table 3.1.3.1 and the mean for IgA antibodies to C1q was found to be 22 U/ml, the standard deviation 25, so the the upper limit of normal was 72 U/ml. The mean value for IgM antibodies to C1q was 44 U/ml, the standard deviation 28 and the

upper limit of normal 100 U/ml. Any value greater than the upper limit of normal was considered to be significantly raised.

3.1.3.2 Comparison of antibody titres to C1q in normal healthy donors and patients with inflammatory disease

Antibodies to C1q may form immune complexes which, in turn, perpetuate inflammation. This study tries to determine if patients with inflammatory disease have a higher than normal level of antibodies to C1q.

In order to determine whether patients with inflammatory disease had higher antibody titres to C1q than normal, 64 patients with inflammatory disease were tested and the results are shown in Table 3.1.3.2. The antibody titres to C1q of different isotype in serum samples of these patients, and normal healthy donors, was compared. There was no statistical significance in IgG antibody titres to C1q found in patients with inflammatory disease when compared with normal healthy donors but IgA and IgM antibody titres to C1q were found to be significantly lower. These findings prompted further investigations to determine if there were differences in titres between types of inflammatory disease.

The patients were then subdivided into groups of disease categories : SLE, RA and HAID. The antibody to C1q levels in each of these categories was compared to those of normal healthy donors and the results shown in Table 3.1.3.2. IgG antibody titres to C1q were significantly higher in patients with SLE when compared to normal healthy donors, while IgA antibody titres to C1q were significantly lower in RA patients. IgM antibody titres to C1q

were significantly decreased in all three disease categories. These results suggest differences in titres of antibodies to C1q depend on type of disease and immunoglobulin isotype.

3.1.3.3 Correlation of serum antibody titres to C1q with age (range 17-89 years) and with biological markers of autoimmune disease activity in patients with various autoimmune inflammatory diseases

Associations of antibody titres to C1q with biological markers of autoimmune disease activity and age would imply antibodies to C1q may contribute to disease activity. This may show measurement of antibody titres to C1q as a possible diagnostic tool for determining inflammatory disease. The aim was to investigate whether associations exist. The biological markers included anti-nuclear antibodies, anti-double stranded DNA antibodies, complement C3, C4, C1 inhibitor and C1q level. 64 patients with inflammatory disease were tested and the results are shown in Table 3.1.3.3.a.

No significant correlations were found except for IgA antibodies to C1q which were found to be correlated with titre of antibodies to double stranded DNA.

The patients were subdivided into groups of disease categories, SLE, RA and HAID and the results are shown in tables 3.1.3.3b-d.

At $p < 0.05$ there were correlations of antibodies to C1q with age and IgA antibodies to C1q with ANA in SLE patients, IgG antibody titres to C1q with C3 and C4 and IgM antibodies to C1q with age in RA patients, and also IgM antibody titres to C1q with C1 inhibitor. In HAID patients IgG antibody

titres to C1q were significantly correlated with age and C1 inhibitor concentrations, and IgA antibodies to C1q with C4 and C1 inhibitor concentrations.

Patients with HAID appear to produce more IgG antibodies to C1q as they get older and when there is a high level of in C1 inhibitor. They also have high levels of IgA antibodies to C1q when there is a high level of C4 and C1 inhibitor present.

While these results suggest there is an association between antibody titres to C1q of certain immunoglobulin isotype and age and various biological markers of disease activity, they are not conclusive evidence that antibodies to C1q contribute to inflammatory disease in general.

3.1.3.4 Correlation of biological markers of autoimmune disease activity in patients with inflammatory disease

After finding that correlations existed between antibodies to C1q and other complement components, it may be useful to determine whether there were associations between the individual markers to try and ascertain their possible influence on inflammatory disease. The results for inflammatory diseases, SLE, RA and HAID are shown in Tables 3.1.3.4a-d respectively. Results of $p < 0.01$ were considered significant.

C3 was correlated with C4 in patients with inflammatory diseases, SLE and RA, but not HAID. C1 inhibitor again featured in HAID patients correlating highly significantly with C3 and also with C3 and C4 in RA. In

patients with SLE, antibodies to double stranded DNA correlated with C1 inhibitor and C1q level.

Generally, inflammatory disease appears to affect the levels of C3, C4 and C1 inhibitor.

3.1.3.5 Comparison of some complement component levels of patients with autoimmune inflammatory diseases

The correlations found between certain complement components where inflammatory disease appeared to affect the levels of C3, C4 and C1 inhibitor prompted this investigation. The levels of these components in patients with autoimmune inflammatory disease were compared to normal and to each other and the results are shown in Tables 3.1.3.5a-b.

There was a significant correlation of SLE with HAID and RA with HAID in C3 which suggests that this complement protein is consumed due to inflammatory disease.

A very high proportion of HAID patients (12/14) had low C3. This suggests that low C3 may be a characteristic of patients with HAID and that they consume large amounts of C3. Activation of C3 causes swelling of blood vessels, known as vasodilation, which leads to inflammation, which in turn stimulates C3 activation. HAID patients may have chronic inflammation which stimulates a constant turnover of C3 therefore leading to the low levels observed.

3.1.3.6 Comparison of the antibody titres to C1q in serum samples of 2 groups of patients ; one group with normal levels, and one group with increased titres of antibodies to double stranded DNA

Increased levels of antibodies to double stranded DNA are characteristic of patients with SLE (96) and are associated with IgG antibodies to C1q (35) but less is known about their association with IgA and IgM antibodies to C1q.

The aim of this section is to determine if there was a quantitative relationship between IgG antibodies to C1q and antibodies to double stranded DNA. A further aim is to examine whether such a relationship applied to the IgA and IgM isotypes of antibodies to C1q because a significant correlation was found between IgA antibodies to C1q and antibodies to double stranded DNA in patients with inflammatory disease (3.1.3.3). Since a positive result for antibodies to double stranded DNA is a criterion from the ACR criteria for the classification of SLE, then a comparative increase of antibodies to double stranded DNA would suggest antibodies to C1q could also become a criterion of SLE.

The serum of 39 patients with increased titres of antibodies to double stranded DNA and 39 patients with normal tittres of antibodies to double stranded DNA titres were tested for antibodies to C1q (Table 3.1.3.6). IgG antibody titres to C1q were found to be significantly higher in patients with increased antibodies to dsDNA titres compared to antibodies to dsDNA normal patients. There was no significant difference in IgA antibodies to C1q or IgM antibody titres to C1q.

There appears to be a relationship between increased IgG antibodies to C1q and antibodies to double stranded DNA.

A relationship has been established between antibodies to C1q and antibodies to double stranded DNA, and therefore with inflammatory disease. Since C reactive protein (CRP) binds avidly to DNA immune complexes from dead and damaged cells and the anti-inflammatory effects of CRP requires the presence of C1q (97), further studies should include associations of antibodies to CRP with inflammatory disease.

3.2 The role of antibodies to CRP in inflammatory disease

Apoptotic cells are cells which are genetically programmed to die. They cleave their DNA into small fragments by their own enzymes. C reactive protein (CRP) is an acute phase protein which is thought to promote the removal of nucleic acid and dead or damaged cells by phagocytosis. CRP may do this by binding to the DNA-histone complexes exposed in dead or damaged cells. After binding, CRP then activates the classical complement pathway via C1, thereby inducing all the known inflammatory, opsonising and complex solubilizing activities of the complement system. All of these processes should reduce chronic inflammation (98,99,100).

Deficiency of C1q is the predominant cause of both the impaired clearance of apoptotic cells and increased predisposition to spontaneous autoimmunity. Apoptotic cells are thought to be a major source of the autoantigens of SLE (101). CRP is thought to carry out a similar immune complex clearance role to that of C1q and the classical complement pathway.

This thesis postulates that antibodies to CRP may be produced in response to increased levels of CRP and may inhibit the ability of CRP to carry out its immune complex clearance process. This could perpetuate inflammation which may lead to inflammatory disease.

An investigation was carried out to determine if antibodies to CRP are associated with inflammatory disease. Initially, a method using ELISA to measure antibodies to CRP had to be developed.

3.2.1 Measurement of antibodies to CRP

There were no standard sera available for IgG, IgA and IgM antibodies to CRP therefore an in-house standard serum was obtained. Sera of 24 patients with autoimmune disease was tested for IgG, IgA and IgM antibody activity to CRP using a checkerboard technique. This involved testing samples at different sample, plate coating and conjugate dilutions to determine the optimum conditions for the assay performance. The serum showing the most antibody activity for each isotype, i.e. the highest optical density, was chosen as a standard. Each of these standards was given an arbitrary value of 100 U/ml. Suitable standard curves were obtained by double diluting the standard for each isotype and was used to measure the IgG, IgA and IgM antibody titres to CRP respectively.

A normal range was established using serum from 30 healthy donors and the levels are listed in Table 3.2.1. The mean titre + 2 standard deviations of these sera samples was regarded as the upper limit of normal.

The mean for IgG antibodies to CRP was found to be 23 U/ml and the upper limit 59 U/ml, the mean value for IgA antibodies to CRP to be 12 U/ml

and the upper limit to be 28 U/ml and, the mean value for IgM antibodies to CRP to be 7 U/ml and the upper limit 24 U/ml. Any value greater than the upper limit of normality was considered to be significantly raised.

