THE ISOLATION AND CHARACTERISATION OF IMMUNE COMPLEXES IN HUMAN BODY FLUIDS

A thesis submitted in fulfilment of the requirements for the degree:

DOCTOR OF PHILOSOPHY

of

The University of London

by

WILLIAM KILGALLON

Department of Immunology
Royal Postgraduate Medical School
Hammersmith Hospital
DuCane Road
LONDON W12 OHS

June 1984

ABSTRACT

Polyethylene glycol (PEG) and ammonium sulphate were used to precipitate preformed immune complexes which had been added to normal human serum. The best compromise between the recovery of these complexes and the coprecipitation of serum proteins was achieved with four per cent PEG. The complexes which were concentrated in this manner were then solubilised in complement buffer and further purified by ligand affinity chromatography.

The characteristics of several affinity columns were first established using radiolabelled immune complexes or heat aggregated human immunoglobulin. Protein-A Sepahrose bound a high proportion of monomeric IgG and IgM in preference to the complexes or aggregates and the yield from this column was low. Purified human Clq and bovine conglutinin, linked to polymethylmetacrylate beads, also bound immune complexes but they would only retain a small proportion of the material added to the column. The problem of efficient extraction was overcome by linking an anti-Clq antiserum to Sepharose. Preformed complexes which had been incubated in serum readily bound to this column and were eluted by the sequential application of an EDTA buffer, sodium chloride and propionic acid.

The immune complexes present in pathological sera were then purified using this method. The majority of the serum samples examined were obtained from patients with Hodgkin's disease although some sera from patients with rheumatoid arthritis and systemic lupus erythematosus were also included. The constituents of these complexes were examined by gel diffusion and SDS polyacrylamide gel electrophoresis. The complexes extracted from patients with Hodgkin's disease were heterogeneous in size but there appeared to be little difference in their composition when compared with those isolated from either the Clq or the conglutinin column. However the complexes isolated from patients with rheumatoid arthritis and systemic lupus erythematosus differed from those found in patients with Hodgkin's disease.

ACKNOWLEDGEMENTS

I would like to thank Dr. B.D. Williams, Dr. P.L. Amlot and Professor J.H. Humphrey for their help in this work.

DEDICATION

To the memory of my late
father, James Kilgallon.

TABLE OF CONTENTS

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 4

DEVELOPMENT AND EVALUATION OF LIGAND SPECIFIC AFFINITY SYSTEMS FOR THE ISOLATION OF IMMUNE COMPLEXES FROM HUMAN SERUM OR PLASMA

CHAPTER 5

IMMUNOCHEMICAL AND PHYSICO-CHEMICAL ANALYSIS OF THE IMMUNE COMPLEXES IN HODGKIN'S DISEASE

INDEX OF FIGURES

CHAPTER 5

INDEX OF TABLES

CHAPTER 5

CHAPTER 1

"To explain all nature is too difficult a task for any one man or even for any one age. 'Tis much better to do a little with certainty, and leave the rest for others that come after you, than to explain all things".

Isaac Newton (1642 - 1727).

INTRODUCTION

1.1 Historical

An immune complex is the result of the reversible non-covalent interaction between an antigen and a specific antibody. The generation of immune complexes is central to the function of the normal humoral and cellular immune systems in that antibody molecules play an adaptive role between the infinitely varied target antigens presented by the environment and the finite number of biological mechanisms capable of dealing with them. It was Paul Ehrlich (1904) who first suggested that there must be a chemical basis for the recognition of antigens by antibodies although it was the studies of Carl Landsteiner (1936) and his associates that led to the proposal that antigens possessed the properties of immunogenicity and a capacity to react with antibodies. Antibodies alone cannot bring about the elimination of foreign antigens but need to interact with effector systems such as the sequence of serum proteins comprising the complement system which stands at this junction between recognition and response. The earliest evidence for the presence of an effector system for the elimination of foreign antigens was provided by the *in vitro* work of Bordet (1896). Since heat inactivated antisera to *Cholera vibrio* only agglutinated but did not lyse bacteria he suggested that the bacteria were agglutinated by specific antibodies, that lysis was brought about by a more labile

substance now known as complement, and that both components were required for the destruction and elimination of infectious agents.

In general terms the formation of immune complexes is beneficial to the host since it leads ultimately to the elimination of foreign antigenic materials; however, under certain circumstances this process can induce injury if they localise in the tissues and activate the complement and kinin systems, or interact with receptors on the surface of phagocytic cells. Apart from tissue injury immune complexes also have the ability to modulate humoral and cellular immune responses by interacting with sub-populations of T and B lymphocytes and also tumour cells bearing surface antigens. The isolation and characterisation of such immune complexes,therefore, presents an opportunity to investigate their role in the pathogenesis of immune complex related diseases.

1.2 Nature of Antigen-Antibody Reaction

When a specific antibody is titrated against a fixed concentration of its complementary antigen *in vitro*, an optimum proportion of both reagents is reached and at this point a precipitate ensues. The first precipitin reaction was described in 1897 by Kraus with microbial culture supernatants and the serum of immunised animals. Later, in 1899, Bordet (1937) performed a similar reaction with milk

antigens and serum of animals injected with these proteins.

The physical and chemical nature of the forces which govern the attraction of antibodies and antigens influence the formation of the complexes. Complementary antigen and antibody molecules interact reversibly in a non-covalent manner and their interaction is influenced by pH, temperature and concentration. The forces of attraction which hold them together are the same as those demonstrated for other systems such as enzyme-substrate or agonist-receptor interactions (Eisen, 1973). The intermolecular attraction of hydrophobic, electrostatic, hydrogen bonding and Van de Waals forces are weak in biophysical terms and operate over limited distances; therefore a prerequisite of the reaction is that the antigen must be in close apposition to the antibody. Since antibody reactions occur in an aqueous environment, hydrophobic and hydrophylic interactions between molecules are important. Antibody molecules appear to possess increased numbers of hydrophobic amino acids in their antigen combining region and therefore, as water molecules are attracted to polar amino acids, less energy may be required to displace this water and allow antigen and antibody to react. This type of interaction is possibly the strongest interaction involved in the complementary binding between antibody and antigen.

Electrostatic interactions are due to the attraction between oppositely charged ionic groups on the two molecules such as ionised amino and carboxyl groups. The force of attraction is inversely proportional to the square of the distance between them. Thus the closer the fit between the molecules, the tighter is the binding and the reaction is facilitated by the molecules having complementary electron-cloud shapes. This interaction is due to the oscillation of induced dipole disturbances and the force of attraction between them is inversely proportional to the seventh power of the distance between them.

An understanding of such intermolecular interactions may be helpful if one wishes to isolate and purify immune complexes. It may also explain why some complexes are more stable than others during the extraction procedures and helps develop ways of dissociating such complexes into their respective components.

1.3 Composition and Fate of Immune Complexes.

The diverse biological activities of immune complexes are dependent not only on the size and valence of the antigen and antibody but also on the ratio of the two reactants (lattice formation), the state of the complement system and the activity of the phagocytic system (reviewed by Weigle,1961; Haakenstad and Mannik,1978). The valence

and isotype of the antibody molecule dictates whether such complexes will activate the complement system and whether they will react with cellular Fc receptors. Immunoglobulin G (IgG), monomeric IgA, IgD and IgE all have a valence of two whereas IgM has a valence of ten and dimeric secretory IgA a valence of four (Metzger, 1970; Spiegelburg, 1974). IgG 1 and 3 can bring about complement activation whereas IgG 2 and 4 are far less efficient in this respect (Ishizaka et al, 1967; Natvig and Kunkel, 1973). Augener (1971) has reported that IgG 4 does not fix C1. IgE and IgA cannot activate the complement system except when aggregated (Gotze and Muller-Eberhard, 1971; Ishizaka et al, 1972a,b). The presence or absence of activated complement components on such immune complexes can determine whether they are rapidly cleared from the circulation by the reticuloendothelial system (RES) via C3b and Fc receptors or whether they persist as soluble complexes (Haakenstad and Mannik,1974). Equally, the presence of complement components on the surface of immune complexes can be exploited to specifically isolate them from biological fluids.

Antigen valence can also influence the biological properties of immune complexes in that a univalent antigen can only bind one antibody molecule and is more likely to persist in the circulation without causing tissue damage (Schmidt et al, 1974). Multivalent antigens however are much more likely to form large lattices, their size being

dependent on the ratio of the reactants (Heidelburger, 1949; Pauling et al, 1944; and Marrack, 1938). Complexes formed in gross antigen excess usually do not fix complement and are less likely to induce inflammatory reactions. Large complexes, however, formed in antibody excess or slight antigen excess do fix complement but because of this they are rapidly removed by phagocytic cells and therefore their ability to inflict tissue damage is limited. Complexes formed between these two extremes appear to display the greatest pathological activity (Dixon, 1963); however, the ability to do this may be influenced by the state of the RES.

The clearance of circulating immune complexes by the RES system takes place mainly in the liver and spleen, being mediated by C3b and Fc receptors respectively. In experimental serum sickness Wilson and Dixon (1971) showed that 99% of immune complexes were cleared by phagocytes, mainly by Kupfer cells in the liver, thus the remaining 1% of the immune complexes were the ones available to produce disease. In circumstances where there is continuous leakage of antigen into the circulation the accumulation of immune complexes could effectively saturate Fc and complement receptors on macrophages, resulting in RES blockade and subsequent deposition of immune complexes in tissues (Haakenstad and Mannik, 1974). An intact complement system, however, is not essential for RES clearance of immune

complexes for Mannik et al, (1971) showed that depletion of complement by cobra venom factor had no effect on the clearance rate of complexes compared to normal animals.

The concept of RES blockade has been studied over a period of years to evaluate disease pathogenesis where circulating immune complexes are prevalent. Frank et al, (1983) extended these studies in a guinea pig model. They showed that normal guinea pig erythrocytes sensitised with rabbit antibody (IgG) were sequestered by the spleen and that the deposition of complement fragments on the erythrocyte surface augmented the clearance. In complement depleted animals the clearance of IgG sensitised erythrocytes was less effective but the cells were cleared by the spleen and rapidly phagocytosed. When IgM was used as the specific anti-erythrocyte antibody, however, the cells were removed and reappeared in the circulation. The explanation of this was that the rapid clearance was mediated by hepatic sequestration and not by phagocytosis. Receptor-specific interactions were therefore critical to the evaluation of the mechanism by which erythrocytes coated with antibody or complement were removed from the circulation. Later studies by Engelfriet and colleagues (1980) clearly demonstrated in normal human volunteers that clearance of autologous erythrocytes coated with IgG anti-Rho (D) antibody was dependent on the interaction of the IgG Fc fragment with the receptor on phagocytic cells

for that fragment of the molecule. When only the $F(ab')_2$ antibody fragment was used to coat the erythrocytes no clearance at all was detected. These experiments highlight the role of Fc receptor-specific clearance in diseases where circulating immune complexes are prevalent and may help evaluate whether persistence of immune complexes in certain disease states is due to RES overload or the interference of Fc receptor function by immune complexes.

1.4 Immune Complex Induced Complement Activation

The complement system comprises a group of twenty plasma proteins whose sequential interaction may be initiated by immunoglobulins, microbial products and proteolytic enzymes. Activation is achieved by the generation and assembly of highly specific enzymes and is accompanied by limited proteolytic cleavage leading to the release of biologically active peptides and the formation of large multimolecular complexes. The most characteristic end product of the complement cascade is the membrane attack system; a protein complex inserted into the cell membrane which induces cell lysis. The complement system may be divided into four functional sections; two pathways for activation (the alternative and classical pathways), an amplification mechanism for augmenting the activating pathways and a final common effector pathway to which the activating sequences are directed and from which the biological activities are derived.

Immune complexes and aggregates of immunoglobulins can activate the classical and alternative complement pathways, depending on the immunoglobulin isotype incorporated. The classical pathway consists of three components C1, C2 and C4. The first component of complement, C1, can be resolved into three sub- components C1q, C1r and C1s. C1q (400,000 daltons) comprises six globular peripheral units held

together by a collagenous tail and exists in a weak association with a tetrameric complex of $C1r_2$-$C1s_2$ (4 x 83,000 daltons) that is formed in the presence of calcium ions (Lepow et al, 1963; Gigli et al, 1976). C1q is believed to bind to a polysaccharide moiety in the CH_2 domain of immunoglobulin molecules (Koide et al, 1977; Winkelhake, 1978) and at least two IgG molecules must be bound by a C1q molecule for complement activation to occur. It has been suggested that one C1q molecule may be capable of binding 12-18 IgG molecules (Schumaker et al, 1976); however, more recent work suggests a valency of six molecules (Tschopp et al, 1980). Arguments still continue as to whether the binding of antigen to IgG causes a structural change in the IgG molecule which results in stronger binding of C1q and therefore complement activation. Schumaker et al, (1976) showed that C1q could bind IgG in serum without initiating further complement activation, the suggestion being that antigen binding does induce allosteric changes in the IgG molecule. This theory is supported by the evidence that only one molecule of IgM is required to fix C1 (Ishizaka et al, 1968) and that in the absence of antigen and aggregation complement fixation by IgM is low (Ishizaka et al, 1967). The more universally accepted hypothesis of C1 activation is that the aggregation of IgG molecules, which occurs after antigen binding, triggers the subsequent activation. Evidence for this comes from the work of Muller-Eberhard (1978), who showed that antibody

binding to polyvalent antigens triggered complement activation, whereas univalent antigens did not. It was also shown that heat aggregated IgG was as effective as antigen induced aggregation in triggering the complement cascade.

C1r acts as a physical link between C1q and C1s and as the activator of C1s (Ziccardi and Cooper, 1976a,b). The activated C1s now cleaves C4 (205,000 daltons) and C2 (117,000 daltons) (Muller-Eberhard, 1975),and interacts with activated C2 to form the C3 convertase C4b2a. The formation of the C3 convertase is very inefficient in comparison to the binding and activation of C1. More than 95% of C1 can be bound in the activated form to excess aggregated antibody; however, only a small percentage of activated C4 binds to aggregates and only some of these actually bind to C2a to form the C3 convertase. Both activated C4 and C2 are labile short-lived molecules and therefore only those activated close to the fixed C1q-IgG complex will be likely to form the C3 convertase. Activated C4 is believed to bind in a covalent manner to the Fd portion of the antibody heavy chain where it associates non-covalently with C2a to cleave C3 (Porter, 1980). Cleavage of C3 by the C4b2a convertase may then release an active acyl group in the C3 chain leading to covalent bond formation with the Fab portion of the antibody or perhaps with C4b bound to the Fab (Porter, 1980).

On the assumption that a soluble immune complex will be composed of Ag2Ab3 prior to complement activation (Mannik et al, 1971; Mannik, 1980) and that the combined molecular weights of the classical pathway components are 1.2×10^6 daltons, it can be easily seen how a relatively small immune complex can have its molecular weight increased to more than 1.5×10^6 daltons, the majority of the constituents being complement proteins.

Activation of the alternative pathway by IgA or IgE containing complexes requires magnesium ions and expression of this pathway is dependent on a positive feedback mechanism in which C3b, the major cleavage product of C3, interacts with two other proteins, B and D, to generate an additional C3 convertase (Figure 1). Cleavage of B to Ba and Bb, and the association of the latter fragment with C3b, results in the generation of another C3 convertase, C3bBb. This convertase, i.e. the alternative pathway C3 convertase, is much more efficient in producing C3 cleavage and the C3b produced by this reaction is now available for the further production of a C3 convertase, thus creating the positive feedback nature of this pathway.

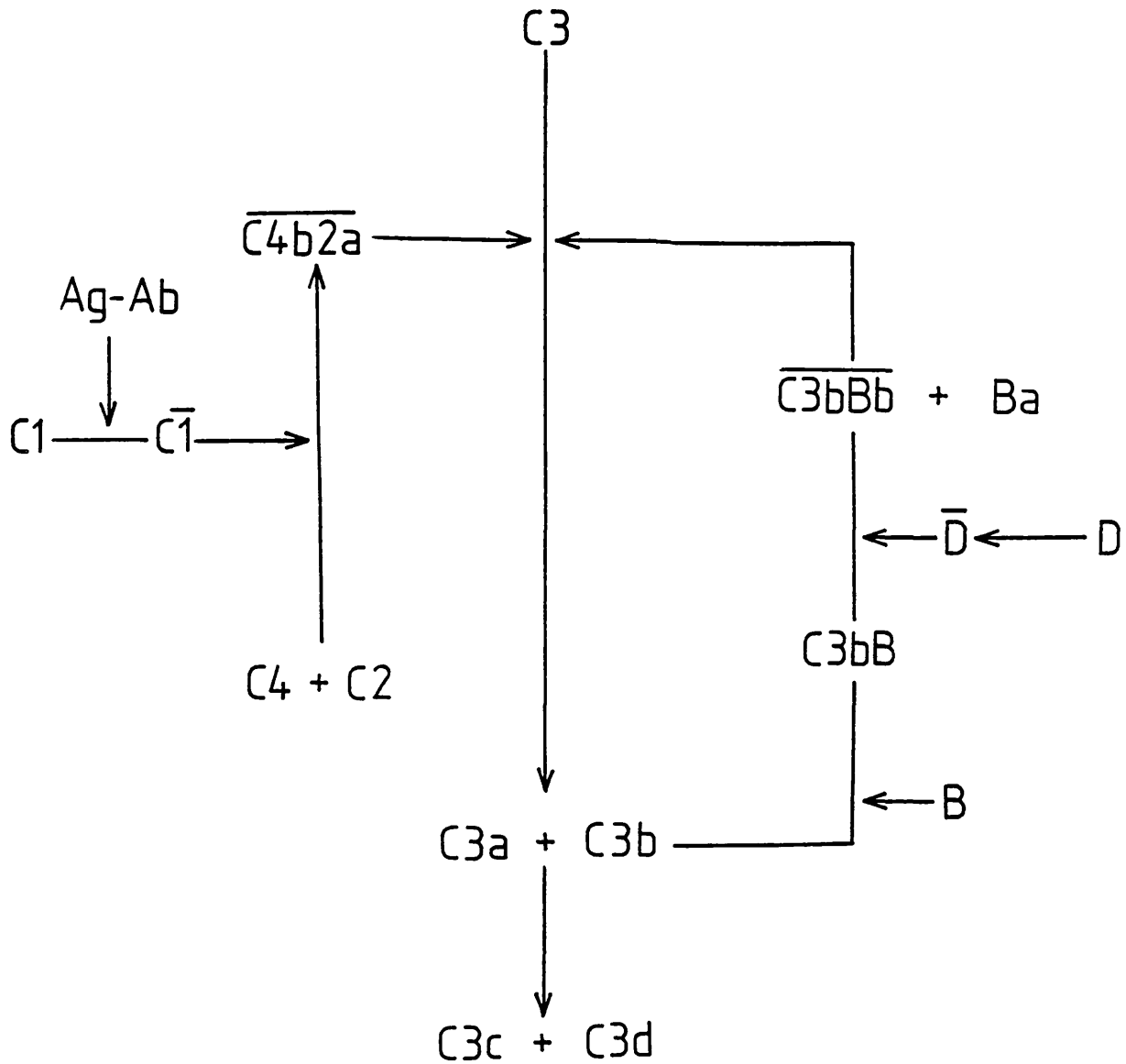
The C3b feedback pathway and the alternative pathway are not synonymous. The positive feedback pathway is the C3 converting system which is utilised by the alternative pathway. The C3 feedback pathway can be activated by the

classical and alternative pathways and therefore activation of either of these pathways is not mutually exclusive. It follows therefore that immune complexes which activate the classical pathway can activate the C3b feedback pathway, thus generating more C3b. The reason why uncontrolled amplification does not occur in normal serum in the absence of activators is due to the instability of the C3bBb enzyme and the action of the control proteins β1H (Factor H) and C3bINA (Factor I). Binding of Factor H to C3b results in decreased binding of Factor B and Bb and in enhanced inactivation of C3b by the proteolytic action of Factor I. The competition between B and Factor H for binding to surface associated C3b is influenced by the amounts of a carbohydrate, sialic acid (Fearon, 1978), and of a glycosaminoglycan, heparin (Kazatchkine et al, 1979), which are present on cell and particle surfaces. Sialic acid, a constituent of some membrane glycoproteins and glycolipids, increases the affinity of cell-bound C3b for Factor H but not for B. Neutral activators of the alternative pathway lack or have small amounts of sialic acid, and removal of this moiety from sheep erythrocytes converts these cells from non-activators to activators. C3b binds much less Factor H when bound to an activator and is therefore relatively protected from the action of Factor I, allowing the amplification loop to proceed. Properdin, after which the pathway was once named, merely plays an accessory role enhancing the stability of the C3 and C5 convertases,

thereby enhancing total alternative pathway activity (Schreiber et al, 1979).

The binding of C3b to the classical (C4b2a) and alternative (C3bBb) pathway C3 convertases modifies these enzymes so that they acquire additional specificity for C5. Cleavage of C5 is the final enzymatic step in the complement sequence and results in the release of a small fragment C5a, and the major fragment C5b binds to C6. The reaction of C5a6 with C7 leads to the formation of C5a67 on the cell membrane; however, fluid phase C5a67 can, for a short period of time, induce bystander lysis of uninvolved cells in the vicinity of the activation site. The C5a67 complex then binds one molecule of C8 and six of C9 to produce the C5-9 complex which brings about the characteristic membrane lesion of complement (Humphrey and Dourmashkin, 1969).

Figure 1



Complement activation to the C3 stage by the classical pathway and the C3 feedback loop

1.5 Mechanisms of Immune Complex Mediated Tissue Damage

The inflammatory process brought about by antigen-antibody complexes is the result of a co-ordinated series of specific and non-specific immunological events. The effector systems involved include the complement system, the coagulation system, prostaglandins and inflammatory cells, including their cell derived chemotactic factors. Extensive interplay exists among the multiple effector systems which can both stimulate and inhibit the resulting inflammatory process. In general, deposition of immune complexes precedes tissue damage, and tissues with a high blood flow, such as kidneys, are particularly susceptible to tissue injury by trapping complexes in their vascular walls. The most likely candidates for tissue deposition are those complexes which by their molecular composition evade phagocytosis and removal by the RES, thus initiating the process of injury. The resulting injury follows a general sequence of cellular events which are triggered by the presence of humoral components such as complement fragments which are generated during complement activation and which interact with specific receptors on a variety of inflammatory cells.

Immune adherence is a process whereby cells including lymphocytes, neutrophils and macrophages bind immune complexes via C3b-C4b receptors. Following characterisation

of complement receptors on cell surfaces, Ross et al, (1973 and 1978) designated the immune adherence (C3b-C4b) receptors as CR1, those which bind soluble or immune complex bound C3d as CR2 and those which bind C3bi as CR3 receptors. The expression of such receptors alone or in combination can relate to various stages of cell maturation; CR2 receptors being present on immature neutrophils and CR1 receptors on mature neutrophils.

Neutrophils are very mobile cells which rapidly respond to sites of tissue injury. They contain at least two types of preformed granules (Henson et al, 1978) and are capable of discharging their contents into phagocytic vacuoles and to the outside of the cell. Toxic oxygen radicals, generated by bursts of oxidative metabolism, are particularly potent mediators of tissue damage. The superoxide anion can be converted, by catalyst-mediated reactions, to hydrogen peroxide, hydroxyl radical and singlet oxygen. Other neutrophil constituents, released as a result of the interaction with immune complexes or complement fragments, include procoagulant activity, pyrogens, proteases (collagenase and elastase), acid hydrolases, peptide induces of the metabolism of the prostaglandin precursor arachidonic acid, platelet activating factor, slow reacting substance of anaphylaxis and kinin forming enzymes. These highly reactive substances are released from neutrophils not only in response to

phagocytosis but also when neutrophils interact with antibody or complement components bound to surfaces. Surface-bound immune complexes which are resistant to phagocytosis have been shown to be extremely potent in inducing release of lysosomal enzymes from neutrophils (Henson, 1976) and therefore sites such as glomerula basement membranes, synovial membranes and blood vessel walls may be damaged by such reactions. Macrophages also undergo immune adherence interactions, for they can bind immune complexes via the C3b receptor; however, Mantovani et al, (1972) demonstrated that Fc receptor activity was also required to mediate ingestion. Following ingestion the complexes are degraded by lysosomal enzymes with subsequent release of hydrolytic enzymes, proteases, oxygen radicals and prostaglandins which participate in the inflammatory process. Macrophages can also be stimulated into action by ongoing complement activation, for Gotze et al, (1979) demonstrated their activation at sites of injury in response to the Bb fragment of Factor B.

The generation of complement fragments at tissue sites stimulates the chemotactic movement of leucocytes. Chemotaxis of leucocytes, defined as the directional migration of cells provoked by a chemical stimulus, is mediated not only by the small molecular weight peptide C5a, but also by the C567 complex (Hugli and Muller-Eberhard, 1978). Recent work by Taylor et al, (1977) has discounted

earlier suggestions that C3a has chemotactic activity and has postulated that contamination of C3a by C5a accounted for chemotactic activity. Neutrophils, eosinophils and basophils display receptors for C5a and the influx of such inflammatory cells to the sites of immune deposition further mediates tissue injury via the release of lysosomal enzymes. Accumulation of eosinophils is associated with IgE-mediated reactions and also the antibody-dependent cellular cytotoxicity (ADCC) killing of parasites (Butterworth et al, 1975; Joseph et al, 1978). They have a similar repertoire of inflammatory mediators as neutrophils, but in addition can release a characteristic basic protein which can directly damage tissues. Receptors for C3a do exist, however, on cells bearing C5a receptors but the C3a ligand does not interfere with the binding of C5a to its receptor.

C3a and C5a both have anaphylatoxin activity with C5a being more active on a molar basis (Hugli and Muller-Eberhard, 1978). An important functional characteristic of the molecules is the presence of arginine at the carboxy terminals; however, this arginine can be cleaved by an enzyme present in plasma, a carboxy peptidase B, which effectively inhibits the action of both C3a and C5a (Hugli and Muller-Eberhard, 1978). Basophils and mast cells interact with C3a and C5a and subsequently release biologically active substances such as heparin, histamine, slow-reacting substance of anaphylaxis (SRS-A) and platelet

activating factor. The subsequent attraction of platelets and release of their vasoactive amines leads to increased vascular permeability allowing immune complexes to become entrapped in the filtering membranes of the vessel walls. Platelets have also been identified in several antibody or immune complex initiated lesions including the Arthus reaction (Cochrane and Koffler, 1973) and have also been shown to have a role in the deposition of immune complexes in vessel walls in serum sickness. Platelet aggregation can also be triggered through the IgG Fc receptor (Henson et al, 1978). Their relative importance in tissue injury was demonstrated by Kniker and Cochrane (1968) who showed that depletion of platelets prevented immune complex disease in the rabbit.

The role of complement in the induction of tissue injury has been demonstrated in experimental situations by complement depletion. Block et al, (1963) studied the effect of complement fixing and non-complement fixing antibodies in the induction of an Arthus reaction in guinea pigs. Only after complement fixation did the full Arthus reaction occur and in the absence of complement fixation, neutrophil infiltration was absent. In other forms of immunological injury, however, such as immune complex mediated nephritis (Henson and Cochrane, 1971) and anti-glomerulo basement membrane (GBM) nephritis (Hawkins and Cochrane, 1968), tissue damage has been shown to develop independently of neutrophils and complement. In these examples the presence of collateral inflammatory mechanisms is suggested.

1.6 Immune Complexes in Human Diseases

1.6.1 Systemic Lupus Erythematosus (SLE)

SLE, the prototype of immune complex disorders in humans, is a chronic systemic inflammatory disease which follows a course of alternating exacerbations and remissions. During periods of disease activity multiple organ system involvement occurs including arthritis, nephritis, vasculitis and pleuritis. The aetiologic agent(s) remains unknown, but the disease is characterised by hyperactivity of B-cells in producing various autoantibodies against cellular and non-cellular components (Theofilopoulos and Dixon, 1979).

SLE has been designated an immune complex disease on the basis of several lines of evidence. Granular deposits of immunoglobulin and complement components have been found in immunofluorescent studies of glomeruli in lupus nephritis. This is distinct from the linear immunofluorescent pattern of anti-GBM disease. Hypocomplementaemia is also a well recognised feature of active disease and evidence suggests that this is due to increased catabolism and consumption of complement components by immune complexes (Schur and Sandson, 1968; Williams et al, 1974). The relationship of immune complexes in SLE to disease activity is still unclear. Studies employing the C1q binding assay and

inhibition of lymphocyte rosette formation reported poor correlation with disease activity (Inman et al, 1980). Furthermore, the Raji cell assay and the monoclonal rheumatoid factor assay showed correlation with non-renal disease,but not with renal disease (Huston et al, 1978). On the other hand, the C1q deviation test (Cano et al, 1977) and the method of augmenting anti-DNA binding by pronase digestion (Bardana et al, 1975) did show concordance with disease activity. This conflicting evidence is possibly due to the presence of a broad spectrum of autoantibodies reactive with DNA and RNA, constituents of soluble nuclear extracts, e.g. the Sm antigen and Ha antigen, and antigenic determinants on T and B cells (Theofilopoulos and Dixon, 1979). Consequently, the immune complexes formed are particularly heterogeneous and therefore their measurement in a variety of immune complex assays, which detect complexes by different ligands, e.g. Fc sites by the C1q binding assay and complement products by the K and Raji cell assays, may not be expected to reveal concordance with disease activity. However, the measurement of immune complexes may still be relevant in disease follow up, for a rise in the level of complexes can precede a relapse of the disease (Zubler and Lambert, 1978).

The most direct evidence for a pathological role of immune complexes in SLE has come from the demonstration of DNA containing complexes in glomeruli (Koffler et al, 1967)

and the presence of single and double stranded DNA in eluates from renal tissue of SLE patients (Koffler et al, 1971). These DNA-anti-DNA complexes may be rapidly localised in glomeruli and other tissues because of the affinity of DNA for collagen and collagen-containing structures (Izui et al, 1976) and therefore this may explain why these complexes are sometimes not detected in sera.

The heterogeneity of antibody production has recently been brought into question (Schwartz, 1983). The suggestion is that antibodies in SLE may be restricted both in their ligand binding properties and their idiotypic diversity. The epitope for many nuclear antibodies is believed to reside in the sugar-phosphate backbone of the nucleic acids and that structural differences in polynucleotide backbones can account for variations in binding specificities among monoclonal antibodies. Present work is involved in the analysis of idiotypic specificity of anti-DNA antibodies and the interesting possibility exists that anti-idiotypic antibodies could in the future be used to suppress the principal serological abnormality of this disease.

1.6.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a systemic recurrent disease which is characterised by immune complex mediated chronic inflammation which appears to underly the arthritis. The antigenic stimulus that initiates the immune response and subsequent inflammation in RA is unknown. Synovial lymphocytes are thought to produce an IgG which is recognised as foreign and therefore stimulates the production of IgG and IgM rheumatoid factors (RF). The subsequent generation of IgG rheumatoid factor complexes results in the activation of the classical complement system. Hay et al, (1979) found a close association between the presence of immune complexes in synovial fluid and the production of antiglobulins, particularly IgG rather than IgM rheumatoid factor. The higher levels of complexes in synovial fluids as opposed to sera, together with the ability of IgG antiglobulins to self-associate (Pope et al, 1975) provide evidence for the local formation of complexes in synovial tissues.

Complement activation of these complexes will generate chemotactic factors and thus attract polymorphs which can release a variety of lysosomal enzymes such as collagenase, elastase and cathepsin G which can degrade collagen and proteoglycan components of cartilage (Janoff, 1975). The synovial fluid contains inhibitors such as α-2 macroglobulin

and α_1 anti-tripsin which can modulate the harmful effects of these enzymes. However, when polymorphs adhere to immune complexes on intact surfaces such as the surface of cartilage and synovial membrane, the influence of enzyme inhibitors is reduced (Ignarro, 1974), resulting in unimpeded tissue degradation. Other mechanisms of activation occur at the margin of the advancing pannus which contains stimulated macrophage-like cells of the synovial lining. These can be activated directly by immune complexes (Allison, 1978) or can be activated by the release of lymphokines, e.g. macrophage activating factor, from sensitised T-cells (Wahl et al, 1975).

The binding of IgM RF to the complexes will generate larger complexes and further enhance complement activation; however, it may also facilitate phagocytosis of complexes with the subsequent release of hydrolytic and lysosomal enzymes which can break down the connective tissue matrix. In the serum, the competition from normal IgG will prevent the formation of large lattices of antiglobulins and thus favour small complexes which would not activate the complement system so effectively. These small complexes may not be pathologically important; however, in patients with hypergammaglobulinaemic purpura, hyperviscosity syndromes and monoclonal gammopathies, as well as widespread RA, the concentration of IgG antiglobulins may reach concentrations high enough to generate large complexes which activate

complement and cause diffuse vasculitis (Pope et al, 1975).

A variety of techniques have demonstrated immune complexes in serum and synovial fluids from patients with RA, with some disparity between levels of complexes and clinical conditions. A positive correlation with clinical features of arthritis was demonstrated using a monoclonal rheumatoid factor radioimmunoassay (Luthra et al, 1975), whereas C1q binding activity (Zubler et al, 1976) correlated with extra-articular disease but not with clinical stage. Monitoring levels of immune complexes in RA may be of value not only diagnostically but also during treatment such as gold therapy where complications of proteinuria, severe rash and mouth ulceration, can be preceded by a rise in immune complex levels (Nineham et al, 1979).

1.6.3 Immune Complexes in Bacterial, Parasitic and Viral Infections

The production of specific antibodies to antigenic determinants on invading organisms is one of the first lines of immunological defence; however, upon degradation of these pathogens soluble antigens can be released into the circulation which can lead to the generation of immune complexes and resultant pathogenic potential.

Some clinical observations of infective endocarditis have been shown to involve immune complexes, for example, Williams and Kunkel (1962) showed that the renal injury was due to the glomerula deposition of immune complexes. This type of renal disease is not restricted to one particular organism for it has been recorded with a variety of infecting organisms including pneumococcus, gonococcus, Haemophilus influenzae, Staphylococcus aureus and S.albus (Williams, 1980). The acute phase of the disease is often accompanied by a rise in rheumatoid factor (RF) which can be reduced to base line levels by successful anti-microbial therapy. The suggestion from such data is that RF was raised against autologous complexed IgG and that perhaps in this or other diseases where RF is produced, the immune complexes provide an ongoing self-immunisation. The physiological significance of the binding of such RF is

still controversial. Messner et al, (1968) showed that IgM RF would interfere with phagocytosis in vitro by preventing the binding of opsonic IgG to the Fc receptors on polymorphonuclearcytes or monocytes. Other work, however, indicates that phagocytosis can be enhanced by the mediation of complement activated RF (McDuffie and Brumfield, 1972).

Evidence for the involvement of immune complexes in the peripheral manifestations of infective endocarditis is less clear; however, deposits of immunoglobulin and complement have been identified by immunofluorescence in the skin lesions of such patients. The presence of immune complexes in skin lesions has also been documented in meningococcal infections. Greenwood et al, (1973) showed the presence of IgM, C3 and gonococcal antigen in purpuric skin lesions; however, isolation of the circulating complexes at the time of the lesions failed to detect the bacterial antigen. This could be due to masking of antigenic sites by antibody and complement or may suggest the presence of idiotype anti-idiotype complexes which may protect the bacteria from the immune system and thus prolong the disease.

The study of immune complexes in parasitic diseases is made difficult due to the presence of shared autoantigens between host and parasite and the ability of the parasite to change its antigenic structure to evade the immune system. Infection with Schistosoma mansoni is an example of this,

whereby the structure of the outer membrane can alter with a turnover rate of 2-3 hours (Wilson and Barnes, 1977). This provides not only a convenient mechanism for escaping immune defences but also a means of continuous parenteral infusion of shed antigens which could produce a state of tolerance. The most widely studied immune complex disease associated with schistosomiasis is the renal disease due to S.mansoni infection. Andrade et al, (1971) reported the presence of glomerulosclerosis, mesangial thickening and glomerulonephritis in patients with S.mansoni infection and later Hoshino-Shimuzi et al, (1976) showed the glomerulo localisation of IgG, IgM, IgE, C3 and S.mansoni antigens in infected patients. The suggestion from this work was that there was preferential renal localisation of parasitic antigen, thus raising the possibility that subsequent antibody binding and complement activation may initiate the immune complex lesions.

This situation has also been postulated in the renal injury by Plasmodium malenae infection. Houba et al, (1976) demonstrated that ^{125}I-antibody to P.malenae showed specific renal localisation when injected into patients with nephritis. An alternative explanation, however, would be that complexes already localised in the kidney had free malarial antigenic sites which could bind ^{125}I-antibody. Although little is known about the physico-chemical composition of the complexed antigens

involved, it appears from renal localisation studies that low affinity antibodies eluted at pH 5.5 to 6.0 may be more often associated with immune complex deposition and subsequent renal damage (Houba, 1975).

Viral diseases present a complex problem, not only because of the generation of immune complexes but because the viral genome can integrate into normal cells (Wecker et al, 1977). The resultant expression of the proteins coded for by the intergrated viral genome brings into question the concept of what is real self and non-self.

Viral hepatitis infection provides an interesting immune complex mediated disease, the clinical picture of which may include polyarteritis and vasculitis due to persistence of hepatitis B antigen (HBag) complexes in vulnerable capillary beds. The underlying question of significance in such disease manifestations is related to the mechanisms of viral persistence, despite evidence of a vigorous humoral immune response to the infecting virus. The persistence of glomerula lesions in chronic HB infection, for example, is believed to be coincidental with a defect in cell mediated immunity (Kohler et al, 1974). In the light of such observations it has been suggested that treatment to stimulate cellular immunity may be more clinically useful in this case. On the other hand, however, cell mediated immunity is believed to be the underlying mechanism of

immune complex mediated hepatic cellular injury in chronic HB infection. Immunoglobulin and complement components have been detected on the surface of hepatocytes in acute hepatitis and cytotoxicity has been shown to function via killer (K) cells bearing Fc receptors (Thompson et al, 1974).

A more recent addition to the hepatitis story has been the so-called e-antigen (eAg). Neurath and Strick (1977) have postulated that the eAg is actually an immunoglobulin directed at hepatitis virus determinants and thus sterically blocks other antibodies directed at significant viral antigenic structures. Moreover, this eAg represents molecules of the IgG 4 sub-class which do not react with C1q nor do they bind macrophages. A situation could therefore be envisaged whereby immune complex formation between eAg and HB virus could hinder the first line of immune defence and therefore lead to persistence and chronicity of the disease.

1.6.4 Significance of Immune Complex Measurement

The accumulation of data over many years concerning the presence of immune complexes in a wide range of diseases has generated increased expectation for their role in disease pathogenesis. The formation of immune complexes is central, not only to the basic immunologic defence from infection, but is also recognised as the final common pathway leading to inflammation in diseases with diverse aetiologies. Conflicting evidence, however, linking the presence of immune complexes with disease pathogenesis has called for a philosophical reassessment of the interpretation of the data.

Perhaps the most important aspect of immune complex detection is the concept of heterogeneity. At the time of sampling the immune complexes present in body fluids are a product of the overall dynamic mechanisms which govern their rate of formation and clearance by the RES. In some diseases, for example SLE, antibodies can be raised against several antigens and therefore complexes can vary in terms of the amount and type of antigen and antibody incorporated. In addition, their size and their ability to remain soluble in body fluids is dependent on the solubilisation effect of the complement system. The complexes therefore will be made up of a wide range of sizes and constituents, both specific and non-specific, some of which having no obvious

pathogenetic role. Even after removal of body fluids immune complexes can still undergo change by dissociation and association due to the non-covalent assembly of the constituents of the complexes. Furthermore, complexes can undergo transient binding to cells such as erythrocytes via C3 receptor binding. The complexes therefore which remain in the fluid phase following removal from the body are the ones which will be selected for immune complex assay and potentially important complexes bound to tissues or cells remain unaccounted for.

The measurement of immune complexes in body fluids introduces more variables in that different assays only detect discrete fractions of total immune complexes. The K binding assay, for example, only detects those complexes with C3bi on their surface and the C1q binding assay only detects those complexes which are capable of activating the classical pathway of complement. The use of heat-aggregated gamma globulin as a standard in such assays is questionable because only those complexes which can mimic activation of complement by HAGG will be detected and also because there is no international standard HAGG. Results obtained therefore in one laboratory will almost certainly differ from those of another. In addition, such assays are non-specific in that they detect complexes irrespective of which antigen they contain.

Although the correlation of immune complex levels with disease activity is a function of physiological and technological parameters, the theory still holds that immune complexes are related to disease activity. A simple interpretation of immune complex levels is difficult and therefore efforts have been directed towards the identification of constituents of these complexes, in particular the underlying antigens. To achieve this objective it is necessary to isolate the complexes from the pathological fluids in which they occur.

1.7 Isolation of Immune Complexes

Immune complexes exist in pathological fluids in very small amounts compared to the vast excess of normal blood proteins. To isolate the complexes, therefore, techniques based on the separation of molecules in terms of molecular size were developed. These included gel filtration, sucrose density gradient centrifugation and precipitation by ammonium sulphate and polyethylene glycol (PEG). Although these products were not preparative for immune complexes, they did provide initial evidence for the existence of immune complexes in certain diseases. Gel filtration of Hodgkin's disease sera, for example, demonstrated the presence of C3 in macromolecular form (Amlot et al, 1976), while Kunkel (1961) and Franklin et al, (1957) demonstrated the presence of IgG-IgM RF and IgG-IgG RF complexes in patients with rheumatoid arthritis using sucrose gradient centrifugation.