3.2.2 Comparison of antibody titres to CRP in healthy donors and patients with inflammatory diseases

Antibodies to CRP may reduce levels of CRP which, in turn, may perpetuate inflammation. This study tried to ascertain if patients with inflammatory disease would show a higher than normal level of antibodies to CRP.

64 patients with inflammatory disease were tested and the results are shown in Table 3.2.2. IgM antibodies to CRP were significantly higher than normal ($p < 0.05$) while IgG and IgA antibodies to CRP were very significantly increased when compared with serum levels in normal healthy donors.

This suggests that antibodies to CRP may inhibit the immune role of CRP. These findings prompted further investigations to determine if there were increased titres between different types of inflammatory disease.

The patients were sub-divided into categories of disease : SLE, RA and HAID (Table 3.2.2). The antibody titres to CRP in each of these categories were compared to those of normal healthy donors. There were significantly higher antibody titres to CRP in all categories of patients. The antibody isotype was especially high for the IgG and IgA antibodies to CRP in SLE when compared to normal healthy donors. However, IgM antibodies titres to CRP in HAID patients was normal.

The results suggest there is a direct link between increased antibody titres to CRP and inflammatory disease and therefore it appears increased antibodies to CRP may have a detrimental effect on the immune capabilities of CRP.

3.2.3 Correlation of serum antibody titres to CRP with age (range 17-89 years) and biological markers of autoimmune disease activity in patients with various autoimmune inflammatory diseases

The aim was to investigate if there was an association of antibody titres to CRP with biological markers of autoimmune disease activity (see below) and also if there was an association of antibody titres to CRP with age. Any associations found would imply antibodies to CRP may contribute to disease activity. The biological markers included anti-nuclear antibodies, anti-double stranded DNA antibodies, complement C3, C4, C1inhibitor and C1q level.

64 patients with inflammatory disease were tested and the results are shown in Table 3.2.3.a. IgG antibody titres to CRP were negatively correlated with age, C3 and C4. IgA was negatively correlated with C1 inhibitor while IgM was positively correlated with antibodies to double stranded DNA.

The patients were then subdivided into groups of disease categories : SLE, RA and HAID and the results are shown in Tables 3.2.3b-d.

IgG antibody titres to CRP were negatively correlated with C3 and C1 inhibitor in HAID patients. IgA antibody titres to CRP were positively correlated with DNA and negatively with C3 and C4 in SLE patients and strongly correlated with C1 inhibitor in HAID patients. IgM CRP titres were

positively correlated with ANA and antibodies to dsDNA in SLE patients and age in HAID patients. No correlations of any kind were found in RA patients.

Decreased levels of C1 inhibitor have been described in patients with autoimmune disease and this decrease was due to complement consumption (109). This present study showed that in HAID patients C1 inhibitor levels decreased significantly as IgG and IgA antibodies to CRP levels increased. This suggests that these antibodies are associated with complement consumption in HAID patients.

The results suggest that, in SLE patients, as the level of antibodies to double stranded DNA increases, DNA immune complexes increase. This raises the level of CRP which, in turn, generates IgM antibodies to CRP. In inflammatory disease, as levels of IgG antibodies to CRP increase C3 and C4 levels decrease, suggesting that IgG antibodies to CRP induce complement consumption.

Measurement of intact components such as C3 and C4 provides only limited information concerning complement activation. The breakdown product C3d is a more useful indicator of complement breakdown and therefore further studies should include associations of C3d level with inflammatory disease.

3.3 C3d as an indicator of inflammatory disease

Raised levels of C3d are an indicator of increased complement activation. Since complement activation is thought to lead to inflammation, the aim of this part of the study is to investigate if there was an increased level of

C3d in patients with inflammatory disease, which would show measurement of C3d as useful in diagnosing various inflammatory diseases. Initially, a method using ELISA to measure C3d level had to be developed.

3.3.1 Correlation of C3d ELISA levels in the plasma of patients with inflammatory disease with C3d DDRIE levels

A method using ELISA was developed, based on a method previously described (102), with the aim to provide a more accurate and quantitative test to measure C3d level in blood plasma than the traditional tried and tested Double Decker Rocket Immuno Electrophoresis (DDRIE) method.

A C3d standard was obtained as described in 2.11.2 and 2.11.3 and normal blood plasma were tested at different sample, plate coating and conjugate dilutions to determine the optimum conditions for assay performance. The C3d standard produced a far higher optical density value than the normal blood plasma suggesting the standard had a far higher concentration of C3d.

The standard was initially diluted to a concentration of 1/500 and doubly-diluted to a final concentration of 1/64 000 to obtain a suitable standard curve. The initial dilution was given an arbitrary value of 100 U/ml, the second dilution 50 U/ml and so on.

A normal range was established using plasma samples from 11 healthy donors. The mean titre +2 standard deviations of these samples were regarded as the upper limit of normal. The mean for normal C3d level was found to be

13 U/ml and the upper limit to be 33 U/ml. Any value greater than the upper limit of normality was considered to be significantly raised.

The results from measurement of 45 patients with inflammatory disease using both methods were compared in order to test the validity of the newly developed C3d ELISA .The results are shown in Figure 3.3.1.

The data from the ELISA method shows almost normal distribution whereas the data from the DDRIE method is skewed. Therefore, in order to normalise the data from the DDRIE method, the log DDRIE data was correlated with the ELISA data. However, the correlation co-efficient remained almost the same (0.352, $p<0.05$).

A significant correlation was found between the two methods suggesting the ELISA method was valid.

3.3.2 Comparison of C3d ELISA levels in the plasma of normal human donors and patients with inflammatory disease

Raised levels of C3d show evidence of complement consumption, which is associated with inflammation. The aim is to determine if raised C3d levels are indicative of inflammatory disease.

Patients with inflammatory disease were tested as a whole and then in the three categories, SLE, renal disease and HAID, and the results are shown in Table 3.3.2. C3d levels were significantly higher in all the disease categories when compared to normal healthy donors. This suggests that increased complement activation is associated with inflammatory disease.

Chapter 4

4 Discussion

Patients with increased levels of autoantibodies often have associated inflammatory disease. This thesis tries to determine if patients with inflammatory disease have a significantly different than normal level of antibodies to C1q, and of antibodies to CRP, and whether this depends on immunoglobulin isotype and on the type of inflammatory disease. Also investigated was whether there are associations of antibodies to C1q, and of antibodies to CRP with age and biological markers of autoimmune inflammatory disease such as anti-double stranded DNA antibodies and complement C3, C4, C1 inhibitor. This thesis has demonstrated that :

- differences in titres of auto-antibodies to C1q and CRP depend on category of inflammatory disease and immunoglobulin type
- an association of antibodies to C1q and antibodies to CRP of certain immunoglobulin isotype with age and various biological markers of disease activity
- patients with increased levels of antibodies to double stranded DNA tend to have increased levels of antibodies to C1q
- antibodies to C1q and antibodies to CRP are strongly associated with C1 inhibitor in HAID patients
- in inflammatory disease, as levels of IgG antibodies to CRP increase, C3 and C4 levels decrease

- there appears to be a direct link between increased antibody titres to CRP and inflammatory disease
- inflammation increases production of C3d in the plasma
- generally, inflammatory disease appears to affect the levels of C3, C4 and C1 inhibitor. In some diseases these levels are reduced in a co-ordinated manner suggesting consumption by the classical pathway. In others, raised levels may suggest increased synthesis as part of the acute phase pathway

Why study antibodies to C1q?

Immune complexes consist of antibodies non-covalently combined with their corresponding antigens (103). They can deposit in tissues, activate the complement pathways and result in a series of inflammatory reactions (104).

Antibodies to C1q have been demonstrated to contribute to the formation of circulating immune complexes (46). C1q molecules bound by immunoglobulins that are part of an immune complex are potential antigens for antibodies to C1q. Antibodies to C1q may amplify the formation of immune complexes by increasing immune complex size. If these circulating immune complexes are trapped, inflammatory reactions can occur (105).

Measurement of antibody titres to C1q may become a valuable tool for the clinical management of patients with inflammatory disease.

Why study antibodies to CRP?

CRP serum levels increase during inflammation which is thought to promote opsonisation, enhance phagocytosis and stimulate leucocyte motility (106). It was postulated that increased levels of antibodies to CRP may inhibit the ability of CRP to carry out the above immune processes therefore causing prolonged diseases such as systemic lupus erythematosus and rheumatoid arthritis. This thesis tries to ascertain if there is also an increased presence of antibodies to CRP in patients with systemic lupus erythematosus and rheumatoid arthritis which would show a correlation between antibodies to CRP and inflammation.

Why study C3d levels?

Activation of the complement cascade is of importance in the pathogenesis of immune damage in rheumatoid arthritis and SLE (107). Levels of complement C3 may be normal or raised in the presence of complement consumption. Since C3 behaves as an acute phase reactant in rheumatoid arthritis, and low levels of C4 are often genetically determined by the possession of C4 null allelic variants (108), then the measurement of fragment C3d, a product of complement breakdown of both the classical and alternative pathways, was considered to be a more reliable method of determining complement activation.

This study tries to develop an ELISA method which would be a more quantitative and sensitive than the traditional DDRIE method and then, using this ELISA method, to determine if there was a higher than normal level of complement activation in patients with SLE and renal disease.

4.1 Antibodies to C1q in patients with inflammatory disease

In order to determine the level of antibodies to C1q present in the blood sera of the normal healthy donors and the patients, the C1q, to be coated onto the ELISA plates, had to be as pure as possible. When antiserum and purified C1q are placed in adjacent wells in an agarose gel, they diffuse out from the wells and form a line of precipitate at the point where antigen and antibody meet (109). As precipitins were formed between all three dilutions of the purified C1q, including the weakest dilution, then it follows that the test sample contained a high concentration of C1q. Analysis by serum protein electrophoresis showed the purified C1q to be free from other main serum proteins, indicating a high a level of purity.

Because C1q has a collagen-like region, there was a concern that antibodies to collagen (not antibodies to C1q) may be combining with C1q, i.e. cross reactivity, and therefore would give a falsely positive result. Glomerular Basement Membrane (GBM) consists of collagen chains and antibodies to GBM are antibodies to collagen. The investigation in this study shows there is no cross reactivity with these antibodies to collagen. This may be because the collagen-like region of C1q may be similar to collagen type II (92,93) while the only antigen significant for GBM antibodies is collagen type IV (111). However, serial measurement by 2 assays of antibodies to C1q and collagen autoantibody titres in the same cohort of SLE patients showed separate disease associations for each autoantibody, indicating that both assays probably detect different antibodies (111). In addition, either native C1q or the collagen-like region of C1q is used for the measurement of antibodies to C1q under

standardised conditions, excluding the possibility of proteolysis by collagenase. Consequently, it is unlikely that anti-collagen antibodies interfere in standardised assays used for the measurement of antibodies to C1q.

It was suggested in 1971 that C1q bound to IgG in SLE (53) and antibodies to C1qs were proved to exist in 1984 (50). Previous studies have found increased titres of IgG antibodies to C1q in 56% (41), 49.4% (91), 34% (40) and 33% (112) of SLE patients.

In 1993 it was found that the occurrence of increased IgA antibodies to C1q in SLE patients remained similar to that in individuals from the general population (64). The present study found similarly ($P > 0.05$). The findings in this study that IgG antibodies to C1q were found to be significantly increased in SLE is supported by previous studies (50).

Previously, IgG and IgA antibodies to C1q were found in less than 5% of the sera from RA patients but found IgA antibodies to C1q in 61% of patients with rheumatoid vasculitis (46). The present study found no significant increase in IgG antibodies to C1q in RA patients.

IgM antibody titres to C1q were very significantly lower in SLE, RA and HAID patients suggesting that inflammatory disease actually inhibits the generation of IgM antibodies to C1q. This was also true of IgA antibodies to C1q in RA.

In patients with SLE, IgG antibody titres to C1q decreased with age. This observation agrees with a previous study (64) which also showed that in serum samples of individuals, randomly selected out of the general population, IgG antibody titres to C1q initially decreased and subsequently increased with age. The decrease in IgG antibodies to C1q may be due to the fact that severe

manifestations of SLE are mostly found in young patients (113) which suggests that IgG antibodies to C1q play a role in the pathogenesis of SLE. IgA and IgM antibodies to C1q showed no significant correlation with SLE patients.

A previous study (64) found that IgA antibody titres to C1q in serum of SLE patients and of randomly selected individuals were low and did not differ significantly between the different age groups. It was postulated that the increased production of autoantibodies in serum of individuals of older age results in defects in the function of suppresser cells and originates from B-cell clones derived from stem cells in the bone marrow (114,115).

Antibodies to C1q may amplify complement activation by immune complexes in tissues, by binding to C1q fixed to immune complexes, enlarging the complexes and promoting further activation (116).

In patients with rheumatoid arthritis, IgG antibody titres to C1q were increased as the complement complements C3 and C4 decreased suggesting IgG antibodies to C1q cause consumption of C3 and C4 leading to the chronic inflammation associated with RA. Patients with rheumatoid disease have low serum complement (117). Low C3 levels are less common and are a pointer to the presence of severe disease (118).

Results in this study show patients with HAID generate more IgG antibodies to C1q as they get older. In the classical pathway C1 inhibitor limits activation of C1 by blocking the C1r and C1s components. The highly significant correlation of IgA antibodies to C1q with C4 and C1 inhibitor, and IgG antibodies to C1q with C1 inhibitor, conflicts with a previous study which found that decrease of C1 inhibitor levels due to consumption were described

in autoimmune diseases such as SLE (119). In contrast to IgG antibodies to C1q in RA, the levels of C4 and C1 inhibitor were raised in the presence of IgA and IgG antibodies to C1q in patients with HAID. There is no clear explanation for this.

There was no evidence that IgA and IgM antibodies to C1q are associated with complement activation or inhibition in patients with SLE and RA. However, a negative correlation of IgM antibodies to C1q with C1 inhibitor in patients with HAID shows C1 inhibitor may decrease in the presence of IgM antibodies to C1q allowing complement activation.

There was a significant correlation between IgA antibodies to C1q and antibodies to double stranded DNA in patients with inflammatory disease which prompted further investigation. This present study discovered that IgG antibody titres to C1q were significantly increased in patients with increased titres of antibodies to double stranded DNA when compared to patients with normal antibodies to double stranded DNA levels.

Several reports have claimed a correlation between autoantibodies to double stranded DNA and disease activity (120,121,122) as well as a positive correlation between antibodies to C1q and antibodies to double stranded DNA (35). This suggests that antibodies to C1q develop as part of an autoantibody response to C1q complexed with other lupus autoantigens and therefore the antibodies to C1q may play a role in the pathogenesis of disease activity.

4.2 Antibodies to C-Reactive Protein in Patients with Inflammatory disease

Many substances react directly with C1q in absence of antibody to activate the classical complement pathway. These include nucleic acids, proteins, lipids and polysaccharides (122).

CRP activates the classical complement pathway upon interaction with polycations as well as phosphorylcholine ligands independent of the presence of antibody, and induces complement-dependant opsonisation by neutrophils and monocytes as well as complement-dependant haemolysis by reactions which initiate at the level of C1q ; like IgG, two molecules are required for activation (123,124).

CRP binds to apoptotic cells and augments the activation of the classical complement pathway. CRP and the classical complement components act together to promote non-inflammatory clearance of apoptotic cells. The anti-inflammatory effects of CRP require the presence of C1q, indicating a pivotal role for the early complement components in this process. Deficiency of C1q fails to protect apoptotic cells from lysis and permits the normally protective effect of CRP to perpetuate the inflammatory response. This impaired handling of apoptotic cells and increased necrosis predisposes individuals to autoimmune disease and autoimmune disorders such as SLE(97).

SLE is a relapsing disease, and whereas CRP increases during acute infections, patients with SLE paradoxically demonstrate low levels of CRP(125).

This study found that patients with inflammatory disease had significantly higher titres of antibodies to CRP of all isotypes tested (IgG, IgA and IgM) in their sera when compared to normal healthy donors. The role of the complement system is to clear apoptotic, dead and dying cells from the tissues in the body. CRP is thought to carry out a similar clearing role to complement and the increased levels of antibodies to CRP provides evidence that these autoantibodies may inhibit this role.

In general, patients with SLE and inflammatory disease showed evidence of associated low C3 and C4 with levels of IgA and IgG antibodies to CRP. Patients with HAID showed associated very low levels of C1 inhibitor with high levels of IgG and IgA antibodies to CRP. These all suggest complement consumption. This disagrees with a previous study that found that antibodies to CRP strongly inhibit the interaction between CRP and C1q and therefore inhibit complement activation (33).

However, the association of IgM antibodies to CRP with antibodies to double stranded DNA in SLE and inflammatory disease may be explained by antibodies to double stranded DNA being produced against DNA from apoptotic , dead and dying cells. The above association provides evidence that IgM antibodies to CRP may inhibit the ability of CRP to bind this DNA and to activate the classical pathway allowing clearance of the DNA complexes. This evidence supports the previous study (33).

Without efficient complement activation, there may be inefficient clearance of immune complexes. These immune complexes will deposit in tissues, and cause injury. This injury may cause the release of autoantigens as an inflammatory reaction and augment the autoimmune response.

One reason for resulting inefficient complement activation may be due to ongoing complement turnover, due to an autoimmune response, leading to depletion of complement components such as C3 and C4. However, while it is not known what antigen initially triggers the autoimmune process, there is evidence that apoptotic cells are the antigen (116).

4.3 C3d levels in Patients with Inflammatory disease

Measurement of levels of C3d in blood plasma are a more useful indicator of complement activation than measurement of C3 and C4 because it is produced as a result of complement activation. This gives information which may be useful in the management of inflammatory disease where complement activation takes place may cause tissue damage.

In a comparison between the ELISA and DDRIE methods, a significant but weak correlation ($p<0.05$) was found. This suggests the ELISA technique was valid, but the weak correlation may be because the ELISA method is usually far more sensitive and quantitative than immuno-electrophoresis techniques such as the DDRIE method.

The ELISA technique was then used to measure levels of C3d and found that patients with SLE, renal disease, HAID and inflammatory disease in general had increased levels of C3d. Therefore it can be concluded that complement activation is increased in patients with inflammatory disease.

If future studies could determine a correlation of abnormal levels of autoantibodies with increased levels of C3d, this would provide further evidence of the association of autoantibodies with inflammatory disease.