One particular modification of these procedures was developed by Male and Roitt (1979). They exploited both the separation of complexed molecules in sucrose density gradients and their decreased solubility in PEG. A PEG gradient was incorporated into the lower three layers of a sucrose gradient to stabilise and precipitate the larger complexes, thus preventing dissociation of low affinity complexes produced by the equilibrium shift during

separation (Kijlstra et al, 1977). However, this method still suffered from the contamination of non-specific, non-complex bound, high molecular weight proteins precipitated by PEG.

The following section discusses the purification of immune complexes by more specific means after initial crude isolation on the basis of molecular size.

1.7.1 Methods based on the Intrinsic Biological Components of Immune Complexes

a) Staphylococcal Protein-A

Protein-A has been reviewed with reference to its use as an immunological reagent (Goding, 1978) and it has long been known that protein-A binds with the Fc region of human IgG 1, 2 and 4 H-chain sub-classes (Forsgren and Sjoquist, 1966 and 1967) although there is recent evidence that binding may occur with sub-groups of IgA and IgM (Lind et al, 1975; Harboe and Folling, 1974). Both Protein-A bearing Staphylococcus aureus bacteria (Kessler, 1975; Natalie et al, 1980) and purified Protein-A Sepharose 4-B (Pharmacia) (Chenais et al, 1977) have been used to isolate immune complexes. Although the binding capacity of Protein-A Sepharose is five times greater than an equivalent weight of bacteria, non-specific adsorption is greater with Sepharose than with bacteria (Goding, 1978). An important prerequisite of this technique is that monomeric IgG should be removed to reduce competition for the binding receptor and therefore to reduce contamination. Gel filtration, rather than PEG or ammonium sulphate precipitation, has been the method of choice prior to adsorption to Protein-A (Heimer et al, 1979). However, various modifications of methods have been employed in recent years (Chenais et al, 1977;

Tucker et al, 1978; Heimer et al, 1979). IgM and polymeric IgG are not removed by gel filtration. However, the contamination from these proteins can be reduced by eluting the bound complexes at pH4 rather than at pH 2.8. Using this schedule 90 per cent of the bound IgG could be recovered.

Normal serum proteins such as complex-bound complement may interfere with the binding of complexes to Protein-A (Scharfstein et al, 1979), presumably through steric hindrance (Nussenzweig, 1980). Rheumatoid factor does not appear to interfere with immune complex binding to Protein-A (Natali et al, 1980).

Bound complexes have been eluted with a variety of eluants ranging from M acetic acid (Chenais et al, 1977) to 2.5 M $MgCl_2$ (Natali et al, 1980). MacSween and Eastwood (1978) investigated the use of several eluants to dissociate in vitro prepared complexes from the immobilised Protein-A. They surprisingly reported that glycine/HCl pH 2.5 removed less than two per cent of the bound ^{125}-I-BSA - anti-BSA complexes, and 0.1M lithium diidosalicylate eluted 82 per cent of the bound complexes, but unfortunately this latter procedure resulted in significant destruction of the antibody. There is no eluant of choice in dealing with the elution of complexes from Protein-A. Eluants

can be chosen on the basis of securing a high yield of complexed material or the preservation of antigen or antibody integrity.

b) Binding to Lectins

Con-A-Sepharose columns have been used by Heimer and Klein (1978) for isolating immune complexes from the sera of patients with Epstein-Barr virus related cancers such as Burkitt's Lymphoma and nasopharyngeal carcinoma. The putative complexed material was eluted with α-methyl-D-mannoside but as whole sera was incubated with the affinity column, it is highly likely that C3, being a glycoprotein and being present in abundance in sera, would compete for the binding sites on Con-A. Also, antigens with lectin binding activity may not bind due to steric hindrance by the antibody molecules. An initial isolation stage, such as gel filtration, should be utilised to separate monomeric glycoprotein containing compounds such as C3 from aggregated ones to reduce competition and increase the yield of specific complexes.

c) Binding to Raji Cells

Raji cells, having receptors for IgG Fc, C3b and C3d, have been used to isolate complement fixing immune complexes from sera (Theofilopoulos et al, 1978). The receptors for IgG Fc are few in comparison to the complement receptors

and are of lower affinity; thus the probability of 7S IgG binding is reduced (Jones and Orlans, 1981). Bound complexes could be iodinated *in situ* and eluted with isotonic citrate buffer pH3 or recovered by immuno-precipitation of cell lysates. The major disadvantage of such a method is that it is difficult to scale up the techniques to increase the yield of complexes, and nuclear antigens such as DNA and RNP may bind to the Raji cell surfaces (Horsfall *et al*, 1981). Consequently the use of this technique has been limited, but it has been pointed out (Amoroso *et al*, 1980) that in situations where the antigen is known the Raji cell technique could be modified to identify the antigen by using specific fluorescent antibodies.

For several years it has been suggested that tumour antigen-antibody complexes are adsorbed *in vivo* on leucocytes (Bansal *et al*, 1976; Hattler and Soehnlen, 1974). Thus, by adopting the concept of the Raji cell technique, a patient could prove a good source of starting material for isolating antigen within the immune complexes. Jaquemin *et al*, (1978) adopted this approach and were able to isolate virus specific antibodies from patients with various leukaemias.

1.7.2 Binding to Complement Components

a) Binding to Solid Phase Clq

The binding of Clq to the CH2 domain of IgG containing complexes was utilised by Svehag and Burger, (1976) to isolate Clq binding complexes. However, as whole serum was used, the competition between free Cl in the serum and solid phase Clq would have limited the recovery of complexed material. Casali and Lambert, (1979) improved this method by linking Clq to Degalan beads and extracting the complexed material with PEG to remove free Clq. In theory this approach could isolate immune complexes which activate the classical complement pathway, thereby only excluding IgG_4, IgA, IgD and IgE complexes.

b) Binding to Solid Phase Bovine Conglutinin (K)

Conglutinin (K) is a normal bovine serum protein which has the capacity to bind inactivated C3b in the presence of calcium ions (Lachmann, 1967). Casali and Lambert, (1979) adsorbed conglutinin on to Degalan beads and used the immune adsorbent column to purify *in vitro* and pathological complexes. Bound complexes were eluted with 0.02M EDTA and the material isolated contained Clq, r, s, IgG, IgM, C3, C3c, and C3d. This method has

the advantage that complexes which have activated both the classical and the alternative pathways of complement can be isolated, and the K does not have the non-specific binding properties of C1q towards polyanions such as DNA. However, because only a sub-population of complexes may carry C3bi, the system would have to be maximised to give a good yield of complexed material.

One disadvantage of using this method is that it has been shown (Pereira et al, 1980; Takahashi et al, 1977) that during complement activation of complexes, antigen dissociation can occur, leaving antibody and C3 peptides bound together in small 7-8S complexes which can bind K. In this way a specific antigen may evade detection.

c) Binding to Liquid Phase Conglutinin

Gupta and Tan, (1981) have recently introduced a liquid phase isolation procedure for immune complexes, based on their interaction with bovine conglutinin (K). Whole serum samples were incubated with K and K-bound immune complexes were precipitated with a rabbit anti-bovine K serum. Complexes were dissociated from the K with 0.02M EDTA pH 7.5 and upon acid treatment at pH3.0, the dissociated antibody was bound to Protein-A Sepharose, leaving the antigen or other components in solution. This technique

has the advantages ascribed to the previous technique of Casali and Lambert, (1979). However, it appears to reach the same end, albeit by more laborious means, and the K is not reusable.

1.7.3 Binding to Anti-Immunoglobulins

Rheumatoid factors of various class and specificity have been used in solid phase systems to isolate immune complexes.

Rheumatoid factors (RF) exist with specificities for all the subclasses of IgG and sometimes for IgD and IgE (Jones and Orlans, 1981) and thus any particular RF will be selective for discrete classes or sub-classes of immunoglobulins. In this respect perhaps the best compromise is to isolate complexes with a solid phase polyclonal RF (Gilead and Sulitzeanu, 1979). However, although IgM rheumatoid factors bind to aggregated or complexed IgG in preference to monomeric IgG, isolation techniques should include steps to initially separate these compounds in order to reduce competition and contamination with monomeric IgG. Gilead and Sulitzeanu (1979) modified their technique in this respect and also removed non-specific complexed material by immunoprecipitation.

Anti-IgM affinity columns have been used to isolate

immune complexes (Jones et al, 1980; Harkiss and Brown, 1980), but isolation of free IgM will always reduce the efficiency of the process. Success in any of these procedures will depend on the degree of complement activation, for if complexes are well coated with C3 then steric hindrance may play a part in reducing the efficiency of the technique.

Perhaps the most useful ligand to exploit in isolating immune complexes is C3. Scharfstein et al, (1979) mixed anti-C3 or anti-C4 antibodies with immune complex containing sera and precipitated the complexed material with Protein-A. The important observation here was that the Protein-A only bound to the Fc of the anti-C3 or anti-C4, and not to the IgG or the preformed complexes. This strengthens the idea that bound complement components mask available Fc sites on complexes and implies therefore that methods which utilise these sites, e.g. Protein-A, must underestimate the amount of total complexes (Nussenzweig, 1980). Pereira et al, (1980) recently reported the use of solid phase $(Fab')_2$ of anti-human C3 to detect immune complexes. This procedure could be developed to isolate the complexes by firstly removing free C3 and any 7-8S IgG-C3 peptide complexes by PEG precipitation, and secondly using a monoclonal antibody that would only detect fixed C3 peptides, C3b, C3c and C3d, and not the native C3. This

would give greater specificity to the method and should also give a greater yield than K-columns which only bind those complexes with available C3bi on their surface.

1.7.4. Antigen-Specific Techniques

Antigen-specific detection systems have been developed for *in vitro* prepared complexes (D'Amelio *et al*, 1981) and for immune complexes in infections such as hepatitis (Pernice *et al*, 1979a, 1979b). The former method is a modification of the K-binding assay (Casali *et al*, 1977) whereby complexes with fixed C3bi bind to solid phase K and are subsequently recognised by the binding of a specific labelled antibody to the antigen in the complex. For reasons already mentioned (1.7.2b) this approach will give an underestimate of the total specific complexes. A better approach is that of Pernice *et al*, (1979a,b), where they incubated whole sera with the solid phase specific antibody to hepatitis B antigen and quantitated the complexes with a second antibody.

Other techniques have been reported whereby the antigen is reacted with its specific labelled antibody following isolation of the complexes by conventional techniques of PEG precipitation (Paganelli *et al*, 1981) or gel filtration and affinity chromatography (Kilpatrick

and Virella, 1980). These approaches are only of use when complexes are being reacted with a range of antibodies to determine the nature of the antigen present in immune complexes following ingestion of food antigens (Paganelli *et al*, 1981) or other known antigens (Gropp *et al*, 1979).

1.7.5 Identification of Antigen or Antibody

As already mentioned, the identification or quantitation of antigen and antibody in model complexes or well defined clinical conditions where the antigen is known is relatively easy and may be useful in the management of such conditions. However, the identification of specific antigens or antibodies in diseases of unknown or uncertain aetiology is more difficult. *In vivo* circulating immune complexes may be extremely heterogeneous and include complexes containing specific antigen which is related to the disease process and immune complexes formed following secondary infections by viruses or bacteria and/or idiotype - anti-idiotype complexes.

Undissociated immune complexes provide a problem in the identification of the specific components, for the antigen and antibody may be relatively covered by a variety of other immunological reactants such as covalently-bound complement components (Porter, 1980)

or immunconglutinins. Despite these difficulties some workers have had limited success in identifying known antigens (Theofilopoulos et al, 1976).

The constituents of isolated immune complexes have been analysed by a variety of immunochemical techniques including immunoprecipitation with specific antisera (Casali and Lambert, 1979), one and two dimensional electrophoresis, SDS-PAGE isoelectric focusing (Maidment et al, 1981), autoradiography (Male and Roitt, 1979) and sucrose density gradient centrifugation (Kilgallon et al, 1982). A recent development has been the electrophoretic transfer of proteins from polyaclamide gels to nitrocellulose sheets (Towbin et al, 1979). In this way unidentified protein bands on PAGE gels can be identified by incubation with appropriate antisera (McMichael et al, 1981). This is in preference to overlaying SDS-PAGE gels with individual patients' sera (Heimer et al, 1979) to identify potential antigens since the SDS in the gel may destroy some antigens (Anderton and Thorpe, 1980).

Recently Maidment et al, (1981) have used a preparative isoelectric focusing technique to analyse complexed material from patients with breast cancer and have demonstrated the presence of putative antigens which not only bind to the IgG of the complex, but also

bind to cell surface antigens present on breast cancer tissue cell lines. Whether the reactive cell surface antigens are tumour specific or organ specific remains to be determined.

Immunisation of rabbits with isolated complexes may result in the production of specific antibodies which could be used as solid phase immunoadsorbents to purify specific antigens. The possibility of finding an association between a particular idiotype and disease exists (Baird et al, 1980; Agnello et al, 1980), although the raising of anti-idiotypic antibodies and their interrelation with respect to specificity presents considerable problems (Sogn et al, 1977; Davie, 1980).

1.8 Objectives

Immune complexes have been detected in a wide range of human diseases and have been implicated in their pathogenetic mechanisms (WHO Bulletin, 1977). Whereas in some diseases such as glomerulonephritis a pathological role has been described for immune complexes, in others, such as Hodgkin's disease, their involvement in the disease process is unclear.

Hodgkin's disease is a lymphoreticular malignancy which spreads by contiguity from one lymphnode chain to another. The neoplastic cell involved in the lymphnodes and tissues is the Reed-Sternberg cell and prognosis correlates well with an increased proportion of normal mononuclear cells to neoplastic cells. Lukes and Butler (1966) classified the histopathological appearance of Hodgkin's disease into lymphocyte predominant, nodular sclerosing, mixed cellularity and lymphocyte depleted forms in order of prognosis; lymphocyte predominant having the best prognosis and lymphocyte depleted the worst.

The presence of immune complexes in Hodgkin's disease was first reported by Amlot et al, (1976). Using Sephadex G-200 gel filtration, they showed that increased quantities of macromolecular C3 were present in plasma of patients with symptomatic, untreated disease. Further work (Amlot et al,

1978) established a good correlation between the presence of immune complexes and the occurrence of symptoms such as fever, night sweats and weight loss. As the presence of these symptoms is associated with dissemination of the disease, it was suggested that the antigen in these complexes may be specific for Hodgkin's disease.

The purpose of this work was to isolate, purify and characterise the immune complexes present in the sera or plasma of patients with Hodgkin's disease in the hope of identifying an antigenic component associated with the disease process. Current techniques for isolating immune complexes were used initially and when their limitations became apparent other techniques were developed.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents

2.1.1 General

All chemicals and reagents were of analytical grade and unless stated otherwise were purchased from BDH Chemical Company, London.

2.1.2 Chromatography

CNBr activated Sepharose-4B, Protein-A Sepharose and SephadeX G200, G25 (fine) and G10 were all purchased from Pharmacia, Uppsala, Sweden.

Degalan Beads (V26) were purchased from Degussa Limited, London and Affi-Gel was purchased from the Pierce Chemical Company, Illinois, USA.

Trichlorofluroethane (Frigen 113 TR-T) was purchased from Hoechst, Germany.

2.1.3 Electrophoresis

Both polyacrylamide gel and immunoelectrophoretic

equipment were purchased from Shandon Instruments Ltd. Coomassie Blue and basic fuschin stains were purchased from Sigma Chemical Company, St Louis, USA.

2.1.4 Radiolabelling

Iodogen (1,3,4,6-Tetrachloro-3α, 6α-diphenyl glycoluril was purchased from Pierce Chemical Company, Illinois, USA and ^{125}I-Na and ^{131}I-Na were both purchased from the Radiochemical Centre, Amersham, Bucks.

2.1.5 Antisera

Antisera to whole human serum and human immunoglobulins G,M,A, and D were purchased from Wellcome Labs., London. Antisera to complement components were purchased from Organon Teknika, Germany (anti-C3 and anti-C3c), and the Red Cross Blood Transfusion service, Netherlands (anti-C3d).

Antisera to human C1q, human fibronectin and C-reactive protein were purchased from Seward Ltd., London.

MATERIALS AND METHODS

2.2 General Methods

2.2.1 Folin Method - Protein Estimation

Protein estimations throughout this whole work were made by the Folin-Ciocalteau method of Lowry et al, (1951). Standards were prepared in duplicate by diluting BSA (200 µg/ml) over the required concentration range in 1 ml aliquots. Duplicate test samples were also diluted to 1 ml. To each sample 5 ml of solution A (1 ml 1% copper sulphate and 1 ml 2% sodium potassium tartrate added to 98ml 2% sodium carbonate) was added. After ten minutes at room temperature 0.5 ml of a 1:2 dilution of Folin-Ciocalteau stock solution was added and after 30 minutes at room temperature the absorbance of the samples was read on a SP 505 spectrophotometer at a wavelength of 750 nm. The protein concentrations of the test samples were determined from the standard calibration curve.

2.2.2 Extraction of Lipids from Serum or Plasma

Lipids were removed from immune complex containing serum or plasma by the addition of one volume of trichlorofluroethane (Frigen 113 TR-T, Hoechst, Germany) to two volumes of serum or plasma. The mixture was

stirred at room temperature for 30 minutes and then centrifuged at 1,500g for 15 minutes at 4°C. The lipid free serum or plasma was collected by vacuum suction and subsequently processed according to the various purification procedures.

2.2.3 Precipitin Reaction (Precipitin Curve)

A classical precipitation reaction was set up to determine the relative proportions of antigen (BSA) and antibody (anti-BSA) that would precipitate at equivalence and thus from this ratio all other ratios of antigen : antibody could be determined.

Concentrations of BSA, ranging from 0.5-50 µg, in a volume of 50 µl of PBS were incubated with 15 µl of rabbit anti-BSA antibody (protein concentration 4.37 mg ml^{-1}) and 10 µl ^{125}I-BSA (4,000 cpm ≡ 8 ng BSA). The total volume of the reaction mixture was made up to 150 µl by the addition of 75 µl PBS or 75 µl normal human serum. After incubation at 37°C for 30 minutes the precipitated material was recovered by centrifugation at 1,500g for 30 minutes, washed in PBS and the quantity of isotope in the precipitate determined. Soluble complexes which remained in the supernatant were precipitated with 50% saturated $(NH_4)_2SO_4$ by incubating for one hour at 4°C and recovered by centrifugation at 1,500g for 30 minutes.

Precipitated material was washed once in 50% saturated $(NH_4)_2SO_4$ prior to isotope counting.

2.2.4 Extraction of Soluble Immune Complexes with $(NH_4)_2SO_4$ and PEG

Saturated $(NH_4)_2 SO_4$ was prepared by adding 1,000 g of $(NH_4)_2 SO_4$ to 1,000 ml distilled water and adjusting the pH to 7.4 with concentrated ammonia solution. The mixture was left at 37°C until the ammonium sulphate was completely dissolved and then allowed to stand at room temperature for precipitation to occur.

Soluble immune complexes of ^{125}I-BSA and anti-BSA were prepared at different degrees of antigen excess in fresh NHS and then incubated at 37°C for 30 minutes. After centrifugation at 1,500g for 30 minutes the soluble complexes were precipitated by the addition of an equal volume of either saturated $(NH_4)_2 SO_4$ (range 10-50% saturation) or PEG 6,000 in 0.1 M PBS containing 0.02M EDTA (range 1-5% $(\frac{W}{V})$. After two hours at 4°C the precipitated complexes were recovered by centrifugation at 1,500g for 30 minutes and the distribution of the ^{125}I-BSA in the pellet and the supernatant determined. The pellet was then redissolved in distilled water and the supernatant dialysed overnight at 4°C against PBS or CFD. Subsequent analysis of protein concentration was achieved

by the Folin-Ciocalteau method. Their molecular size and composition was determined by sucrose density gradient centrifugation, immunoelectrophoresis and gel electrophoresis (PAGE).

2.2.5 Latex Agglutination Test (Ortho R A Test)

A qualitative and semi-quantitative latex slide test for rheumatoid factor was carried out on serum samples and isolated immune complexes. The test was dependent upon the agglutination of latex particles coated with human gamma globulin by the rheumatoid factor present in the test samples. One drop (25 µl) of the test sample was mixed with one drop of the latex suspension (Ortho) on a glass slide, this was gently tilted to produce an even suspension and agglutination observed microscopically as coarse or fine aggregation, was verified using positive and negative controls.

A semi-quantitative procedure was adopted whereby the test sample was diluted until it no longer produced agglutination. The highest dilution in which visible agglutination occurred was the end point. The approximate rheumatoid factor concentration (CRF) was given by the following formula :-

CRF in IU ml $^{-1}$ = Titre of last positive dilution x sensitivity in 1U ml $^{-1}$

Sensitivity for this test was 1.25 IU ml $^{-1}$

2.2.6 Sucrose Density Gradient Centrifugation

Sucrose gradients were prepared over the range 10-50% $(\frac{W}{W})$ in a variety of buffers at pH 7.4 and pH 3.0. A stock of 50% $\frac{W}{W}$ sucrose was prepared by mixing 500 g sucrose with 500 ml of the appropriate buffer containing 0.01% sodium azide. The buffers used were PBS or CFD, (either with or without 0.02M EDTA, pH 7.4), 40%, 30%, 20% and 10% sucrose were prepared in the appropriate buffer and the accuracy was checked using an optical refractometer. As the dilution of sucrose does not follow a linear progression, the relative amounts of 50% $\frac{W}{W}$ sucrose to add to the buffer was determined by the following table:-

	50%	40%	30%	20%	10%
ml 50% sucrose	20	15.3	11	7	3.4
ml Buffer	0	4.7	9	13	16.6
Refractive index	1.4201	1.3999	1.3813	1.3639	1.3478

0.9 ml samples of each dilution were layered on top of each other, beginning with 50% sucrose, in a 5.5 ml plastic centrifuge tube (MSE). After 6-7 hours at 4°C the tubes were centrifuged in a 6 x 5 cm rotor on an MSE 65 centrifuge at 30,000 rpm = 105,000 g at 4°C. The gradients were fractionated under a manual calibrated

pressure system. Molecular weight determinations were calculated by the method of Martin and Ames (1961).

2.2.7 Assays for Immune Complexes

a) Clq Binding Assay (Clq BA) (Zubler et al, 1976)

Purified human Clq (section 2.3.1) was radiolabelled by the Iodogen reaction (section 2.2.8b) and on the day of testing a portion of the ^{125}I-Clq was centrifuged at 18,000 g for 40 minutes at 4^{o}C to remove any aggregates and then diluted in CFD containing 1% BSA. To prevent ^{125}I-Clq being incorporated into the Clqrs complex 200 µl 0.2M EDTA was added to 50 µl samples of test sera and incubated at 37^{o}C for 30 minutes. After transferring the mixture to an ice bath 50 µl ^{125}I-Clq (10,000 cpm ≡ 2 ng Clq) and 1 ml of 3% $\left(\frac{W}{V}\right)$ PEG-6000 were added slowly with gentle stirring. Following incubation for 60 minutes on ice the precipitated material was harvested by centrifugation at 1,500 g for 20 minutes at 4^{o}C and the radioactivity in the precipitate counted. Calibration curves were produced for the binding of ^{125}I-Clq to HAGG in NHS over the concentration range 3 to 200 µg HAGG. Precipitation of ^{125}I-Clq was calculated as a percentage of the TCA precipitable ^{125}I-Clq added to control serum and results were expressed as HAGG equivalents,

b) Conglutinin Binding Assay (K-BA) (Casali et al, 1977)

Purified bovine conglutinin (K) (section 2.3.2) was diluted to 5 µg ml^{-1} in CFD containing 0.02% sodium azide which had been brought to pH 9.0 by the addition of 2 M NaOH. Disposable polypropylene tubes (Beckman) were coated with 250 µl (1.25 µg) of K by incubating at 37°C for 3 hours and following vacuum aspiration of the contents with two rinses, the coated tubes were incubated with 1% BSA at pH 9.0 for 1 hour at room temperature. Following further aspiration and rinsing the tubes were stored at 4°C.

K-coated tubes were washed twice with CFD containing 0.05% Tween-20 (CFD-Tween) prior to assay. Tubes were filled with 25 µl of test sera or NHS containing HAGG over the concentration range 3 to 200 µg HAGG and 255 µl CFD-Tween, then incubated for one hour at room temperature. In a second step, after washing three times with CFD-Tween, 250 µl of a ^{125}I-labelled rabbit anti-human IgG antibody diluted in CFD-Tween was added and the tubes incubated for 4 hours at room temperature. The tubes were washed three times in CFD-Tween and the bound radioactivity counted. Results were expressed as µg equivalents of HAGG.

2.2.8 Radiolabelling Procedures

a) Chloramine-T Method

Chloramine-T (sodium salt of the N-monochloro derivative of p-toluene sulphonamide) is a mild oxidising agent that slowly releases hypochlorous acid in aqueous solution. Protein iodination involves the oxidation of NaI to cationic iodine and its subsequent incorporation into tyrosine residues.

Proteins were radiolabelled with ^{125}I-Na by a modification of the chloramine-T method of Hunter and Greenwood (1962). Fifty µg protein in 100 µl phosphate buffered saline (PBS) was added to 500 µCi of ^{125}I-Na. Thirty µg chloramine-T (1 mg ml^{-1}) was added to start the reaction and the vial was gently agitated for two minutes. The reaction was stopped by the addition of 30 µl sodium meta-bisulphite (2 mg ml^{-1}). Protein bound iodine was separated from free iodine by gel filtration on a Sephadex G-25 column. The radioactive protein peak was pooled and stored in 500 µl aliquots at -20^{o}C. Iodine incorporation was in the range of 20-50% and specific activities were in the range 0.5 to 2 µCi µg^{-1}.

b) Iodogen Method

Iodination was carried out by the method of Fraker and Speck (1978).

Iodogen (1,3,4,6-Tetrachloro-3α,6α-diphenylglycoluril was dissolved in chloroform (250 μg ml $^{-1}$) and 100 μl aliquots were dried under nitrogen onto the surface of 50 x 1 ml reaction vials. Fifty μg protein and 500 μCi ^{125}I-Na or ^{131}I-Na were added and after 30 minutes' incubation at room temperature the protein was removed from the reaction vessel, diluted with complement fixation diluent (CFD) (Oxoid, Ltd., London) containing 0.25M NaCl and dialysed against CFD overnight at 4°C. Iodine incorporation was in the order of 50-80% giving specific activities in the range 1-10 μCi μg^{-1}.

2.3 Purification of Specific Proteins

2.3.1 Purification of Human C1q

C1q was purified from fresh normal human serum by the method of Volanakis and Stroud (1972) with some modifications (Zubler et al, 1976). The following reagents were prepared at 4°C:

2 litres 0.1M	EDTA di-sodium salt (pH 4.4)
2 litres 0.1M	EDTA tetra sodium salt (pH 10.0)
Solution A	200 ml EDTA pH 7.4 (1.1 mixture of di and tetra sodium salt).
Solution B	1500 ml 0.005 M EDTA pH 7.4 (1:19 dilution of solution A)

Solution C	500 ml final volume. 0.1M EDTA pH 7.4 diluted with cold distilled water to a relative salt concentration (RSC) of 0.04 (conductivity of 2.75 µmhos at 4°C). Approximate dilution of 1:3.
Solution D	2 x 4,000 ml required. 0.1M EDTA pH 5.0 diluted with cold distilled water to a RSC of 0.078 (conductivity of 5.55 µmnos at 4°C) Approximate dilution of 1-0.5.
Solution E	20 ml 0.01 M EDTA 0.75M NaCl pH 5.0
Solution F	20 ml 0.01 M EDTA 0.3 M NaCl pH 7.5

(the relative salt concentration (RSC) refers to a solution of NaCl giving the same electrical resistance at 0°C).

One pint (560 ml) of fresh normal human blood was allowed to clot at room temperature for 90 minutes and then at 4°C for 4 hours. Serum was collected by centrifugation at 1500 g at 4°C and free light density lipids were removed by centrifugation at 30,000 g for 2 hours at 4°C. Fifty ml solution A was added to 200 ml serum, the pH was adjusted to pH 7.4 at 4°C with 0.1M HCl, and the serum was incubated at 37°C for 10 minutes followed by immediate chilling to 1°C.

Ice-cold 0.005 M EDTA pH 7.5 (solution B) was added until a RSC of 0.04 (conductivity of 2.75 µmhos at 1°C) was achieved. This mixture was allowed to stand for 2 hours with occasional stirring. The precipitate was

harvested by centrifugation at 1,000g for 30 minutes, washed twice with 200 ml cold solution C, (RSC 0.04, conductivity of 2.75 µmhos) at 4°C, and dissolved in 10 ml solution E.

The purified material was dialysed overnight against solution D at 4°C, the precipitate was washed twice with 20 ml solution D and finally dissolved in solution F.

Purity of the Clq preparation was investigated by immunoelectrophoresis and SDS-PAGE. Concentration of Clq was based on the extinction coefficient @ 1% of 0.682 (Reid et al, 1972). Approximately 20 mg Clq were purified from 200 ml NHS without contamination with other globular proteins such as IgG.

2.3.2 Purification of Bovine Conglutinin (K)

a) Preparation of Yeast

The method is based on that of Hadding (1967) with some modifications (Lachmann and Hobart, 1979). One pound (lb) of fresh baker's yeast was suspended in 2 litres of PBS and autoclaved for 30 minutes at 120°C. The yeast was centrifuged and washed three times in PBS until the supernatant was clear and then resuspended in

250 ml PBS containing 0.1 mol l^{-1} mercaptoethanol (1.7 ml) and incubated at 37°C, with stirring, for 2 hours. After centrifugation to remove the bulk of the mercaptoethanol the yeast was suspended in 500 ml 0.02 ml l^{-1} iodoacetamide in 0.01 M phosphate buffer pH 7.2 containing 0.85% NaCl and stirred for two hours at room temperature. The yeast was centrifuged and washed three times in PBS, suspended in two litres of PBS and autoclaved for 30 minutes at 120°C. After repeated centrifugation and washing in PBS until the supernatant was free of protein and clear, the yeast was finally suspended in 1 litre of complement fixation diluent (CFD) containing azide and stored at 4°C.

b) Bovine Conglutinin (K) Purification

Adult bovine serum, with a conglutinating titre of > 1:5,000 was heated at 56°C for 30 minutes to inactivate complement. One ml of the yeast preparation, already described, and 5 µl of 40% $CaCl_2$ solution were added per 5 ml of bovine serum and the suspension stirred at 4°C for 1 hour. After centrifugation the conglutinated yeast was washed three times in PBS, then suspended in saline containing 0.01M EDTA pH 7.4 (0.5 ml for each 1 ml of yeast used) and stirred at room temperature for ten minutes. After centrifugation the yeast was incubated again in saline containing EDTA and the supernatant containing conglutinin from these two incubations was

harvested. The pooled supernatant was adjusted to pH 5.4 with dilute H_3PO_4 and dialysed against 0.01M phosphate buffer pH 5.4 overnight at 4°C. The euglobulin precipitate was dissolved in 10 ml CFD. This conglutinin containing fraction was further purified by another cycle of adsorption and elution from yeast. Lipids were removed from the conglutinin preparation by ether extraction (Lachmann, 1962). One part diethyl ether was added to three parts conglutinin solution and the mixture was gently shaken for 15 minutes at room temperature. The mixture was frozen to -70°C, allowed to thaw, and then centrifuged at 1,500g. The ether was removed together with the lipid interface. Ether extraction had no effect on conglutinin activity or titre.

c) Conglutination

(i) Preparation of Sheep EA

Conglutinin titre was estimated by the conglutination of sensitised sheep red blood cells, prepared as described by Lachmann and Hobart (1979).

0.5 ml of packed fresh sheep erythrocytes were washed three times in PBS and resuspended to 10% by volume in CFD. To 5 ml 10% sheep erythrocytes (E) were added 100 µl of rabbit anti-sheep erythrocyte antibody (Wellcome)

and the mixtures incubated at 37°C for 15 minutes. After washing three times in cold CFD the antibody coated erythrocytes (EA) were resuspended to 10% by volume and tested for complement lysis with guinea pig serum. To 100 µl of 1% EA was added 200 µl of a 1:50 dilution of guinea pig serum and the mixture was incubated at 37°C. Complete lysis in less than five minutes, compared to control EA with no guinea pig serum, was taken as proof of efficient sensitisation of the sheep erythrocytes.

(ii) EA-143 KAF Cells

EA-143 cells were prepared using C6 deficient rabbit serum. 4.5 ml of 1% EA was added to 0.5 ml C6 deficient rabbit serum and after 15 minutes at 37°C the reaction mixture was plunged into ice, subsequently washed three times in CFD and resuspended to 0.5% by volume.

(iii) Conglutination Titration

Doubling dilutions of 200 µl volumes of the purified bovine K were prepared in duplicate, starting at 1:100, and to these, 100µl 0.5% EA143 cells were added. After overnight incubation at 4°C conglutination was examined visibly in indirect light compared to control EA-143 cells incubated in buffer. The conglutination reaction could be reversed by the addition of 0.01 M EDTA and the conglutinin titre was expressed as the last K dilution capable of conglutination.

2.3.3 Purification of C3

A globulin fraction of fresh NHS was obtained by warming the serum to 37°C and adding 20 g anhydrous Na_2SO_4 to each 100 ml of serum. Flocculation occurred at room temperature and the precipitate was washed in 20% Na_2SO_4. The precipitate was resuspended in 11% Na_2SO_4 in PBS pH 6.0 containing 0.01M EDTA, 0.005M NaN_3 and 0.005 M phenyl methyl sulphonyl fluoride (PMSF) (to

inhibit proteolysis). The mixture was stirred at room temperature for two hours and after centrifugation the precipitate was discarded and the supernatant was dialysed at 4°C against PBS pH 5.6 containing 0.01M EDTA, 5 x 10^{-3} M NaN_3 and 5 x 10^{-4} M PMSF.

The dialysed material was diluted with ice-cold distilled water to achieve a conductivity of 2.1 µmhos cm^2 and after two hours at 4°C the precipitate was removed by centrifugation and the supernatant was diluted further to a conductivity of 0.8 µmhos cm^2. After a further two hours at 4°C the insoluble fraction was harvested and redissolved in a minimal volume of 0.015M phosphate buffer containing 0.01M EDTA and 0.005M NaN_3.

The C3 containing fraction was then purified by chromatographic techniques. The redissolved precipitate was loaded onto a DEAE-cellulose column equilibrated with the above buffer (1.5 ml bed volume per ml of initial serum). After washing through several column volumes a linear gradient was established with NaCl rising to a final concentration of 0.3 mol l^{-1}. C3 eluted early in the gradient and was located by rocket electrophoresis. The C3 containing fractions were pooled and dialysed against 0.01M phosphate buffer pH7 to remove the EDTA.

The purified material was then loaded onto a hydroxylappatite column (2 ml bed volume per 5 ml initial serum volume) equilibrated with 0.01M phosphate buffer pH 7.9. A linear gradient of phosphate buffer, rising to a final concentration of 0.2 ml l^{-1}, was established and again the C3 located by rocket electrophoresis. Purity was checked by Ouchterlony, immunoelectrophoresis and SDS-PAGE analysis.

2.3.4 Preparation of Heat Aggregated Human IgG (HAGG)

HAGG was prepared according to the method of Greenwood et al, (1971) with some modifications as follows.

A 2% solution of human IgG in 0.158M PBS pH 9 prepared by DEAE cellulose chromatography (section 2.4.5) was heated at 43°C for 15 minutes and then centrifuged at 1,500 g for 15 minutes. The supernatant was subsequently centrifuged at 40,000 rpm (15,000g) at 4°C for 90 minutes and the pelleted protein was dissolved in a small volume (5-10 mls) of PBS. A 2M sodium sulphate solution was added to the dissolved aggregates until a final molarity of 0.2M. sodium sulphate was achieved. After stirring for 30 minutes insoluble material was pelleted by centrifugation at 1,500g for 30 minutes at 4°C and the supernatant was made to 0.4M Na_2SO_4 by the further addition of 2M Na_2SO_4. Following centrifugation at

1,500g for 30 minutes at 4°C the pelleted aggregates were dissolved in PBS, dialysed overnight at 4°C against PBS and stored in 0.5 ml aliquots at -70°C. The aggregates thus prepared were of 30-35S in molecular size and maintained their discrete molecular weight size over several months of storage at -70°C.

2.3.5 Isolation of IgM Rheumatoid Factor

Fifty ml of serum from a seropositive rheumatoid patient was extensively dialysed against 0.002M phosphate buffer at pH 6.3. The euglobulin precipitate was harvested by centrifugation, washed once in 0.002M phosphate buffer and solubilised in 0.5M NaCl. Molecular weight separation was effected by Sephadex G-200 gel filtration. The exclusion protein peak was pooled, concentrated by Amicon ultra filtration and tested for purity by gel diffusion and immuno electrophoresis.

2.4 Affinity Chromatographic Techniques

2.4.1 Cyanogen Bromide (CNBr) Activated Affinity Columns

2.4.1 a) CNBr-activated Sepharose-4B Affinity Columns

The following general procedure was applied to the coupling of rabbit Immunoglobulin G, rabbit anti-human C3

and rabbit anti-human C1q (Seward) to CNBr-activated Sepharose-4B (Pharmacia). Coupling was performed by the method of Cuatrecasus and Anfinsen (1971).

Immunoglobulin fractions of antisera were prepared by precipitation with 50% $(NH_4)_2SO_4$ and subsequent dialysis against 0.1M $NaHCO_3$ buffer at pH 8.3.

1 gm (dry weight) of CNBr-Sepharose-4B (Pharmacia) was swollen and washed for 15 minutes on a glass filter with 10^{-3} M HCl solution (200 ml). The protein to be coupled was dissolved in 0.1M $NaHCO_3$ buffer pH 8.7 containing 0.5M NaCl and about 5-10 mg protein was incubated per ml swollen gel for 2 hours at room temperature or overnight at $4^{o}C$. Unbound protein was removed by washing in buffer and remaining active groups were reacted with 1M ethanolamine at pH 8.0 for 2 hours at room temperature. Non-covalently bound material was removed by alternating rinses of coupling buffer pH 8.3 followed by 0.1M acetate buffer pH 4 containing 0.5M NaCl until the eluates were free of protein.

Appropriate material dissolved in CFD was incubated with these columns for 30 minutes at room temperature, unbound material was removed by extensive washing with CFD and subsequent elution of bound material was effected by the sequential application of CFD containing 0.02M

EDTA, O.5M NaCl and M propionic acid (rabbit IgG and rabbit anti-human C1q columns) or just M propionic acid (rabbit anti-C3 column). All acid eluates were immediately neutralised with 2M NaOH and dialysed against PBS or CFD.

b) BSA-Sepharose 4B-Cl Affinity Column

For elution of antigen or antibodies by chaotrophic agents, the antibody molecules must be distorted. If the initial covalent binding of antibody to the Sepharose is by multiple linkage, then the antibodies are not so readily distorted and the elution of the complementary antigen or antibody can only occur under much more rigorous conditions. As CNBr activated Sepharose prepared by Pharmacia is very highly activated, the following protocol was established to obtain a less activated Sepharose surface thus leading to light substitution of bound ligand.

An 18 ml slurry of Sepharose 4B-Cl was washed well in distilled water over sintered glass and suspended as a thick suspension in ice water. The following procedure was performed in a fume cupboard. CNBr was added at a rate of 20 mg ml^{-1} to sedimented Sepharose, keeping the volume as low as possible. The solution was stirred on ice and the pH was maintained at pH 10.5 by the addition

of 4N NaOH. After eight minutes the slurry was washed extensively on a pre-cooled sintered glass filter with cold distilled water.

BSA was added as soon as possible in fresh 0.1M $NaHCo_3$, pH 8.4 at 2 mg ml^{-1} Sepharose. After 4 hours agitation at room temperature, or overnight at $4^{o}C$, the slurry was washed on a sintered glass filter to remove the free BSA and then suspended in M ethanolamine pH 8.0 or any primary amine. After one hour the slurry was washed with $NaHCO_3$ buffer and stored at $4^{o}C$ until required.

Before using the column it was pre-washed with the eluting buffer M Propionic acid followed by PBS to remove any loosely bound BSA. The Protein-A isolated anti-BSA was incubated with the BSA Sepharose column at a flow rate of 0.5 ml per minute. Unbound material was removed by washing with PBS and specific anti-BSA antibody molecules were recovered by elution with 1M propionic acid into tubes containing 1M Tris buffer, followed by dialysis overnight against PBS.

2.4.2 Sepharose-C3 Immunoadsorbent for the Isolation of Anti-C3 Antibodies

The immunoadsorbent was prepared as described by

Pepys et al, (1976). Sepharose CL-4B was washed once in CFD and approximately 5 ml of packed Sepharose was incubated with 50 ml of fresh NHS for 1 hour at 37°C. The Sepharose -C3 was packed into a column and mixed with PBS pH 7.4 and 0.05M glycine-HCl pH 2.2 to remove unbound proteins.

A rabbit anti-human C3 antiserum was incubated with Sepharose-C3 at room temperature for one hour. The column was mixed in PBS to remove unbound proteins and anti-C3 antibodies were eluted with alternate washes of 0.05M glycine-HCl pH 2.4 and PBS containing M NaCl. The eluates were dialysed against PBS and concentrated by pressure ultra filtration on an XM 50 Diaflo membrane (Amicon). Purity was checked by Ouchterlony and immunoelectrophoretic analysis.

2.4.3 Degalan Affinity Columns

Bovine K, human C1q or rabbit anti-BSA antibodies were coupled to polymethylmetacrylate (PMMA) beads (Degalan V26, London) by incubation in 0.05M carbonate buffer, pH 9.0 at 37°C for 3 hours. Unbound protein was removed by extensive washing with 0.5M NaCl in a sintered glass funnel and the coated beads were then stored at 4°C in CFD containing 0.01M NaN_3. The binding capacity of the beads was estimated by extracting

bound protein from a known weight of beads with 0.1 M NaOH and assaying the protein by the Folin method. Between 1% and 5% of the added protein bound to the beads, i.e.20 mg protein added to 5 g dry weight of Degalan beads resulted in a binding of between 40-200 µg protein per gram of dry beads.