Chapter 5

5 Conclusions

Differences in titres of antibodies to C1q vary according to type of inflammatory disease, age and various biological markers of autoimmune disease activity. There is a positive correlation between increased antibodies to C1q and antibodies to double stranded DNA suggesting that antibodies to C1q develop as part of an autoantibody response to C1 complexed with other autoantigens. Overall, antibodies to C1q of different isotype correlate with some, but not all, autoantibodies. This would imply a causal link other than polyclonal activation of B-lymphocytes.

There was evidence of associated levels of C3, C4 and C1 inhibitor with high levels of IgA and IgG antibodies to CRP suggesting induction of complement consumption. However, IgM antibodies to CRP may inhibit the clearing ability of CRP, which would inhibit complement activation. IgM antibodies to CRP may bind with CRP at the site of apoptotic cells, preventing DNA-histone bound CRP from activating the classical complement pathway via C1.

Complement activation was increased in patients with inflammatory disease. This was evident because of the associated increased C3d levels in these patients.

The results show there is strong evidence that antibodies to C1q and antibodies to CRP of certain immunoglobulin isotype form immune complexes which induce complement consumption. The resulting complement

deficiency of C3 would impair the ability of complement to clear immune complexes. An autoimmune response is then triggered and so the cycle goes on leading to chronic inflammation and inflammatory disease. Future studies should try to determine how and why this inflammatory cycle is initiated. Certainly, it appears that measurement of antibodies to CRP and possibly antibodies to C1q, may prove a tool for diagnosing inflammatory disease.

Table 1.1.1 *Components of the complement pathway*

Name	Function
Mannose-binding protein (MBP)	Binds to bacterial polysaccharides, activates MASP
MBP-associated protein (MASP)	Activates C4 and C2
C1q	Binds to Ig in antibody-antigen complexes, activates C1r
C1r	Activates C1s
C1s	Removes C4a fragment and thus activates C4 ; removes C2a and thus activates C2
C4	Combines with C2
C2	Cleaves C3 and C5
C3	Combines with C5
Factor B	Combines with $C3(H_2O)$; cleaves C3 and C5
Factor D	Activates factor B when B is in complex with C3b or $C3(H_2O)$
C5	Anchoring molecule for C6
C6	Anchoring molecule for C7
C7	Anchoring molecule for C8
C8	Anchoring molecule for C9
C9	Forms pores in the membrane

Table 1.2.8 *Occurrence of IgG antibodies to C1q in autoimmune and renal diseases**

Disease	Occurrence of C1q (%)
HUVS	100
MPGN	88
Lupus nephritis	83
SLE	34
Rheumatoid vasculitis	32
Sjögrens syndrome	13
RA	4

*Data adapted from (35,38,40,46,62,63)

Table 3.1.2 *Determination of cross-reactivity with C1q by anti-collagen antibodies in arbitrary units/ml*

Antibodies to C1q Immunoglobulin type	Anti-GBM positive 1	Anti-GBM positive 2	Antibodies to C1q positive control	Antibodies to C1q negative control
IgG	27	0	1500	0
IgA	0	0	62	8
IgM	0	1	148	40

Table 3.1.3.1 *Antibody titres to C1q in normal healthy donors in arbitrary units/ml*

No.	IgG	IgA	IgM
1	50	13	52
2	50	13	54
3	57	13	32
4	50	45	35
5	70	13	46
6	188	19	31
7	45	13	77
8	35	13	45
9	105	13	19
10	115	13	28
11	51	13	17
12	180	13	106
13	85	13	37
14	105	17	41
15	57	138	60
16	38	13	20
17	66	43	62
18	35	17	6
19	24	13	76
20	50	13	97
21	27	13	53
22	38	16	0
23	41	13	8
24	78	25	20
25	100	13	83
26	60	13	69
27	110	27	24
Mean + 2 SD	71+84	22+50	44+56
Upper limit	155	72	100

Table 3.1.3.2 *Comparison of antibody titres to C1q in normal healthy donors and patients with inflammatory diseases. The figures are in medians and interquartile range. The asterisks depict the significance between normal human donors and patients.*

	IgG	IgA	IgM
Normal (n=30)	57 (41-100)	13 (13-17)	41 (20-62)
Inflammatory diseases (n=64)	78 (24-262)	10 ** (4-20)	17 *** (15-23)
SLE (n=27)	139 * (32-386)	9 (4-20)	18 ** (16-26)
RA (n=23)	34 (0-190)	9 ** (2-13)	17 *** (15-20)
HAID (n=14)	80.5 (50-232)	13 (7.5-27)	18 ** (16-29)

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

Table 3.1.3.3a *Correlation of antibody titres to C1q in serum of inflammatory disease patients with biological markers of autoimmune disease activity (correlation coefficient r)*

Variable	IgG	IgA	IgM
Age	n.s.	n.s.	n.s.
ANA	n.s.	n.s.	n.s.
DNA Ab	n.s.	0.56 **	n.s.
C3	n.s.	n.s.	n.s.
C4	n.s.	n.s.	n.s.
C1 inhibitor	n.s.	n.s.	n.s.
C1q level	n.s.	n.s.	n.s.

Table 3.1.3.3b *Correlation of antibody titres to C1q in serum of SLE patients with biological markers of autoimmune of disease activity (correlation coefficient r)*

Variable	IgG	IgA	IgM
Age	-0.38 *	n.s.	n.s.
ANA	n.s.	0.44 *	n.s.
DNA Ab	n.s.	n.s.	n.s.
C3	n.s.	n.s.	n.s.
C4	n.s.	n.s.	n.s.
C1 inhibitor	n.s.	n.s.	n.s.
C1q level	n.s.	n.s.	n.s.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

n.s not significant

Table 3.1.3.3c *Correlation of antibody titres to C1q in serum of RA patients with biological markers of auto immune disease activity (correlation coefficient r)*

Variable	IgG	IgA	IgM
Age	n.s.	n.s.	-0.46 *
ANA	n.s.	n.s.	n.s.
C3	-0.50 *	n.s.	n.s.
C4	-0.46 *	n.s.	n.s.
C1 inhibitor	n.s.	n.s.	n.s.
C1q level	n.s.	n.s.	n.s.

Table 3.1.3.3.d *Correlation of antibody titres to C1q in serum of HAID patients with biological markers autoimmune of disease activity (correlation coefficient r)*

Variable	IgG	IgA	IgM
Age	0.67 **	n.s.	n.s.
DNA Ab	n.s.	n.s.	n.s.
C3	n.s.	n.s.	n.s.
C4	n.s.	0.78 ***	n.s.
C1 inhibitor	0.78 ***	0.97 ***	-0.64 *
C1q level	n.s.	n.s.	n.s.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

n.s not significant

Table 3.1.3.4a *Correlation of biological markers of autoimmune disease activity in patients with inflammatory disease*

	Age	ANA	DNA	C3	C4	C1inh
ANA	n.s.					
DNA	-0.39 *	n.s.				
C3	n.s.	n.s.	-0.41 *			
C4	n.s.	n.s.	n.s.	0.38 **		
C1inh	n.s.	0.30 *	n.s.	n.s.	n.s.	
C1q level	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table 3.1.3.4b *Correlation of biological markers of autoimmune disease activity in patients with SLE*

	Age	ANA	DNA	C3	C4	C1inh
ANA	n.s.					
DNA	n.s.	n.s.				
C3	n.s.	n.s.	n.s.			
C4	n.s.	n.s.	n.s.	0.65 ***		
C1inh	n.s.	n.s.	0.57 **	n.s.	n.s.	
C1q level	n.s.	n.s.	0.75 ***	n.s.	n.s.	n.s.

Table 3.1.3.4c *Correlation of biological markers of autoimmune disease activity in patients with RA*

	Age	C3	C4	C1inh
C3	n.s.			
C4	n.s.	0.71 ***		
C1inh	n.s.	0.68 ***	0.64 ***	
C1q level	n.s.	n.s.	n.s.	n.s.

Table 3.1.3.4d *Correlation of biological markers of autoimmune disease activity in patients with HAID*

	Age	ANA	C3	C4	C1inh
ANA	n.s.				
C3	n.s.	n.s.			
C4	n.s.	n.s.	n.s.		
C1inh	0.59 *	n.s.	0.999 ***	0.64 *	
C1q level	n.s.	n.s.	n.s.	n.s.	n.s.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

n.s not significant

Table 3.1.3.5a *Levels of complement components in patients with various autoimmune inflammatory diseases (median and interquartile range) and controls (normal range)*

	Normal range (n=30)		SLE (n=27)	RA (n=23)	HAID (n=14)
C3	0.88-1.82		0.97 * ** (0.77-1.45)	1.31 **** (1.18-1.83)	0.21 (0.10-0.59)
C4	0.18-0.45		0.12 (0.08-0.22)	0.18 (0.14-0.27)	0.14 (0.10-0.24)
C1 inhibitor	0.16-0.46		0.27 (0.20-0.33)	0.35 *** (0.24-0.39)	0.11 (0.10-0.26)
C1q level	63-158		63 (30-158)	63 (24-135)	143 (65-353)

Significant difference between * SLE and RA ; P<0.05, ** SLE and HAID ; P< 0.001, *** RA and HAID ; P< 0.05, **** RA and HAID ; P< 0.001

Table 3.1.3.5b *Proportion of patients with autoimmune inflammatory disease with lower than normal complement component levels*

	SLE	RA	HAID
C3	10/23 (43%)	2/23 (9%)	12/14 (86%)
C4	17/24 (71%)	11/22 (50%)	9/14 (64%)
C1 inhibitor	2/22 (9%)	3/18 (17%)	2/3 (66%)
C1q level	13/27 (48%)	12/23 (52%)	3/14 (21%)

Table 3.1.3.6 *Comparison of antibody titres to C1q in serum samples of patients with increased titres of anti dsDNA antibodies and patients with normal anti DNA antibody titres. The figures are in medians and interquartile range.*

	Increased anti dsDNA antibodies	Normal anti dsDNA antibodies
IgG antibodies to C1q	206 * (33-458)	58 (3-139)
IgA antibodies to C1q	12 (3.5-47)	8 (4-19.5)
IgM antibodies to C1q	17 (15.5-22.5)	16.5 (15-20.5)

* $p < 0.05$

Table 3.2.1 *Antibody titres to CRP in normal healthy donors in arbitrary units/ml*

No.	IgG	IgA	IgM
1	23	8	3
2	34	11	13
3	33	6	10
4	38	17	11
5	32	10	0
6	23	20	0
7	19	9	20
8	23	3	21
9	16	22	19
10	9	11	4
11	17	10	4
12	17	3	8
13	22	11	0
14	36	6	18
15	29	27	7
16	48	19	7
17	58	22	10
18	60	20	3
19	7	25	36
20	60	16	0
21	24	1	0
22	0	0	0
23	5	0	9
24	0	15	0
25	0	11	0
26	0	2	0
27	35	26	7
28	0	1	0
29	0	7	0
30	21	12	4
mean +2 SD	23+36	12+14	7+17
upper limit	59	28	24

Table 3.2.2 *Comparison of serum antibody titres to CRP (medians and interquartile range) in normal healthy donors and patients with inflammatory diseases. The asterisks depict the significance between normal human donors and patients.*

	IgG	IgA	IgM
Normal (n=30)	22.5 (6.5-34)	11 (5-19)	4 (0-10)
Inflammatory diseases (n=64)	50 *** (31-75)	25.5 *** (11-45)	8 * (4-19)
SLE (n=27)	54 *** (41-82)	32 *** (14-50)	8 * (5-19)
RA (n=24)	32 * (25-55)	22 * (6-41)	6 * (1-17)
HAID (n=14)	59 ** (29-93.5)	22 * (10-48)	9 (5-17)

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

Table 3.2.3a *Correlation of antibody titres to CRP in serum of inflammatory disease patients with levels of variables of disease activity (correlation coefficient r)*

Variable	IgG	IgA	IgM
Age	-0.383 *	n.s.	n.s.
ANA	n.s.	n.s.	n.s.
DNA	n.s.	n.s.	0.47 **
C3	-0.29 *	n.s.	n.s.
C4	-0.36 **	n.s.	n.s.
C1 inhibitor	n.s.	-0.32 *	n.s.
C1q level	n.s.	n.s.	n.s.

Table 3.2.3b *Correlation of antibody titres to CRP in serum of SLE patients with levels of variables of disease activity (correlation coefficient r)*

Variable	IgG	IgA	IgM
Age	n.s.	n.s.	n.s.
ANA	n.s.	n.s.	0.40 *
DNA	n.s.	0.46 *	0.56 **
C3	n.s.	-0.43 *	n.s.
C4	n.s.	-0.39 *	n.s.
C1 inhibitor	n.s.	n.s.	n.s.
C1q level	n.s.	n.s.	n.s.

* $p < 0.05$

** $p < 0.01$

n.s not significant

Table 3.2.3c *Correlation of antibody titres to CRP in serum of RA patients with levels of variables of disease activity (correlation coefficient r)*

Variable	IgG	IgA	IgM
Age	n.s.	n.s.	n.s.
ANA	n.s.	n.s.	n.s.
C3	n.s.	n.s.	n.s.
C4	n.s.	n.s.	n.s.
C1 inhibitor	n.s.	n.s.	n.s.
C1q level	n.s.	n.s.	n.s.

Table 3.2.3.d *Correlation of antibody titres to CRP in serum of HAID patients with levels of variables of disease activity (correlation coefficient r)*

Variable	IgG	IgA	IgM
Age	n.s.	n.s.	-0.55 *
DNA Ab	n.s.	n.s.	n.s.
C3	-0.54 *	n.s.	n.s.
C4	n.s.	n.s.	n.s.
C1 inhibitor	-0.82 ***	-0.85 ***	n.s.
C1q level	n.s.	n.s.	n.s.

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

n.s not significant

Table 3.3.2 *Comparison of C3d ELISA levels in the plasma of normal healthy donors and patients*

Disease Category	Median (Interquartile range)
Normal (n=11)	13 (2-23)
Inflammatory disease (n=45)	23 (17-30) *
SLE (n=15)	23 (17-31) *
Renal (n=15)	24 (21-30) *
HAID (n=15)	22 (16-27) *

* $p < 0.05$

Classical Pathway

Antigen-antibody complexes Cell Surface Carbohydrate

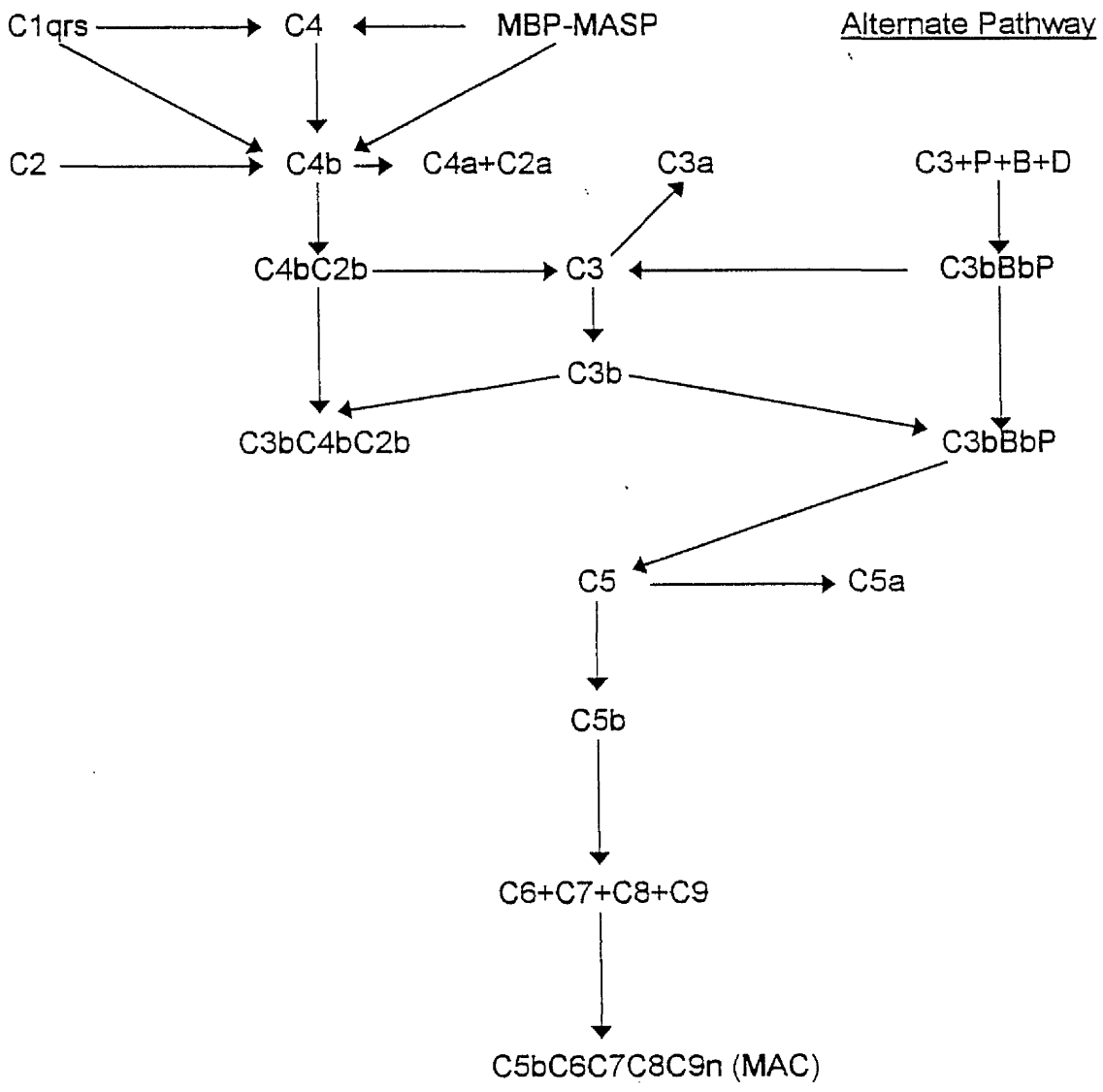


Figure 1.1.2 The complement pathway.
MBP is mannose-binding protein and MASP is MBP-associated serine protease. P, B and D are properdin, factor B and factor D respectively. MAC is membrane attack complex.