Immune complex containing serum or plasma was treated with trichlorofluroethane (Frigen) to remove lipids and extracted with PEG as already described. The PEG precipitate was redissolved in CFD and incubated with either the Clq or K-Degalan column for one hour at 4°C Unbound material was removed by rinsing the K-column with CFD and the Clq-column with CFD containing 0.02M EDTA. Bound material was eluted from the Clq column with CFD containing 0.02M EDTA and 0.5M NaCl, and from the K-column with CFD containing 0.02M EDTA. The eluted material was concentrated by pressure filtration (Amicon), dialysed against CFD at 4°C and stored at -70°C.

2.4.4 Protein-A Sepharose 4B

Purification of rabbit anti-BSA serum: one gram of freeze dried Protein-A Sepharose Cl-4B was reconstituted in PBS to a swollen gel of volume 3.5 ml and packed into a 5 ml disposable syringe plugged with glass wool. Several column volumes of PBS were passed through the

column to remove any unbound material. Whole rabbit serum was repeatedly passed through the column at a flow rate of 0.5 ml per minute. After removing all unbound protein by extensive washing with PBS the bound IgG molecules were eluted with 0.1M citrate buffer at pH 3.0, immediately neutralised with M NaOH and dialysed overnight at 4°C against PBS.

2.4.5 DEAE-Cellulose Chromatography

Equilibration of the DEAE cellulose DE-52 was achieved by adding the pre-swollen material to 0.01M phosphate buffer pH 7.6. After 2-3 hours at 4°C the slurry was poured into a column and packed by pumping the same phosphate buffer through at a flow rate of 25 ml per hour until the ionic strength of the effluent was the same as that of the original buffer.

A 50% ammonium sulphate precipitate of human serum was dialysed extensively against the equilibrating buffer and then applied to the column. The unbound proteins were eluted with a linear gradient of NaCl (final concentration 0.5M).

2.5 Immunochemical Techniques

2.5.1 Immunodiffusion (Ouchterlony, 1958)

1.5% agarose (Miles-Servac) in PBS was heated and poured onto a glass plate (10 ml agarose per 8 x 8 cm plate). Wells of varying size (1,2 or 3 mm) were cut into the gel using hollow metal tubing followed by vacuum suction to remove the small cylinders of gel. After filling the wells with appropriate materials the plates were allowed to stand for 2-3 days in a humid container at room temperature. The plates were then covered with a multi-layer of wet Whatman filter papers and absorbent paper and compressed with a lead weight. After five minutes the plate was dried under a stream of hot air and incubated in PBS overnight. The washed plate was stained in Coomassie Brilliant Blue solution (0.2% Coomassie Blue in 10% acetic acid and 48% methanol) and destained in the same solvent system without the dye.

2.5.2 Electro-Immunodiffusion (Laurell, 1965)

One per cent agarose solution was prepared at 100°C by heating 1 gm agarose with 90 ml glycine/barbital/Tris buffer (GBT high voltage electrophoresis buffer) pH 8.6 and 10 ml 0.1M EDTA pH 8.6. The GBT buffer was prepared by mixing equal volumes of solution A

and Solution B. Solution A was made by dissolving 13 gm sodium barbital and 2.07 gm of barbituric acid in one litre of distilled water. Solution B was prepared by mixing 14 gm glycine and 11.3 gm Tris in one litre of distilled water.

10 ml of 1% agarose was cooled down to 56°C, mixed with 150-300 µl of an appropriate antiserum and immediately poured onto a 8 x 8 glass plate, avoiding air bubbles. The gel was allowed to set at room temperature and 2 mm wells were cut by suction 5 mm apart at the cathodal side of the plate. The plate was placed in a water cooled electrophoretic chamber (Shandon, Southern, UK), a voltage of 20 v applied and test samples and standards were dispensed into the wells using a Drummon micropipette. The voltage was increased to 200 v and electrophoresis was allowed to proceed for 2-3 hours. The plate was pressed, dried, washed and stained as already indicated (2.5.1) and rocket heights were measured from the anodal border of the original well to the nearest half millimetre. The concentration of the test samples was read from a calibration curve of rocket heights produced by a range of standard antigen concentrations.

2.5.3 One dimensional Immunoelectrophoresis

Immunoelectrophoresis was performed on a 8 x 8 cm glass plate containing 10 ml of 1% agarose in GBT-EDTA buffer at pH 8.6. Several 2 mm wells were cut in the centre of the plate and filled with the appropriate test samples. One well was reserved for the bromophenol blue marker solution. The electrophoresis (Shandon, Southern, UK) was allowed to proceed for 2-3 hours at 4°C with a constant current of 12 mA. After this troughs were cut and then filled with the appropriate antisera. The plates were then placed in a humid container for two days at 4°C. Precipitin lines were visualised against incident light and by permanent staining with Coomassie Blue as described earlier (2.5.1).

2.5.4 Two dimensional Immunoelectrophoresis

The first immunoelectrophoretic run was performed in a similar manner to that described above but the wells were cut about 10 mm from the cathodal side of the plate and only two test samples were run on each plate. After completion of the first dimension two sections of agarose were removed from the plate, leaving two 10 mm strips of agarose containing the electrophoresed protein. Eight ml of warm (45°C) agarose, containing 180-300 µl of the appropriate antiserum was poured onto the remainder of

the plate and electrophoresed at 100 v for 3-4 hours. The plate was then pressed, washed and stained.

2.5.5 SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) provides a versatile, gentle high resolution method for fractionation and physico-chemical characterisation of molecules on the basis of size, conformation and net charge. The polymerisation reaction can be controlled to provide uniform gels of varying pore sizes.

Polyacrylamide gels were prepared by dissolving acrylamide monomer and cross-linking agent N.N-methylene bis acrylamide (BIS) in aqueous buffer solutions. Proportions were such that on addition of catalyst - accelerator compounds and the exclusion of oxygen gels were formed after 20-30 minutes. The gels consisted of a 3-dimensional network of long hydrocarbon chains cross-linked at intervals by methylene groups. Gelation of the acrylamide monomer and 'BIS' were induced by the addition of ammonium persulphate and N,N,N.N-tetramethyl-ethylenediamine (TEMED) as chemical catalyst-accelerators.

The PAGE equipment (Shandon, Southern, UK) consisted of a power pack with facility for constant voltage or current and an electrophoresis tank designed to take

vertical slab gels prepared between two glass plates. The PAGE procedure was based on the method of Laemmli (1970). Gels of varying percentages were prepared according to the following schedules:-

Two clear glass plates were held together via plastic spacers to form a gel template. The gel was filled to within 15 mm from the top and a 0.1% SDS solution was used to overlay this gel. After 20-30 minutes the overlayer was poured off and a stacking gel was applied together with a multi-sample template. After 15-20 minutes the template was removed, revealing discrete sample troughs. The prepared gel was positioned on the vertical electrophoresis tank and the buffers in the top and bottom reservoirs were brought into intimate contact with the gel by removing the bottom spacer from the glass plates and by removing trapped air bubbles from the gel base.

Stock Solutions

Solution A 36.3g Tris + 48 ml M HCl + 0.33 ml TEMED and made up to 100 ml, pH 8.9.

Solution B 9g Tris + 0.59 ml TEMED + HCl and made up to 100 ml pH 6.5.

Solution C 30g acrylamide + 0.8g N, N-BIS-methylene acrylamide made up to 100 ml.

Ammmonium Persulphate	2% made up fresh when required.
SDS	10%.
Well buffer	3g Tris + 14.4g glycine + 10 ml of 10% SDS made up to 1 litre, pH 8.3.

Preparation of Gels

	5%	6%	7.5%	8.5%	10%
Solution A (ml)	5.0	5.0	5.0	5.0	5.0
Solution B (ml)	-	-	-	-	-
Solution C (ml)	6.7	8.04	10.0	11.3	13.3
2% Ammonium persulphate (ml)	2.8	2.8	2.8	2.8	2.8
10% SDS (ml)	0.4	0.4	0.4	0.4	0.4
Distilled Water (ml)	25.1	23.8	21.8	20.5	18.5

Stacking Gels

	ml
Solution B	1.7
Solution C	1.0
10% SDS	0.1
Distilled Water	6.2
2% Ammonium persulphate	1.0

2.5.5 a Preparation of Samples

Samples and molecular weight standards were prepared in a reduced or non-reduced state according to the following protocol.

	Reduced	Non-reduced
Samples (1mg ml^{-1}) (µl)	80	80
Dithiothreitol (30mg ml^{-1}) in 10% SDS (µl)	20	20 µl 10% SDS only
100°C for 2 mins	+	-
Indole acetic acid (60 mg ml^{-1}) 100°C for 2 mins.	20	20
50% sucrose in Bromophenol Blue	1 drop	1 drop

Each sample was loaded through the well buffer into the base of each trough using a 1 ml syringe and needle. Electrophoresis was carried out at a constant voltage of 100 v for 4-6 hours.

2.5.5 b Fixing and Staining Gels

(i) Protein Staining

The gel was fixed in 10% trichloroacetic acid (TCA) overnight and stained for 2 hours at room temperature in 0.2% Coomassie Blue in 5% methanol and 10% acetic acid. Destaining was carried out in the same solvent system

without stain.

(ii) Carbohydrate Staining

Schiff Reagent

a) 1g basic fuchsin was dissolved in 20 ml boiling water for 5 minutes and cooled to 50°C.

b) After paper filtration 20 ml 1N HCl was added and after a further cooling to 25°C 1g sodium/potassium metabisulphite was added. The solution was placed in the dark for 14-24 hours.

Procedure

After fixing the gel in 2% acetic acid for 1 hour at room temperature it was placed in 0.2% periodic acid for 45 minutes at 4°C. Without rinsing, the gel was placed immediately in Schiff reagent again for 45 minutes at 4°C. The gel was retained at room temperature in 2 or 3 changes of 10% acetic acid. For faster destaining the gel was placed in a freshly prepared solution containing 50 ml 1N HCl and 5g potassium metabisulphite in 1 litre of distilled water.

2.5.6 Autoradiography

Destained gels were photographed and then wrapped in cling film (Alcan, London) and non-porous plastic sheeting, using a glass plate as support. Kodak auto-process X-ray film was then placed in contact with the gel for varying periods of time, the exposure time being dependent on the level of radioactivity in the gel.

CHAPTER 3

STUDIES ON THE FORMATION AND EXTRACTION OF IN VITRO PREPARED IMMUNE COMPLEXES

3.1 Introduction

The formation of immune complexes is a unique biochemical process and since the original work of Heidelberger and Kendal (1935) the theoretical basis of this reaction has been well studied and reviewed (Goldberg, 1953.; Steensgaard and Johansen, 1980). Immune complex formation differs in mechanism from most other biological ligand binding reactions, such as enzyme substrate or receptor drug interactions, because of the multivalency of the antigen and the divalency of the antibody. The result of such a reaction is the generation of complexes of varying composition as apposed to the generation of a single discrete product as in enzyme reactions.

To be able to prepare *in vitro* immune complexes which simulate those found *in vivo* in association with various diseases, it is important to consider how the valency of the antigen and antibody can influence the final product. The simplest interaction occurs between a monovalent antigen or hapten and a specific antibody and under these conditions only two different complexes are possible, namely $Ag_1\ Ab_1$ and Ag_2Ab_1.

Multivalent antigens can be divided into those with differing non-repeating determinants and those where all the antigenic determinants are alike. Antibody interactions with the latter are difficult to treat theoretically because cyclical complexes can be generated and also because of the possibility that antibody can bind to the antigen by both antibody combining sites (Day, 1972). The interaction of antibodies with antigens displaying different non-repeating determinants are, however, considered to be typical of the immune complex formation which occurs *in vivo*. Large multivalent antigens can be utilised in some experimental situations to simulate the antigenic determinants on viruses (hepatitis) or micro-organisms e.g. flagellin. However, these interactions differ from those described in that the antibody molecules will tend to cover the surface of the particle and unless these are shed from the surface, lattice formation will not occur.

The reaction between an antigen with different non-repeating determinants and a divalent antibody can proceed in three ways after the formation of the initial Ag_1Ab_1 complex. The AgAb complex may accept another antigen giving Ag_2Ab_1, another antibody giving Ag_1Ab_2 or may react with another complex giving a larger complex. Although other mutual interactions are possible the combination of these three are sufficient to account

for the formation of all possible complexes (Steensgaard and Johansen, 1980). The generation of such complexes over a range of antigen concentrations accounts for the composition of complexes which occur at various points in the AgAb precipitation reaction. Antibody excess complexes are those where the molar ratio of Ag : Ab is less than 1, antigen excess complexes exist where the Ag : Ab ratio is greater than 1 and in between intermediary complexes exist where the Ag : Ab ratio approaches 1. These intermediary complexes form large antigen-antibody lattices which precipitate as their sheer physical size outweighs the natural ionic and hydrophobic forces which keep them in solution.

When the interaction between antigen and antibody takes place in NHS, however, other factors such as the complement system come into play and the activation of this can influence the precipitation reaction. It has been known for many years that complement could bind to immune complexes, for Muir (1909) referred to complement as "that labile substance of normal human serum which is taken up by the combination of antigen and its anti-substance (immune body)". In later years Maurer and Talmage (1953) demonstrated that immune precipitates, prepared in NHS, contained more protein that that accounted for by antigen and antibody. The suggestion was therefore made that

complement components bound to the complexes in such a way as to prevent precipitation. More recent work by Czop and Nussenzweig (1976) described this phenomenon as solubilisation and showed that the process required sufficient amounts of C3 and the alternative pathway of complement. Takahashi et al, (1977) further showed that an alternative pathway-dependent C3-convertase was assembled on immune complexes and that solubilisation was Mg^{2+}, C3, B, P, and factor D dependent. Although these authors demonstrated that solubilisation could not occur through the classical pathway alone they did show that in the absence of Ca^{2+}, C4 or C2, it was much less effective and they therefore postulated that the role of the classical pathway was to prime the complexes by depositing C3b molecules on the antibody which would then become the nucleus for the assembly of the alternative pathway C3 convertase.

Following progressive accumulation of C3 and C4 fragments onto the antigen-antibody lattice a secondary reaction takes place which can also occur in medium devoid of serum proteins and does not require free divalent cations. The tight binding of bulky complement peptides to the antibody molecules appears to interfere with antigen-antibody bonds either by steric hindrance and/or the induction of allosteric changes in the combining site

of the antibody. In addition if the binding of the C3 fragments is in the Fc region of the antibody then interference with lattice formation could occur by inhibiting non-specific Fc-Fc interactions which promote precipitation (Nisonoff and Pressman, 1958). Immune precipitates can also be solubilised by incubation with monovalent antibody fragments (Fab) against antigenic determinants on molecules within the immune precipitate. In contrast, $F(ab')_2$ fragments have the reverse effect due to their ability to cross link antigenic determinants. In view of this, Czop and Nassinzweig (1976) postulated that the C3 and C4 fragments bound to the antibody in the immune precipitate must be functionally monovalent to bring about solubilisation.

In this chapter the solubilising effects of the complement system have been exploited to generate soluble immune complexes in NHS. These complexes have been used to study the efficiency of PEG and $(NH_4)_2SO_4$ to precipitate them from serum and to evaluate the effects of these two agents on the dissociation and denaturation of these complexes. Although PEG (Zubler et al, 1977 ; Chia et al, 1979) and ammonium sulphate (Chenais et al, 1977) have been used to precipitate immune complexes from pathological fluids no formal study of their comparative efficiency has been reported.

3.2 Precipitin Reactions between BSA and Rabbit Anti-BSA Antibodies

3.2.1 Variation of Methods

Precipitin curves were set up in PBS, NHS, or EDTA-NHS as described (2.2.3) by incubating BSA (0.5 - 50 µg) with a fixed concentration of affinity purified (2.4.1b) anti-BSA antibodies. A trace amount of ^{125}I-BSA was added to all tubes for the purpose of measuring the amount of antigen precipitated. The protein content of the precipitates was estimated by the Folin method. Solubilised complexes were precipitated with 50% saturated $(NH_4)_2SO_4$ as described in section (2.2.4).

In some experiments the amount of antigen and antibody were reduced by a factor of **4** and the amount of NHS or EDTA-NHS was increased by a factor of **2** to investigate the solubilising effect of NHS.

3.2.2. Results

Figure 1 shows a typical precipitin curve for the reaction between BSA and anti-BSA antibodies. At equivalence there was no free antigen or antibody detectable in the supernatant but as the BSA concentration increased beyond this point there was a progressive increase in the amount of soluble complexes detected. These appeared to plateau at 10 x antigen excess. Superimposable precipitin curves were obtained in the presence of small amounts of NHS. When the concentration of complexes was reduced however, and the amount of NHS increased, the precipitation reaction could be abolished (Figure 2). This solubilisation of insoluble complexes was abolished by the addition of 0.01M EDTA to the NHS or by using heat-inactivated human sera (heated for 30 minutes at 56^{o}C).

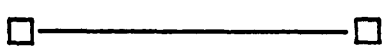
In the region of antibody excess it was possible to estimate the valency of the BSA. At several points from equivalence to antibody excess the ratio of antigen to antibody in the precipitate was calculated on the basis that at antibody excess all the antigen is complexed. These values were then plotted against the amount of antigen added (Figure 1). The calculated ratios were based on the following formula (Hudson and Hay, 1976):-

$$\frac{\mu g \text{ Antigen}}{\text{molecular weight of antigen}} : \frac{\mu g \text{ Antibody}}{\text{molecular weight of antibody}}$$

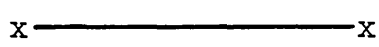
At each point the protein content due to the antigen was subtracted from the total protein in the precipitate to give the antibody content of the precipitate.

Figure 1

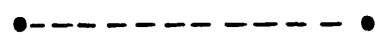
Quantitative Precipitin Reaction between ^{125}I-BSA and Rabbit Anti-BSA Antibodies.



Molar Ratio Antigen/Antibody



Insoluble complexes



Soluble complexes

Figure 1

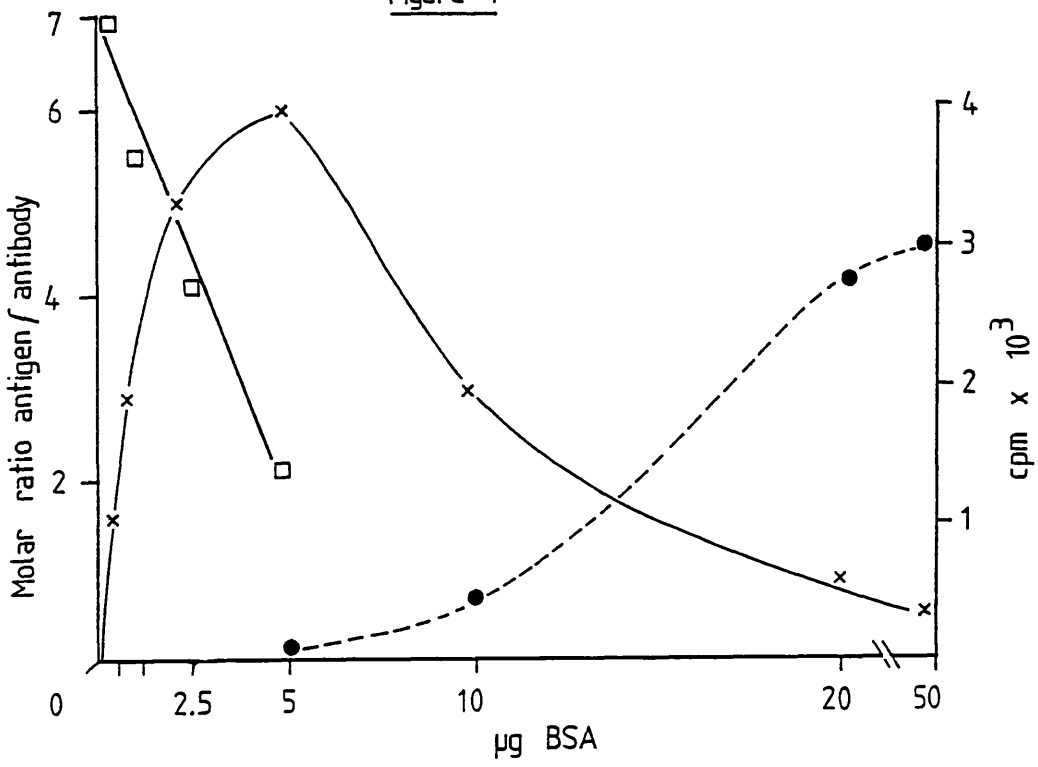

7
6
4
2
0
Molar ratio antigen / antibody
4
3
2
1
cpm x 10^3
2.5
5
10
20
50
µg BSA

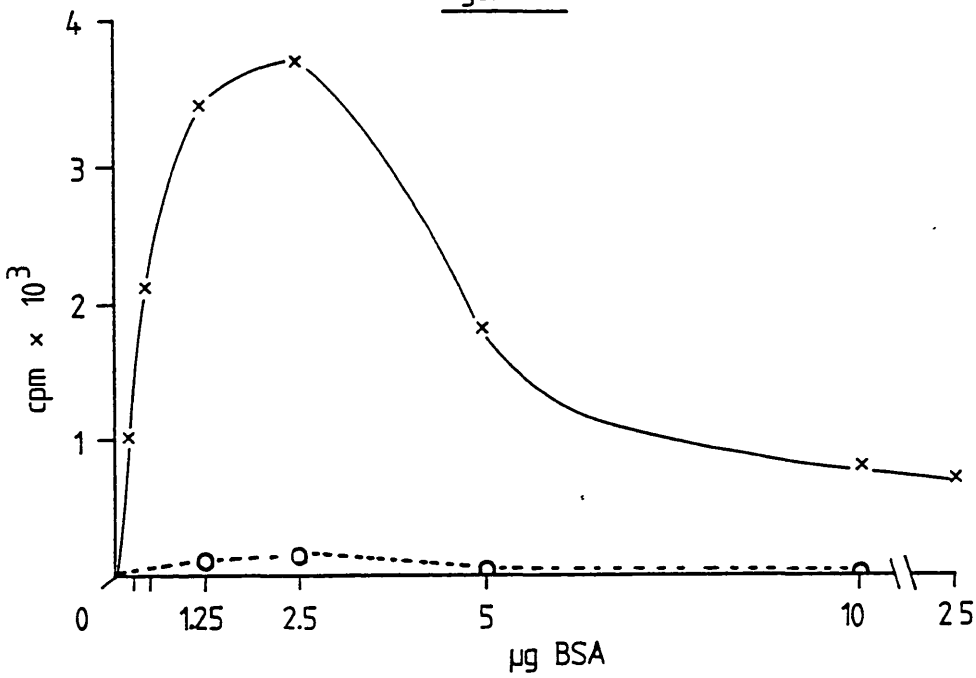
Figure 2

Quantitative Precipitin Reaction between ^{125}I-BSA and Rabbit Anti-BSA Antibodies in NHS, EDTA-Sera and Heat Inactivated Sera.

X————————X EDTA -Sera or heat inactivated sera

o----------o NHS

Figure 2



3.2.3 Discussion

The classic precipitin reaction originally described by Heidelberger and Kendall (1935) forms the basis of all quantitative studies of antigen-antibody interactions. The precipitin curve is divided into three parts : the zone of equivalence, the zone of antigen excess and the zone of antibody excess.

At the limit of antibody excess it was assumed that the multi-valent antigen was saturated with antibody and at this point (Figure 1) the antigenic valency was estimated as approximately 7 determinants. At the zone of equivalence all the antigen and antibody was complexed; however, precipitation progressively decreased in the region of antigen excess, due to the effect of free antigen breaking up or preventing the formation of lattices between antigen and antibody molecules. In the region of antigen excess there was a progressive increase in soluble complexes which appeared to plateau off at tenfold antigen excess. Sucrose gradient analysis of these complexes formed in buffer showed that at equivalence all the complexed material existed as an insoluble precipitate whereas at tenfold antigen excess the complexes were soluble with sedimentation coefficients between 8 - 19S with the peak at 13S.

Figure 2 demonstrates how NHS influences the precipitin reaction and how its effects can be reversed by conditions which inactivate the complement system. The major consequence of the interaction between complement and insoluble immune complexes is the generation of soluble macromolecules which may persist in plasma as circulating soluble immune complexes. The involvement of complement in the solubilisation process was considered likely since precipitates of BSA and anti-BSA (Figure 2) dissolved rapidly when incubated in fresh NHS but not in serum which had been heated to destroy complement (Miller and Nussenzweig, 1975). Nussenzweig and co workers have shown that the solubilisation of antigen-antibody aggregates is a general phenomenon applicable to a wide range of different antigen-antibody systems. It is however, influenced by the affinity of antibody and by the source of serum used for the solubilisation process. This function of complement does not involve proteolysis of complement fragments into the antigen-antibody lattice.

The ability to generate large soluble immune complexes in NHS was explcited throughout this study to simulate the in vivo formation of large complement solubilised complexes which would be coated with a variety of complement fragments.

In the next section we inyestigated the effects of varying concentrations of PEG and ammonium sulphate on their ability to precipitate solubilised complexes from serum.

3.3 Extraction of BSA - anti-BSA Complexes from NHS

3.3.1 Variation of Methods

Soluble BSA - anti-BSA complexes were formed in fresh NHS at 2 fold, 5 fold and 10 fold antigen excess by incubating 250 µl anti-BSA with 125 µg, 250 µg and 500 µg BSA (1 mg ml^{-1}) in 3 ml NHS for one hour at 37°C. ^{125}I-BSA was added in trace amounts to monitor the extraction procedure. After centrifugation at 1,500g for thirty minutes at 4°C, 150 µl aliquots of the respective soluble complexes were pipetted in duplicate into 1 ml glass tubes with conical bases (Dreyer tubes). The immune complexes were extracted with saturated $(NH_4)_2SO_4$ over the range 20 - 50% saturation and PEG over the range 1 - 5% by the addition of equal volumes of either $(NH_4)_2SO_4$ or PEG. Non-specific binding and extraction of ^{125}I-BSA was monitored by incubating ^{125}I-BSA with 3 ml NHS, with adjustments made for the addition of an appropriate volume of PBS. After two hours at 4°C the precipitates were harvested and washed once in the appropriate concentration of $(NH_4)_2SO_4$ or PEG. The precipitates were monitored for total protein content and the amount of antigen by counting the ^{125}I-BSA present in the precipitate. The extraction of complexed material was calculated for each set of duplicates as follows:-

$$\frac{\left[\text{cpm precipitated}\right] - \left[\text{non-specific precipitation (cpm)}\right] \times 100}{\left[\text{Total cpm added}\right] - \left[\text{non-specific precipitation (cpm)}\right]}$$

3.3.2 Results

Table 1 summarises the extraction of solubilised immune complexes of BSA - anti-BSA by $(NH_4)_2SO_4$ and PEG. An enrichment index was calculated at each extraction point by dividing the percentage yield of extracted complexes by the percentage of total protein precipitated.

Extraction of complexes with PEG, at all concentrations used and over the whole range of antigen excess, resulted in a higher enrichment index than that produced by parallel extractions with $(NH_4)_2SO_4$. This was due mainly to the smaller quantities of protein precipitated from serum by the addition of PEG rather than an increase in the overall efficiency of immune complex extraction.

The major difference between PEG and $(NH_4)_2SO_4$ with regard to their ability to precipitate immune complexes from serum is shown in Figure 3. Two per cent PEG selectively extracted 76 per cent of the high molecular weight complexes formed at twofold antigen excess but only a relatively low percentage (16.2%) of small complexes formed at tenfold antigen excess. Four per cent PEG, however, extracted more of the tenfold antigen excess complexes (70.5%) as well as precipitating nearly all the large complexes (97.5%).

$(NH_4)_2SO_4$ precipitated immune complexes to a similar degree over the whole range of antigen excess. However, because of the precipitation of more non-specific proteins, the enrichment index was much lower than that observed with PEG extraction.

TABLE 1

Extraction of $^{125}_{I}$BSA - anti-BSA complexes from NHS by PEG and $(NH_4)_2SO_4$

Degree of Ag Excess	% PEG	% Extraction	Enrichment Index	% $(NH_4)_2SO_4$	% Extraction	Enrichment Index
2.5 x	5	94.1	11.5	50	92.9	5.1
	4	97.5	40.6	45	92.9	6.7
	3	92.5	61.7	40	83.2	8.3
	2	76.6	78.2	35	63.2	6.3
	1	50.1	98.2	30	44.8	16.0
				20	3.7	2.2
5 x	5	98.9	13.3	50	93.7	5.2
	4	91.0	42.1	45	88.8	6.4
	3	79.7	53.1	40	86.1	8.6
	2	44.2	45.1	35	68.7	6.9
	1	6.9	13.7	30	28.1	10.1
				20	8.7	5.7
10 x	5	97.0	13.05	50	93.4	5.6
	4	70.5	29.4	45	92.3	7.4
	3	42.2	28.1	40	91.1	9.1
	2	16.2	16.5	35	66.6	6.7
	1	10.7	21.1	30	40.1	14.3
				20	5.3	3.5

$$\text{Enrichment Index} = \frac{\% \text{ extraction of complexes from NHS}}{\% \text{ total protein extracted from NHS}}$$

Figure 3

Extraction of ^{125}I-BSA-anti-BSA Complexes from NHS. Efficiency of Extraction is Expressed as a Percentage of the Total Complexed Antigen.

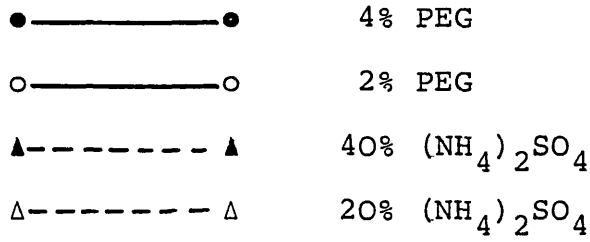
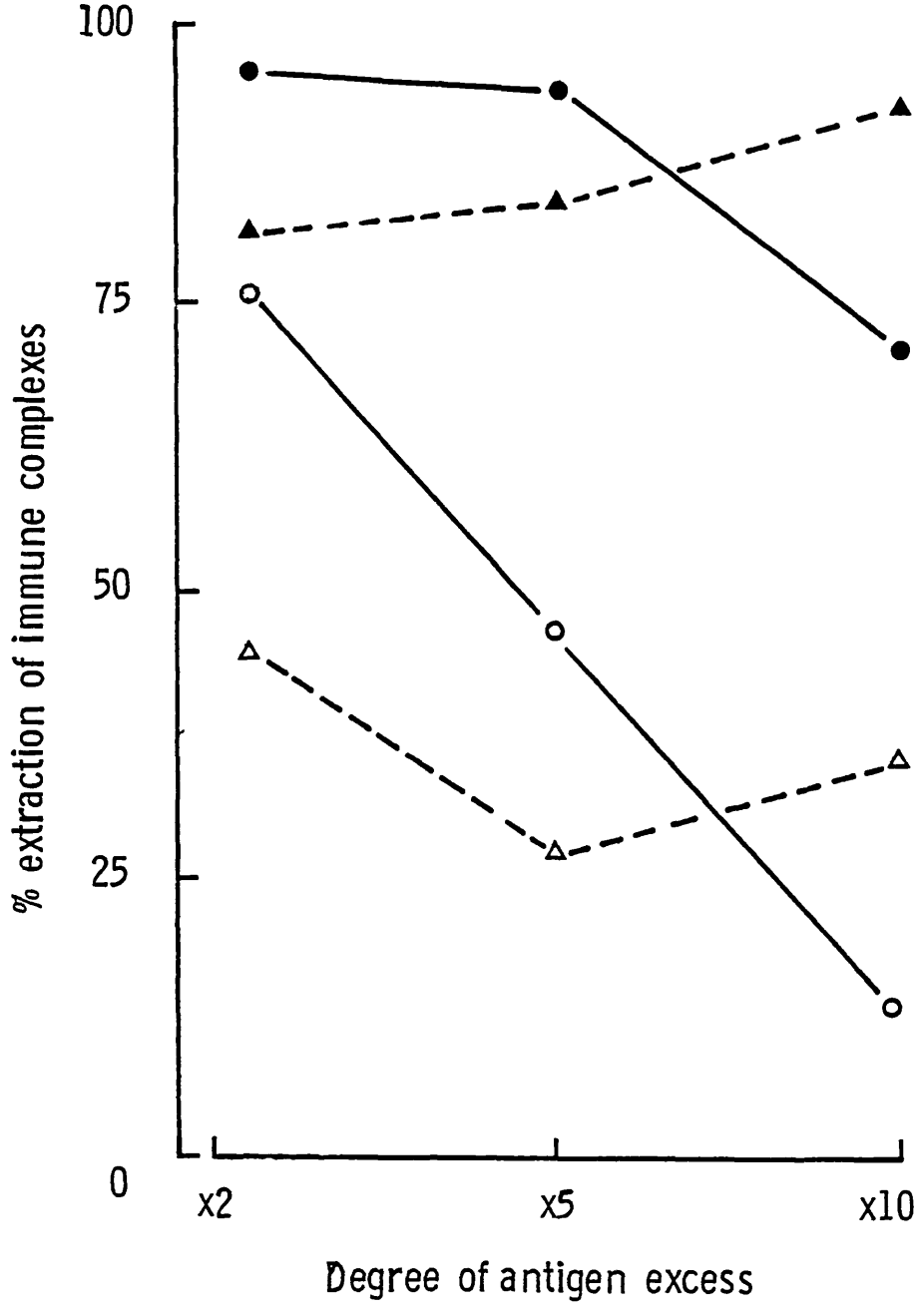


Figure 3


100
75
50
25
0
% extraction of immune complexes
x2
x5
x10
Degree of antigen excess

3.3.3 Discussion

Since the early description of the use of uncharged water-soluble polymers for reversible protein precipitation (Albertson, 1960), polyethylene glycol has been used to precipitate proteins according to their molecular weights (Chesebro and Svehag, 1968; Zubler et al, 1977) and to enhance the immune precipitation of antigens and antibodies (Harrington et al, 1971). Similarly, since the original work by Farr (1958) on the physico-chemical precipitation of immunoglobulins by $(NH_4)_2SO_4$ this agent has been used to precipitate antigen-antibody complexes. However, a direct quantitative and qualitative comparison between $(NH_4)_2SO_4$ and PEG on their ability to precipitate immune complexes has not been reported. The important difference between the two reagents is that PEG precipitates proteins in a manner which is much more dependent on their molecular weight and consequently less protein is precipitated by PEG, as compared to $(NH_4)_2SO_4$. Although the exact mechanism by which PEG reversibly precipitates proteins is not known, two explanations have been proposed : Albertson (1960) suggested that polymer-protein associations masked surface properties which govern protein solubilities while Laurent (1963) suggested that PEG sterically excludes the protein from part of the solvent (solvent exclusion effect) and that this brings the protein to its limit of solubility.

The findings of this particular study emphasized the difference between PEG and $(NH_4)_2SO_4$ with respect to their ability to extract immune complexes from serum and the results provide a clear indication as to which concentration of PEG should be used to selectively isolate immune complexes. It was necessary however, to establish a compromise between quantitative isolation of immune complexes and the non-specific precipitation of normal serum proteins. With this in mind PEG was chosen for its ability to precipitate complexes formed over a wide range of Ag : Ab ratios (Figure 3, Table 1) over the range of antigen excess studied.

The final consideration of criterion investigated in this section was the influence of the extraction techniques on the sizes of the complexes in question.

3.4 Precipitation of Immune Complexes from Serum by $(NH_4)_2SO_4$ and PEG.

3.4.1. Variation of Method

Soluble immune complexes of ^{125}I-BSA - anti-BSA were prepared in fresh NHS at 2 and 5 fold antigen excess and extracted with either 4% PEG or 40% $(NH_4)_2SO_4$ as described (2.2.4). The precipitated complexes were redissolved in CFD and these, and the supernatants, were dialysed against CFD for 2 - 3 hours at room temperature or overnight at 4^{o}C. Sucrose density gradient profiles (2.2.6) were obtained for the complexes before extraction, those remaining in the supernatant after extraction and the redissolved complexes.

3.4.2. Results

Figures 4 and 5 show the molecular size of soluble complexes prepared at 2 and 5 fold antigen excess respectively. There is a progressive decrease in complex size with increasing free antigen concentration. $(NH_4)_2SO_4$ at 40% saturation effectively precipitated the soluble complexes, irrespective of size, and also precipitated some free ^{125}I-BSA. The size of the redissolved complexes formed at 5 times antigen excess was larger after precipitation and resuspension in buffer.

Similar results were obtained when 4% PEG precipitated complexes were redissolved in buffer (Figures 6 and 7). Very little free ^{125}I-BSA was precipitated by PEG and the complexes precipitated were larger than 13S. The redissolved complexes showed a pattern similar to those after $(NH_4)_2SO_4$ extraction in that the 5 fold excess complexes appeared larger after extraction.

Figure 4

Sucrose Density Centrifugation of ^{125}I-BSA - Anti-BSA Complexes at Twofold Antigen Excess Precipitated by 40% $(NH_4)_2SO_4$.

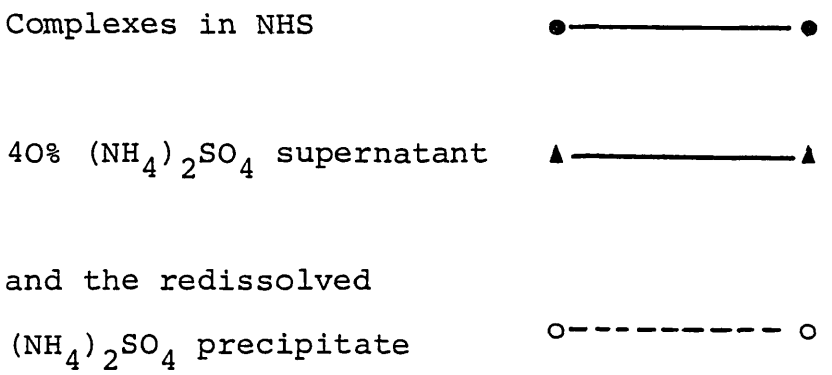


Figure 4

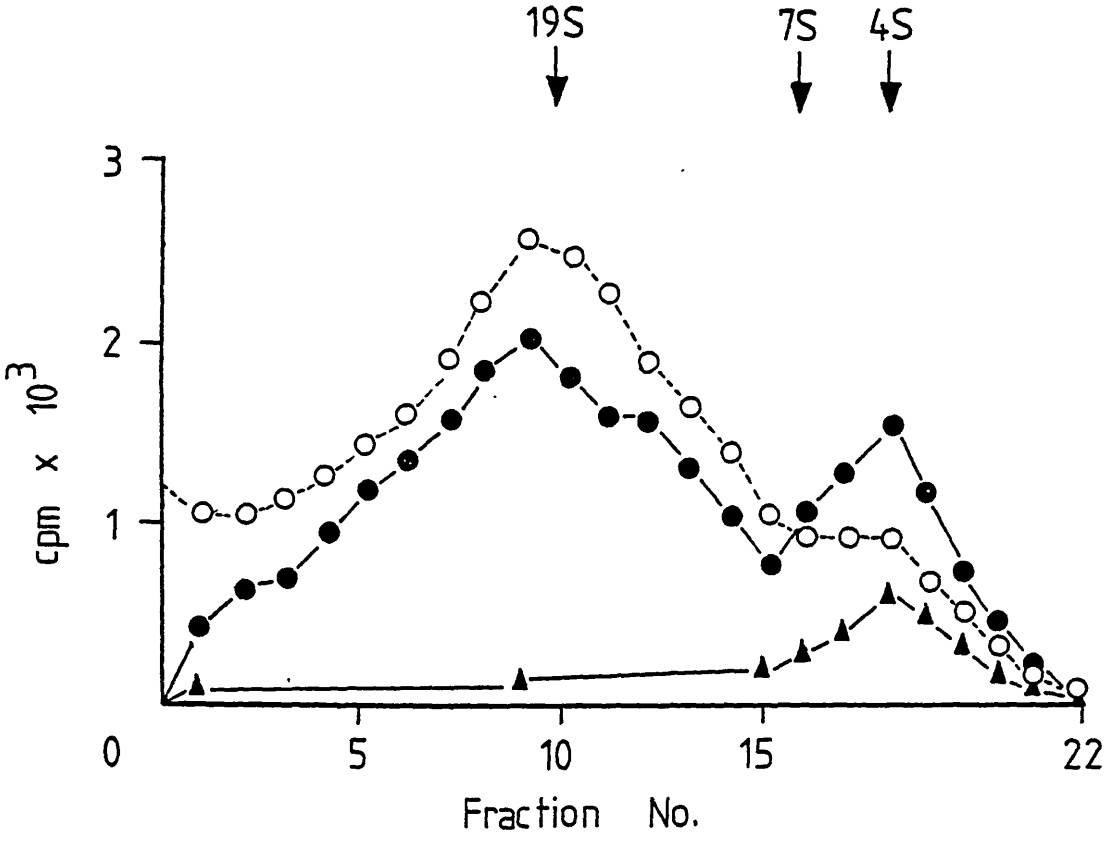


Figure 5

Sucrose Density Centrifugation of ^{125}I-BSA - Anti-BSA Complexes at Fivefold Antigen Excess Precipitated by 40% $(NH_4)_2SO_4$.