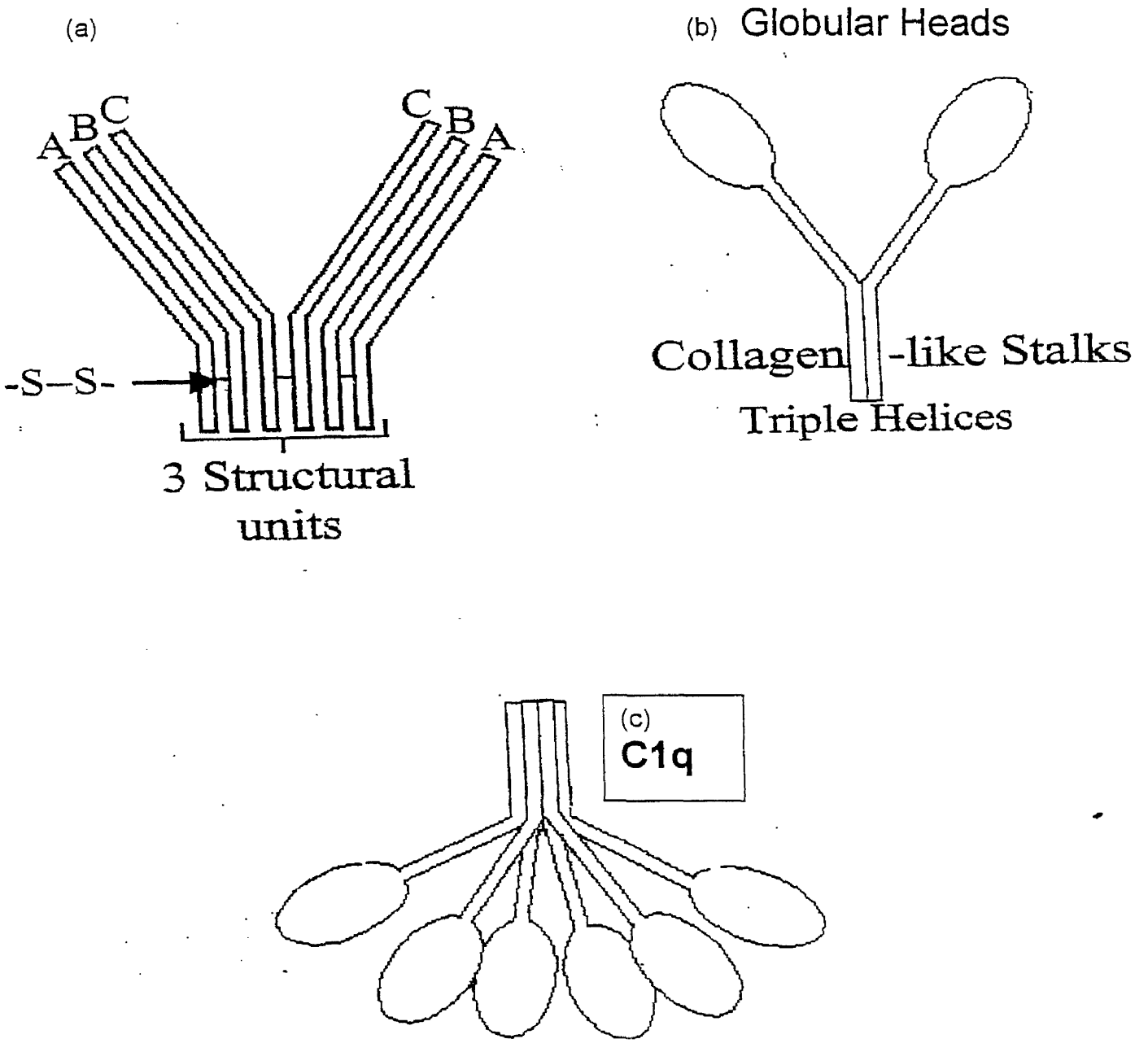


Figure 1.2.1 The structure of complement component C1q
(a) Interchain and intrachain disulphide bonding in two collagen-like stalks, each formed by three peptide chains, A, B and C.
(b)A doublet of chains showing globular heads attached to the collagen-like stalks containing triple helices.
(c) A whole C1q molecule, which consists of six globular heads attached to six collagen-like stalks.

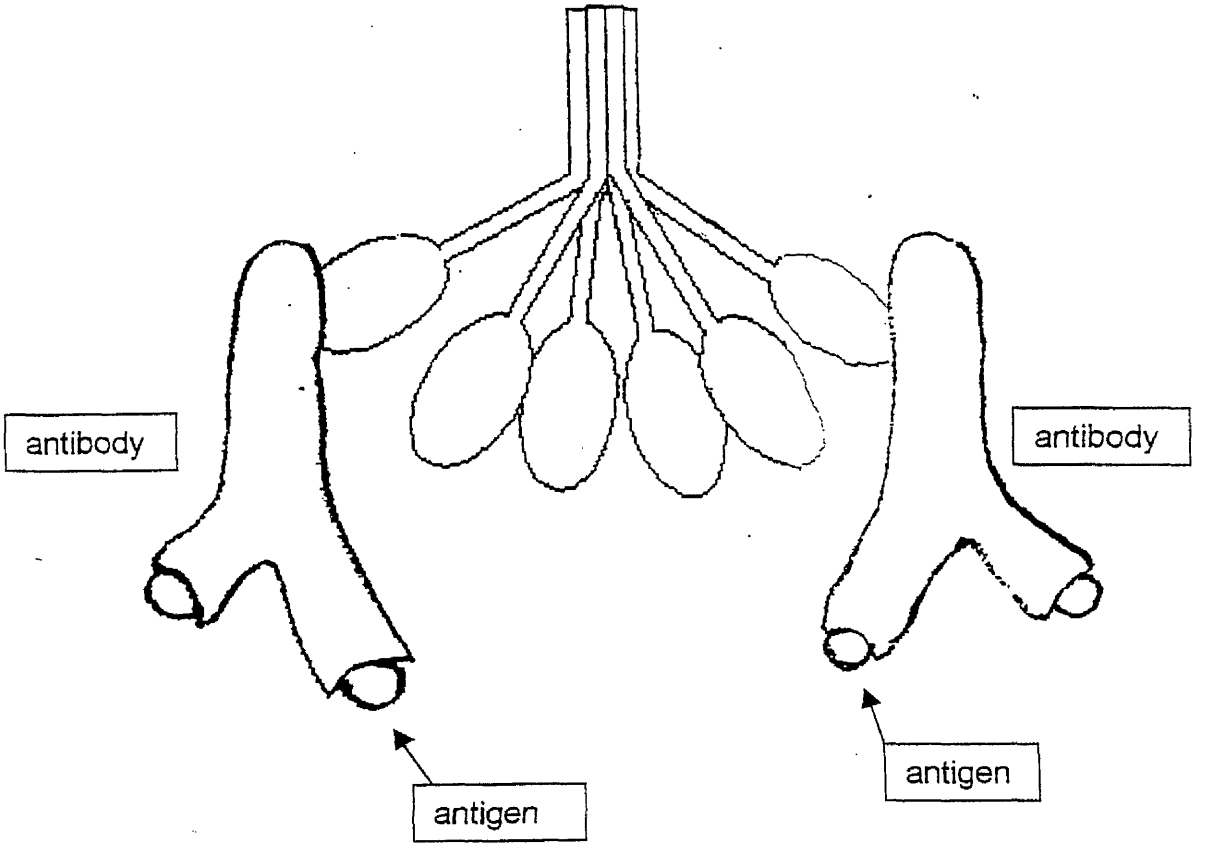


Figure 1.2.4 Interaction of C1q with IgG
The C1q component bridges two membrane bound IgG molecules. The antibodies interact via their Fc regions with binding sites on the globular heads of the C1q component.

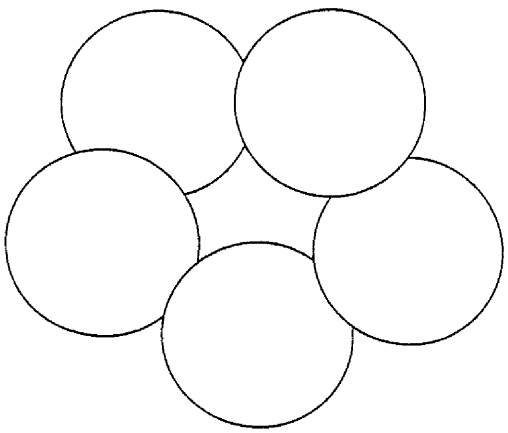


Figure 1.3 Structure of C-reactive protein.
The CRP molecule consists of five identical, non-covalently bound subunits. Each subunit is 187 amino acid residues long.