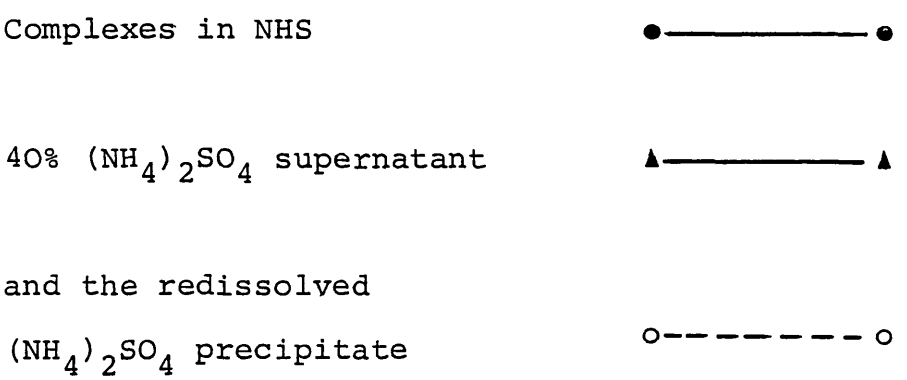


Figure 5

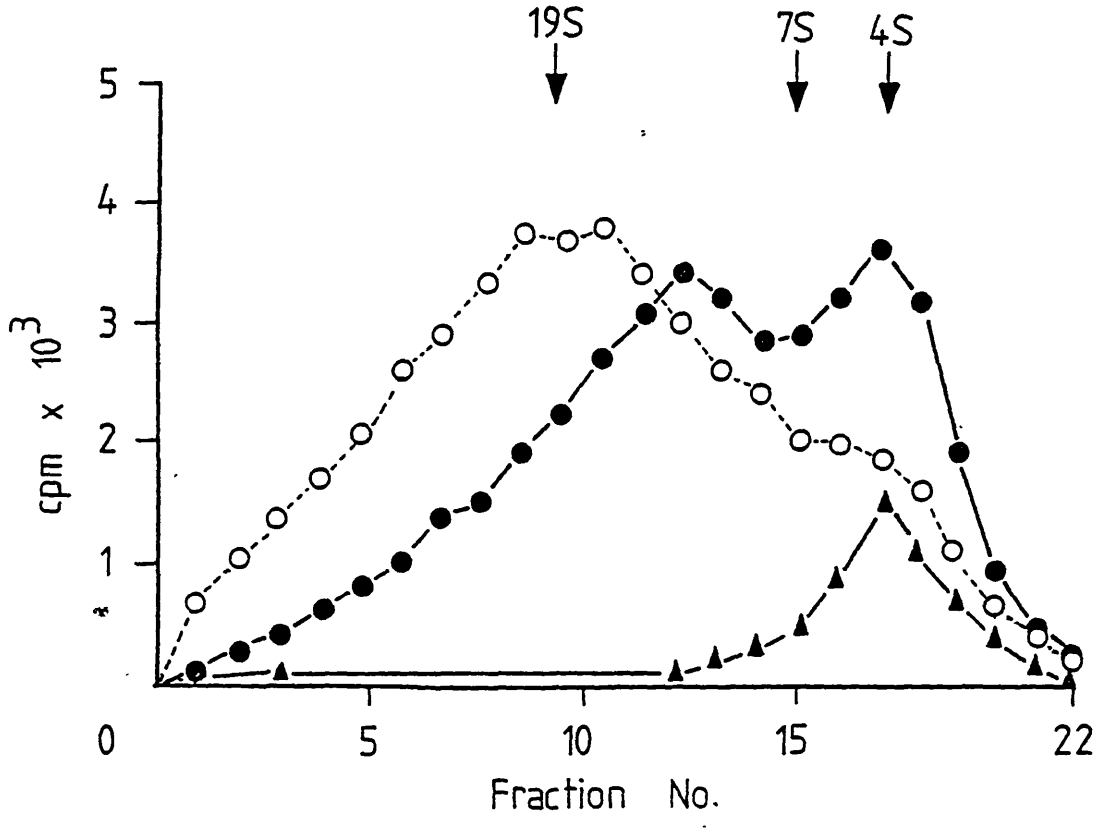


Figure 6

Sucrose Density Centrifugation of ^{125}I-BSA - Anti-BSA Complexes at Twofold Antigen Excess Precipitated by 4% PEG.

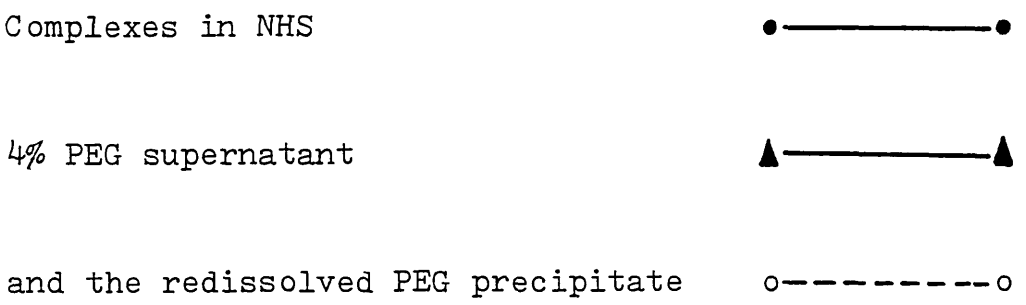


Figure 6

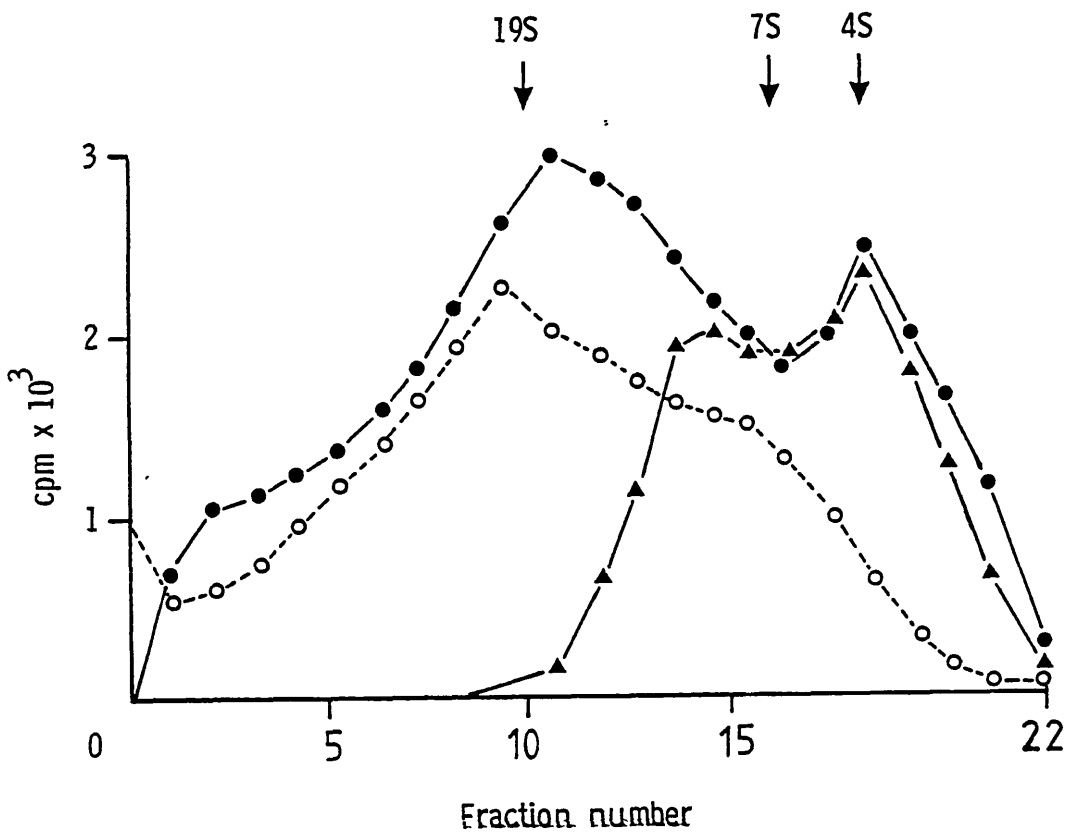


Figure 7

Sucrose Density Centrifugation of ^{125}I-BSA - Anti-BSA Complexes at Fivefold Antigen Excess Precipitated by 4% PEG.

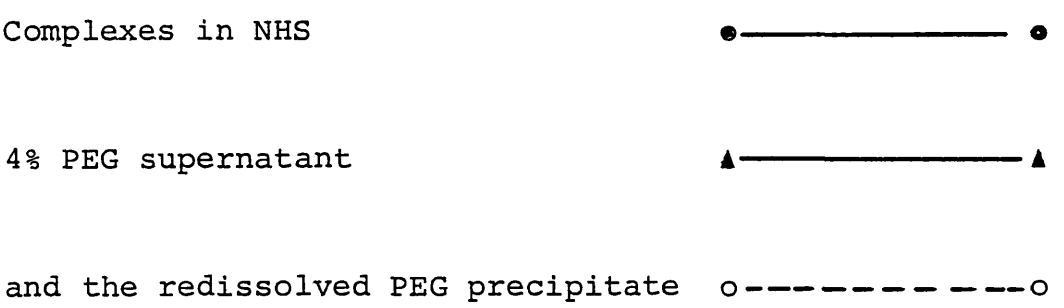
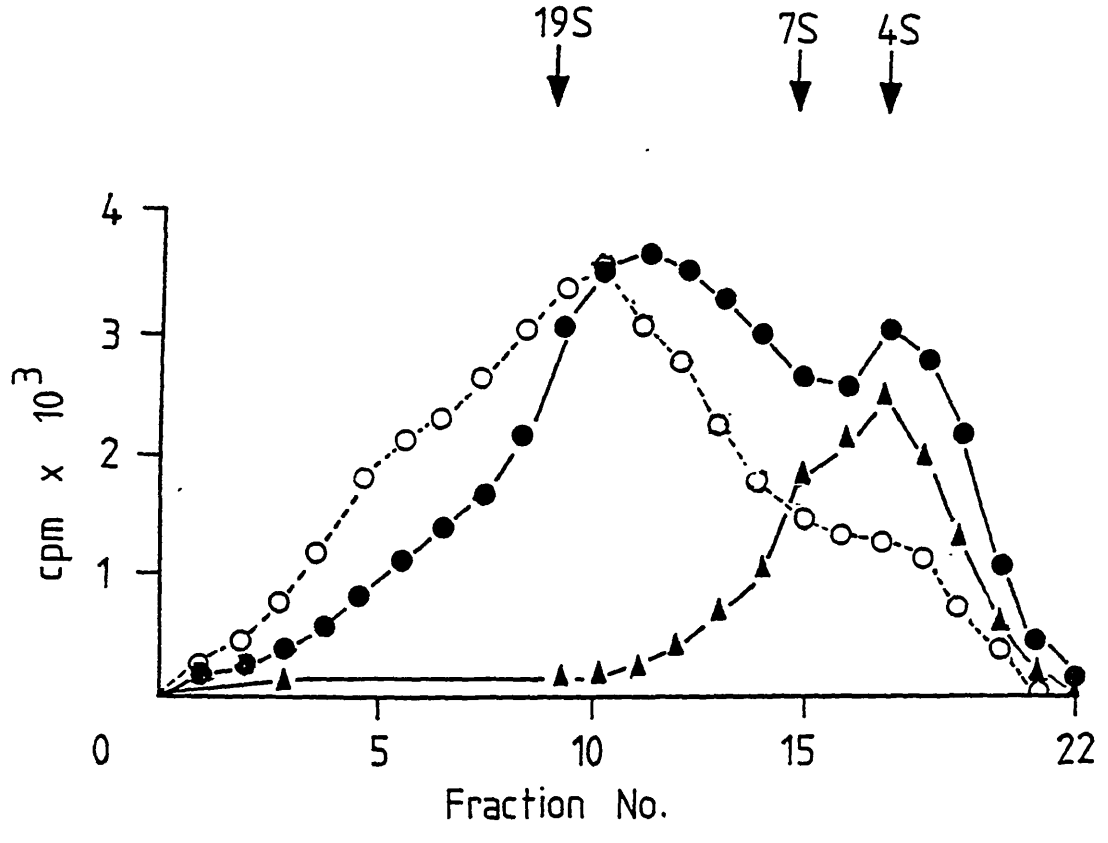


Figure 7



3.4.3. Discussion

This particular section examines two aspects of the precipitation of immune complexes by PEG and $(NH_4)_2SO_4$:-

(1) Does either reagent produce dissociation of antigen and antibody within the precipitated complex?

(2) Is there significant denaturation of the precipitated proteins?

It has already been reported that PEG has little tendency to denature proteins, even when used at room temperature (Polson et al, 1964) and, furthermore, the precipitation of a given protein by PEG is not temperature dependent, so that precise temperature control is not critical (Juckes, 1971; Hao and Wickerhauser, 1978). Miekka and Ingham (1978) compiled an interesting report on the influence of self-assocation of proteins on their precipitation by PEG. By manipulating pH and salt concentrations they were able to enhance the precipitation of β-lactoglobulin A and chymotrypsin by PEG by exploiting the physical conditions which promoted self-association. With respect to immune complex formation the association of antigen and antibody can be manipulated by altering the relative concentration of either reactants or by altering the pH of the reaction mixture. Acid pH produces dissociation

of the components leading to less precipitation whereas neutral pH would allow association and therefore enhance the precipitation.

Neither $(NH_4)_2SO_4$ (Figures 4 and 5) nor PEG (Figures 6 and 7) caused significant dissociation of labelled antigen from antibody during the extraction procedure. However, in the case of fivefold antigen excess complexes, when the small complexes were separated from the free antigen a new equilibrium was set up. There was a consistent alteration however in the molecular weight profile of the fivefold antigen excess complexes after precipitation from serum with PEG, the complexes being significantly larger after precipitation. This change was not observed with the twofold antigen excess complexes. Associated with the change in profile was the observation that PEG did not precipitate unbound BSA. Its failure to do this must have produced a change in the molar ratio of antigen to antibody and the complexes therefore were able to reassociate in conditions which were now closer to those of twofold antigen excess complexes, thus producing larger molecules. It was estimated that 20-30% of the albumin remained in solution after extraction and when the equilibrium between antigen and antibody was established in vitro the molecular weight profile of the mixture of immune complexes produced closely mirrored that shown in Figure 6. The

reassociation of antigen and antibody also implies that little or no denaturation of these proteins occurred following PEG or $(NH_4)_2SO_4$ precipitation.

In attempting to answer the question concerning the effects of $(NH_4)_2SO_4$ and PEG on denaturation of protein, it was clear from the sucrose density centrifugation profiles that the precipitated complexes retained their molecular profiles after extraction by PEG or $(NH_4)_2SO_4$. This finding suggests therefore that neither agent had a significant effect on either the antigen or antibody.

From this study it was concluded that neither PEG nor $(NH_4)_2SO_4$ had damaged either antibody or antigen and that any changes in molecular size were due to the establishment of new equilibria, rather than the physical effects of the two reagents on either antigen or antibody

CHAPTER 4

DEVELOPMENT AND EVALUATION OF LIGAND SPECIFIC AFFINITY SYSTEMS FOR THE ISOLATION OF IMMUNE COMPLEXES FROM HUMAN SERUM OR PLASMA

4.1 Introduction

At present there is a considerable quantity of circumstantial evidence which suggests that immune complexes play an important part in the pathogenesis of a number of human diseases (WHO Bulletin, 1977; Lambert et al, 1978). In some of these diseases e.g. glomerulonephritis (Wilson and Dixon, 1976) immune complexes may well be involved in the initiation or perpetuation of tissue injury but in many other diseases their pathogenetic role has yet to be clearly established. The demonstration of immune complexes in serum or in tissues is not sufficient evidence that the complexes are causally related to the disease. Much of the problem in ascribing a pathogenetic role to the immune complexes is that they are present in a wide variety of diseases which have different clinical features and patterns of organ involvement and secondly, correlation between immune complexes in serum and clinical course is often poor (WHO Bulletin, 1977). Part of this discrepancy between detectable immune complexes and disease may be accounted for by the fact that non-specific means were used to demonstrate these substances in serum and it is therefore possible that this material does not represent true antigen-antibody complexes. It is also possible that in many chronic inflammatory diseases interaction

between acute phase proteins, immunoglobulin and complement may generate macromolecules which are recognised by one or more of the assays currently used to detect immune complexes in serum. The prediction that any immune complex which is relevant to the disease process would be expected to contain either a specific exogenous antigen or an endogenous antigen has led several workers to overcome the problem of non-specific association of serum proteins by attempting to identify specific antigens within the immune complexes.

Rheumatoid arthritis, SLE and hepatitis are among the diseases where the putative antigen in the complexes has been identified. Male et al, (1980) used a combination of sucrose-PEG density gradient centrifugation and immuno-electrophoretic analysis to identify components within the immune complexes found in the synovial effusions of patients with rheumatoid arthritis. They hypothesised that IgG is the major, if not the sole, antigen responsible for provoking and maintaining the pathological changes in rheumatoid arthritis. However, a number of antibodies with specificities against nuclear antigens, collagen and viral components also occur in these patients and equally these antigens could play a pathogenetic role. The significance of immune complexes in the sera of patients with SLE remains controversial and is complicated by a

variety of autoimmune responses directed at both cellular and non-cellular antigens (Glass and Schur, 1978). Although DNA - anti-DNA complexes have been detected in SLE, Izui et al, (1977) suggested that other antigen-antibody systems were involved and he was therefore reluctant to ascribe a primary role to the DNA binding to basement membrane.

Almeida and Waterson (1969) demonstrated the presence of hepatitis B surface (HBs) antigen complexes by electron microscopy and later Takekoshi et al, (1979), using physico-chemical techniques, detected immune complexes of IgG and the cryptic 'e' antigen (HBe) of the hepatitits B virus. More recently an antigen specific immune complex binding assay has been developed (D'Amelio et al, 1981) to detect HBs - anti-HBs complexes and this approach may further clarify the role of these complexes in the disease process.

Although there are many methods available for immune complex detection (Zubler and Lambert, 1977) only a few laboratories have exploited these detection systems to extract immune complexes from sera or other body fluids. This chapter examines three approaches to the problem of immune complex extraction, namely the use of Protein-A Sepharose, bovine conglutinin and human C1q. Since none of these methods proved to be particularly effective in

isolating immune complexes from body fluids a fourth method was developed which made use of the binding between anti-Clq antibodies linked to Sepharose with immune complex bound Clq.

Protein-A has been used to isolate immune complexes from sera after preliminary extraction procedures, e.g. PEG and ammonium sulphate precipitation or gel filtration, had partially purified the high molecular weight immune complex-containing material (Chenais et al, 1977; Tucker et al, 1978). Subsequent purification however has been complicated by the fact that Protein-A does not bind all sub-classes of IgG and may also bind monomeric IgM and IgA (Vidal and Conde, 1980).

The use of Clq as a receptor for the isolation of immune complexes was first described by Svehag and Burger (1976). However, these first attempts to isolate immune complexes were hindered by competition between free Cl in serum and the agarose-bound Clq, this competition leading to low yields of extracted material. Casali and Lambert (1979) improved this technique by precipitating immune complexes from EDTA serum with PEG, thereby removing bound Clq from Clq present in serum. They also extended the technique by using bovine conglutinin as a ligand, either alone or in combination with human Clq.

Logically the most appropriate method for isolating immune complexes from serum or plasma would be to use the same ligand for extraction as utilised in the assay to detect them. Bearing this in mind this chapter examines the use of four different affinity systems (Protein-A, Clq and K columns and anti-Clq column) for their ability to isolate in vitro prepared complexes from NHS with respect to yield and composition of product, specificity of interaction and potential usefulness in the isolation of pathological complexes.

4.2 Investigation of the Use of Protein-A Sepharose in the Purification of Immune Complexes.

4.2.1 Variation of Methods

4.2.1a Elution of Bound Complexes from Protein-A Sepharose

Soluble immune complexes were prepared in NHS at two-fold antigen excess by incubating 200 µl anti-BSA 3.46 mg ml^{-1} with 100 µl BSA (1 mg ml^{-1}) and a trace amount of ^{125}I-BSA, as described (2.2.4). After 4% PEG precipitation the redissolved complexes were incubated with a 5 ml bed volume Protein-A Sepharose column (2.4.4) and the bound complexes were eluted sequentially from the column with 0.1M citrate buffer, pH3 followed by 0.1M HCL-glycine pH 2.4. On a few occasions the Protein-A bound complexes were eluted with 0.1M lithium diiodosalicylic acid (LIS).

4.2.1b Analysis of Purified Complexes

At various stages of the extraction procedures aliquots were taken and subjected to sucrose density gradient ultracentrifugation. Eluted complexes were also analysed by SDS-PAGE (2.5.5) and autoradiography (2.5.6).

4.2.2. Results

a) Elution of Bound Complexes from Protein-A

Solubilised immune complexes were not bound efficiently by the Protein-A column and more than 50% of immune complexes passed through the column without binding (Table 1). Citrate buffer at pH 3.0 removed only 30.2% of the bound complexes whereas 0.1M glycine - HCl pH 2.4 eluted a further 51.4%. When the columns were washed with only glycine-HCL, pH 2.4 the total recovery of labelled antigen in all cases was greater than 93%. ^{125}I-BSA alone failed to bind to the column.

b) Analysis of Purified Complexes

Figures 1 and 2 show the sucrose gradient profiles of ^{125}I-BSA - anti-BSA complexes at different stages during the extraction and purification procedures. Immune complexes at twofold antigen excess (Figure 1) showed a characteristic profile with large molecular weight complexed material and a small proportion of free antigen. Upon extraction with 40% $(NH_4)_2SO_4$ or 4% PEG the redissolved complexes occupied similar molecular profiles as the complexes in whole serum, but with no free antigen present. Elution with glycine-HCl pH 2.4 followed by neutralisation

changed the molecular profiles of the complexes (Figure 2). Irrespective of whether the complexes were precipitated by $(NH_4)_2SO_4$ or PEG, the acid eluted complexes were of a smaller molecular size (8-16S) with a high proportion of free antigen.

SDS-PAGE and autoradiographic analysis of the acid eluted complexes (Figure 3) demonstrated the presence of IgG and free ^{125}I-BSA. Upon reduction and alkylation the IgG produced two bands which corresponded with the heavy and light chains. The apparent increase in molecular weight of BSA was the result of the reduction and alkylation procedure altering the BSA in a way that hindered the uptake of SDS, thus affecting its electrophoretic mobility (Weber and Osborn, 1975).

4.2.3 Discussion

In this study, 0.1M glycine-HCl pH 2.4 proved to be most effective in removing complexes which had bound to the Protein-A (95.6%), whereas citrate buffer pH 3.0 only removed 30.2% of the complexes (Table 1).

The molecular weight profiles of the eluted complexes (Figure 2) indicate that a certain degree of antibody damage has taken place during the elution procedure with acid. On the other hand, however, some antibody was lost during ultrafiltration of the elutes on Amicon filters and it is therefore possible that this may reflect the changes in antigen-antibody ratio, rather than antibody damage by low pH. Loss of antibody by either means, however, would result in an equilibrium shift to antigen excess, thus generating small complexes. It is important to neutralise the eluate very quickly since this minimises the effect of the acid buffer on the antibody. When immune complexes of known sizes were acidified and neutralised immediately there was no alteration in their molecular profile. Similar molecular weight profiles to those shown in Figures 1 and 2 were obtained for complexes formed in heparinised plasma, suggesting that fibrinogen or other components of the clotting cascade did not appear to interfere with complex formation, extraction or elution.

Lithiumdiiodosalicyclic acid (LIS) efficiently released bound complexes from the Protein-A column (Table 1). However, after dialysis against PBS, no association of antigen or antibody was detected by sucrose gradient centrifugation and it was concluded that LIS had a pronounced damaging effect on the antibody.

MacSween and Eastwood, (1978) came to a similar conclusion about the effect of LIS on antibody activity and also reported that it had no damaging effect on antigen (BSA).

SDS-PAGE of labelled antigen-antibody complexes purified from Protein-A, coupled with autoradiography clearly shows that the antigen within the immune complex could be isolated and identified by a combination of these techniques (Figure 3). The sensitivity of each method is such that it allows both antigen and antibody to be identified, however it must be emphasised that the amount of protein isolated from a Protein-A column far exceeds the quantities of BSA - anti-BSA added to the serum (in the order of ten times more). This discrepancy is further compounded by the observation that only 50% of the labelled antigen is isolated from the serum. The dense banding pattern for both the reduced and non-reduced IgG must therefore reflect the co-purification of non-specific human IgG. This was confirmed by immunodiffusion analysis which showed that the main component in the isolated material was human IgG.

Throughout the course of these experiments the binding capacity of the Protein-A column for immuno-globulin, far exceeded the amount of immune complexes added to it. Therefore it was likely that the complexes which

failed to bind to the column (Table 1) were prevented from doing so due to the binding of other proteins such as complement components to their surface, thus rendering their Fc sites unavailable for interaction with Protein-A.

One of the more recent drawbacks to the use of Protein-A in purifying immune complexes has been the discovery that in addition to binding IgG 1, 2 and 4 it also binds IgM, IgA and probably IgD and IgE (Vidal and Conde, 1980). Monomeric immunoglobulins, not involved with the complexes, would therefore be able to compete with complexed IgG for binding to Protein-A. Chenais et al, (1977) incorporated a gel filtration step after PEG extraction to remove monomeric immunoglobulins; however, free IgM and dimeric IgA could still contaminate the system. Rather than introduce time-consuming gel filtration methods, which would not have totally solved the problem, it was decided to investigate the use of other methods to extract immune complexes from serum.

Table 1

Binding and Elution of Immune Complexes from Protein-A Sepharose

% Bound	40.1	55.3	46.2
% eluted with			
0.1M Citrate buffer pH3.0	30.2	-	-
0.1M Glycine/HCl pH2.4	51.4	95.6	-
Litium diiodosalicylic acid (LIS) pH7.2	-	-	94.2

Figure 1

Sucrose Density Centrifugation of ^{125}I-BSA - Anti-BSA Complexes at Twofold Antigen Excess following $(NH_4)_2SO_4$ and PEG Extraction. Complexes in NHS ●——●

redissolved complexes after 40% $(NH_4)_2SO_4$ extraction o- - - - - - - - - o

redissolved complexes after 4% PEG extraction ▲- - - - - - - ▲

Figure 1

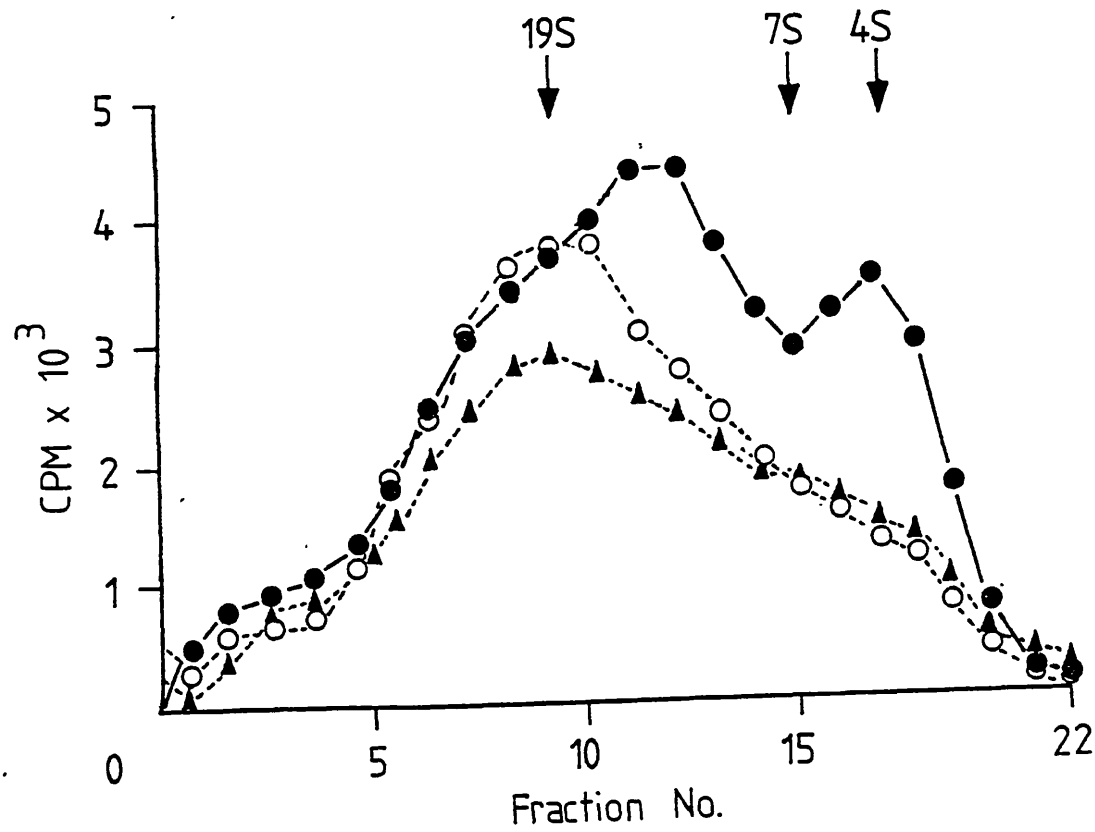


Figure 2

^{125}I-BSA - Anti-BSA Complexes Extracted with 4% PEG or 40% $(NH_4)_2SO_4$ and Eluted with Glycine - HCl pH 2.4 from a Protein-A Sepharose column.

$(NH_4)_2SO_4$ extracted complexes eluted at pH 2.4 and neutralised ●——————●

PEG extracted complexes eluted at pH 2.4 and neutralised o- - - - - - - - - - - -o

Figure 2

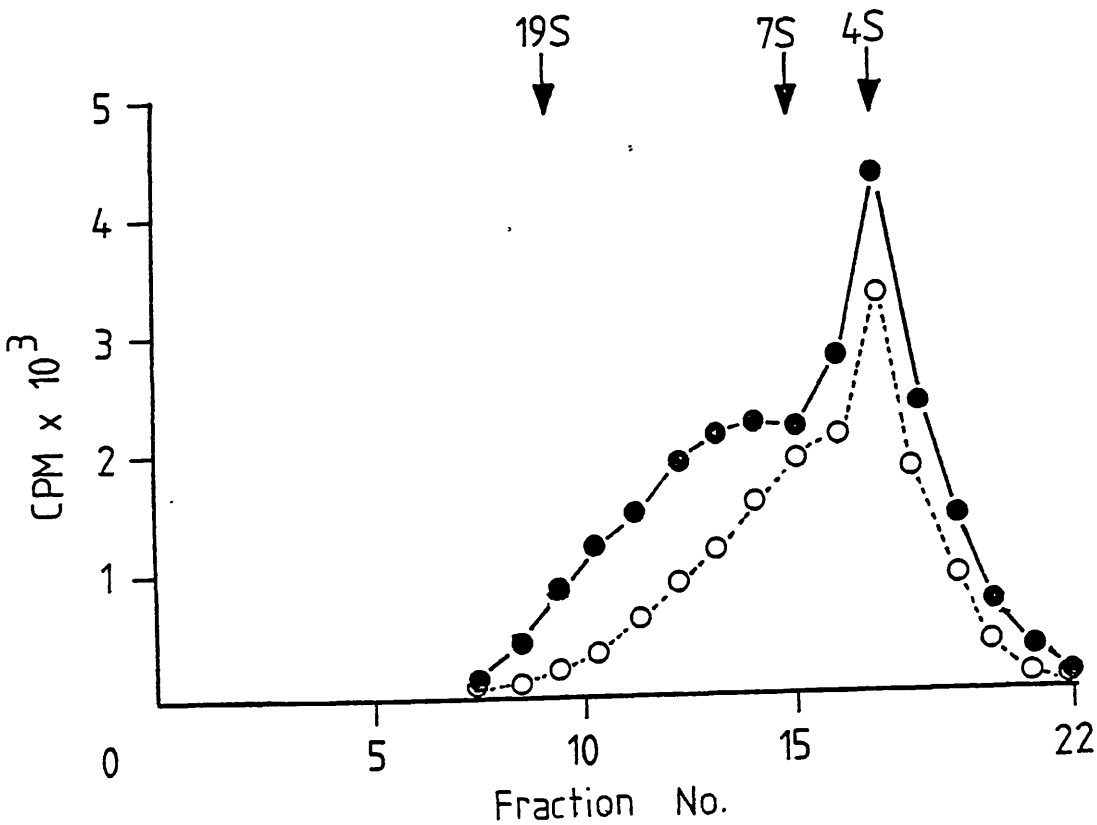


Figure 3

SDS-PAGE (7.5% gel) and Autoradiographic Analysis of Acid Eluted ^{125}I-BSA - Anti-BSA Complexes from a Protein-A Sepharose column.

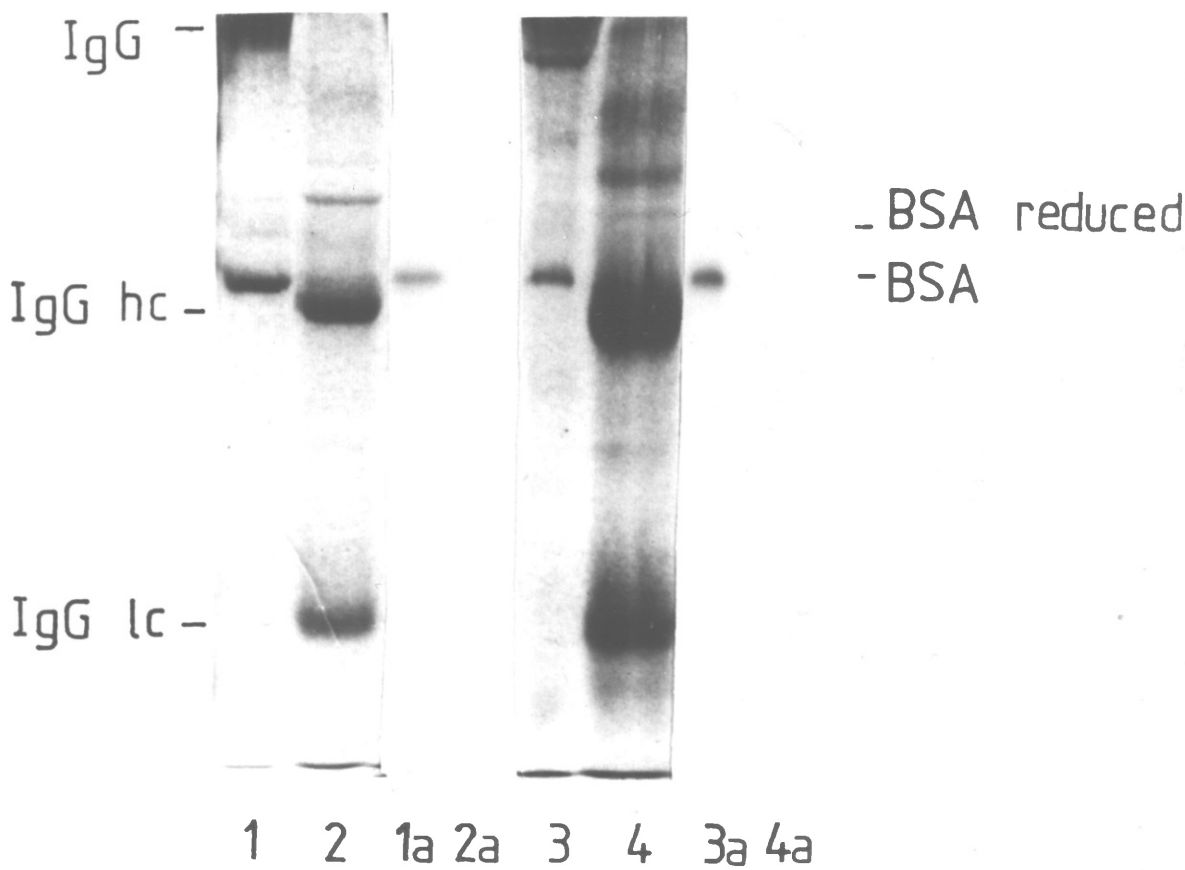
4% PEG extracted complexes followed by acid elution.

- Column 1 not reduced
- 2 reduced and alkylated
- 1a autoradiogram of column 1
- 2a autoradiogram of column 2

40% $(NH_4)_2SO_4$ extracted complexes followed by acid elution

- 3 not reduced
- 4 reduced and alkylated
- 3a autoradiogram of column 3
- 4a autoradiogram of column 4

Figure 3



4.3 Investigation of Immobilised Bovine Conglutinin (K) and Human Clq as Ligands for the Purification of Immune Complexes.

4.3.1 Variation of Methods

a Human Clq and Bovine (K) Affinity Columns

These columns were prepared and used as described in the Materials and Methods section (2.4.3).

b Dissociation of Purified Immune Complexes

Antigen or antibody labelled BSA - anti-BSA complexes, purified by the Clq-Degalan or K-Degalan column, were analysed on sucrose density gradients prepared in CFD, pH 7.4, and acetate - EDTA buffer pH 3.0. Some of the acid gradients were neutralised after fractionation and an excess of specific antisera to human C3, human IgG or human Clq added; the same antisera were also added to samples fractionated at pH 7.4.

4.3.2. Results

4.3.2a Purification of Human Clq

The yield of human Clq, based on the extinction coefficient@1% 0.685 (Reid et al, 1972) was in the range 8 - 10 mg Clq per 100 mls NHS; assuming a Clq concentration of 150 $\mu g\ ml^{-1}$, this is equivalent to an extraction efficiency of 50 - 75%. Immunoelectrophoretic analysis revealed a single cathodal precipitin line using an anti-whole human serum and this line gave a reaction of immunological identity with the precipitin line against an anti-human Clq sera (Seward). SDS-PAGE analysis of three different human Clq preparations (Figure 4) revealed similar degrees of purity and banding patterns. The human Clq preparations comprised two non-covalent bands at 60,000 and 42,000 daltons which upon reduction and alkylation yielded three molecular weight bands of 29,000, 27,000 and 22,000 daltons. Apart from slight contamination with low molecular weight bands in reduced gels, the data compared well with that of Yonemasu and Stroud (1972).

The functional property of each Clq preparation was assessed by its ability to agglutinate latex particles coated with human IgG and by its ability, after radiolabelling with ^{125}I, to associate with Clr and Cls. This association was demonstrated by a molecular shift from

9.5S (monomeric Clq) to 16S (Cl) on sucrose density gradients. Storage of Clq at -70°C for several months did not affect its ability to reassociate with Clr and Cls.

4.3.2 b Purification of Bovine Conglutinin (K)

The yield of bovine conglutinin was estimated from the conglutination of EAC 142 cells (2.3.2 c) and expressed as specific activity ratios. Each conglutinin preparation gave a single precipitin line against anti-whole bovine serum.

Table 2 shows the increase in K titre during various stages of the purification process. The total protein concentration was determined according to the Folin method and the specific activity ratios were calculated by the method of Lachmann and Richards (1964). During the course of the purification the average conglutinin titre was increased by a factor of over 700 and when expressed as a function of total protein isolated, the increase was in the order of 7000. The purified K maintained its functional ability after storage at -70°C for more than twelve months.

4.3.2 c Preparation of K and Clq-Degalan Affinity Columns

Initially anti-BSA antibody was bound to Degalan

beads, as described (2.4.3), in order to determine the optimum binding conditions and also to examine the ability of bound antibody to bind specific antigen (BSA) and release it under the appropriate elution conditions. Antibody binding to the beads was calculated by extracting the antibody from a known volume of beads with 0.1M NaOH and estimating the protein content by the Folin method. It was found that to bind between 200 - 700 µg anti-BSA (IgG) per gram of beads 10 to 20 fold excess of anti-BSA (IgG) had to be provided. When radiolabelled BSA was added to the beads, after the appropriate washing more than 98% could be eluted from the column by 0.1M LIS or glycine-HCl pH 3.0.

Similar findings, in terms of the amount of protein bound per gram of beads, were obtained when K and Clq were incubated with the Degalan beads. Calculations based on the binding of K Clq and IgG to the beads (assuming an approximate surface area of IgG molecules of a micron squared)allowed calculations to be made between the area occupied by the bound protein in relation to the total surface of the beads. From these calculations less than 5% of the bead surface was occupied by protein.

4.3.2d Purification of Immune Complexes on K and C1q-Degalan Columns

The yield of ^{125}I-BSA-anti-BSA complexes extracted from both columns was low (Table 3); however, enough material could be purified for analysis of the complexes by immunodiffusion and SDS-PAGE studies. The C1q column lost its ability to bind immune complexes after two or three elution procedures and therefore fresh columns had to be prepared when needed. The K-Degalan column was more stable and the activity of the column was maintained for several weeks at 4°C. The amount of immune complexes purified from this column was higher than that of the C1q-Degalan column (Table 3).

The material eluted from each column contained much more protein than could be attributable to the total amount of complexed antigen and antibody added to the columns (Table 3). Evidently immune complexes prepared in NHS bound various complement components to their surface and these molecules were therefore co-purified during the purification procedures. This was confirmed by Ouchterlony and immunoelectrophoretic analysis of the eluted complexes which

revealed the presence not only of BSA and rabbit anti-BSA, but also C3, C3c and C3d. In addition human IgG, IgM and albumin were identified.

The following controls were applied to the C1q and K-Degalan columns. ^{125}I-BSA-anti-BSA complexes formed at equivalence in NHS and extracted with 4% PEG were incubated with uncoated Degalan beads and beads coated with rabbit immunoglobulin. More than 98% of the radioactivity was recovered unbound in both cases and no detectable protein was eluted in the EDTA, NaCl or acid washes. When NHS was extracted with 4% PEG and incubated with the C1q and K columns no protein could be detected in any of the three elutions. Throughout the remainder of this study PEG extracts of normal serum samples, which had been stored for the same length of time as the pathological sera, were regularly incubated with the columns. ^{125}I-BSA alone failed to bind to the columns.

SDS-PAGE and autoradiographic analysis also demonstrated that high molecular weight complexes containing radioactive antigen (BSA) could be purified by the K column (Figure 5).

When the complexed material was reduced and alkylated IgG heavy and light chains were clearly visible and the ^{125}I-BSA was dissociated from its specific antibody.

The ability of complexes to bind to the K-column was related to the size of the complex formed (Figure 6). ^{125}I-BSA - anti-BSA complexes were prepared in NHS over the range 5 fold antigen to 2 fold antibody excess, precipitated with 4% PEG and incubated with the K-column. Binding of ^{125}I-BSA - anti-BSA complexes to the K-column was expressed as a percentage of the total antigen-bound complexes added to the column. The peak binding occurred with the complexes prepared at equivalence and the amount which bound decreased with complexes formed in antigen and antibody excess.

4.3.2 e Dissociation of Purified Immune Complexes

Antigen labelled and antibody labelled immune complexes were prepared in NHS, extracted with 4% PEG and purified on the Clq or K column. The purified complexes were then analysed by sucrose density gradient centrifugation in CFD-EDTA at pH7.4 and 0.2M acetate buffer pH3, containing 0.15M NaCl and 0.02M EDTA. Under these conditions almost complete dissociation was achieved for both antigen labelled (Figure 7a) and antibody labelled

(Figure 7b) immune complexes. However, when unlabelled complexes were purified in the same way and then labelled with ^{125}I after purification, an increase in their apparent size was observed after incubation in acid buffer (Figure 7c). Even though some fragments were produced their size did not correspond to either BSA or anti-BSA, most of the radioactivity remaining in the fractions containing molecules whose size was >19S. When the same acid treated gradient fractions were neutralised and an excess of anti-human C3 antiserum added, a shift of C3 containing material was observed from the high molecular weight region (pH 7.4) to the lower molecular weight region (acid neutralised gradient). Precipitation of similar gradient fractions with anti-human IgG and Clq antisera did not result in a net shift of radioactivity to the low molecular weight region.

4.3.3 Discussion

Techniques for purifying immune complexes from pathological fluids should fulfil two criteria before being applied to the isolation of immune complexes namely, specificity of interaction and efficiency of extraction. These are important requirements since only small amounts of immune complexes are present in pathological fluids when compared to the amounts of other

proteins which could be co-purified during the isolation procedure. Whether both of these criteria can be met has been examined using *in vitro* prepared complexes solubilised in normal human sera.

The combination of a physico-chemical isolation procedure followed by extraction through binding to immobilised K and Clq provides a means of purifying immune complexes and avoids the use of adverse physico-chemical conditions. Bovine conglutinin (K) binds to fixed C3bi on immune complexes (Lachmann *et al*, 1973; Linscott *et al*, 1978) and therefore can be used to purify immune complexes which have activated either the classical or alternative complement pathways. Clq, on the other hand, binds to the CH_2 domain on IgG or IgM (Porter, 1980) and will only react with immune complexes which activate the classical pathway.

Immobilised conglutinin proved to be a good method for isolating immune complexes; however, because of the inefficiency of binding of the conglutinin to Degalan beads the yield of immune complexes isolated by this means was low (Table 3). To increase the yield larger amounts of coated beads had to be used (up to 20g). However, since large volumes of buffer were needed to elute the larger bead volume, we were unable to prevent the unavoidable loss of the eluted material, nearly 50%, which occurred

following ultrafiltration. Similar criticisms could also be applied to the Clq column, but since the yields obtained from this were even smaller the losses after ultrafiltration were proportionally much greater.

Large molecular weight complexes appeared to be bound preferentially by conglutinin (Figure 6). One possible explanation for this could relate to the spatial arrangement of conglutinin molecules on the surface of the Degalan beads. Since only 5% of the total bead surface was coated with conglutinin, it could be argued that only the large complexes which had activated the complement system would have bound sufficient C3b onto the lattice of the complex to allow multivalent attachment and binding. The smaller complexes, being less efficient activators of the complement system, would not therefore be able to satisfy the optimum conditions for binding.

Many experiments consistently showed that the yield of immune complexes from the Clq Degalan column was less than that of the K column. The precise reasons for this are not clear but two factors may have contributed to this. Firstly, bound Clq may not exchange as efficiently with the immune complexes as fluid phase Clq, secondly there may be interference to binding of the Clq attached to the Degalan beads due to the binding of complement

components to the immune complex-bound IgG and C1q. If the latter were true, then the chances of isolating complement solubilised complexes in serum would be further limited.

Because of the difficulties encountered with these two methods a new method was developed which attempted to isolate those complexes that had already bound C1q.

When complexes were isolated from the K or Clq columns it was possible to detect labelled antigen and antibody within the complex; however, the ^{125}I was predominantly bound to those molecules on the surface. Figure 6 (Chapter 5) demonstrates that the majority of the isotope was bound to a protein band whose molecular weight was 68K daltons and this protein would correspond to one of the fragments produced following C3 binding and subsequent cleavage with C3b INA (Factor I). The method of generating these complexes, viz. incubating in serum at 37°C for 30 minutes, would also lead to further degradation of the C3 molecules bound to the complexes by some of the proteolytic enzymes produced following clotting. This interpretation of the experimental data is supported by the observation that these fragments were precipitated from the gradient fractions by an anti-C3 antiserum (Figure 7d). Their appearance only after neutralisation in a low pH buffer also suggests that they are bound in a non-covalent

manner to the surface of the complex. Finally the shift in the size of the complex after acid incubation suggests that once complement activation has occurred the molecules are more liable to aggregation or denaturation.

When either an anti-rabbit IgG or an anti-human C1q antisera was added to the purified immune complexes no shift in their profile occurred after acid centrifugation and neutralisation. These findings therefore suggest that even though the IgG molecule was susceptible to iodination the binding of iodine to a larger molecule, viz. rabbit antibody, was prevented by the attachment of other serum proteins to the complex.

Figure 4

SDS - PAGE (10% gel) of Three Different Human C1q Preparations.

Columns	1	human IgM reduced and alkylated
	2	human C1q
	2a	human C1q reduced and alkylated
	3	human C1q
	3a	human C1q " " "
	4	human C1q
	4a	human C1q " " "

Figure 4

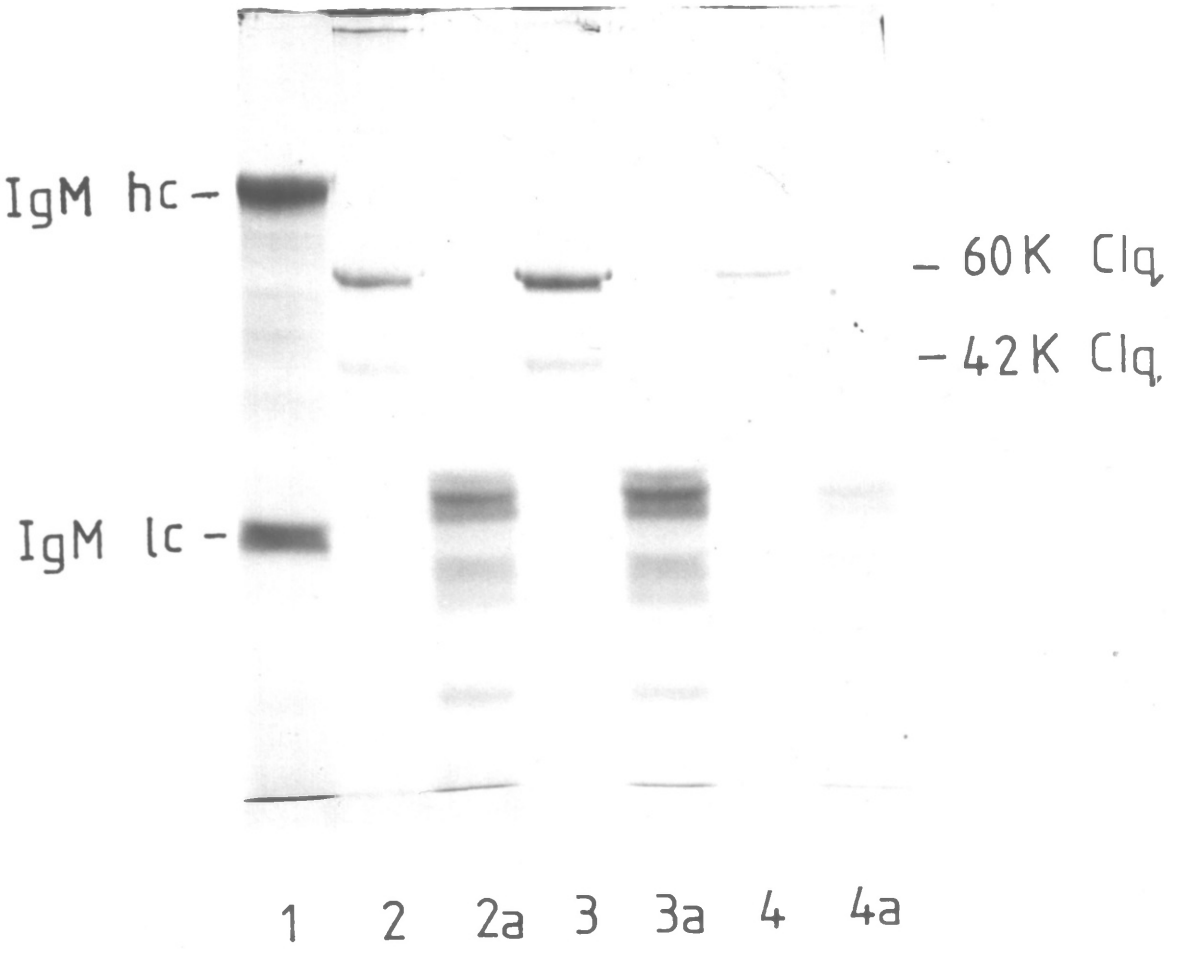


Table 2

Purification of Bovine Conglutinin (K)

	Reciprocal K titre (Kt)	Protein$_{l}$ (mg ml^{-1})	$\frac{Kt \times 5}{Protein}$
neat Bovine Sera	7,000	16.2	2,200
after 1st dialysis	64,000	0.28	1,142,000
2nd euglobulin preparation	6,400,000	3.46	9,248,000
after ether extraction	5,120,000	1.7	15,058,000

Table 3

Efficiency of Binding of ^{125}I-BSA - Anti-BSA* Complexes to Clq and K Degalan Columns.

	Percentage recovered	Quantity of protein in eluate
Clq column	8.9% = 11.8 µg	120 µg
K column	15% = 20 µg	155 µg

* ^{125}I-BSA - anti-BSA complexes were prepared in NHS at 37°C and after PEG extraction and resuspension 133 µg of immune complexes were added to the columns.

Figure 5

SDS-PAGE (7.5% gel) and Autoradiographic Analysis of K - column Purified ^{125}I-BSA - Anti-BSA Complexes Prepared in NHS.

Column 1 not reduced

" 1a reduced and alkylated

" 2 autoradiogram of column 1

" 2a autoradiogram of column 1a

Figure 5

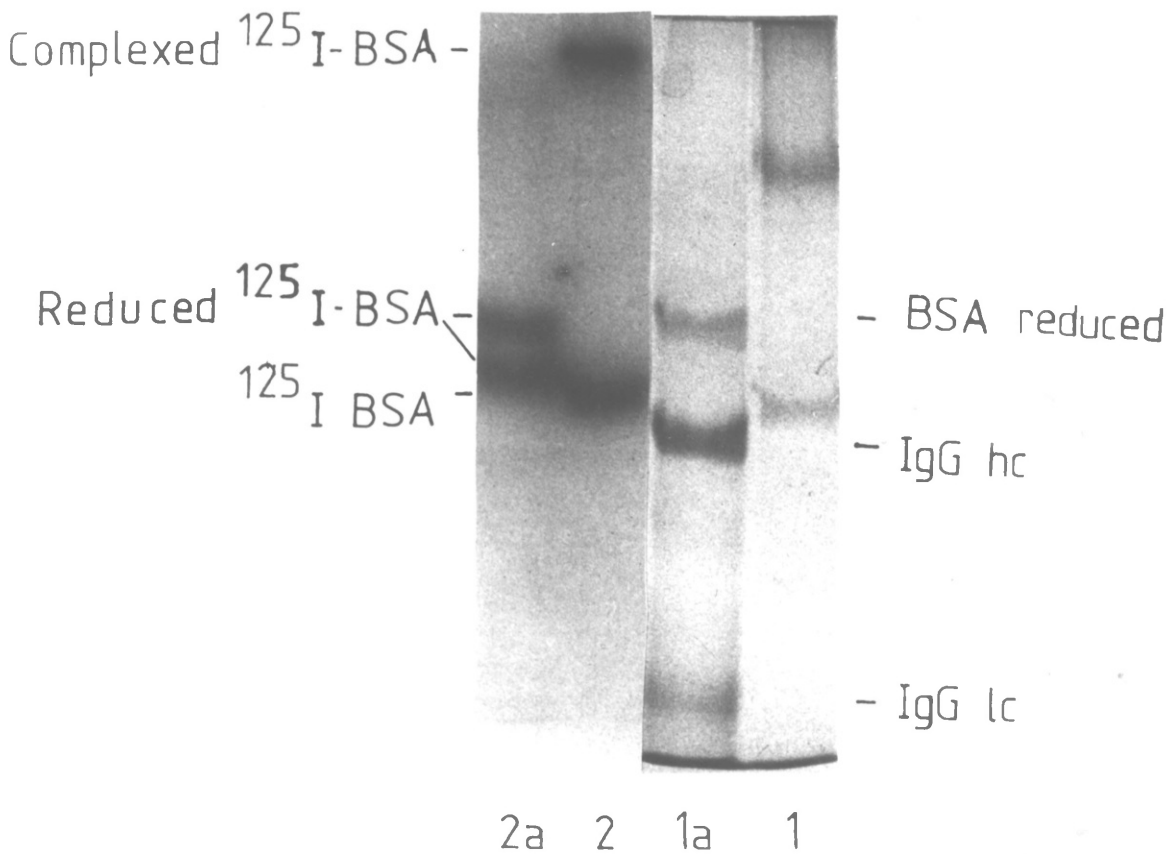

Complexed ^{125}I-BSA
Reduced ^{125}I-BSA
^{125}I BSA
BSA reduced
IgG hc
IgG lc
2a
2
1a
1

Figure 6

^{125}I-BSA-Anti-BSA Complexes Prepared in NHS and Purified by the K-Column

Binding is expressed as a percentage of the total antigen-bound complexes added to the column.

Figure 6

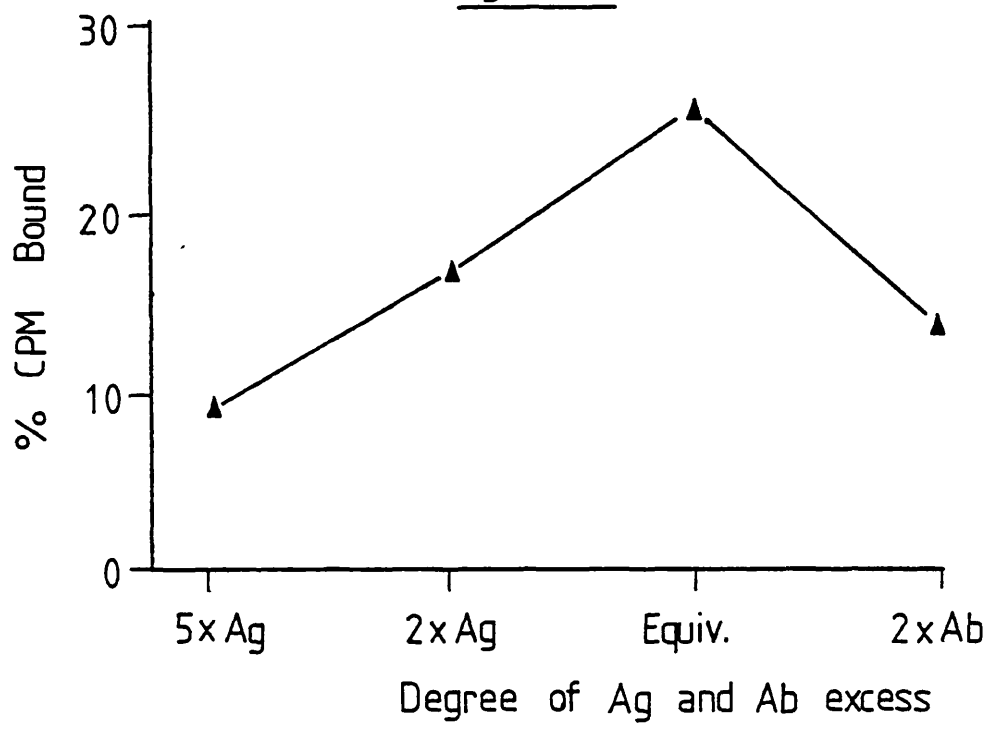

30
20
10
0
% CPM Bound
5x Ag
2x Ag
Equiv.
2x Ab
Degree of Ag and Ab excess

Figure 7

Dissociation of K - Column Purified BSA - Anti-BSA Complexes Prepared in NHS.

a Antigen labelled immune complexes

b Antibody labelled immune complexes

c Complexes labelled after purification

d Complexes labelled after purification and precipitated with anti-human C3 sera.

●———● Sucrose gradient at pH 7.4

o———o Sucrose gradient at pH 3.0

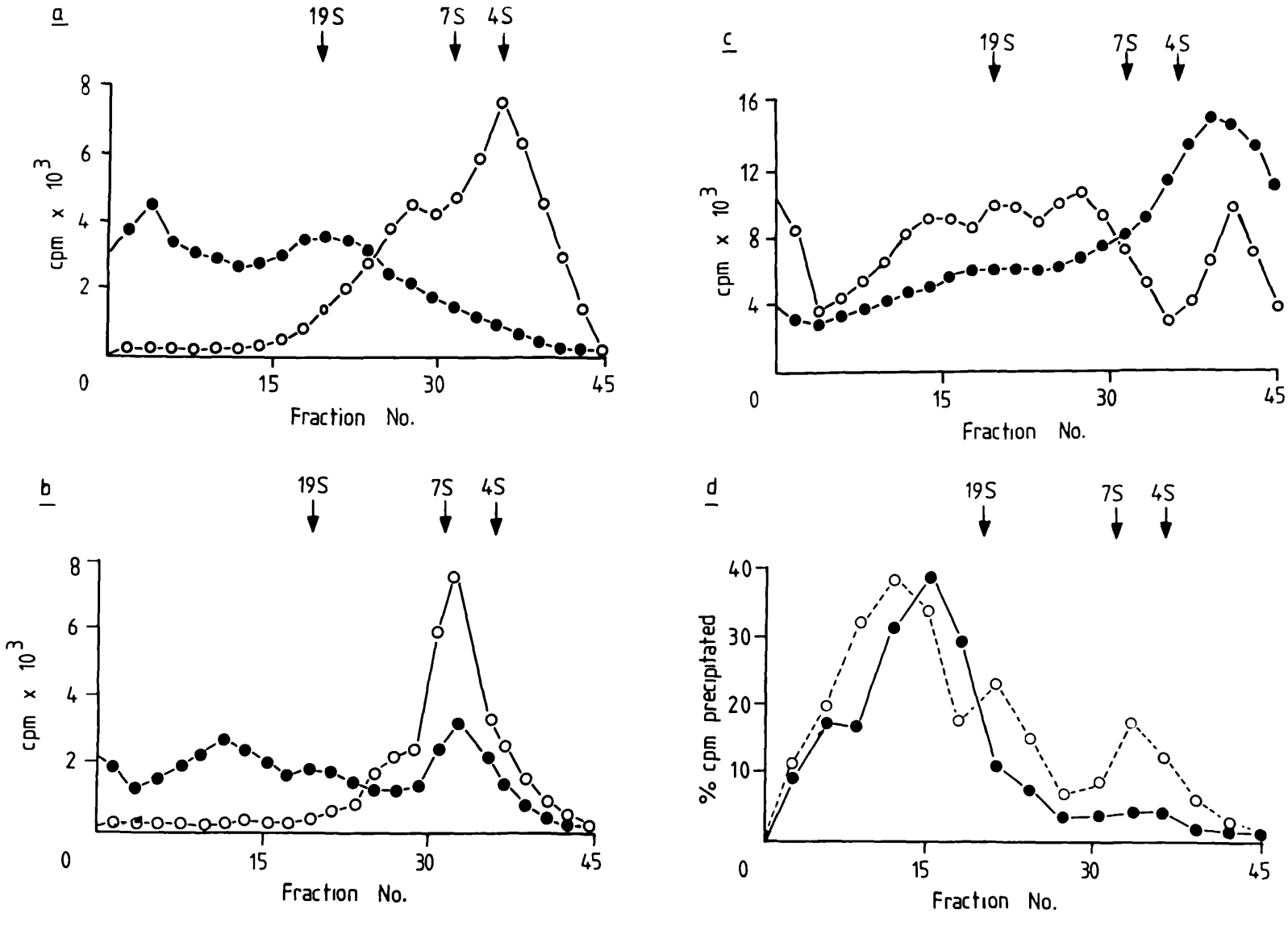


Figure 7

4.4 Anti-Clq Column : Ligand Specific Purification of Immune Complexes from Human Serum or Plasma. Analysis of the Interaction between Clq and Immune Complexes.

4.4.1 Variation of Methods

4.4.1a Preparation of ^{125}I-Clq - BSA-Anti-BSA and ^{125}I-Clq - HAGG Complexes

^{125}I - Clq was incubated with 100 µg BSA and 360 µg anti-BSA antibody (equivalence) or 250 µg HAGG in CFD at 37°C for 30 minutes. Insoluble ^{125}I- Clq - BSA-anti-BSA complexes were recovered by centrifugation and were either studied alone or after incubation with 3 ml of NHS at 37°C for 30 minutes. Complexes were extracted by PEG as described earlier.

4.4.1b Anti-Clq Affinity Column

The anti-Clq column was prepared as described earlier (2.4.1). PEG extracted and redissolved complexes were poured into a 1.5 ml volume column and left for 30 minutes at room temperature. The unbound material was removed by extensive washing with CFD. Subsequent elution of bound material was effected by the sequential application of CFD buffer containing 0.02M EDTA, 0.5M NaCl and 1M propionic acid. Propionic acid eluates were immediately neutralised with 2M NaOH.

4.4.2 Results

4.4.2a Preparative Enrichment of Immune Complexes with PEG

Further to the results of the comparison between $(NH_4)_2SO_4$ and PEG to precipitate immune complexes (Chapter 3); PEG precipitated much less extraneous protein and it did not precipitate free C1q from EDTA plasma or EDTA-treated sera, as demonstrated by Ouchterlony analysis (Figure 8). EDTA at a final concentration of 0.02M was added before PEG precipitation to dissociate the C1 molecule into its components. When more sensitive quantitative methods were used it was found that 4% PEG could precipitate 10-15% of radiolabelled C1q.

The addition of 4% PEG to sera of patients with seropositive RA, which did not have detectable immune complexes, removed most of the RF activity and it could be subsequently identified in the redissolved precipitate.

4.4.2b Affinity Binding between C1q bound to HAGG and anti-C1q

^{125}I-HAGG was incubated in either NHS or EDTA serum at 37°C for thirty minutes then extracted with 4% PEG and incubated with the anti-C1q column (Table 4). Conditions in

EDTA serum should only allow C1q to react with the HAGG but inhibit subsequent activation and binding of other complement components and in these conditions 63% of the ^{125}I-HAGG bound to the anti-C1q column and most of the bound material was eluted with 0.5M NaCl. In marked distinction, when another sample of the same ^{125}I-HAGG was incubated with NHS only 6% of the added material bound to the column. The maximum binding ever achieved for ^{125}I-HAGG in NHS (29.2%) was never as high as that in EDTA sera or plasma. ^{125}I-HAGG alone did not bind to the anti-C1q column, nor did ^{125}I-HAGG or ^{125}I-C1q-HAGG bind to a Sepharose column coated with a non-specific immunoglobulin (rabbit IgG).

4.4.2c Binding of Preformed Immune Complexes to the anti-C1q Column

BSA-anti-BSA complexes incubated in NHS bound to the anti-C1q column in similar proportions to HAGG (i.e. 15-30% of total complexes). BSA-anti-BSA complexes formed as described precipitated in EDTA serum and therefore their binding in the absence of subsequent complement components could not be tested. BSA-anti-BSA complexes formed in NHS and processed as described above were predominantly eluted in the NaCl and acid washes from the anti-C1q column (Table 5). A comparison of the amount of specific antigen or antibody isolated relative to the total protein eluted in

the various washes, expressed as the proportion of specific labelled material, showed that the acid eluate had the highest yield (Table 5). Increasing the salt concentration to 1M did not dissociate the acid elutable material from the column. Neither free antigen nor free antibody, when incubated in NHS, bound to the anti-C1q column. ^{125}I-BSA-anti-BSA complexes formed in NHS did not bind to a rabbit IgG-Sepharose-4B column. No protein from NHS activated with zymozan and precipitated with PEG as described bound to the anti-C1q column.

4.4.2d Binding of Immune Complexes to the Anti-C1q Column.

Figure 9 shows the molecular weight profiles of ^{125}I-BSA-anti-BSA complexes prepared at equivalence in NHS, precipitated with 4% PEG and redissolved in CFD. When incubated with the anti-C1q column 15-30% of the complexes bound. The unbound complexes had a similar molecular weight profile to those which bound to the anti-C1q column. The failure of the majority of these complexes to bind to the column was not due to saturation of the column, for they also failed to bind when they were reapplied to either a fresh or a regenerated anti-C1q column.

4.4.2e Complexes not Binding to the Anti-C1q Column do Bind to an Anti-C3 Column

^{125}I-C1q was incorporated into BSA-anti-BSA complexes at equivalence and HAGG to further investigate the molecular characteristics of the material which failed to bind to the anti-C1q column. Table 6 shows that high percentages of ^{125}I-C1q bound to the anti-C3 column after previously not binding to the anti-C1q column. It is self-evident that ^{125}I-C1q was present in complexes that did not bind to an anti-C1q column.

4.4.2f Characteristics of Anti-C1q Column Purified Immune Complexes.

By Ouchterlony and SDS-PAGE analysis of the isolated BSA-anti-BSA complexes we were able to demonstrate the expected presence of BSA and rabbit anti-BSA immunoglobulin G (See also Table 5) but in addition there were human serum proteins :- human IgG, IgM, albumin, C3, C3c, C3d and C1q in both of the EDTA and NaCl eluates. Only BSA, anti-BSA, human C1q and C3d were identified in the acid eluates. When either stored or fresh NHS, free of immune complexes, was put through the extraction and isolation procedures described, no detectable protein was recovered from the anti-C1q column.

Figure 10 demonstrates that the molecular weight profiles of the complexes eluted with EDTA and NaCl were similar to each other and to the complexes precipitated from EDTA-treated sera with PEG. Immune complexes eluted with acid were of a smaller molecular size. Subsequent experiments have shown that acid treatment and subsequent neutralisation of preformed immune complexes of known molecular size does not generate smaller size complexes.

4.4.2g Binding of IgM Rheumatoid Factor and Immune Complexes to a Rabbit Ig Column and a Rabbit Anti-C1q Column

A rabbit Ig column was prepared in exactly the same way as that described for the anti-C1q column (Sections 2.4.1 and 4.4.1b). ^{125}I-BSA-anti-BSA complexes (100µg total), formed in NHS and PEG extracted did not bind to a rabbit Ig Sepharose column, but did bind to and were eluted from a rabbit anti-C1q column (Table 7). Immune complex containing HD and SLE sera were PEG extracted as described and incubated with both affinity columns. No protein was eluted from the rabbit Ig column; however, complexed material was eluted from the rabbit anti-C1q column in both cases. NHS sera treated in the same way failed to bind to either column.

Sera from patients with seropositive (sero +ve) RA and

seronegative (sero -ve) RA and sera from patients with active SLE were kindly supplied by Professor G. Panayi, Guy's Hospital Medical School, London SE1. The seronegative sera did not contain IgM or IgG rheumatoid factor (RF) activity as measured by a sensitive ELISA method (Faith et al, 1982).

The seronegative and immune complex negative (IC-ve) samples from patients with RA, PEG extracted as described, failed to bind to both columns (Table 7); however, the seropositive IC negative RA samples did bind to and elute from the rabbit Ig column as well as the anti-C1q column. The eluted material was positive for RF activity by the latex agglutination test and most of the activity occurred in the acid eluates. Immunodiffusion analysis of the acid eluates demonstrated the presence of IgM. IgM RF therefore represents a source of contamination in both the NaCl and acid eluates. Of particular note was the observation that more protein was isolated from the anti-C1q column compared to the rabbit Ig column after incubation with the seropositive IC positive samples obtained from RA patients. RF activity in the EDTA eluates from the anti-C1q column must have been isolated either because of its incorporation into the complexes or because of its subsequent binding to complexes already immobilised on the column.

Figure 8

Ouchterlony Demonstration that Free Clq is not Precipitated by 4% PEG.

wells 1 NHS

2 4% PEG precipitate of NHS

3 EDTA Serum

4 4% PEG precipitate of EDTA - Serum

5 Rabbit anti-human Clq.

Figure 8

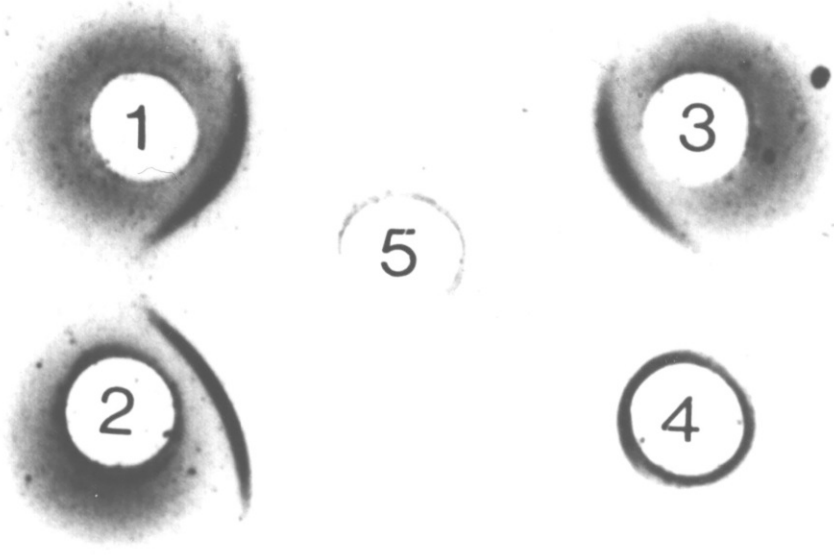


Table 4

Binding of ^{125}I-HAGG to the Anti-Clq Column

^{125}I-HAGG incubated* in:-

	EDTA Serum	NHS
% total cpm bound	63.4	6.1
% total cpm eluted with		
0.02M EDTA	19.7	38.2
0.5 M NaCl	70.9	37.9
M Propionic acid	9.4	23.9

* ^{125}I-HAGG incubated at 37°C for 30 minutes. When incubated in saline <0.3% total cpm bound to the anti-Clq column.

Table 5

Distribution of Protein and BSA - Anti-BSA Complexes in the Three Eluates from the Anti-Clq Column

Eluting Solutions	Antigen labelled complexes		Antibody labelled complexes	
	% BSA eluted+	µg BSA/mg protein*	% anti-BSA+	µg anti-BSA/mg protein*
0.02M EDTA	9.2	2.3	14.1	3.4
0.5M NaCl	43.9	16.1	42.1	19.5
M acid	46.9	51.0	43.8	62.0

* µg antigen and antibody were calculated from recovery of ^{125}I and total protein was measured by the method of Lowry et al, (1951)

\+ % of total label eluted from anti-Clq column

Figure 9

Sucrose Density Centrifugation of ^{125}I-BSA - Anti-BSA Complexes which did not bind to an Anti-C1q Column.

Complexes formed at equivalence and extracted with 4% PEG ●——————●

Complexes which did not bind to the anti-C1q column o——————o

Figure 9

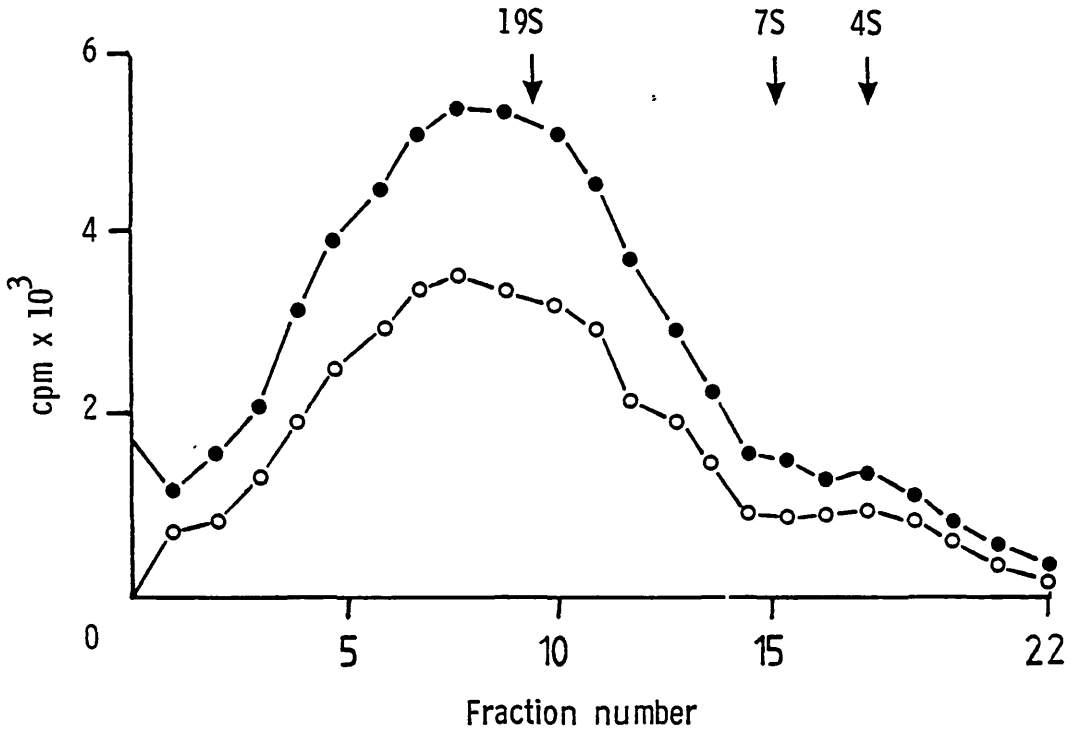


Table 6

Binding of ^{125}I-C1q - Immune Complexes Sequentially to the Anti-C1q and Anti-C3 Columns.

	% bound cpm/total cpm	
	^{125}I-C1q-BSA-anti-BSA*	^{125}I-C1q-HAGG*
Anti-C1q column	19.6	29.2
Anti-C3 column+	56.3	32.9

* incubated in NHS at 37°C for 30 minutes

\+ cpm not binding to anti-C1q column subsequently applied to the C3 column

Figure 10

^{125}I-BSA - Anti-BSA Complexes formed at Equivalence in NHS, PEG Extracted and Eluted from an Anti-Clq Column with :

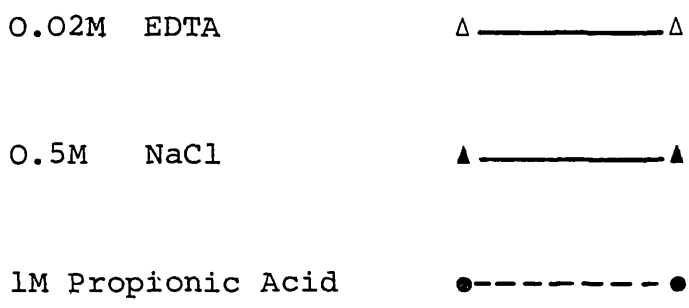


Figure 10

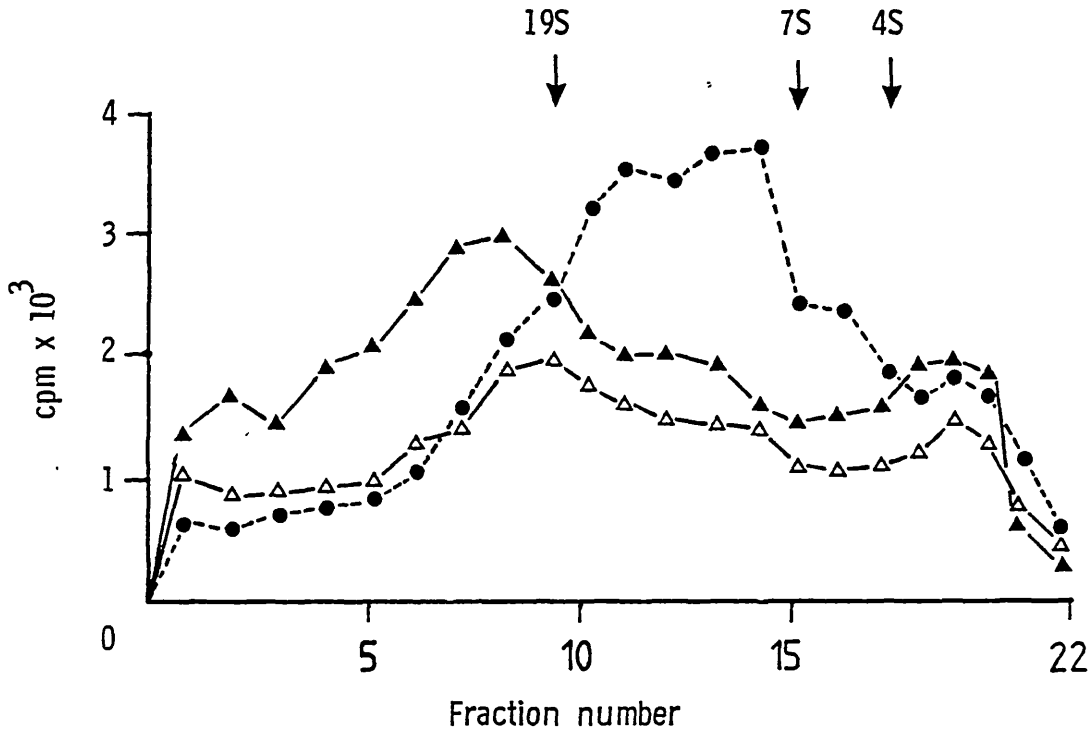


Table 7

Binding of IgM Rheumatoid Factor and Immune Complexes to a Rabbit Ig Column and a Rabbit Anti-C1q Column

a Concentration of rheumatoid factor in IU ml^{-1} (section 2.2.5)

b $\mu g\ ml^{-1}$ of protein eluted from the columns as measured by the method of Lowry et al,(1951)

Two different samples were processed from each disease type. Each serum sample was divided into two equal aliquots prior to PEG extraction and after resuspension the concentrated material was applied to each column.

Table 7

Columns	Rabbit Ig			Rabbit Anti- C1q		
Samples	EDTA	NaCl	Acid	EDTA	NaCl	Acid
Sero+ve RA (IC-ve)	-	(10)[a]	(80)	-	(6)	(80)
	-	28[b]	147	-	63	110
" "	-	(6)	(160)	-	(6)	(160)
	-	41	180	-	83	140
Sero+ve RA (IC+ve)	-	(6)	(30)	(10)	(10)	(30)
	-	39	74	710	560	180
" "	-	(2.5)	(30)	(2.5)	(6)	(30)
	-	50	63	520	341	130
Sero-ve RA (IC-ve)	-	-	-	-	-	-
Sero-ve SLE (IC+ve)	-	-	-	300	146	64
" " "	-	-	-	410	100	38
Sero-ve HD (IC+ve)	-	-	-	630	420	110
" " "	-	-	-	480	260	140
NHS	-	-	-	-	-	-
^{125}I-BSA-anti-BSA complexes in NHS	-	-	-	410	160	52
	-	-	-	370	110	20

4.4.3 Discussion

Clq binding assays are frequently used to detect immune complexes in pathological sera. When therefore attempts were made to isolate these complexes from sera it was logical to utilise Clq as an affinity ligand on an immunoadsorbent column. The first attempts to do this, however, were hindered by the competition between free Cl in serum and the agarose bound Clq for immune complex bound Clq (Svehag and Burger, 1976). In an attempt to overcome this polyethylene glycol was used to precipitate immune complexes from the serum, which were then redissolved and incubated with Degalan beads to which had been attached purified Clq (Casali and Lambert, 1979). Although immune complexes could be purified from pathological sera by using this technique, the quantities isolated were small. An additional disadvantage which we found with this method was the loss of activity of the Clq after two or three isolation procedures.

This work describes the use of an anti-Clq immunoadsorbent column to remove Clq binding immune complexes from serum or plasma. An $(NH_4)_2SO_4$ cut of monospecific anti-Clq serum was coupled to the CNBr Sepharose-4B in order to bind specific anti-Clq antibody molecules randomly

on the solid phase with other non-specific Ig to act as spacers. The effective light substitution of anti-Clq molecules allowed easier elution of bound immune complexes from the column.

An essential preliminary step involved the separation of free Clq from Clq bound to immune complexes and this was achieved by precipitation of bound Clq from EDTA-serum or EDTA-plasma with 4% PEG. The resolubilised material was then applied to the anti-Clq column and eluted in three stages. Three eluting solutions were used in order to examine the bonds between the solid phase anti-Clq antibody and immune complex material bound to the column. Thus 0.02M EDTA dissociated bonds dependent on divalent cations, especially calcium ions; 0.5M NaCl should dissociate binding between Clq and antibody; while M propionic acid should dissociate anti-Clq antibody binding to the Clq molecule. The efficiency of the system has been examined using heat aggregated IgG (HAGG) and preformed immune complexes of BSA - anti-BSA.