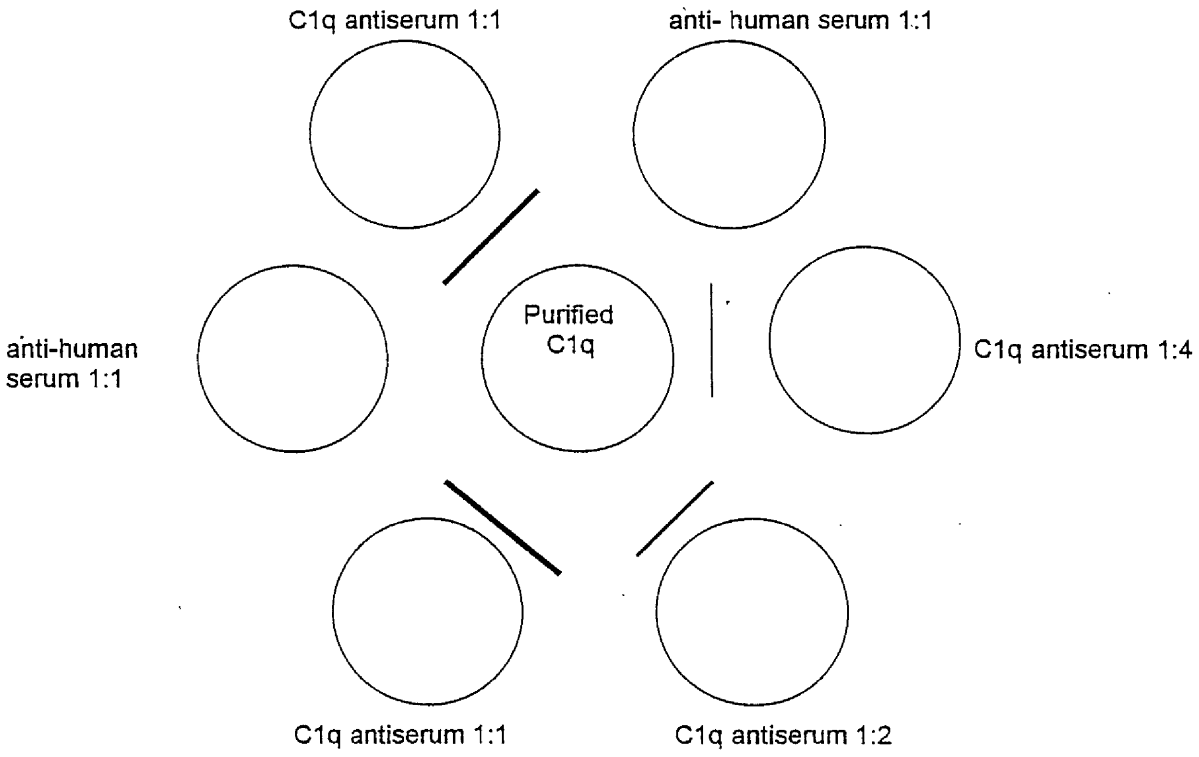
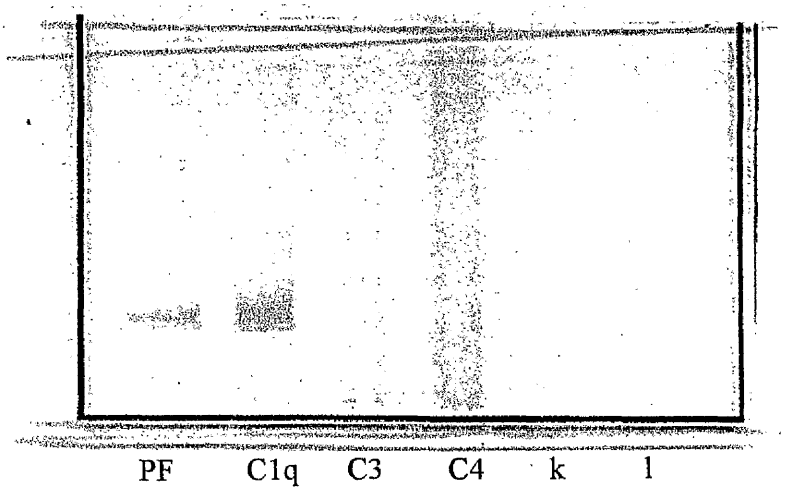


Figure 3.1.1.1 Double-diffusion technique to determine evidence and quality of purified C1q. Precipitins were formed between the purified C1q and the C1q antiserum at all the dilutions, the strongest at 1:1, the next strongest at 1:2, and the weakest at 1:4. No precipitins were formed between the purified C1q and the anti-human serum.



Key ; PF protein fixation

C1q antiserum to C1q

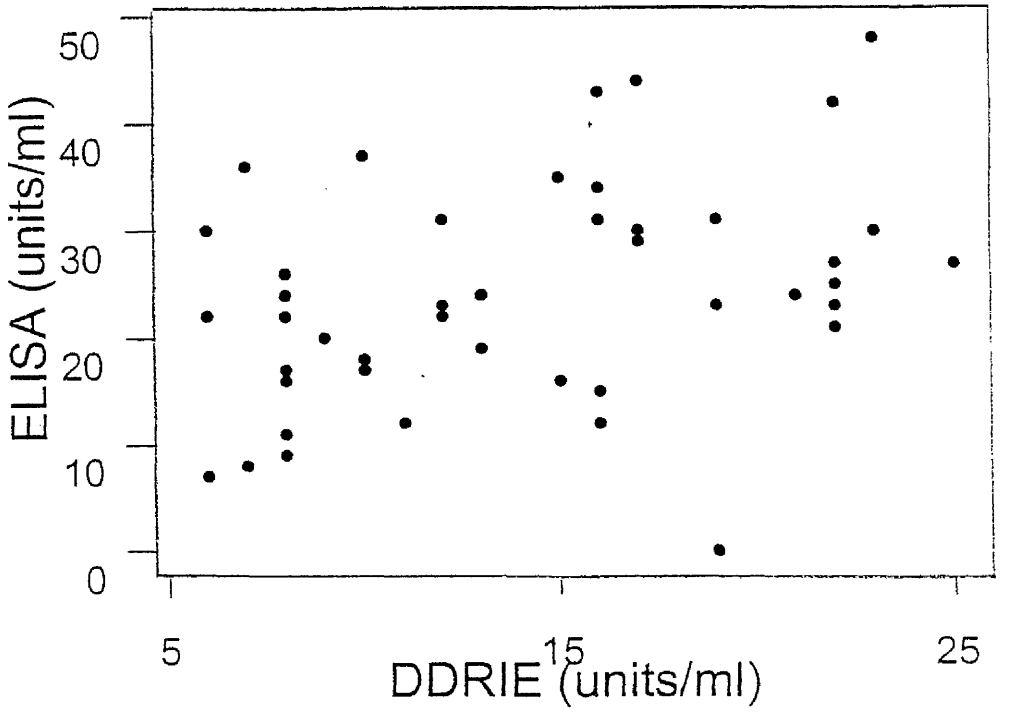
C3 antiserum to C3

C4 antiserum to C4

k antiserum to kappa

l antiserum to lambda

Figure 3.1.1.2 Serum Protein Electrophoresis method to determine the quality of purified C1q.
The electrophoresis gel shows a single protein band in the protein-fixed migration. Protein analysis by immunofixation with C1q antiserum and C3, C4, kappa and lambda antisera identified this band as C1q. Immunofixation with C3,C4, kappa and lambda showed no discernible bands.



Correlation coefficient : 0.354 ($p<0.05$)

Figure 3.3.1 Graph showing correlation of C3d DDRIE with C3d ELISA. The units are arbitrary and are defined differently in the two assays and should not be directly compared.