HAGG bound to Clq alone, by incubation in EDTA serum, was soluble and was purified with a high degree of efficiency from the anti-Clq column. As expected the majority dissociated in 0.5M NaCl (Table 4). When HAGG was incubated with NHS, however, very much less of the aggregate

was isolated from the column, the majority passing unbound through the column. In examining the possible explanations for this observation,viz. displacement of bound Clq or interference/masking of the Clq by other complement components or proteins, we were able to show that when labelled Clq was pre-incubated with HAGG and the complexed material added to serum, the labelled Clq remained part of the complexes although they failed to bind to the anti-Clq column (Table 6). The non-binding complexes, however, could bind to the anti-C3 affinity column and similar results were obtained when BSA - anti-BSA complexes were used (Table 6). It was likely therefore that after incubation in NHS other proteins, especially C3 and its breakdown products, interfered with binding to the anti-Clq column. In order for BSA - anti-BSA complexes to be solubilised NHS is necessary, and solubilisation is dependent upon adequate levels of C3, factor B, factor D and properdin (Czop and Nussenzweig, 1976). It has been shown that solubilisation depends on intercalation of C3b onto the immune complex lattice. It is likely therefore that molecules such as C3 and C4 and their degradation products sterically interfere with the binding between Clq and anti-Clq on the solid phase column.

Although small quantities of either HAGG or BSA - anti-BSA were added to the serum, much larger amounts of

protein were isolated from the affinity column, particularly in the EDTA and NaCl eluates (Table 5). The isolation of this protein, generated by complement activation, was however dependent on the presence of immune complexes, since no protein bound or was isolated where a PEG extract of zymozan activated NHS was applied to the column. The majority of these serum proteins which were assembled on the immune complexes were susceptible to elution with 0.02M EDTA; even though the mechanisms by which these proteins bind to the complexes and to each other are not known, their EDTA sensitivity suggests that divalent cations are necessary. The generation of fragments containing macromolecular C3 following the addition of immune complexes to NHS probably accounts for the high incidence of positive results obtained when these fragments were identified on gel chromatography. Of four separate assays used to examine the incidence of immune complexes in Hodgkin's disease, the macromolecular C3 assay was more frequently positive when compared with the Clq binding assay, K binding assay and raised levels of plasma C3d (Amlot et al, 1978).

Of the complexes which bind Clq and are available to interact with the anti-Clq antibody a proportion are eluted from the column with 0.5M NaCl. This fraction does however contain many complement components and other non-complement serum proteins such as IgM, IgG and albumin.

The presence of IgG and IgM in in vitro prepared complexes may represent immunoconglutinins or RF, although IgM RF was not detected in the initial sera or PEG extracts. The albumin may be present simply because of its association with IgM and IgG (Mannik, 1967; Hauptman and Sobczak, 1976).

The binding of IgM RF to rabbit Ig represents a source of contamination in isolated complexes from seropositive pathological sera (Table 7). This was most prominent in the NaCl and acid eluates where IgM RF was isolated due to its interaction with immobilised rabbit anti-C1q antisera. PEG extracted NHS and seronegative IC negative sera failed to bind to either the rabbit Ig or the rabbit anti-C1q column. IC positive seronegative HD and SLE sera only bound to the anti-C1q column but not to the rabbit Ig column. IgM RF present in the EDTA eluate must have been either (a) already incorporated into the complexes which were isolated due to their ability to bind to anti-C1q antibodies or (b) co-purified by binding to available human IgG Fc sites on immune complexes already bound to the column. The anti-C1q column was initially developed to isolate immune complexes from Hodgkin's disease sera which do not contain IgM RF; however, this contamination can be reduced by using $F(ab')_2$ fragments of the anti-C1q antibody rather than the whole IgG. On the other hand, pepsin agglutinators in serum samples could still cause contamination by binding to

the $F(ab')_2$ fragments.

The complexes isolated in the acid eluates were smaller than those found in the EDTA or NaCl eluates. The difference was not due simply to an alteration in the ratios of antigen and antibody following extraction, since complexes of known molecular weight profiles showed no alteration in their molecular size following PEG extraction, acid treatment and neutralisation. They may therefore represent complexes which have either a high binding affinity for C1q or those complexes in which interference of binding by complex bound C3b has been reduced following the cleavage of this molecule into its small fragment C3d. Supporting this idea is our finding that antisera to C3d reacted with these complexes, whereas antisera to C3, C3b or C3c did not.

The combination of PEG precipitation of EDTA serum or plasma followed by affinity chromatography on an anti-C1q affinity column is an efficient and reproducible method for isolating immune complexes. Only a small percentage ($\leq 30\%$) of the complexes present were isolated by this method and the EDTA and NaCl eluates contained relatively large amounts of serum proteins which bound to the complexes following complement activation. Nevertheless, the acid eluate was relatively free from these proteins and is clearly the fraction of choice when studies are undertaken to identify

the antigen or examine the specificity of the antibody in an immune complex. It also offers a considerable advantage in that SDS-PAGE of this eluate is free from co-purified serum proteins and their peptide fragments.

Two points of interest arise out of this study concerning the isolation of C1q binding complexes. Firstly, a minority (ranging from 15-30%) of immune complexes having bound C1q have accessible C1q on their surface and yet a larger proportion (50-60%) have C3 or C3 breakdown products on their surface. Secondly, the stabilisation of the C1q to the immune complex by subsequently bound C3 or C4 components is unlikely to allow free interchange between the bound C1q and ^{125}I-C1q as normally performed in the C1q binding assay (C1q BA). These observations lend support to the proposal that C3 in a macromolecular form is a good marker and indicator of immune complexes (Amlot et al, 1976) and has obvious implications in the assessment of immune complexes by assays which recognise C1q (Zubler et al, 1976) as opposed to those that recognise C3 and its activated products (Theofilopoulos et al, 1978; Casali et al, 1977). Thus to obtain a good assessment of immune complexes in pathological sera a combination of assays should be employed in order to detect total immune complexes.

CHAPTER 5

IMMUNOLOGICAL AND PHYSICO-CHEMICAL ANALYSIS OF THE IMMUNE COMPLEXES IN HODGKIN'S DISEASE

5.1 Introduction

Immune complexes have been demonstrated in a wide variety of malignancies (Teshima et al, 1977) where more than 60% of sera from cancer patients showed reduced levels of C3 and C1q together with elevated levels of circulating immune complexes. In other studies (Jerry et al, 1976) immune complexes in melanoma patients were noted to be frequently associated with advanced disease and were shown to drop to undetectable levels following chemotherapy Similar data was obtained by Hoffken et al,(1977) in a study of breast cancer patients where patients identified clinically as having a poor prognosis after mastectomy had elevated levels of complexes compared to those of good prognosis.

The role of immune complexes in malignant disease appears to be less well defined than the classic immune complex diseases, since the patient often succumbs to the cancer before the development of immune complex lesions. Generally manifestation of glomerulonephritis and vasculitis are not common in cancer patients; however, there is suggestive evidence that the association is not simply a fortuitous one. Retrospective post-mortem studies have revealed the presence of immunoglobulin and complement deposits in Hodgkin's disease renal tissue by

immunofluorescence (Sutherland et al, 1974a). In this study only 2 of 23 post-mortem kidneys were positive for immune complexes. Another piece of evidence linking nephrotic syndrome with the deposition of tumour-related complexes was reported by Lewis et al, (1971). In this study immunoglobulins eluted from glomeruli in a patient with nephrotic syndrome associated with bronchial carcinoma showed specific reaction with surface membranes of the tumour cells. Equally, many reports have demonstrated the disappearance of proteinuria following the removal of tumours by surgery or by chemotherapy and these have lent support to the hypothesis that renal disfunction in neoplastic disease may be a specific event rather than a fortuitous one (Cantrell, 1969; Sherman et al, 1972).

Several reports in the literature which draw attention to the association between neoplasm and vasculitis have been reported. Frequently the vasculitis precedes the diagnosis of the tumour and it may even be the first sign of a neoplasm or evidence of recurrent disease. Examples of this pattern of signs and symptoms of vasculitis are prevalent in patients with Hodgkin's disease and CNS vasculitis, and lymphoreticular neoplasms associated with hypersensitivity vasculitis. Although there is only weak association between Hodgkin's disease and central nervous system (CNS) vasculitis there is suggestive evidence for the varicella-zoster as the aetiologic agent of the

vasculitis (Rosenblum and Hadfield, 1972). Post-mortem findings in such patients revealed a histologic picture of panarteritis with infiltrations by lymphocytes and monocytes; however, viral particles were not found and neoplastic cells could not be found in the CNS (Greco et al, 1976).

Hypersensitivity vasculitis can be seen with a variety of neoplastic diseases (Schroeter et al, 1971) including Hodgkin's disease, other lymphomas, multiple myeloma and carcinoma. It is not certain that the underlying lymphoma is responsible for the hypersensitivity vasculitis. However, it is worth noting that cutaneous vasculitis may precede the clinical presentation of tumour by weeks to years. Another association between neoplasm and systemic necrosis is the observation that a patient with hairy cell leukaemia and polyarteritis nodosa subsequently developed vasculitis (Hughes et al, 1979). This patient also had circulating immune complexes. This observation together with the high percentage of cancer patients who have immune complexes (Theofilopoulos et al, 1976; Teshima et al, 1977), suggests that immune complexes play a significant role in the pathophysiology of the vasculitic syndromes seen in patients with cancer.

The expression of antigens on tumour cells should direct humoral and cellular immune responses to the task of killing

the tumour cells. Under certain circumstances, however, the stimulation of the immune system and the formation of immune complexes can actually promote or assist the growth and establishment of the tumour. The mechanisms involved in such defences include T-cell specific killing, B-cells bearing Fc receptors, natural killer (K) cells and antibody dependent cell-mediated cytotoxicity (ADCC).

The antigen-specific recognition unit on T-cells shows the same degree of specificity as the antibody combining site and evidence indicates that T-cells recognise cell surface antigens in conjunction with major histocompatibility products (Taniguchi et al, 1976). It is possible, therefore, that circulating free antigen or antigen complexed with antibody could interfere with such killing mechanisms (Williams, 1980).

The other forms of target killing involve the recognition of antibody bound to Fc receptors on the target cells by Fc receptors on K-cells. ADCC can be inhibited or enhanced by immune complexes, depending on the molar ratio of the antigen and antibody involved. In antigen excess ADCC is inhibited due to its binding of immune complexes to Fc receptors on K-cells; however, in antibody excess ADCC is enhanced due to the presence of free antibody combining sites in the immune complexes bound to the K-cells which can

attack the tumour target cells (Perlmann et al, 1972). The blocking effects of immune complexes are also directed at K-cells bearing Fc receptors. These receptors can become saturated with Fc sites on complexed antibody, thus allowing the tumour cells to evade surveillance.

Evidence has accumulated over recent years indicating that the immune response to tumours can be compromised by the production of "unblocking" antibodies which can abrogate the blocking effect of immune complexes. Hellstrom et al, (1971) showed that sera from tumour-free or cured patients could unblock other sera from patients with progressive tumours of the same type. The current hypothesis is that these unblocking antibodies could be anti-gamma globulins or even rheumatoid factors directed against conformational features of the complexed antibody, or perhaps against their idiotypes (Williams, 1980). These proposed gamma globulins could interact with Fc determinants on the antibodies in the complexes to prevent them binding to K-cells, thus allowing the K-cells to destroy the tumour cells.

A more general phenomenon, which has an immunoregulatory role, is the concept of idiotypy whereby an antibody binding site acts as an antigen to raise anti-idiotype antibodies which then regulate or control immune interactions (Jerne, 1974). Hartman and Lewis (1974) demonstrated in a patient with malignant melanoma, an inverse relation between

antibodies directed against Fab IgG determinants and the presence of anti-tumour antibodies. The binding of anti-idiotype antibodies to tumour specific antibodies could therefore lead to unimpeded progression of tumour growth by generating idiotype-anti-idiotype complexes which would then bind to Fc receptors on the K-cells.

Because of the importance of immune complexes in human disease, attempts have been made over recent years to isolate them from pathological sera in the hope of identifying antigens involved in the pathogenetic processes. Heimer and Klein (1976) showed that 60% of patients with Burkitt's lymphoma had immune complexes as defined by the complement consumption method. The complexed material fractionated between 7S and 19S and almost all of the complexes could be removed by insoluble rheumatoid factor, thus indicating that immunoglobulin was a major component of the complexes. The most interesting observation, however, was that the complexes could bind to conconavalin-A and could be dissociated with alpha-methyl-D-mannoside, suggesting the presence of glycoprotein antigens in the complexes.

The search for specific antigens in immune complexes has been assisted over recent years by the development of preparative isolation techniques (reviewed by Jones and Orlans, 1981). Antigens have been successfully isolated from immune complexes in situations where the nature of the

antigen was already known. In one study, DNA was identified in the complexes of patients with SLE (Theofilopoulos and Dixon, 1979) and in another, the antigenic glycoprotein gp70 was identified from a murine leukaemia using a combination of gel filtration and Protein-A chromotography. Unfortunately the techniques used also isolated other large molecular weight proteins such as IgM, IgA and aggregated IgG along with IgG containing complexes (Vidal and Conde, 1980). Casali and Lambert (1979) used PEG precipitation followed by affinity chromatography on a bovine conglutinin (K) column to identify the putative antigen present in immune complexes isolated from the serum of patients with Kala-Azar. This method had the advantage of using a more specific ligand, C3bi, for isolating immune complexes than those described hitherto.

The presence of immune complexes per se does not necessarily mean that they are pathogenic, for complex formation is part of the normal immunological defence mechanism against foreign antigenic material. In some disease states, however, such as Hodgkin's disease, there is good correlation between the presence of immune complexes and the progression of the disease, even though the classical immune complex clinical features of vasculitis and renal damage are rarely associated with the disease (Amlot et al, 1978).

Unfavourable prognosis in Hodgkin's disease is associated with a greater proportion of neoplastic Sternberg-Reed cells to mononuclear cells and with the occurrence of symptoms such as fever, night sweats and weight loss (symptoms suggesting dissemination of disease). Furthermore, the incidence of immune complexes increases in those types of Hodgkin's disease carrying poor prognosis (Amlot et al, 1976, 1978). Pursuing the rationale that an association between immune complexes and disease manifestation might allow one to identify a disease specific antigen, attempts were made to isolate these complexes and identify their molecular components. Autoimmune disorders such as rheumatoid arthritis (RA) and SLE were also studied since a comparison of the constituents of the isolated complexes would allow the possible identification of a Hodgkin's disease specific antigen. In the case of RA the molecular components of the complexes have been identified (Male et al, 1980) and have been shown to be composed entirely of autologous proteins.

In this final chapter the results of earlier isolation procedures will be presented together with a critical appraisal of three different ligand affinity columns: the C1q and K -Degalan columns (Casali and Lambert, 1979) and the anti-C1q column (Kilgallon et al, 1982) in the characterisation of immune complexes in Hodgkin's disease.

5.2 Modification of Methods

5.2.1 Analysis of Purified Immune Complexes

Immune complexes were isolated from the serum, plasma and synovial fluid samples either by precipitation with 4% PEG (2.2.4) or by a combination of PEG precipitation followed by affinity precipitation on Protein-A Sepharose (2.4.4), Clq or K-Degalan (2.4.3), or anti-Clq bound to Sepharose (2.4.1). The constituents of the purified IC were then examined by SDS-PAGE (2.5.5). Standards for determining molecular weights were obtained commercially from Pharmacia and included Thyroglobulin (330K), Ferritin (200K), Albumin (67K), Catalase (60K), lactic dehydrogenase (36K) and a Ferritin sub-unit (18K) The components of immune complexes were subsequently identified using immunoprecipitation and immunoelectrophoretic procedures using a range of specific antisera. Molecular weight profiles of the purified immune complexes were determined by sucrose density gradient centrifugation (2.2.6) using radiolabelled BSA, human IgG and IgM as the 4S, 7S and 19S molecular weight markers respectively.

5.3 Results

5.3.1 Analysis of PEG Precipitates from the Sera of Patients with Hodgkin's Disease

Ouchterlony and immunoelectrophoretic analysis of 4% PEG precipitates obtained from the sera of patients with Hodgkin's disease, or from healthy controls, revealed the presence of IgG, IgM, IgA, alpha-2-macroglobulin, albumin, C3, C3c, C3d, C4 and transferrin. Clq was only found in the precipitates of immune complex containing sera and CRP was present only in the Hodgkin's disease sera. Although between 70 - 97% of *in vitro* immune complexes, prepared over a wide range of molecular sizes, could be isolated by 4% PEG (Chapter 3, Table 1) the majority of the protein was derived by the precipitation of normal serum proteins. The ratio of specific to non-specific protein was always in excess of 1 : 50. It was not surprising therefore that analysis of such extracts on SDS-PAGE (Figure 1) failed to distinguish normal and pathological sera. Upon reduction and alkylation the banding patterns were compatible with the heavy and light chains of IgM and IgG and C3 breakdown products.

5.3.2. Analysis of Protein-A Sepharose Purified Hodgkin's Disease Complexes.

Following the addition of PEG (4%) the precipitated

material was redissolved and the immune complexes were purified on a protein-A Sepharose column. Ouchterlony and SDS-PAGE (Figure 2) analysis of the purified material showed that IgG was the main protein recovered by this procedure. Although up to 55% of labelled complexes would bind to Protein-A once again the majority of the IgG isolated was derived from the serum IgG precipitated by PEG and the ratio of non-specific to specific antibody was estimated to be in excess of 30 : 1.

5.3.3. Yields of Immune Complex (IC) Material from Clq-Degalan, Anti-Clq and K-Degalan Columns

Serum samples containing Clq binding complexes were processed and portions of the redissolved 4% PEG precipitates applied either to the Clq-Degalan or anti-Clq column. The material which bound was subsequently eluted and was measured for protein. Although the two columns had different binding capacities, the binding capacity of each column was not exceeded. The figures in Table 1 therefore represent the maximum extraction. Two to seven-fold more material was extracted by the anti-Clq column than the Clq-Degalan column. There was only rough agreement between the amount of IC material extracted by each column with each other or with the estimate of IC in the original sample by the Clq binding assay. Using pre-formed BSA-

anti-BSA complexes (400 μg) dissolved in fresh normal human serum, the isolated material probably exceeded twice that weight. If we assume that there was at least 1 mg of complexed material after the BSA - anti-BSA immune complexes had been incubated with fresh NHS, then approximately 8% of the original complexes were isolated by the Clq-Degalan ligand and 20% by the anti-Clq column. This agreed with previous estimates of recovery based on radiolabelled antigen (Kilgallon, Amlot and Williams, 1982).

In Table 2, similar figures are shown for the K-Degalan column. Generally smaller amounts of IC were isolated from pathological sera and this accords with lower values of HAGG equivalents by the conglutinin binding assay.

It should be borne in mind that the expression of unknown IC material in sera or plasma in terms of HAGG equivalents is at best only a rough estimate of the relative amounts of IC between samples tested by the same assay. When BSA - anti-BSA complexes were used as a standard a greater proportion of IC was retrieved on the K-Degalan column than with either anti-Clq or Clq-Degalan. Using the previous estimates of total complexed material, probably some 50% of IC were isolated by the K-Degalan column.

When sera or plasma samples not containing immune complexes were processed, whether from normal subjects or patients with Hodgkin's disease, no detectable protein bound to any of the columns.

5.3.4 Components of Isolated Immune Complexes

Table 3 lists the components identified in eluates from the anti-C1q column. The eluting fluids shown were used sequentially to elute material from IC-containing sera binding to the anti-C1q column. Two of the initial rheumatoid arthritis (RA) samples were seropositive synovial fluids and the remaining samples were seropositive RA sera. In interpreting results it should be remembered that most protein was present in the EDTA eluates and the least in the acid eluates, whereas the proportion of specific antigen and antibody followed the opposite pattern (Kilgallon et al, 1982).

IgM was regularly detected in immune complex material isolated from RA patients but not at all in those complexes isolated from patients with SLE. IgM occurred predominantly in the EDTA and NaCl eluates of BSA-anti-BSA and SLE complexes. IgG was detected in some of the RA complexes, in the EDTA, NaCl and acid eluted material. IgA was not detected in any of the eluted IC material.

Results from Table 7 of Chapter 4 suggest that the IgG and IgM present in the NaCl and acid washes of the anti-C1q column from seropositive RA patients are rheumatoid factors which were isolated due to their interaction with rabbit immunoglobulin on the column. However, the IgG and IgM present in the EDTA eluates must have been isolated either because the rheumatoid factors were already incorporated into the complexes which bound to the column via the C1q ligand, or because they bound to available IgG Fc sites on complexes which were already immobilised on the column, i.e. co-purification.

The presence of human serum albumin in the IC isolates could be explained by its affinity for IgM and IgG (Mannik, 1967) and its detection correlated well with that of IgM. The large molecular weight protein alpha-2-macroglobulin did not bind to the anti-C1q column and C4, transferrin and ferritin were rarely found in the isolated material.

C3 components were regularly detected but it was interesting that they were not detected in any of the acid eluates from pathological sera. In the SLE patients this was not surprising, for sera were obtained during acute exacerbation of the disease when the C3 levels were markedly reduced (10-30%). C3 and C3d were regularly detected in the BSA-anti-BSA acid eluates.

C-reactive protein (CRP) also bound to the column in a calcium dependent fashion, for it was readily eluted with EDTA but was only identified in those sera or heparinised plasma samples which were not treated with EDTA before extraction by PEG. Fibronectin, a "sticky" molecule of large molecular weight, was frequently bound to complexed material. However, recent evidence indicates that C1q binds fibronectin via its collagenous tail and can be co-purified with it (Menzel et al, 1981; Anderson et al, 1981).

Table 4 compares the proteins subsequently identified which bound to the K-Degalan, C1q-Degalan or anti-C1q column from HD serum samples. IC containing material was eluted from the K-Degalan column with EDTA, while the C1q-Degalan and anti-C1q columns were eluted with NaCl. IgG was the major immunoglobulin present in immune complexes from patients with HD, for it was identified in every case by each of the affinity columns. IgA was only occasionally detected and IgM was only isolated on the anti-C1q column. None of the HD sera or HD isolated complexes contained IgM rheumatoid factor and therefore the presence of IgM may simply be a reflection of the increased yield of complexes from this column as compared to the yields from the C1q and K columns. The molecular association between IgM and

albumin (Mannik, 1967) is emphasized because albumin was only identified in the fractions eluted from the anti-C1q column and not from the K or C1q-Degalan columns. C3 and its breakdown components were regularly isolated and C4 identified in a minority of the eluates from the Degalan columns.

CRP was frequently identified and eluted with EDTA from the C1q-Degalan and K-Degalan columns. It has already been shown to elute from the anti-C1q column where PEG precipitation was not carried out in the presence of EDTA.

5.3.5 SDS-PAGE Analysis of Isolated Immune Complexes

Immune complex material isolated from each of the affinity columns was applied to SDS-PAGE. Examples of the banding patterns of immune complexes isolated from Hodgkin's disease (HD) and rheumatoid arthritis (RA) patients are seen in Figures 3 and 4. Figure 3 shows material eluted with 0.02M EDTA and Figure 4 material eluted with 0.5M NaCl (except for the K column where the EDTA eluted material was used both times). The same amount of protein material (50µg) was applied to each sample well, except in Figure 4 where reduced amounts of complexed proteins were eluted from the columns with 0.5M NaCl.

The protein banding pattern differed between HD and rheumatoid arthritis and yet again from BSA-anti-BSA complexes (not shown). The banding pattern was very similar between immune complexes isolated from different patients with HD and, as can be seen from Figure 3, is remarkably similar whether complexes were isolated from K-Degalan, C1q-Degalan or anti-C1q columns. Identification of protein bands on SDS-PAGE gels was achieved by combining immunoprecipitation data on the isolated complexes with the known molecular weights of the identified components and their breakdown products. The most common components were immunoglobulin heavy and light chains and complement proteins.

The clearer and larger number of bands seen on SDS-PAGE of EDTA eluates correspond to the greater amounts of protein found in these eluates.Almost all of the bands could be accounted for by IgM, IgG and the breakdown products of C3. The synovial fluid sample (R+) obtained from a patient with seropositive RA and isolated on the anti-C1q column, contained IgM, IgG heavy and light chains as well as probable C3 breakdown products of the α chain (approx. molecular weight 78K, 70K, 60K, 47K and 42K daltons) and the β chain at 75K daltons (Harrison and Lachmann, 1980)(Index 1). By way of contrast the seronegative serum sample (R-) from an RA patient had no IgM, only IgG heavy and light chains with a restricted range of probable C3 breakdown

products (α chain at 70K and 47K daltons) with no identifiable β chain. C1q (29K daltons) was detected and two other protein bands at 80K and 48K daltons were not identified. The most abundant component was the 78K dalton α chain fragment of C3. This was absent in the seronegative RA serum sample (R-) and was only present in trace amounts in the isolated HD complexes.

The isolated complexed material from the seropositive synovial fluid sample was positive for rheumatoid factor (RF) activity by latex agglutination and showed the presence of IgM by Ouchterlony and immunoelectrophoretic analysis. As IgM RF cannot be eluted from rabbit Ig with 0.02M EDTA (Table 7 of Chapter 4) the suggestion is that it must have been already incorporated into the complexes or alternatively co-purified with existing complexes by binding to available human IgG Fc sites. IgM was not identified in the seronegative serum sample (sample R-, Figure 3). IgM present in the NaCl eluate (sample R+, Figure 4) could be due to contamination as IgM RF can bind to rabbit Ig and be eluted with 0.5M NaCl (Table 7, Chapter 4).

Although the banding pattern in the HD samples differed from the RA samples, similar components were identified. IgG was the major immunoglobulin and IgM was only identified after elution from the anti-C1q column (samples 4 and 5 of

Figures 3 and 4). Probable C3 breakdown components of the α chain (approx. molecular weight 78K, 70K, 60K, 47K and 42K daltons) were detected in all cases along with a 75K dalton fragment which would correspond to the β chain. C1q was not detected in any EDTA eluate. The dominant bands in the HD samples were fragments which would correspond to the C3 β chain (75K daltons), the C3 α chain (70K and 60K daltons) and IgG heavy chain (55K daltons). Of particular note is the small molecular weight band (sample 5 of Figure 3) from a HD patient. It has an approximate molecular weight of 22K daltons and corresponds to the band described by Begent et al, (1980). It was only seen in EDTA eluates. This band corresponds to monomeric C-reactive protein.

Comparison of samples 1 and 2 (Figure 3) showed a remarkable similarity in the bands derived from the isolation of immune complexes from a single serum HD sample using the C1q and K-Degalan columns. This particular patient's sera was positive in both the K binding assay and the C1q binding assay. Sera positive only by the C1q binding assay did not bind to the K column and vice versa.

As was previously seen by Ouchterlony analysis, very little protein was eluted from C1q-Degalan columns by the subsequent use of NaCl (samples 2 and 3 in Figure 4). Sequential elution of HD complexes from the anti-C1q column

with NaCl produced more protein and the major constituents were identified as C3 and IgG. IgM was identified less often. In addition, C1q could be identified in NaCl eluates. In none of the isolated HD complexes was a band identified corresponding to the molecular weight of ferritin.

Antigenic analysis of NaCl eluates from the anti-C1q column showed that IgM RF represented a source of contamination. The presence of IgM (70K daltons) in the NaCl eluate (sample R+ of Figure 4) could have been due to the binding and elution of IgM RF to rabbit IgG (anti-C1q). The presence of IgM RF was confirmed by latex agglutination. All other samples were negative for IgM RF activity.

5.3.6 Sucrose Density Gradient Profiles of Immune Complexes

Immune complex material isolated from the K-Degalan, C1q-Degalan and anti-C1q columns ranged in size from 8S to 40S. There was no notable size difference in complexes eluted from one as opposed to another column. Attempts to dissociate ^{125}I-labelled complexes from pathological sera were unsuccessful. It can be seen in Figures 7a and 7b of Chapter 4 that BSA-anti-BSA complexes prepared with previously labelled antigen or antibody showed the anticipated dissociation at low pH. However, if purified

immune complexes from affinity columns were radiolabelled *in toto*, whether they were from HD (Figure 5) or BSA-anti-BSA complexes (Figure 7c of Chapter 4), then there was a net shift to a higher molecular weight profile at low pH.

SDS-PAGE and autoradiographic analysis of these labelled immune complexes (Figure 6) showed that in HD complexes purified by the anti-C1q column the majority (70%) of the radiolabel resided in a 68K dalton band, which corresponds with a breakdown product of the α' chain of C3. IgG heavy and light chains were also labelled. In the acid eluted complexes (sample 'a' of Figure 7) trace amounts of activity were seen corresponding to the 68K dalton band (C3), the 55K dalton band (IgG heavy chain) and the 25K dalton band (IgG light chain) together with some activity at the top of the gel corresponding to a molecular weight greater than 200K daltons. This latter band could be unidentified large molecular weight material but it more probably represents acid aggregated IgG or complement components.

Figure 1

SDS-PAGE (8.5%) of 4% PEG Precipitated Material from Sera of Patients with Hodgkin's Disease and Normal Subjects

Columns 1 HD (CC)* not reduced

1a HD (CC) reduced and alkylated.

2 HD (CT) not reduced

2a HD (CT) reduced and alkylated

3 Human IgG standard not reduced

3a Human IgG standard reduced and alkylated

4 HD (CJ) with complexes not reduced

4a HD (CJ) " " reduced and alkylated

5 Control sera (DH) not reduced

5a Control sera (DH) reduced and alkylated.

* Patients' initials.

Figure 1

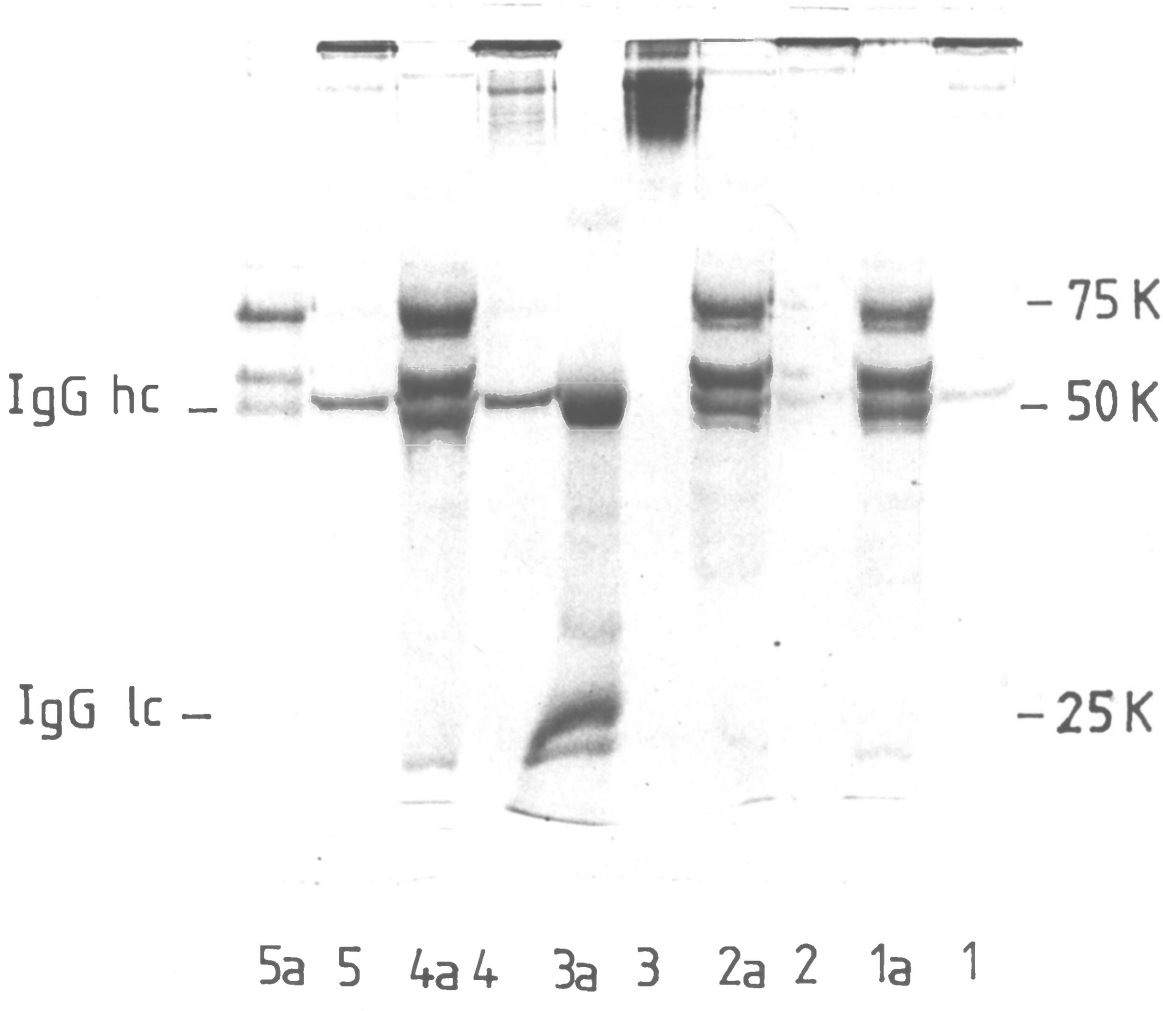


Figure 2

SDS-PAGE (7.5 % gel) of 4% PEG Precipitated and Protein-A Purified Complexes from Hodgkin's Disease Sera and NHS.

Columns	1	HD (JB)	not reduced
	1a	HD (JB)	reduced and alkylated
	2	HD (MS)	not reduced
	2a	HD (MS)	reduced and alkylated
	3	Human IgG standard	not reduced
	3a	Human IgG standard	reduced and alkylated
	4	HD (RR) without complexes	not reduced
	4a	HD (RR) without complexes	reduced and alkylated
	5	Control sera (ML)	not reduced
	5a	Control sera (ML)	reduced and alkylated

Figure 2

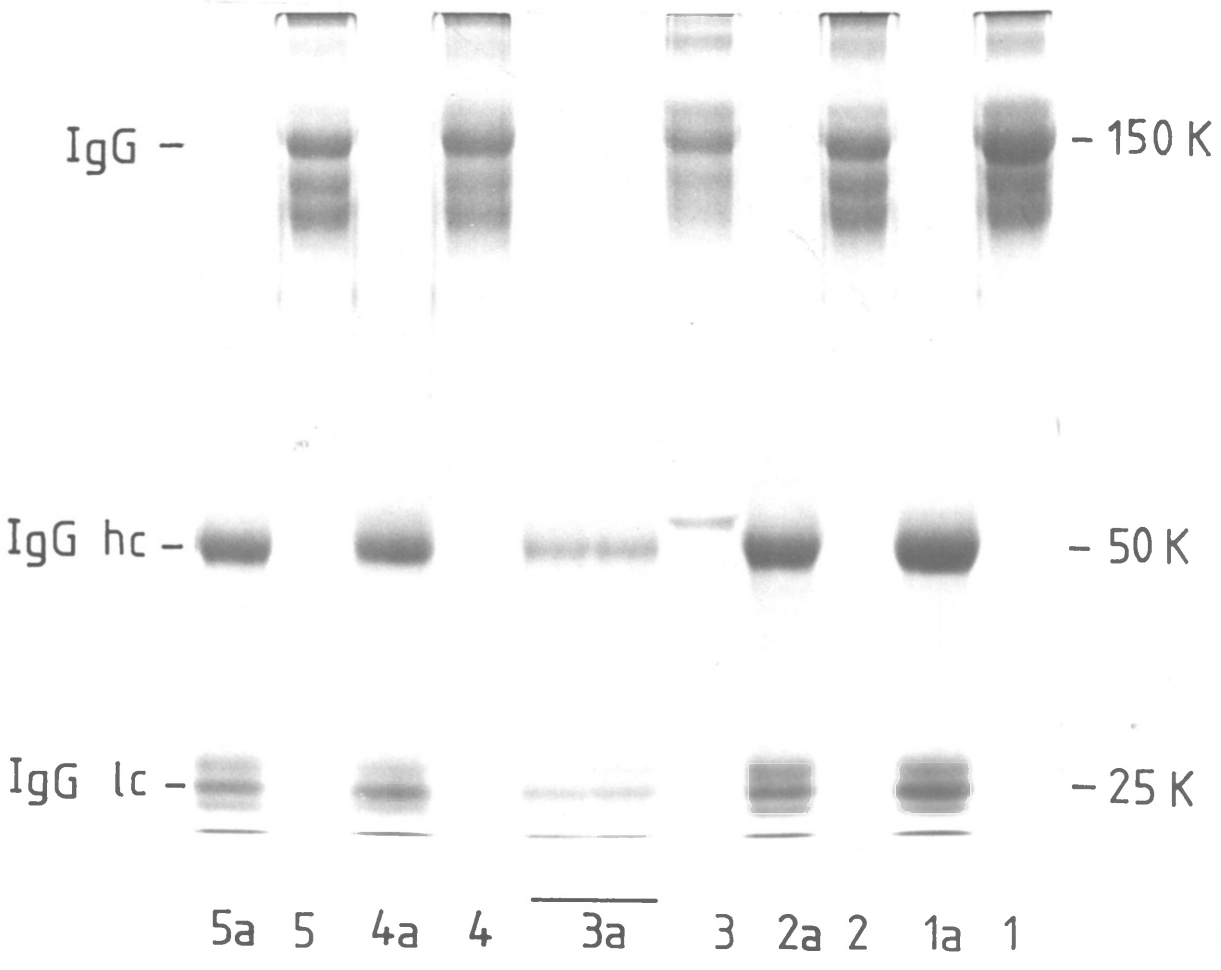


Table 1

Extraction of Clq Binding Complexes from Pathological Sera by the Anti-Clq and the Clq-Degalan Columns.

Patient with HD	Anti-Clq column (µg/ml*)	Clq-Degalan column (µg/ml*)	HAGG equivalents in µg/ml* by Clq BA +
C.D.	116	64	165
P.B.	201	47	80
Y.M.	26	0	60
R.L.	243	86	120
A.McQ.	390	51	140
BSA αBSA	193	78	400**

* µg of protein measured by method of Lowry et al., (1951) extracted per ml. of original serum or plasma.

** based on actual amount of complexed BSA and ∝ BSA

+ Clq binding assay (Amlot et al., 1978)

Table 2

Extraction of K Binding Complexes by the K Column.

Patient (HD)	K-Degalan column (µg/ml*)	HAGG equivalent in µg/ml of KgBA +
K.K.	43	5
J.S.	32	6
M.S.	72	10
C.T.	53	18
F.M.	86	40
BSA αBSA	120	100*+

*µg/ml of original serum or plasma and measured by Lowry et al., (1951)

**based on actual amount of complexed BSA and αBSA

+ KgBA - conglutinin binding assay (Amlot et al., 1978)

Table 3 Proteins Identified in Eluates of Immune Complex Material Binding to the Anti-C1q Column

Human Proteins identified	Immune complex material purified from: BSA-αBSA in NHS (n=5) [a]			Hodgkin's disease (n=9) [a]			Rheumatoid Arthritis (n=6) [a]			SLE (n=3) [a]		
	EDTA [b]	NaCl [b]	Acid [b]	EDTA	NaCl	Acid	EDTA	NaCl	Acid	EDTA	NaCl	Acid
IgM	5 [c]	5	-	4	7	2	6	4	4	-	-	-
IgG	5	5	5	9	9	9	2	4	4	3	3	3
IgA	-	-	-	-	-	-	-	-	-	-	-	-
Albumin	5	-	3	6	6	-	6	4	4	-	-	3
C_3	5	5	3	7	7	-	2	-	-	-	-	-
C_3c	5	5	-	7	5	-	4	2	-	-	-	-
C_3d	5	5	5	1	3	-	4	2	-	-	-	-
C1q	5	5	5	6	6	3	6	6	4	-	-	3
C4	3	-	-	-	-	-	-	-	-	-	-	-
Transferrin	-	-	-	(2)	-	-	-	-	-	-	-	-
CRP	-	-	-	(2) [d]	-	-	(2)	-	-	(2)	-	-
α2-macrogl.	-	-	-	-	-	-	-	-	-	-	-	-
Fibronectin	2	2	2	4	4	-	2	3	4	-	-	1
Ferritin	-	-	1	-	-	1	-	-	-	-	-	-

(a) No. of patient samples.

(b) Eluting fluid used sequentially on column bound material

(c) No. of samples positive cfd. to samples applied.

(d) Figures in brackets are samples not EDTA treated before application to an anti-C1q column.

Table 4 Proteins Identified from HD Immune Complex Material Binding to K-Degalan, Clq-Degalan or αClq Column.

Human Proteins identified:	Immune complex material purified on: K-Degalan column (n=7)[a]	Clq-Degalan column (n=6)	αClq column (n=9)
IgM	-	-	7
IgG	7	6	9
IgA	1	2	-
Albumin	-	-	6
C3	7	6	7
C_3c	7	6	5
C_3d	1	4	3
Clq	-	-	6
C_4	2	2	-
Transferrin	2	2	-(2)[b]
CRP	5	4	-(2)[b]
α2 macrog.	-	-	-
Fibronectin	5	2	4
Ferritin	-	-	1

(a) No. of different patient samples applied.

(b) Only detected where EDTA not used in preparation.

Figure 3

SDS-PAGE (10%) of EDTA Eluted Complexes

Immune complex material eluted with 0.02M EDTA then reduced and alkylated. Subscript abbreviations stand for:

R+	=	seropositive RA synovial fluid sample off the anti-C1q column
R-	=	seronegative RA serum sample off the anti-C1q column
1	=	HD (MS) off K column
2	=	HD (MS) off Clq-Degalan column
3	=	HD (RL) off Clq-Degalan column
4	=	HD (RL) off the anti-C1q column
5	=	HD (PB) off the anti-C1q column
STD	=	standards

Patient MS had both KgBA and ClqBA immune complexes in his serum, while RL and PB only had ClqB immune complexes.

Figure 3

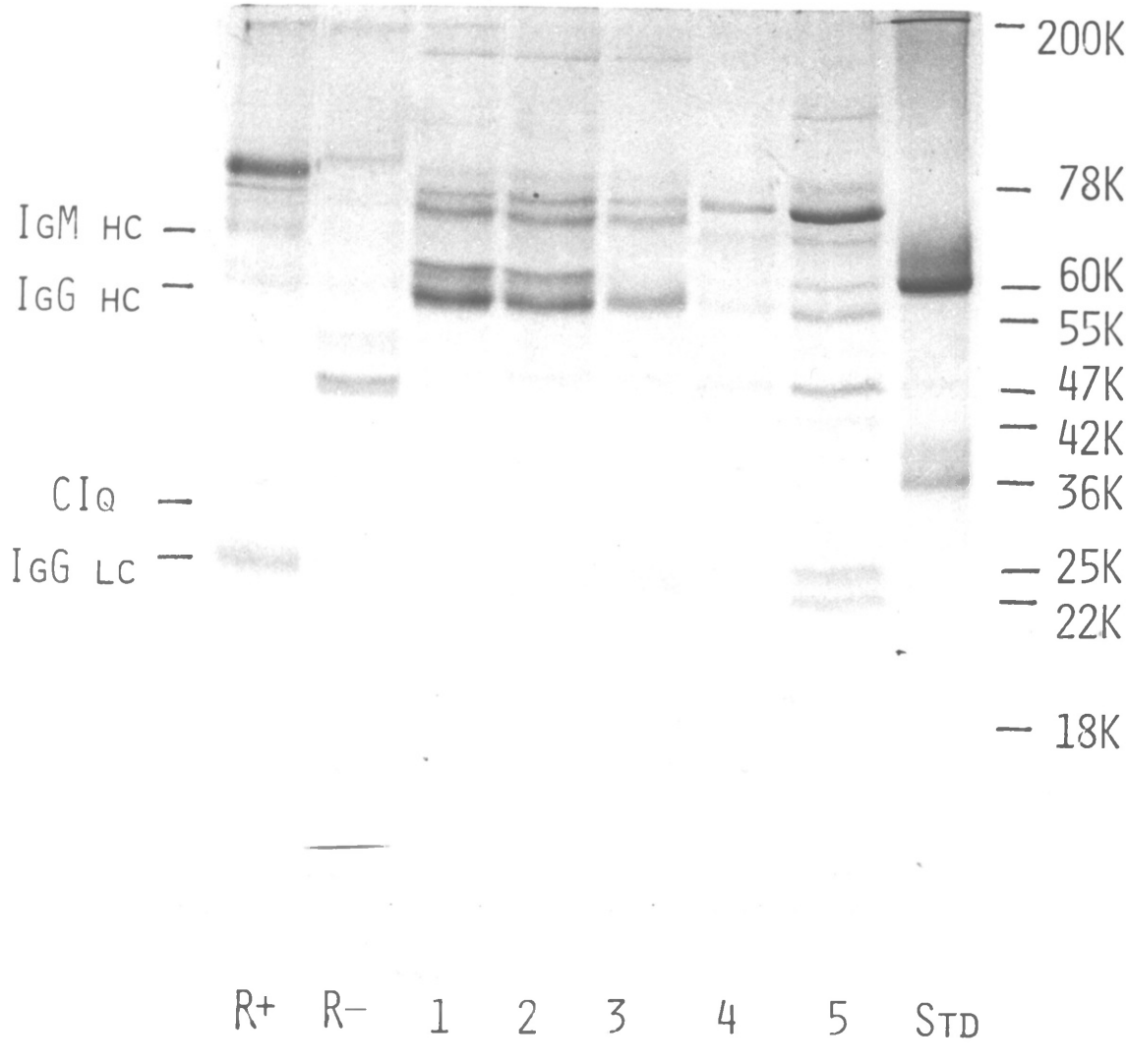

IgM HC
IgG HC
C1q
IgG LC
200K
78K
60K
55K
47K
42K
36K
25K
22K
18K
R+
R-
1
2
3
4
5
STD

Figure 4

SDS-PAGE (10%) of NaCl Eluted Complexes

Immune complex material eluted with 0.5M NaCl then reduced and alkylated. Subscript abbreviations stand for:

R+	=	seropositive RA synovial fluid sample off the anti-C1q column
R-	=	seronegative RA serum sample off the anti-C1q column
1	=	HD (MS) off K column
2	=	HD (MS) off Clq-Degalan column
3	=	HD (RL) off Clq-Degalan column
4	=	HD (RL) off the anti-C1q column
5	=	HD (PB) off the anti-C1q column
STD	=	standards

Figure 4

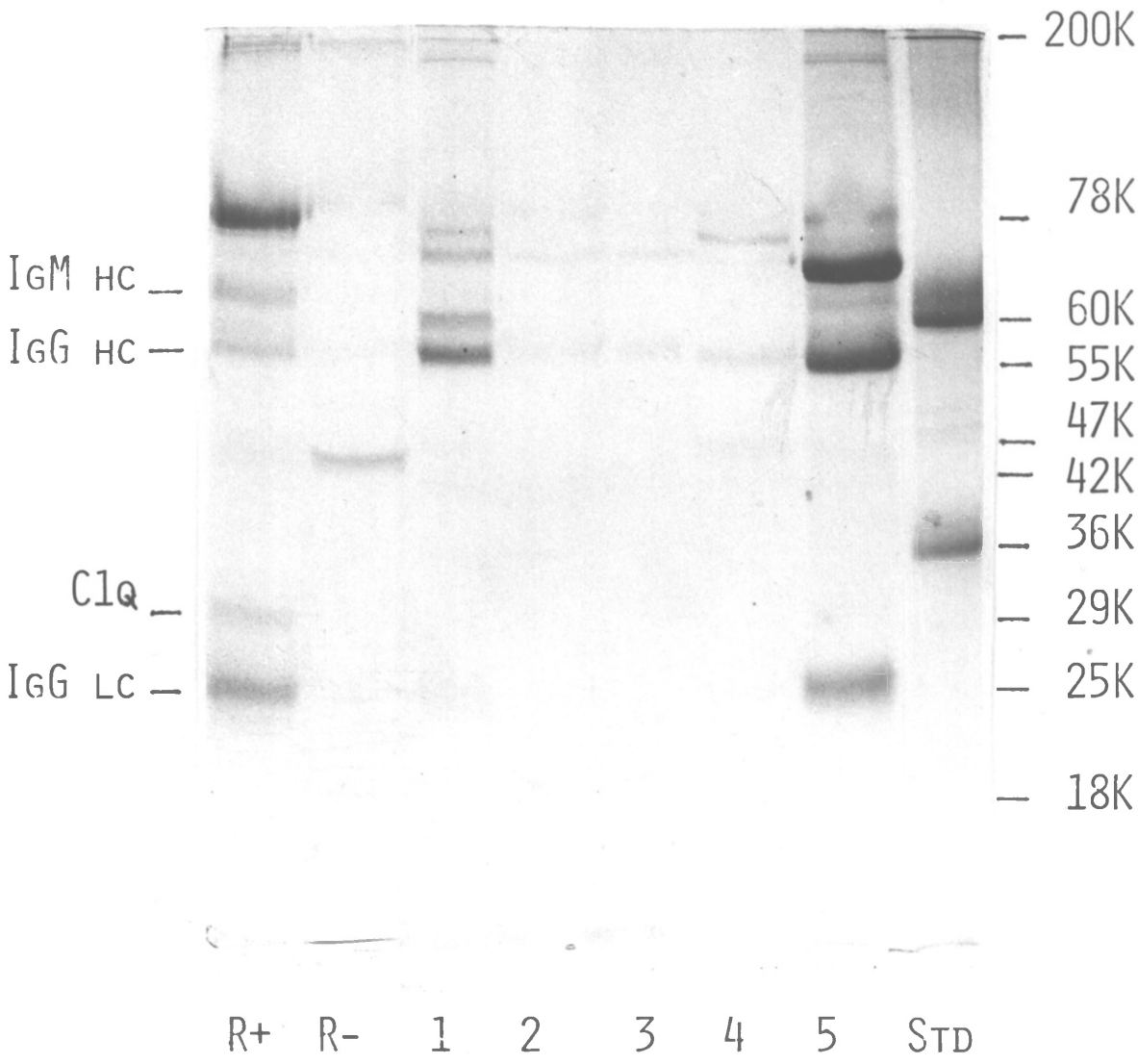

IgM HC
IgG HC
C1q
IgG LC
200K
78K
60K
55K
47K
42K
36K
29K
25K
18K
R+
R-
1
2
3
4
5
STD

Figure 5

Surose Density Gradient Profiles of Immune Complexes Isolated on the K Column at Neutral and Acid pH.

Hodgkin's desease complexes iodinated after purification

●——————● gradient at pH 7.4

o——————o gradient at pH 3.0

Figure 5

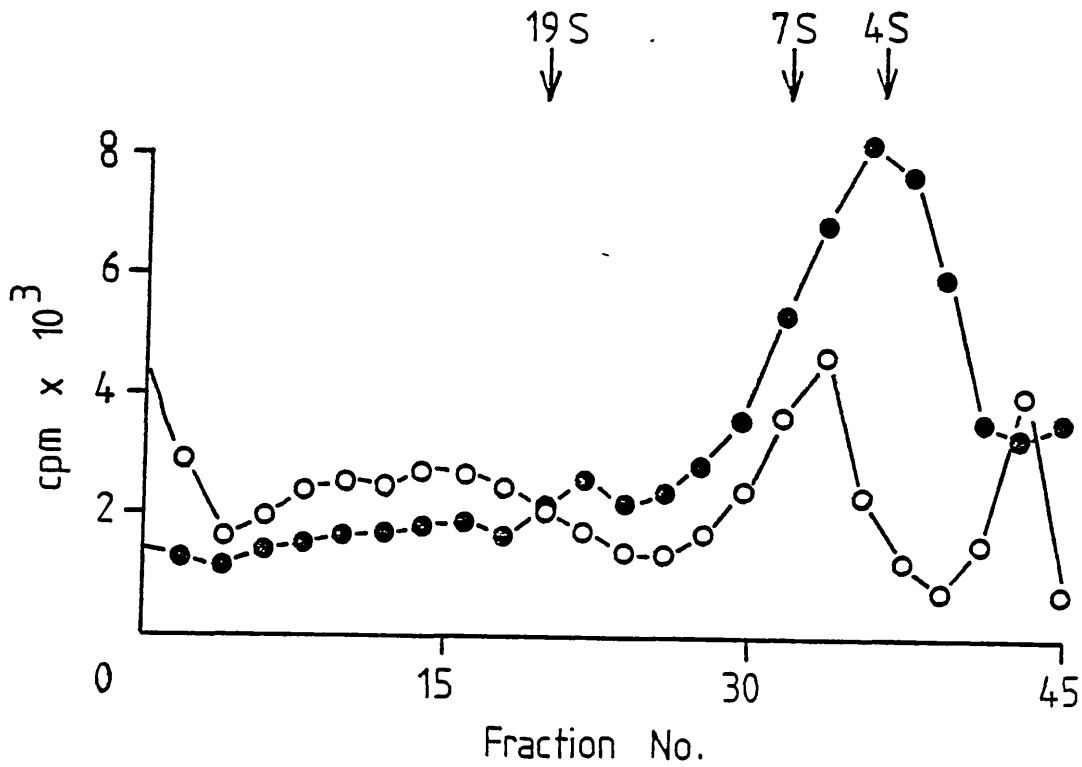


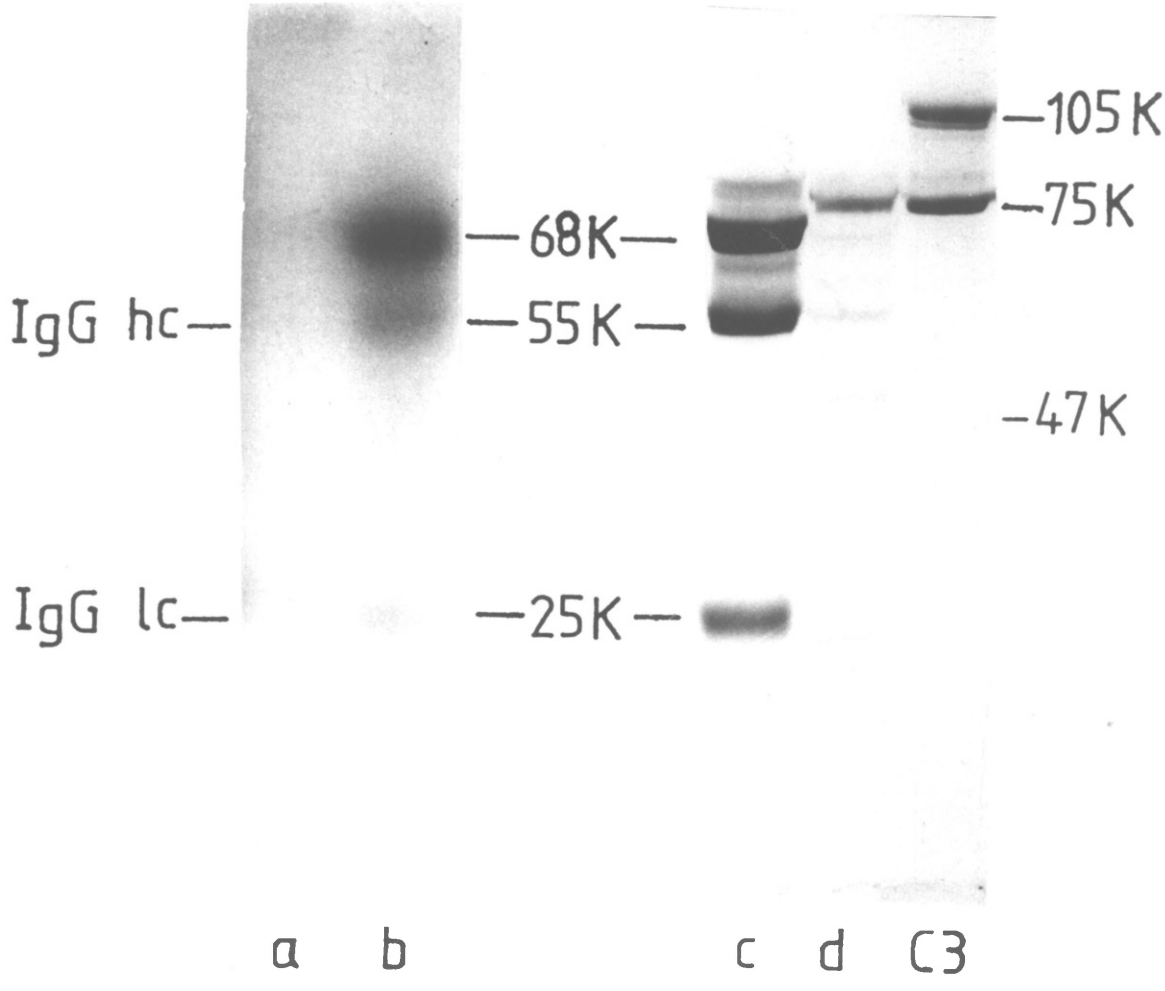
Figure 6

SDS-PAGE (8.5% gel) and Autoradiographic Analysis of Hodgkin's Disease Complexes Eluted from an Anti-C1q Column

a	Autoradiograph of acid eluted complexes
b	Autoradiograph of NaCl eluted complexes
c	NaCl eluted complexes
d	EDTA eluted complexes
C3	standard C3 preparation

All samples were reduced and alkylated.

Figure 6



5.4 Discussion

SDS-PAGE analysis of material precipitated by PEG from the sera of patients with Hodgkin's disease did not show any difference when compared to the material precipitated from NHS (Figure 1). The identification of any specific protein would have been masked by the vast excess of normal serum proteins which were precipitated out of solution by PEG.

Protein-A Sepharose chromatography offered an opportunity to isolate IgG containing immune complexes and therefore improve the chances of identifying a specific antigen. The initial experiments with ^{125}I-BSA - anti-BSA complexes (Chapter 4) showed that only half of the added complexes would bind to Protein-A and since this phenomenon only occurred in fresh NHS, interference by bound complement components was considered the most likely explanation for this. When this isolation technique was applied to the complexes in the sera of patients with Hodgkin's disease SDS-PAGE analysis (Figure 2) showed that the major component was IgG and its concentration was much greater than the concentration of the complexed IgG. IgM was present in trace amounts. Although gel filtration steps could have been taken to remove these components the disadvantages of using time consuming steps in examining a large number of samples outweighed the advantage of such procedures.

In an attempt to overcome the problems encountered with the Protein-A column three other affinity columns were used. The yields obtained and the identity of the constituents within the complexes were compared. Isolation of immune complexes via the Clq ligand was achieved by both the Clq-Degalan and the anti-Clq column. The major difference between these two methods was performance, for the yield of IC with the Clq-Degalan column was much lower by two to sevenfold. The reasons for the relative inefficiency of the Clq-Degalan were threefold. Firstly, the solid phase Degalan (PMMA) beads had a limited capacity to adsorb protein. Secondly, human Clq is a labile protein and its binding activity was severely reduced after two to three column runs. Thirdly, the mechanism by which binding between the Clq-Degalan matrix and IC occurred was probably via free Clq binding sites on available Fc receptors rather than interchange of Clq in a complex with solid phase Clq. This last point probably accounts for the relatively low proportion (estimated 8%) of total IC isolated via the Clq-Degalan column. On the other hand the anti-Clq column isolated IC by binding to available Clq sites on the surface of IC. An estimated 20% of total IC were isolated by this means which represented significantly more than via the Clq-Degalan column but still only accounted for a minority of total IC. Despite the difference in yield between these two columns the isolated material was similar (Figures 3 and 4, and Table 4).

Our inability to demonstrate C1q in isolates from the C1q and K-Degalan columns could be due to several factors. Firstly, the low yields of complexed material isolated from these columns, secondly, the steric position of C1q within the complexes and thirdly, the consequences of C1 binding by the HD complexes. Fust et al, (1978) outlined three different consequences of C1 binding: (a) C1 binding with no activation, (b) transient fixation of C1 with activation and (c) strong binding of C1 with activation. Although the second mechanism has only been shown to occur in vitro, it is nevertheless possible that within heterogenous immune complexes present in HD, a proportion of these complexes could activate C3 even though the affinity of C1q binding was low. This would tend to produce C3 containing complexes, but C1q would not be identified.

The regular demonstration of C1q in eluates from the anti-C1q column was expected in C1q binding immune complexes, but it was possible that small amounts of undissociated C1 or C1q were still present in the PEG precipitates of EDTA-treated serum samples and that this was concentrated after elution of the anti-C1q column. Furthermore, the presence of C1 or C1q on the column may have allowed reverse binding of IgM.

The K-Degalan column isolated immune complexes (IC) because of the affinity between bovine conglutinin (K) and

C3bi present on the surface of IC. Binding of K to PMMA beads was no more efficient than C1q but it was reliably stable and the K-column could be used many times without loss of activity. An estimated 50% of total IC was isolated by the K-column and presumably represented the larger amounts of C3bi bound to the complexes. This may also explain why detection of IC estimated as HAGG equivalents is more sensitive using the conglutinin binding assay (KgBA) than the C1q binding assay (C1qBA). If the preparation of HAGG used is efficient in complement activation, then the more frequent presence of C3bi on its surface should mean smaller amounts are required for detection than C1q in the C1qBA.

There was a discrepancy between the efficiency shown by the C1q column in isolating IC and the frequency with which the KgBA and the C1qBA detect IC in vivo. In Hodgkin's disease the C1qBA was positive four times more often than the KgBA (Amlot et al, 1978). Usually the one assay is positive to the exclusion of the other, suggesting different types of complexes. Similar findings have been observed in RA and leprosy (Casali et al, 1977) as well as SLE (Eisenberg et al, 1977). In SLE during acute exacerbations of the disease the C3 level can fall dramatically and complexes may not be sufficiently coated with C3, as in the patients in this study, to react with K. This situation does not hold, however, for HD, RA or leprosy in which

usually there are elevated levels of C3. There are three possible explanations for this discrepancy. Firstly, the C3 coating of KgB complexes is a potent ligand for cells of the RES, thus favouring their removal in vivo. KgB complexes therefore may only be detected in the circulation when the RES is "blocked". Secondly, the detection system in the KgBA is via immunoglobulin and like C1q the immunoglobulin molecule may be masked by complement components. Immune complexes may bind, therefore, to K but may not be detected by the anti-Ig. Thirdly, there are likely to be important differences between in vitro prepared complexes which are solubilised by NHS and IC occurring in vivo. The answer to this discrepancy has not been ascertained but the first and third explanations are favoured as they are mutually compatible. The fact that pathological sera positive for IC only by the C1qBA did not bind to the K-column argues against the second explanation. C3 is an important component for solubilising in vitro prepared IC and the heavy coating of C3 and its breakdown products on in vitro prepared complexes favour its binding to K,while the same C3 ligand would favour its removal in vivo.

The main difference between the three columns was that IgM, albumin and C1q were found in the eluates from the anti-C1q column, but not from the C1q and K- Degalan columns.

The explanation for this may be due simply to the low yields of protein observed in comparison to the anti-C1q column. It is most unlikely that the IgM isolated from immune complexes in Hodgkin's disease represented RF binding to rabbit immunoglobulin since all sera and complexes isolated from Hodgkin's disease sera were negative for RF activity.

The situation in rheumatoid arthritis samples is very different. Table 7 (Chapter 4) clearly demonstrates that IgM RF can be eluted from a rabbit Ig column with NaCl and acid. No RF activity, however, could be eluted with EDTA. The suggestion, therefore, is that in the seropositive immune complex positive RA samples IgM RF present in the EDTA eluates must have been either incorporated into the complexes which were isolated due to their binding to anti-C1q antibodies or because of its subsequent binding to complexes already immobilised on the column (Figure 3, Table 3). The presence of IgM RF in the NaCl and acid eluates, however, could well be due to non-complex bound RF being co-isolated via its interaction with rabbit immunoglobulin. This disadvantage could be overcome by using $F(ab')_2$ fragments of the anti-C1q antibody; however, pepsin agglutinators in rheumatoid sera and synovial fluid could still cause contamination by binding to the $F(ab')_2$ fragments.

Comparison of the components of immune complexes from

different disease states may help to elucidate their pathological roles. In the synovium where there is local synthesis of IgG and self-associating IgG RF, the pathogenetic mechanisms of tissue damage are established. These complexes activate the complement system and via the release of chemotactic and biologically active C3 and C5 breakdown components, there is an ingress of polymorphonuclear leucocytes and mononuclear cells into the areas of acute inflammatory reaction. The subsequent release of enzymes such as collagenase and proteinase leads to cartilage breakdown and collagen degradation. The demonstration of fibronectin in RA complexes may also have significance in this respect, for it has a proven affinity for collagen (Engvall et al, 1978) and may therefore facilitate the adherence of complexes to collagen fibre in the joint capsule and cartilage matrix. The resulting local enzyme release by cells will therefore be concentrated in the joint capsule, collagen fibres in the cartilage matrix and even blood vessels.

The presence of IgM rheumatoid factor may have variable biological effects, depending on the system in question. Although IgM binding to immune complexes will increase their size, the result of such an interaction can be enhancement or suppression of complement fixation, depending on the properties of the complexes (Schmid et al, 1970). Hurd et al (1970) showed that soluble complexes could only be

phagocytosed by polymorphs *in vitro* after the addition of IgM RF. The suggestion from this was that the interaction of IgM RF with the complexes altered them in such a way as to facilitate phagocytosis and possibly enzyme release. Similar work relating to the role of IgM RF came from Yeatts *et al*, (1978) who showed that only after the addition of IgM RF to soluble aggregates of IgG could platelets be induced to release biologically active amines. This could facilitate their vascular deposition.

The major feature of rheumatoid arthritis is the absence of renal disease. This is probably a reflection of both the size and the composition of the circulating immune complexes in the blood. In the joint the local synthesis of IgG antiglobulins can give rise to large self-associated complexes; however, in the circulation this situation is rare because of the relative abundance of autologous IgG which can competitively inhibit this self-association. The immune complexes thus generated are small, readily dissociable, non-complement fixing and therefore less likely to have a pathogenetic role.

The ability of complement to solubilise immune complexes is an important factor, not only in rheumatoid arthritis but also in other diseases where immune complexes have pathogenetic roles. Miller and Nussenzweig (1975) were the first to demonstrate solubilisation of antigen-antibody

precipitates. The alternative pathway of complement was responsible for this phenomenon although its efficiency was greatly enhanced by the participation of the classical pathway due to the initial generation of C3b. This process *in vivo* would produce solubilisation of immune precipitates, favour their removal from sites of deposition and reduce their capacity to induce inflammation. Assays which measure this particular activity of the complement system have shown that in man its function is impaired in a variety of immune complex mediated diseases, or those diseases which are associated with hypocomplementaemia (Schifferli *et al*, 1981).

More recently Schifferli *et al*, (1980, 1982) have drawn attention to another property of the complement system, viz. its ability to inhibit the precipitation of immune complexes incubated at 37°C in normal human serum. Unlike solubilisation of immune precipitates, which depends on an intact alternative pathway, the prevention of precipitation has an absolute requirement for the intact classical pathway. The biological significance of this process is not clear; however, it is possible that the failure to prevent the precipitation of immune complexes formed within the circulation may play an important part in the pathogenesis of immune complex diseases and in particular those associated with a variety of inherited complement deficiency syndromes.

It has recently been reported (Naama et al, 1983a,b) that the sera of patients with RA, with normal or raised complement levels, were unable to maintain antigen-antibody complexes in solution. Furthermore, the addition of rheumatoid sera to normal sera containing immune complexes inhibited the solubilising effect of complement. This inhibition was most easily demonstrated in heat inactivated sera, was absent from samples obtained from patients with seronegative arthritis and occurred most frequently in the sera of patients who exhibited the extra-articular features of RA. Neither the mechanism of action of this inhibitor nor its nature has been fully established, although it has been proposed that it acts by preventing the binding and/or activation of the first component of complement. The association of this inhibitor with extra-articular features of RA, and the recognition that a more aggressive clinical course of the disease with the presence of nodules or vasculitis are associated with high titres of rheumatoid factor, suggests that the inhibitory factor may be associated with rheumatoid factor and may therefore be important in disease pathogenesis.

The observations made in this work concerning the immune complexes in RA bear close agreement with previous articles. Male et al (1980,1981) reported that immune complexes in RA were composed mainly of IgG and IgM with only moderate amounts of C3. However, when

complement-fixing immune complexes were isolated by the conglutinin column a larger proportion of C3 products were identified along with IgG and IgM. This data along with that presented here is consistent with the view that antiglobulins provide the main complement-fixing antibody system in RA.

The complexes identified in SLE (Table 3) were a reflection of the degree of complement activation in the disease. All patients studied were hypocomplementaemic and thus the absence of complement components in the complexed material was expected. The major organ of involvement in these patients was the kidney and an examination of the urine deposits from each individual revealed proteinuria with both hyaline and granular casts. The components identified in the IC were IgG, C1q and albumin. IgM RF was not present in the complexes or the original sera.

Immunofluorescent and ultastructural examination of biopsy and autopsy materials from SLE patients has established the involvement of immune complex deposits in the kidney disease associated with SLE. The presence of immunoglobulin and complement components in the glomerula basement membrane have been taken to indicate that activation of the complement system is involved in production of the renal lesion. The exact mechanism by which complexes deposit in the kidney is still not clear.

Koffler et al, (1971) has suggested that C1q can bind to DNA in the kidney and bring about complement activation because of the known ability of C1q to precipitate DNA and associate with ss-DNA in the kidney. Another suggestion put forward by Izui et al, (1976) is that DNA antigenic components can bind to collagen structures in the kidney and thus bring about the formation of immune complexes in situ and subsequent complement activation. This situation may also apply to the deposition of immunoglobulin and complement in the dermal-epidermal functional layers of the skin in patients with active SLE (Gilliam et al, 1974).

The complement system functions to solubilise immune complexes so as to reduce their size, facilitate their removal by the RES and prevent their precipitation and deposition in tissues. In SLE the presence of hypocomplementaemia and a defect in Fc receptor-mediated clearance (Frank et al,1983) provides a favourable environment for the deposition of immune complexes and thus the eventual tissue damage. This is in contrast to RA where no RES blockade occurs. Baatrup et al, (1983) have demonstrated the existence of incompletely complement solubilised immune complexes in SLE. SLE sera was shown to inhibit the complement mediated solubilisation of in vitro prepared immune complexes by normal human serum (NHS). Furthermore, endogenous immune complexes in SLE sera could be solubilised by the addition of NHS. The suggestion from

this work was that the disease activity in SLE sera may be not only a reflection of high levels of immune complexes and autoantibodies but may also be the result of the incomplete solubilisation of phlogistic immune complexes.

Fibronectin was only found in one acid eluate from SLE complexes. However, it could be postulated that its affinity for collagen facilitates the deposition of complexes in renal glomeruli and may indeed act as an immunoadsorbent for more C1q containing complexes. This is supported by recent evidence (Kono et al, 1983) which demonstrates that fibronectin can bind C1q in SLE cryoglobulins and that the binding is calcium dependent.

Although Hodgkin's disease complexes contained similar molecular components to RA complexes, i.e. IgG, IgM, C1q and C3, their expression in terms of disease activity is markedly dissimilar. The major symptoms associated with immune complexes in Hodgkin's disease are those of fever and night sweats; the hypothesis being that the ingestion of complexes by leucocytes stimulates the synthesis and release of endogenous pyrogen. In patients with such symptoms the C1q binding assay was positive four times more frequently than the K binding assay (Amlot et al, 1978). This evidence suggests that those complexes with C3 components on their surface may be the ones which are more susceptible to phagocytosis and thus eventual pyrogen release. In vitro

studies have shown that antigen-antibody complexes are chemotactic to and can be phagocytosed by leucocytes with subsequent degranulation and release of permeability factors (Root and Wolff, 1968). These findings can be related to the fact that leucocyte alkaline phophatase levels correlate with relative disease activity (Bennet et al, 1968), and that leucocytosis in the absence of infection may be associated with poor prognosis in this disease. Equally, Sheagren et al, (1967) demonstrated that clearance of aggregated albumin by the RES is more rapid in the later stages of the disease than in the earlier stages. Hence, there may well be a close interrelation between phagocytosis of immune complexed material and the development of symptoms such as fever and night sweats.

The presence of vasculitis and glomerulonephritis in Hodgkin's disease is not a frequent occurrence; however, IgG and C3 deposits have been demonstrated in two out of twenty three post-mortem kidneys and five out of twenty two kidneys biopsied during staging laparotomies (Sutherland et al, 1974a). In addition, immunoglobulin and complement deposits have been demonstrated in renal biopsies of patients with a variety of cancers (Sutherland et al, 1974b); however, their role in disease pathogenesis has not been clarified. The exact mechanism of deposition resulting in such minimal change nephropathy is not known but it is possible that local increases in glomerula permeability

brought about by other stimuli, for example lymphokines, could facilitate the deposition of immune complexes in Hodgkin's disease and other cancers where nephritis is not a common complication. One particular view relating to such minimal change nephropathy was put forward by Moorthy et al, (1976). These authors observed no specific glomerula abnormalities in HD renal tissue, of which the majority was from patients with mixed cellularity HD. They suggested that the presence of functional glomerula damage without tissue damage could be attributable to lymphokines released from lymphoid tissues involved in the mixed cellularity lesions of Hodgkin's tissue. The link between glomerulonephritis or nephrotic syndrome to disorders of cell-mediated immunity has not been firmly established in Hodgkin's disease although suggestions supporting this view have been reported (Cohen et al, 1974)

Analysis of acid eluates of Hodgkin's disease complexes revealed the presence of immunoglobulin and complement components; however, as very little material was isolated more sensitive methods were required to identify the components. SDS-PAGE followed by autoradiography (Figure 6) showed feint lines for IgG heavy and light chains and a dense band at 68K daltons which was compatible with a fragment of the α chain of C3. This observation adds support to the suggestion that C3 breakdown products are the most accessible proteins for radiolabelling in complement

activated IC and that aggregation of these labelled components at acid pH could explain why dissociation of immune complexes apparently did not occur (Chapter 4, Figure 7; Chapter 5, Figure 5).

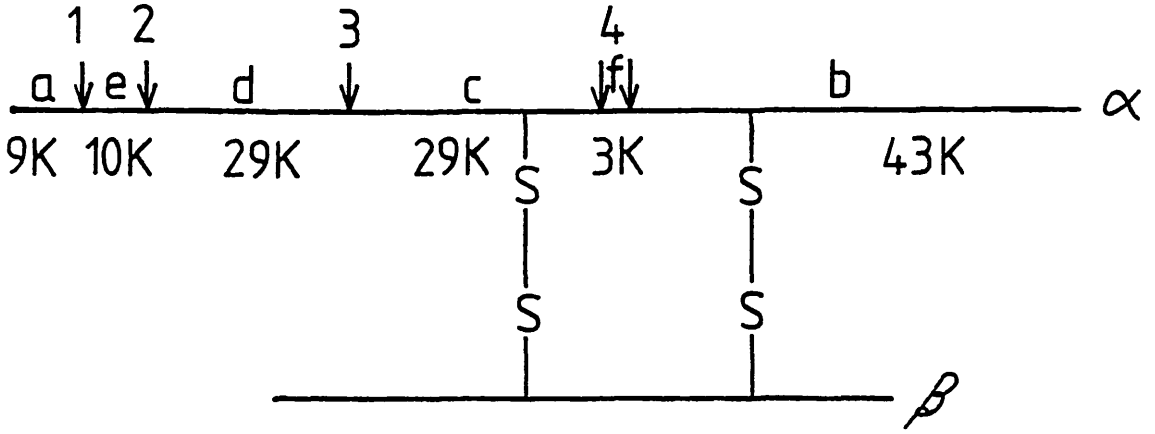
Another approach to the specific identification of protein bands in SDS-PAGE is the immunoblotting technique (Towbin et al, 1980). This offers increased sensitivity for the detection of known serum constituents but may still fail to detect foreign antigenic material present as only a minor constituent of the total complexed material. Recently Maire et al, (1983) used this technique to identify components of IC from sera of patients with SLE. SDS-PAGE of isolated IC showed a qualitative similarity between different patients studied and, following immunoblotting, immunoglobulin and complement components were identified. In one out of five patients CRP was detected in the isolated complexes. This strengthens the observation in this work that CRP can be present in IC. However, whether it reacts with complement coated IC or forms a complex with microbial polysaccharides or other cell constituents with subsequent binding of complement components remains unanswered.

All the physical characteristics of IC isolated from HD patients were compatible with the characteristics of complement activated immune complexes. They behaved in a similar manner to in vitro IC during isolation but they

displayed different SDS-PAGE banding when compared to *in vitro* IC and IC from other diseases. Although similar serum components were identified in RA, SLE and HD complexes, their manifestation in terms of pathological disease was quite different. This difference was not simply quantitative in terms of IC components but was more a reflection of subtle changes in the composition of the IC.

No disease specific component was identified in the HD complexes. This may have been because such a component was in such low concentrations that it failed to be detected by the methods used. Alternatively, if the antigenic component was a lipid or lipoprotein it is possible that the use of Frigen in the original extraction process could have removed the antigen along with other lipids. The use of Frigen to extract lipids from sera prior to PEG extraction facilitated the resuspension of the PEG precipitates. From this work it is clear that the acid eluted material from the anti-C1q column was the most relevant for the identification of foreign antigenic components; however, more sensitive techniques will have to be employed in this pursuit for the antigenic components of immune complexes are only present in minute amounts compared to the vast excess of complexed proteins.

INDEX I



The sequence of α chain fragments within the intact α chain of C3. The fragment designation is indicated above and its size below its position in the chain. Points of action of of serum proteases are indicated by arrows.

1) C3 Convertase ($\overline{C42}$, $\overline{C3bBb}$) producing C3a+C3b;

2) unidentified protease possibly releasing C3e from surface bound C3 and, in the fluid phase, generating C3d and C3e;

3) unidentified protease releasing C3c from C3d;

4) C3b INA and β 1H dependent proteolysis (i) giving rise to C3bi from C3b, with the possible release of a small fragment C3f (α^f); (ii) acting on intact C3 to produce an altered C3 molecule, possibly C3x.

from Harrison and Lachmann (1980)

Concluding Remarks

The work presented in this thesis describes a new method for isolating immune complexes from pathological fluids. In an *in vitro* system, whereby complexes of BSA and anti-BSA were added to normal human serum, we observed the production of large amounts of high molecular weight material which could be subsequently purified by a variety of ligand affinity columns. The isolated macromolecules comprised mainly complement proteins, IgM, IgG, C-reactive protein and fibronectin. The generation of these macromolecules was dependent upon the activation of the classical pathway of the complement system by the immune complexes.

The anti-C1q affinity column was then used to isolate immune complexes from the sera of patients with Hodgkin's disease and Systemic Lupus Erythematosus and from the sera and synovial fluid of patients with Rheumatoid Arthritis. In most instances the isolation of immune complexes was specific and dependent on C1q binding to the complexes. In patients with rheumatoid arthritis, however, rheumatoid factor was also isolated either because it was incorporated into the complexes formed in serum or because of direct binding to the anti-C1q antibody bound to the immunoadsorbent column.

Most of the macromolecular material generated following

complement activation was non-specific and was dependent on the presence of true immune complexes in the sera. Activation of the alternative pathway of complement alone could not generate such macromolecules. An examination of the eluates obtained from the anti-C1q column showed that the majority of the specific antibody and antigen was isolated in the acid wash. This finding would therefore suggest that any attempt to identify the specificity of the antibody present in the isolated complexes should be directed towards this eluate.

SDS polyacrylamide gel electrophoresis was used to examine the protein bands isolated from both the artificially prepared complexes and the complexes isolated from patients with RA, SLE and Hodgkin's disease. No protein bands were identified which were specific to any disease category. Although formal demonstration of the identificatin of the proteins was not carried out, the molecular weights of the isolated proteins were consistent with the known molecular weights of the various fragments of the complement system, IgM and IgG. Qualitative differences between the complexes were identified and some of these differences may have been relevant to the part played by these complexes in determining both the extent and site of tissue injury.

REFERENCES

AGNELLO,V., ARBETTER,A., KASEP,G. et al. (1980) Evidence for a subset of rheumatoid factors that cross-react with DNA-histone and have a distinct cross-idiotype. J.Exp.Med. 151,1514.

AGNELLO,V., KOFFLER,D. and KUNKEL,H.G. (1973) Immune complex systems in the nephritis of Systemic Lupus Erythematosus. Kidney Int. 3,90.

ALBERTSON,P.A. (1960) Partition of cell particles and macromolecules; distribution and fractionation of cells, viruses, microsomes, proteins, nucleic acids, and antigen-antibody complexes in aqueous polymer two-phase systems. Almquist and Wiksell, Uppsala.

ALLISON,A.C. (1978) Macrophage activation and non-specific immunity. Int.Rev.exp.Path. 18,304.

ALMEIDA,J.D. and WATERSON,A.P. (1969) Immune complexes in hepatitis. Lancet 2,983.

AMLOT,P.L., SLANEY,J.M. and WILLIAMS,B.D. (1976) Circulating immune complexes and symptoms in Hodgkin's disease. Lancet i,449.

AMLOT,P.L., PUSSEL,B., SLANEY,J.M. and WILLIAMS,B.D. (1978) Correlation between immune complexes and prognostic factors in Hodgkin's disease. Clin.exp.Immunol. 31,166.

AMOROSO,P., VERGANI,D., WOJCICKA,B.M., McFARLANE,I.G. et al (1980) Identification of biliary antigens in circulating immune complexes in primary biliary cirrhosis. Clin.exp.Immunol. 42,95.

ANDERSON,B., ENTWISTLE,R., PUYAT,L. et al (1981) Fibronectin associated with C1q in a C1q isolation procedure. Immunol.Comm. 10, (8),687.

ANDERTON,B.H. and THORPE,R.C. (1980) New methods of analysing for antigens and glycoproteins in complex mixtures. Immunology Today 1,122.

ANDRADE,Z.A., ANDRADE,S.A. and SADGURSKY,M. (1971) Renal changes in patients with hepatic splenic schistosomiasis. Am.J.Trop.Med.Hyg. 20,77.

AUGENER,W., GREY,H.M., COOPER,N.R., et al (1971) The reaction of aggregated immunoglobulins with C1. Immunochemistry 8,1011.

BAATRUP,G., PETERSEN,I., JENSENIUS,J.C. and SVEHAG,S.A. (1983) Reduced complement-mediated immune complex solubility capacity and the presence of incompletely solubilized immune complexes in SLE sera. Clin.exp.Immunol. 54,439.

BAIRD,L.G., TACHOVSKY,T.G., SANDBERG-WOLLHEIM,M., KOPROWSKI,H. and NISONOFF,A. (1980) Identifcation of a unique idiotype in cerebrospinal fluid and serum of a patient with multiple sclerosis. J.Immunol. 124,2324.

BANSAL,S.C., BANSAL,B.R., and BOLAND,J.P.(1976) Blocking and unblocking serum factors in neoplasia. Curr.Top.Microbiol.Immunol. 75,45.

BARDANA,E.J., HARBECK,R.J., HOFFMAN,A.A. et al (1975). The prognostic and therapeutic implications of DNA: anti-DNA immune complexes in SLE. Am.J.Med. 59,515.

BEGENT,R.H.J., TUCKER,D.F. and KEEN,J. (1980). A serum factor with potential as a tumour marker in malignant lymphoma. Brit.J.Cancer. 41,No.3,481.

BENNETT,J.M.,NATHANSON,L. and RUTENBURG,A.M. (1968) Significance of leucocyte alkaline phosphatase in Hodgkin's disease. Arch.Intern.Med. 121,338.