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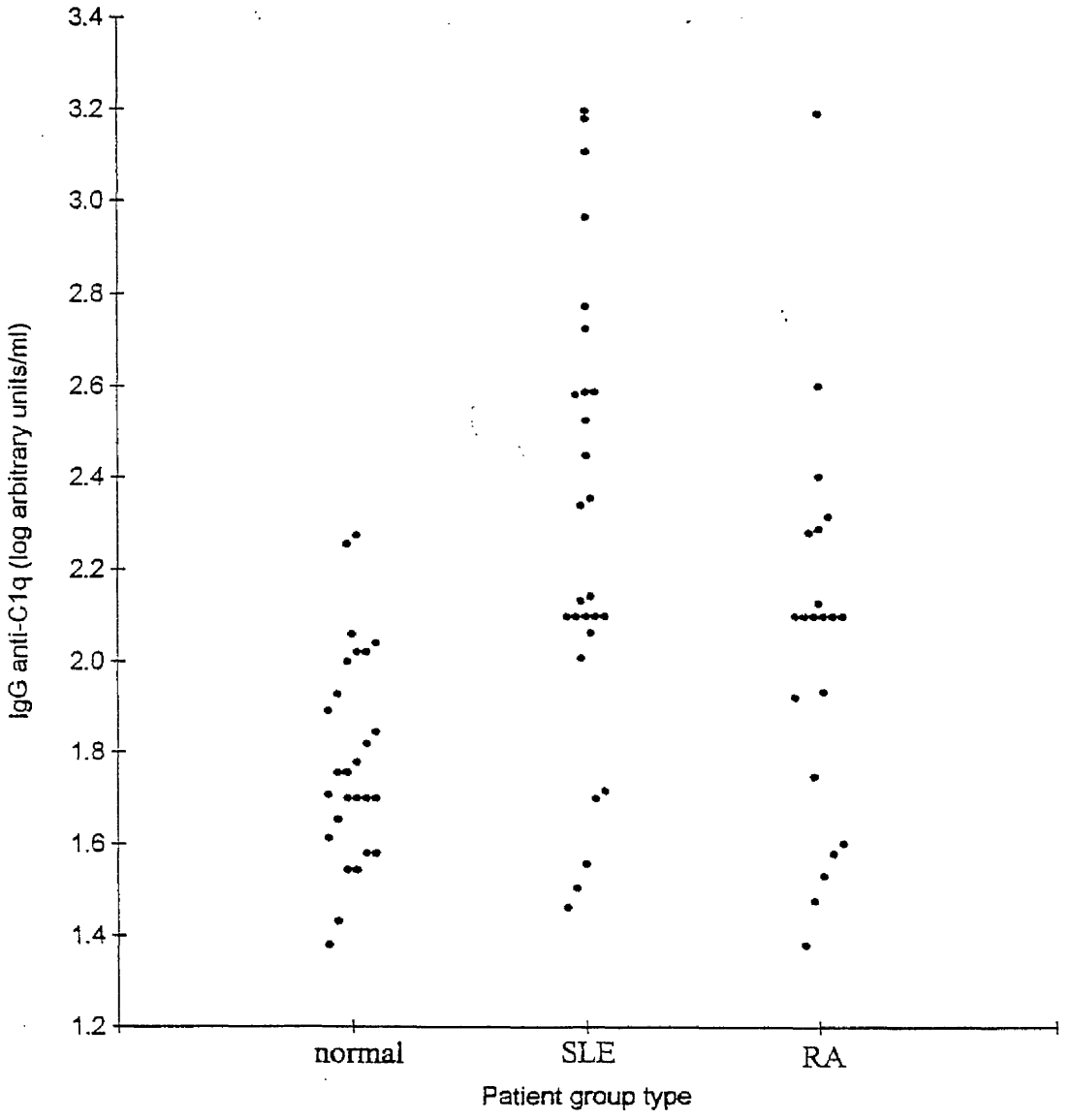
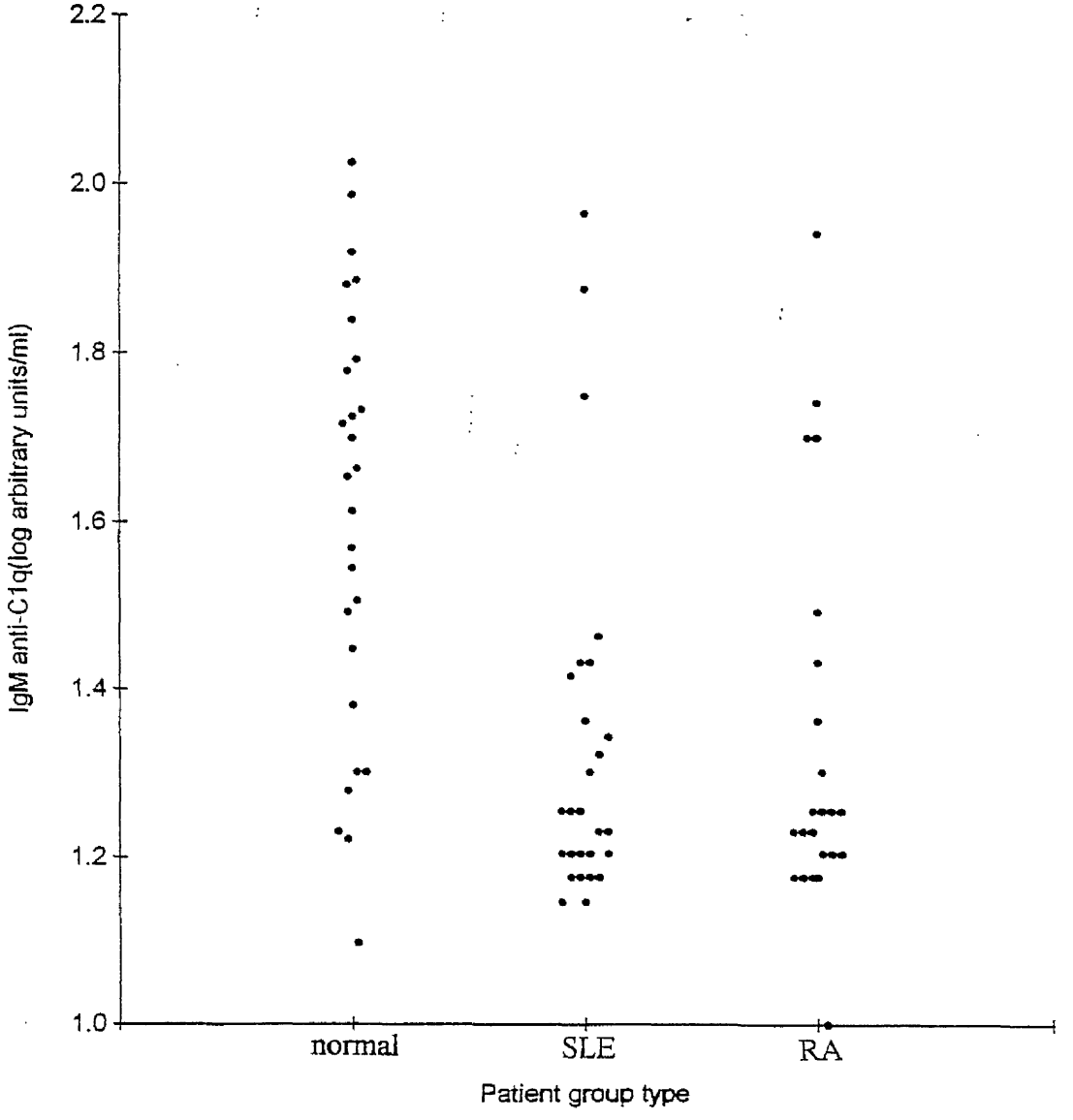
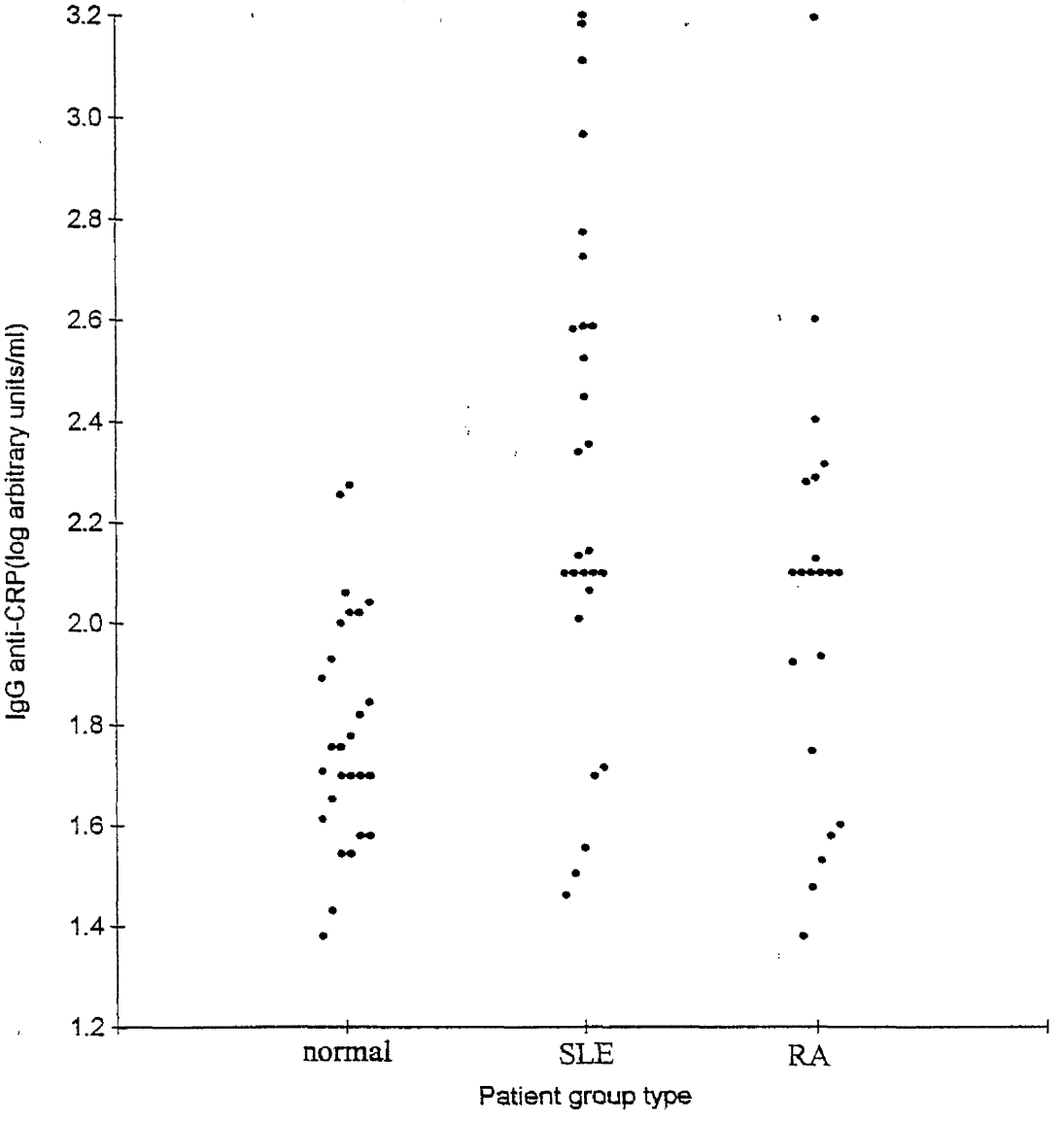


Graph (i) IgG antibody titres to C1q in normal healthy donors, SLE patients and RA patients



Graph (ii) IgM antibody titres to C1q in normal healthy donors, SLE patients and RA patients



Graph (iii) IgG antibody titres to CRP in normal healthy donors, SLE patients and RA patients