BLOCK,K.J., KOWRILSKY,F.M., OVARY,Z. and BENACERRAF,B. (1963) Properties of guinea pig 7S antibodies. J.Exp.Med. 117,965.

BORDET,J.(1896) Sur le mode d'action des sérums préventifs. Ann.Inst.Pasteur. 10,193.

BORDET,J.(1937) Traité de l'immunité dans les maladies infectieuses. 2nd Ed. Masson.

BUTTERWORTH,A.E., STURROCK,R.F. and HOUBA,V. (1975) Eosinophils as mediators and antibody damage to Schistosomula. Nature 256,727.

CANO,P.O., JERRY,L.M., SLADOWSKI,J.P. and OSTERLAND,C.K. (1977) Circulating immune complexes in SLE. Clin.exp.Immunol. 29,197.

CANTRELL,E.G. (1969) Nephrotic syndrome cured by removal of gastric carcinoma. Brit.Med.J. 1,739

CASALI,P.,BOSSUS,A.,CARPENTIER,N.A. and LAMBERT,P.H. (1977) Solid phase enzyme immunoassay or radioimmunoassay for the detection of immune complexes based on their recognition for conglutinin: conglutinin binding test Clin.exp.Immunol. 29,342.

CASALI, P. and LAMBERT,P.H. (1979) Purification of soluble immune complexes from serum using polymethylmetacrylate beads coated with conglutinin or C1q. Clin.exp.Immunol. 37,295.

CHENAIS,F., VIRELLA,G., PATRICK,C.C. and FUDENBERG,H.H. (1977) Isolation of soluble immune complexes by affinity chromatography using staphylococcal Protein-A Sepharose as substrate. J.Immunol.Methods 18,183.

CHESEBRO,B. and SVEHAG,S.E. (1968) Precipitation of human serum proteins by polyethylene glycol. Clin.Chim.Acta. 20,527.

CHIA,D., BARNETT,E.V., YAMAGATA,J., KNUTSON,D.,RESTIVO,C. and FURST,D. (1979) Quantitation and characterisation of soluble immune complexes precipitated from sera by polyethylene glycol. Clin.exp.Immunol. 37,399.

COCHRANE,C.G. and KOFFLER,D. (1973) Immune complex disease in experimental animals and man. Adv.Immunol. 16,185.

COHEN,S., FISHER,B., YOSHIDA,T. et al (1974) Serum migration-inhibitory activity in patients with lymphoproliferative diseases. N.Eng.J.Med. 290,882.

CUATRECASUS,P. and ANFINSEN,C.B. (1971) Affinity chromatography. Meth.Enzym. 22,345.

CZOP,J. and NUSSENZWEIG,V. (1976) Studies on the mechanism of solubilisation of immune precipitates by serum. J.Exp.Med. 143,615.

D'AMELIO,R., BRIGHOUSE,G., BARNET,M. and LAMBERT, P.H. (1981) Antigen-specific detection of soluble immune complexes in conglutinin binding assays. Clin.exp.Immunol. 45,283.

DAVIE,J.M. (1980) Anti-idiotypic reagents. J.Immunol.Methods. 38,1

DAY,E.D.(1972) Advanced Immunochemistry. Williams and Williams Co., Baltimore.

DIXON,F.J. (1963) The role of antigen-antibody complexes in diseases. Harvey Lecture 58,21.

EHRLICH,P. (1904) Gesammelte arbeiten zur immunitatsforschung. Hilschwald (Berlin).

EISEN,H.N. (1973) in "Microbiology" (B.D. Davies et al, editors) p.359, Harper, New York.

EISENBERG,R.A., THEOFILOPOULOS,A.N. and DIXON,F.J. (1977) Use of bovine conglutinin for the assay of immune complexes. J.Immunol. 118,1428.

ENGELFRIET,C.P., VON DEM BORNE,A.E.G.Kr., FLEER,A. et al (1980) In vivo destruction of erythrocytes by complement-binding and non complement-binding antibodies. In "Progress and Clinical and Bichemical Research" vol.43, 213. eds. S.G. Sandler et al. pub. A.R. Liss Inc.

ENGVALL,E., RUOSLAHTI,E. and MILLER,E.J. (1978) Affinity of fibronectin to collagens of different genetic types and to fibrinogen. J.Exp.Med. 147,1584.

FAITH,A., PONTESILLI,O., UNGER,A., PANAYI,G.S. and JOHNS,P. (1982) ELISA assays for IgM and IgG rheumatoid factors. J.Immunol.Methods 55,169.

FARR,R.S. (1958) A quantitative immunological measure of the primary interaction between I-BSA and antibody. J.Infect.Dis. 103,239.

FEARON,D.T. (1978) Regulation by membrane sialic acid of βlH-dependent decay - dissociation of amplification C3 convertase of the alternative complement pathway Proc.Nat.Acad.Sci. U.S.A. 75,1971.

FORSGREN,A. and SJOQUIST,J. (1966) "Protein-A" from Staphylococcus aureus. I. Pseudo-immune reaction with human gamma globulin. J.Immunol. 97,822.

FORSGREN,A. and SJOQUIST,J. (1967) "Protein-A" from Staphylococcus aureus. III. Reaction with rabbit gamma globulin. J.Immunol. 99,19.

FRAKER,P.J. and SPECK,J.C. (1978) Protein and cell membrane iodinations with a sparingly soluble chloroamide. Biochem. Biophys. Res. Comm. 80,849.

FRANK,M.A., LAWLEY,T.J., HAMBURGER,M.I. and BROWN,E.J.(1983) Immunoglobulin G Fc receptor-mediated clearance in autoimmune diseases. Annals Int.Med. 98,206.

FRANKLIN,E.C., HOLMAN,H.R., MULLER-EBERHARD, H.J. and KUNKEL,H.G. (1957) An unusual protein component of high molecular weight in the serum of certain patients with rheumatoid arthritis. J.Exp.Med. 105,425.

FUST,G., MEDGYESI,G.A., RAJNAVOLGI,E., CSECSI-NAGY,M. CZIKORA,K. and GERGELY,J. (1978) Possible mechanisms of the first step of the classical complement activation pathway: binding and activation of C1. Immunology 35,873.

GIGLI,I., PORTER,R.R. and SIM,R.B. (1976) The unactivated form of the first component of human complement, C1. Biochemical J. 157,541.

GILEAD,Z. and SULITZEANU,D. (1979) A technique for the purification of immune complexes using rheumatoid factor. J.Immunol.Methods 30,11.

GILLIAM,J.N., CHEATUM,D.E., HURD,E.R. et al (1974) Immunoglobulin in clinically uninvolved skin in SLE: association with renal disease. J.Clin.Invest. 53,1434.

GLASS,D. and SCHUR,P.H. (1978) In "Autoimmunity" ed. N.Talal. p.569, Academic Press, N.Y.

GODING,J.W., (1978) Use of staphylococcal Protein-A as an immunological reagent. J.Immunol.Methods 20,241.

GOLDBERG,R.J. (1953) A theory of antigen-antibody reactions. II. Theory of multivalent antigen with multivalent antibody. J.Am.Chem.Soc. 75,3127.

GOTZE,O., BIANCO,C. and COHN,Z.A. (1979) The induction of macrophage spreading by factor B of the properdin system. J.Exp.Med. 149,372.

GOTZE,O. and MULLER-EBERHARD,H.J. (1971) The C3-activator system: an alternative pathway of complement activation. J.Exp.Med. 134,90.

GRECO,F.A., KOLINS,J., RAJJOUB,R.K. and BRERETON,H.D. (1976) Hodgkin's disease and granulomatous angiitis of the central nervous system. Cancer 38,2027.

GREENWOOD,B.M., BROWN,J.C., De JESUS,D.G. and HOLBOROW,E.J. (1971) Immunosuppression in murine malaria. II. The effect on reticuloendothelial and germinal centre function. Clin.exp.Immunol. 9,345.

GREENWOOD,B.M., WHITTLE,H.C. and BRYCESON,A.D.M. (1973) Allergic complications of meningococcal disease. II. Immunological investigations. Brit.Med.J. 2,737.

GROPP,C., HAVEMANN,K., SCHEURER,A. and GRAMSE,M. (1979) Studies of identification of antigens in immune complexes of lung cancer patients. Behring Inst.Mitt. No.64,114.

GUPTA,R.C. and TAN,E.M. (1981) Isolation of circulating immune complexes by conglutinin and separation of antigen from dissociated complexes by immobilised Protein-A. Clin.exp.Immunol. 46,9.

HAAKENSTAD,A.O. and MANNIK,M.(1974) Saturation of the reticuloendothelial system with soluble immune complexes. J.Immunol. 112,1939.

HAAKENSTAD,A.O. and MANNIK,M.(1978) In "Autoimmunity" ed. N.Talal p.278. Academic Press,N.Y.

HADDING,U. (1967) Protides of the Biological Fluids. ed. Peeters,H. p.17, Elsevier.

HAO,Y.L. and WICKERHAUSER,M. (1978) In "Proceedings of the International Workshop on Technology for Protein Separation and Improvement of Blood Plasma Fractionation". U.S.Dept.Health, Education and Welfare Publication No. (NIH) 78-1422, p.372.

HARBOE,M. and FOLLING,I. (1974) Recognition of two distinct groups of human monoclonal IgM. Scand.J.Immunol. 3,471.

HARKISS,G.D. and BROWN,D.L. (1980) The use of C1q, conglutinin and low affinity rabbit IgM antibody to human Fc in a ligand cocktail radioassay for detecting and characterising immune complexes in pathological sera. Clin.exp.Immunol. 39,576.

HARRINGTON,J.C., FENTON,J.W. and PERT,J.H.(1971) Polymer induced precipitation of antigen-antibody complexes: "Perciplex" reactions. Immunochemistry 8,413.

HARRISON,R.A. and LACHMANN,P.J. (1980). Novel cleavage products of the third component of human complement. Molecular Immunol. 17,219.

HARTMAN,D. and LEWIS,M.G. (1974) Presence and possible role of anti-IgG antibodies in human malignancy. Lancet 1,1318.

HATTLER,B.G. and SOEHNLEN,B. (1974) Inhibition of tumour-induced lymphocyte blastogenesis by a factor or factors associated with peripheral leucocytes. Science 184,1374.

HAUPTMAN,S.P. and SOBCZAK,G. (1976). Origin of immunoglobulin albumin complex. Nature 263,64.

HAWKINS,D. and COCHRANE,C.G. (1968) Glomerula basement membrane damage in immunological glomerulonephritis. Immunology 14,665.

HAY,F.C., NINEHAM,L.J., PERUMAL,R. and ROITT,I.M. (1979) Intra-articular and circulating immune complexes and antiglobulins (IgG and IgM) in rheumatoid arthritis: correlation with clinical features. Ann.Rheum.Dis.38,1.

HEIDELBERGER,M. (1949). Quantitative absolute methods in the study of antigen-antibody reactions. Bacteriological Reviews. 3,49.

HEIDELBERGER,M. and KENDALL,F.E. (1935) The precipitin reaction between type III pneumococcus polysaccharide and homologous antibody. J.Exp.Med. 61,559.

HEIMER,R., ABRUZZO,J.L. and GLICK,D. (1979) The examination of antigens in highly purified immune complexes. Immunol.Commun. 8,545.

HEIMER,R. and KLEIN,G. (1976) Circulating immune complexes in sera of patients with Burkitt's lymphoma and nasopharyngeal carcinoma. Int.J.Cancer 18,310.

HEIMER,R. and KLEIN,G. (1978) The affinity of soluble immune complexes for concanavalin-A. Scand.J.Immunol. 7,315.

HELLSTROM,I., HELLSTROM,K.F., SJOGREN,H.O. et al (1971) Serum factors in tumour-free patients cancelling the blocking of cell-mediated tumour immunity. Int.J.Cancer 8,185.

HENSON,P.M.(1976) Activation and desensitisation of platelets by platelet-activating factor derived from IgE-sensitised basophils. J.Exp.Med. 143, 937.

HENSON,P.M. and COCHRANE,C.G. (1971) Acute immune complex disease in rabbits. J.Exp.Med. 133, 554.

HENSON,P.M., GINSBERG,M.H. and MORRISON,D.C. (1978) Mechanisms of mediator release by inflammatory cells. In "Membrane Fusion" eds. G.Poste and G.L. Nicolson, p.407-508, Elsevier Press.

HOFFKEN,K., MEREDITH,I.D., ROBINS,R.A. et al (1977) Circulating immune complexes in patients with breast cancer. Brit.Med.J. 2,218.

HORSFALL,A.C., VENABLES,P.J.W., MUMFORD,P. and MAINI,R.N. (1981) Interpretation of the Raji cell assay in sera containing anti-nuclear antibodies and immune complexes. Clin.exp.Immunol. 44,405.

HOSHINO-SHIMIZU,S., de BRITO, T., KANAMURA,H.Y. et al (1976) Schistosoma mansoni antigen detection in renal glomeruli. Trans.R.Soc.Trop.Med.Hyg. 70,492.

HOUBA,V. (1975) Immunopathology of nephropathies with malaria. W.H.O. Bull. 52,199.

HOUBA,V., LAMBERT,P.H., VOLLER,A. et al (1976) Clinical and experimental investigation of immune complexes in malaria. Clin.Immunol.Immunopathol. 6,1.

HUDSON,L. and HAY,F.C. (1976). Practical Immunology. Oxford, Blackwell Scientific.

HUGHES,G.R.V., ELKON,K.B., SPILLER,R. et al (1979) Polyarteritis nodosa and hairy-cell leukaemia. Lancet 1,678.

HUGLI,T.E. and MULLER-EBERHARD,H.J. (1978) Anaphylatoxins: C3a and C5a. Adv.Immunol. 26,1.

HUMPHREY,J.H. and DOURMASHKIN,R.R. (1969) The lesions in cell membranes caused by complement. Advances Immunol. 11,75.

HUNTER,W.M. and GREENWOOD,F.C. (1962) Preparation of ^{131}I-labelled human growth hormone of high specific activity. Nature 194,495.

HURD,E.R., LOSPALLUTO,J. and ZIFF,M. (1970) Formation of leucocyte inclusions in normal polymorphonuclear cells incubated with synovial fluid. Arth.Rheum. 13,724.

HUSTON,K.A., GUPTA,R.C., DONADIO,J.V. et al (1978) Circulating immune complexes in SLE. Association with other immunological abnormalities but not with changes in renal function. J.Rheumatol. 5,423.

IGNARRO,L.J. (1974) Release of neutral protease and beta glucuronidase from human neutrophils in the presence of cartilage treated with various immunological reactants. J.Immunol. 113,298.

INMAN,R.D., FONG,K.I., PUSSELL,B.A. et al (1980) The C1q binding assay in SLE. Discordance with disease activity. Arthritis Rheum. 23,1282.

ISHIZAKA,T., ISHIZAKA,K., SALMON,S. and FUDENBERG,H.H. (1967) Biological activities of aggregated gamma globulin. VIII. Aggregated immunoglobulins of different classes. J.Immunol. 99,82.

ISHIZAKA,T., SIAN,C.M., and ISHIZAKA,K. (1972a) Complement fixation by aggregated IgE through alternative pathway. J.Immunol. 108,848.

ISHIZAKA,T., SOTO, C.M. and ISHIZAKA,K. (1972b) Characteristics of complement fixation by aggregated IgE. J.Immunol. 109,1290.

ISHIZAKA,T., TADA,T. and ISHIZAKA,K. (1968) Fixation of C' and C'1a by rabbit gamma-G and gamma-M antibodies with particulate and soluble antigens. J.Immunol.100,1145.

IZUI,S., LAMBERT,P.H. and MIESCHER,P.A. (1976) In vitro demonstration of a particular affinity of glomerula basement membrane and collagen for DNA. A possible basis for a local formation of DNA-anti-DNA complexes in SLE. J.Exp.Med. 144,428.

IZUI,S., LAMBERT,P.H. and MIESCHER,P.A. (1977) Determination of anti-DNA binding test. Elimination of non-specific binding of DNA to non-immunoglobulin basic protein by using an anionic detergent. Clin.exp.Immunol 26,(3), 425.

JANOFF,A. (1975) At least three human neutrophil lysosomal proteases are capable of degrading joint connective tissue. Ann.N.Y.Acad.Sci. 250,402.

JAQUEMIN,P.C., SAXINGER,C and GALLOW,R.C. (1978) Surface antibodies of human myelogenous leukaemia leucocytes reactive with specific type-C viral reverse transcriptases. Nature 276,230.

JERNE,N.K. (1974) Towards a network theory of the immune system. Ann.Immunol.(Inst.Pasteur) 125C,373.

JERRY,L.M., ROWDEN,G., CANO,P.O. et al (1976) Immune complexes in human melanoma. Scand.J.Immunol. 8,845.

JONES,V.E., COWLEY,P.J., ALLEN,C. and ELSON, C.J. (1980) The isolation of immune complexes containing IgM (RF) and recovery of IgG (RF) from the complexes. J.Immunol.Methods 37,1.

JONES,V.E. and ORLANS,E. (1981) Isolation of immune complexes and characterisation of their constituent antigens and antibodies in some human diseases: A review. J.Immunol.Methods 44,249.

JOSEPH,M., CAPRON,A., BUTTERWORTH,A.E. et al (1978) Cytotoxicity of human and baboon mononuclear phagocytes against schistosomula in vitro. Induction by immune complexes containing IgE and S.mansoni antigens. Clin.exp.Immunol. 33,48.

JUCKES,I.R. (1971) Fractionation of proteins and viruses with polyethylene glycol. Biochim.Biophys.Acta. 229,535.

KAZATCHKINE,M.D., FEARON,D.T., SILBERT,J.E. and AUSTEN,K.F. (1979) Surface associated heparin inhibits zymozan-induced activation of the human alternative complement pathway by augmenting the regulatory action of the control proteins on particle-bound C3b. J.Exp.Med. 150,1202.

KESSLER,S.W. (1975) Rapid isolation of antigens from cells with a staphylococcal Protein-A antibody adsorbent: parameters of the interaction of antigen-antibody complexes with Protein-A. J.Immunol. 115,1617.

KIJLSTRA,A., KNUTSON,D.W., VAN DER LELIJ,A. and VANES,L.A. (1977) Characteristics of soluble immune complexes prepared from oligovalent DNP-conjugates and anti-DNP antibodies. J.Immunol.Methods 17,263.

KILGALLON,W., AMLOT,P.L. and WILLIAMS,B.D. (1982) Anti-C1q column: ligand specific purification of immune complexes from human serum or plasma. Analysis of the interaction between C1q and immune complexes. Clin.exp.Immunol. 48,705.

KILPATRICK,J.M. and VIRELLA,G. (1980) Isolation and characterisation of soluble insulin-anti-insulin immune complexes formed *in vitro* and *in vivo* in sera from patients with diabetes mellitus. Clin.exp.Immunol. 40,445.

KNIKER,W.T. and COCHRANE,C.G.(1968) The localisation of circulating immune complexes in experimental serum sickness. J.Exp.Med. 127,119.

KOFFLER,D., AGNELLO,V. and KUNKEL,H.G. (1971) Systemic lupus erythematosus: prototype of immune complex nephritis in man. J.Exp.Med. 134,169.

KOFFLER,D., SCHUR,P.H. and KUNKEL,H.G. (1967) Immunological studies concerning the nephritis of SLE. J.Exp.Med. 126, 607.

KOHLER,P.F., CRONIN,R.E., HAMMOND,W.S. *et al* (1974) Chronic membranous glomerulonephritis caused by hepatitis B antigen-antibody complexes. Ann.Int.Med. 81,448.

KOIDE,N., NOSE,M. and MURAMATSU,T. (1977) Recognition of IgG by Fc receptor and complement: effects of glycosidase digestion. Biochem.Biophys.Res.Commun. 75,838.

KONO,I., SATURAI,T., KABASHIMA,K. *et al* (1983) Fibronectin binds to C1q: possible mechanisms for their co-precipitation in cryoglobulins from patients with SLE. Clin.exp.Immunol. 52,305.

KRAUS,M. (1897) In "Basic and Clinical Immunology" p.13, by Fudenburg,H.H., Sites,D.P., Caldwell,J.L. and Wells, J.V. 2nd edition, Lange, 1978.

KUNKEL,H.G., MULLER-EBERHARD,H.J., FUDENBERG,H.H. and TOMASI,T.B. (1961) Gamma globulin complexes in rheumatoid arthritis and certain other conditions. J.Clin.Invest. 40,117.

LACHMANN,P.J.(1962) A comparison of some properties of bovine conglutinin with those of rabbit immunoconglutinin. Immunology 5,687.

LACHMANN,P.J. (1967) Conglutinin and immunoconglutinins. Adv.Immunol. 6,179

LACHMANN,P.J., ELIAS,D.E. and MOFFETT,A.(1973) Conglutinin and immunoconglutinins. In "Biological Activities of Complement". ed. D.G. Ingram. p.202, S.Karger, Basle.

LACHMANN,P.J. and HOBART,M. (1979) Complement Technology. In "Handbook of Experimental Immunology". 1.5A.20 ed. D.M. Weir, Blackwell Scientific Publication.

LACHMANN,P.J. and RICHARDS,C.B. (1964) An estimate of some molecular parameters of bovine conglutinin. Immunochemistry 1,37.

LAEMMLI,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227,680.

LAMBERT,P.H., DIXON,F.J., ZUBLER,R.H. et al (1978) A WHO collaborative study for the evaluation of eighteen methods for detecting immune complexes in serum. J.Clin.Lab.Immunol. 1,1.

LANSTEINER,K. (1936) The Specificity of Serological Reactions. Pub.by Thomas.

LAURELL,C.B. (1965) Antigen-antibody crossed immunoelectrophoresis. Anal.Biochem. 10,358.

LAURENT,T.C. (1963) The interaction between polysaccharides and other macromolecules. Biochem.J. 89,253.

LEPOW,I.H., NAFF,G.B., TODD,E.W., PENSKY,J. and HINZ,C.F. (1963) Chromatographic resolution of the first component of human complement into three activities. J.Exp.Med. 117,938.

LEWIS,M.G., LOUGHRIDGE,L.W. and PHILIPS.T.M. (1971) Immunological studies in nephrotic syndrome associated with extra renal malignant disease. Lancet 2,134.

LIND,I., HARBOE,M. and FOLLING,I. (1975) Protein-A reactivity of two distinct groups of human monoclonal IgM. Scand.J.Immunol. 4,843.

LINSCOTT,D.W., RANKEN,R. and TRIGLIA,R.P. (1978) Evidence that bovine conglutinin reacts with an early product of C3b degradation and an improved conglutinin assay. J.Immunol. 121,658.

LOWRY,O.H., ROSEBROUGH,N.J., FARR,A.L. and RANDALL,R.J. (1951) Protein measurement with the Folin phenol reagent. J.Biol.Chem. 193,265.

LUKES,R.J. and BUTLER,J.J. (1966) The pathology and nomenclature of Hodgkin's disease. Cancer Res. 26,1063.

LUTHRA,H.S., McDUFFIE,F.C., HUNDER,G.G. and SAMAYOA,E.A. (1975) Immune complexes in sera and synovial fluids of patients with rheumatoid arthritis. J.Clin.Invest. 56,458.

MacSWEEN,J.M. and EASTWOOD,S.L. (1978) Recovery of immunologically active antigen from staphylococcal Protein-A antibody adsorbent. J.Immunol.Methods 23,259.

McDUFFIE,F.C. and BRUMFIELD,H.W. (1972) Effect of rheumatoid factor on complement mediated phagocytosis. J.Clin.Invest. 51,3007.

McMICHAEL,J.C., GREISIGER,L.M. and MILLMAN,I. (1981) The use of nitrocellulose blotting for the study of hepatitis B surface antigen electrophoresed in agarose gels. J.Immunol.Methods 45,79.

MAIDMENT,B.W., PAPSIDERO,L.D., NEMOTO,T. and MING CHU,T. (1981) Recovery of immunologically reactive antibodies and antigens from breast cancer immune complexes by preparative isoelectrofocusing. Cancer Res. 41,795.

MAIRE,M.A., BARNET,M., CARPENTIER,N., MIESCHER,P.A. and LAMBERT,P.H. (1983) Identification of components of immune complexes purified from human sera. Clin.exp.Immunol. 51,215.

MALE,D.K. and ROITT,I.M. (1979) Analysis of the components of immune complexes. Mol.Immunol. 16,197

MALE,D.K. and ROITT,I.M. (1981) Molecular analysis of complement-fixing rheumatoid synovial fluid immune complexes. Clin.exp.Immunol. 46,521.

MALE,D.K., ROITT,I.M. and HAY,F.C. (1980) Analysis of immune complexes in synovial effusions of patients with rheumatoid arthritis. Clin.exp.Immunol. 39,297.

MANNIK,M. (1967) Binding of albumin to gamma A-myeloma proteins and Waldenstrom macroglobulins by disulphide bonds. J.Immunol. 99,899.

MANNIK,M. (1980) Physicochemical and functional relationships of immune complexes in mice by administration of excess immune complexes. J.Invest.Dermatol. 74, (5), 333.

MANNIK,M., AREND,W.P., HALL,A.P. and GILLIAND,B.C. (1971) Studies on antigen-antibody complexes. I. Elimination of soluble complexes from rabbit circulation. J.Exp.Med. 133,713.

MANTOVANI,B., RABINOVITCH,M . and NUSSENZWEIG,V. (1972) Phagocytosis of immune complexes by macrophages. J.Exp.Med. 135,780.

MARRACK,J.R. (1938) Medical Research Council (G.B.) Special Rep.Ser. SRS-230.

MARTIN,R.G. and AMES,B.N. (1961) A method for determining the sedimentation behaviour of enzymes: application to protein mixtures. J.Biol.Chem. 236,1372.

MAURER,P.H. and TALMAGE,D.W. (1953) The effect of complement in rabbit serum on the quantitative precipitin reaction. J.Immunol. 70,135.

MENZEL,E.J., SMOLEN,J.S., LIOTTA,L. and REID,K.B.M. (1981) Interaction of fibronectin with C1q and its collagen-like fragment. Febs Lett. 129, (1), 188.

MESSNER,R.P., LAXDAL,T., QUIE,P.G. et al (1968) Serum opsonins, bacteria and polymorphonuclear leucocyte interactions in subacute bacterial endocarditis. J.Clin.Invest. 47,1109.

METZGER,H. (1970) Structure and function of gamma-M macroglobulins. Adv.Immunol. 12,57.

MIEKKA,S.I. and INGHAM,K.C. (1978) Influence of self-association of proteins on their precipitation by polyethylene glycol. Arch.Biochem.Biophys. 191,526.

MILLER,G.W. and NUSSENZWEIG,V. (1975) A new complement function: solubilisation of antigen-antibody aggregates. Proc.Nat.Acad.Sci. 72,418.

MOORTHY,A.B., ZIMMERMAN,S.W. and BURKHOLDER,P.M. (1976) Nephrotic syndrome in Hodgkin's disease. Evidence for pathogenesis alternative to immune complex deposition. Am.J.Med. 61,471.

MUIR,R. (1909) Studies on Immunity. Oxford University Press, London.

MULLER-EBERHARD,H.J. (1975) Complement. Ann.Rev.Biochem. 44,697.

MULLER-EBERHARD,H.J. (1978) Complement: molecular mechanisms, regulation and biologic function. In "Molecular Basis of Biological Degradative Processes" ed.R.D.Berlin et al. p.65-114. New York, Academic Press.

NAAMA,J.K., MITCHELL,W.S. and WHALEY,K. (1983a) Inhibition of complement-mediated solubilisation of antigen-antibody complexes by sera from patients with rheumatoid arthritis. Clin.exp.Immunol. 54,429.

NAAMA,J.K., MITCHELL,W.S., ZOMA,A. et al(1983b) Complement-mediated inhibition of immune precipitation in patients with immune complex diseases. Clin.exp.Immunol. 51,292.

NATALI,P.G., WALKER,L. and PELLEGRINO,M.A. (1980) Isolation of soluble immune complexes from serum using Protein-A bearing Staphylococcus aureus bacteria. Separation of the antigen from immune complexes and production of antisera. Clin.Immunol.Immunopathol. 15,76.

NATVIG,J.B. and KUNKEL,H.G. (1973) Human immunoglobulins: classes, sub-classes, genetic variants and idiotypes. Adv.Immunol. 16,1.

NEURATH,A.R. and STRICK,N. (1977) Host specificity of a serum marker for hepatitis B: evidence that "e" antigen has the properties of an immunoglobulin. Proc.Nat.Acad.Sci.(Wash.), 74,1702.

NINEHAM,L.J., HAY,F.C., MALE,D.K. et al (1979) Immune complexes in rheumatoid arthritis: correlations with clinical features and effects of gold. In "Protides of the Biological Fluids" ed. H.Peeters. 26,179.

NISONOFF,A. and PRESSMAN,D. (1958) Loss of precipitating activity of antibody without disruption of binding sites. Science (Washington,D.C.) 128,659.

NUSSENZWEIG,V. (1980) In "Immunology 80", Progress in Immunology, IV, eds. M. Fougereau and J. Dausset. p.1044 Academic Press, London.

OUCHTERLONY,O. (1958) Diffusion in gel methods for immunological analysis. Progress in Allergy 5,1.

PAGANELLI,R., LEVINSKY,R.J. and ATHERTON,D.J. (1981) Detection of specific antigen within circulating immune complexes: validation of the assay and its application to food antigen-antibody complexes formed in healthy and food-allergic subjects. Clin.exp.Immunol. 46,44.

PAULING,L., PRESSMAN,D and CAMPBELL,D.H. (1944) Evidence for framework theory of serological precipitation. J.Am.Chem.Soc. 66,330.

PEPYS,M.B., BELL,A.J. and ROWE,I.F. (1976) Sepharose-C3. Preparation and use as an immunosorbent. Scand.J.Immunol. Supl. 3, p.75.

PEREIRA,A.B., THEOFILOPOULOS,A.N. and DIXON,F.J. (1980) Detection and partial characterisation of circulating immune complexes with solid-phase anti-C3. J.Immunol. 125,763.

PERLMANN,P., PERLMANN,H. and BIBERFIELD,P. (1972) Specifically cytotoxic lymphocytes produced by preincubation with antibody-complexed target cells. J.Immunol. 108,558.

PERNICE,W., SCHMITZ,H., SCHINDERA,F., BEHRENS,F. and SEDLACEK,H.H. (1979a) Antigen-specific detection of immune complexes in patients with hepatitis B, influenza A and rubella. Behring Inst.Mitt. No. 64,102.

PERNICE,W., SODOMANN,C.P., LUBEN,G., SEILER,F.R. and SEDLACEK,H.H. (1979b) Antigen-specific detection of HBsAg-containing immune complexes in the course of hepatitis B virus infection. Clin.exp.Immunol. 37,376.

POLSON,A., POTGIETER,G.M., LARGIER,J.F., MEARS,G.E.F. and JOUBERT,F.J. (1964) The fractionation of protein mixtures by linear polymers of high molecular weight. Biochim.Biophys.Acta. (Amst.) 82,463.

POPE,R.M., MANNIK,M., GILLILAND,B.C. and TELLER,D.C. (1975) The hyperviscosity syndrome in rheumatoid arthritis due to intermediate complexes formed by self-association of IgG-rheumatoid factors. Arth.Rheum. 18,97.

PORTER,R.R. (1980) The complex proteases of the complement system. Proc.Roy.Soc.Lond.B. 210,477.

REID,K.B.M., LOWE,D.M. and PORTER,R.R. (1972) Isolation and characterisation of C1q; a sub-component of the first component of human complement. Biochem.J. 130,749.

ROOT,R.K and WOLFF,S.M. (1968) Pathogenetic mechanism in experimental immune fever. J.Exp.Med. 128,309.

ROSENBLUM,W.I. and HADFIELD,M.G. (1972) Granulomatous angiitis of the nervous system in cases of herpes zoster and lymphosarcoma. Neurology 22,348.

ROSS,G.D., JAROWSKI,C.I., RABELLINO,E.M. and WINCHESTER,R.J. (1978) The sequential apperance of Ia-like antigens and two different complement receptors during the maturation of human neutrophils. J.Exp.Med. 147,730.

ROSS,G.D., POLLEY,M.J., RABELLINO,E.M. and GREY,H.M. (1973) Two different complement receptors on human lymphocytes J.Exp.Med. 138,798.

SCHARFSTEIN,J., CORREA,E.B., GALLOW,G.R. and NUSSENZWEIG,V. (1979) Human C4-binding protein. Association with immune complexes in vitro and in vivo. J.Clin.Invest. 63,437.

SCHIFFERLI,J.A., BARTOLOTTI,S.R. and PETERS,D.K. (1980) Inhibition of immune precipitation by complement. Clin.exp.Immunol. 42,387.

SCHIFFERLI,J.A., MORRIS,S.M., DASH,A. and PETERS,D.K.(1981) Complement-mediated solubilisation in patients with SLE, nephritis and vasculitis. Clin.exp.Immunol. 46,557

SCHIFFERLI,J.A., WOO,P. and PETERS,D.K. (1982) Complement-mediated inhibition of immune precipitation. I. Role of the classical and alternative pathways. Clin.exp.Immunol. 47,555.

SCHMID,F.R., ROITT,I.M. and ROCHA,M.J. (1970) Complement fixation by a two-component antibody system: IgG and IgM RF. Paradoxical effect related to IgG concentration. J.Exp.Med. 132,673.

SCHMIDT,D.H., KAUFMAN,B.N. and BUTLER,V.P. (1974) Persistence of hapten-antibody complexes in the circulation of immunised animals after a single intravenous injection of hapten. J.Exp.Med. 139,278.

SCHREIBER,R.D., PANGBURN,M.K., LESAVRE,P.H. and MULLER-EBERHARD,H.J. (1979) Initiation of the alternative pathway of complement: recognition of activators by bound C3b and assembly of the entire pathway from six isolated proteins. Proc.Nat.Acad.Sci.(U.S.A.) 75,(8), 3948.

SCHROETER,A.L., COPEMAN,P.W.M., JORDAN,R.E. et al (1971) Immunofluorescence of cutaneous vasculitis associated with systemic disease. Arch.Dermatol. 104,254.

SCHUMAKER,V.N., CALCOTT,M.H., SPIEGELBERG,H.L. and MULLER-EBERHARD,H.J. (1976) Ultracentrifuge studies of the binding of IgG to the C1q sub-unit of the first component of complement. Biochemistry. (Wash.) 15,5175.

SCHUR,P.H. and SANDSON,J. (1968) Immunological factors and clinical reactivity in SLE. N.Eng.J.Med. 278,533.

SCHWARTZ,R.S. (1983) Monoclonal lupus autoantibodies. Immunology Today 4,No.3. p.68.

SHEAGREN,J.N., BLOCK,J.B. and WOLFF,S.M. (1967) Reticuloendothelial system phagocytic function in patients with Hodgkin's disease. J.Clin.Invest. 46,855.

SHERMAN,R.L., SUSIN,M., WEKSLER,M.E. et al (1972) Lipoid nephrosis in Hodgkin's disease. Am.J.Med. 52,699.

SOGN,J.A., COLIGAN,J.E. and KINDT,T.J. (1977) The use of idiotypes as markers for antibody variable regions in the rabbit. Fed.Proc. 36,214.

SPIEGELBERG,H.L. (1974) Biological activities of immunoglobulins of different classes and sub-classes. Adv.Immunol. 19,259.

STEENSGAARD,J. and JOHANSEN,A.S. (1980) Biochemical aspects of immune complex formation and immune complex disease. Allergy 35,457.

SUTHERLAND,J.C., MARKHAM,R.V. and MARDINEY,M.R. (1974b) Subclinical immune complexes in the glomeruli of kidneys post-mortem. Am.J.Med. 57,536.

SUTHERLAND,J.C., MARKHAM,R.V., RAMSEY,H.E. and MARDINEY,M.R. (1974a) Sub-clinical immune complex nephritis in patients with Hodgkin's disease. Cancer Research 34,1179.

SVEHAG,S.E., and BURGER,D. (1976) Isolation of C1q-binding immune complexes by affinity chromatography and desorption with a diaminoalkyl compound. Acta.Path.Microbiol.Scand. Sect.C. 84,45.

TAKAHASHI,M., TACK,B.F. and NUSSENZWEIG,V. (1977) Requirements for the solubilisation of immune aggregates by complement. J.Exp.Med. 145,86.

TAKEKOSHI,Y., TANAKA,M., MIYAKAWA,Y. et al (1979) Free "small" and IgG-associated "large" hepatitis B e antigen in the serum of two patients with membranous glomerulonephritis. N.Eng.J.Med. 300,814.

TANIGUCHI,M., HAYAKAWA,K. and TADA,T. (1976) Properties of antigen-specific suppressive T cell factor in the regulation of antibody response of the mouse. II. In vitro activity and evidence for the I region gene product. J.Immunol. 116,542.

TAYLOR,J.C., CRAWFORD,I.P. and HUGLI,T.E. (1977) Limited degradation of the third component of human complement by human leucocyte elastase (HLE): partial characterisation of C3 fragments. Biochemistry 16,3390.

TESHIMA,H., WANEBO,H., PINSKY,C. et al (1977) Circulating immune complexes detected by ^{125}I-C1q deviation test in sera of cancer patients. J.Clin.Invest.59,1134.

THEOFILOPOULOS,A.N. and DIXON,F.J. (1979) Immune complexes in human diseases. Am.J.Pathol. 100,No.2, 531.

THEOFILOPOULOS,A.N., EISENBERG,R.A. and DIXON,F.J. (1978) Isolation of circulating immune complexes using Raji cells. Separation of antigens from immune complexes and production of antiserum. J.Clin.Invest. 61,1570.

THEOFILOPOULOS,A.N., WILSON,C.B. and DIXON,F.J. (1976) The Raji cell radio-immune assay for detecting immune complexes in human sera. J.Clin.Invest. 57,169.

THOMPSON,A.D., COCHRANE,M.A.G., McFARLANE,I.G. et al (1974) Lymphocyte cytotoxicity to isolated hepatocytes in chronic active hepatitis. Nature 252,721.

TOWBIN,H., STAEHELIN,T. and GORDEN,J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc.Nat.Acad.Sci. 76, No.9, 4350.

TSCHOPP,J., VILLIGER,W., LUSTIG,A., JATON,J.C. and ENGEL,J. (1980) Antigen-independent binding of IgG dimers to C1q as studied by sedimentation equilibrium, complement fixation and electron microscopy. Eur.J.Immunol. 10,529.

TUCKER,D.F., BEGENT,R.H. and HOGG,N.M. (1978) Characterisation of immune complexes in serum by adsorption on staphylococcal Protein-A: model studies and application to sera of rats bearing a gross virus-induced lymphoma. J.Immunol. 1, No.5, 1644.

VIDAL,M.A. and CONDE,F.P. (1980) Studies of the IgM and IgA contamination obtained by eluting IgG from Protein-A Sepharose columns with pH steps. J.Immunol. Methods 35,169.

VOLANAKIS,J.E. and STROUD,R.M. (1972) Rabbit C1q: Purification, functional and structural studies. J.Immunol.Methods 2,25.

WAHL,L.M., WAHL,S.M., MERGENHAGEN,S.E. and MARTIN,G.R. (1975) Collagenase production by lymphokine-activated macrophages. Science 187,261.

WEBER,K. and OSBORN,M. (1975) In "The Proteins" 3rd edition Vol.1, eds. Neurath,H. and Hill,R.L.

WECKER,E., SCHIMPLE,A. and HUNIG,T. (1977) Expression of MuLV GP71-like antigen in normal mouse spleen cells induced by antigenic stimulation. Nature 269,598.

WEIGLE,W.O. (1961) Fate and biological action of antigen-antibody complexes. Adv.Immunol. 1,283.

WHO Report of a scientific group (1977) The role of immune complexes in disease. WHO technical report series No.606

WILLIAMS,D.G., PETERS,D.K., FALLOWS,J. et al (1974) Studies of serum complement in the hypocomplementaemic nephritides. Clin.exp.Immunol. 18,391.

WILLIAMS,R.C. (1980) Immune complexes in clinical and experimental medicine. Harvard Univ. Press, Mass.

WILLIAMS,R.C. and KUNKEL,H.G. (1962) Rheumatoid factor, complement and conglutinin aberrations in patients with sub-acute bacterial endocarditis. J.Clin.Invest. 41,666.

WILSON,C.B. and DIXON,F.J. (1971) Quantitation of acute and chronic serum sickness in the rabbit. J.Exp.Med. 134,7s.

WILSON,C.B. and DIXON,F.J. (1976) In "The Kidney" eds. Brenner,B.M. and Rector,F.C. Vol.II,p.864. Saunders, Pennsylvania.

WILSON,R.A. and BARNES,P.E. (1977) The formation and turnover of the membranocalyx and the tegument of Schistosoma mansoni. Parisitology 74,61.

WINCHESTER,R.J. (1975) Characterisation of IgG complexes in patients with rheumatoid arthritis. Ann.N.Y.Acad.Sci. 256,73.

WINKELHAKE,J.L. (1978). Immunoglobulin structure and effector functions. Mol.Immunol. 15,695.

YEATTS,R.P., TURNER,R., COLLINS,R. et al (1978) Soluble and insoluble immune complex-platelet interactions in rheumatoid inflammation. Ann.Rheum.Dis. 37,421.

YONEMASU,K. and STROUD,R.M. (1972) Structural studies on human C1q: non-covalent and covalent sub-units. Immunochemistry 9,545.

ZICCARDI,R.J. and COOPER,N.R. (1976a) Physicochemical and functional characterisation of the C1r sub-unit of the first complement component. J.Immunol. 116,496.

ZICCARDI,R.J. and COOPER,N.R. (1976b) Activation of C1r by proteolytic cleavage. J.Immunol. 116,504.

ZUBLER,R.H. and LAMBERT,P.H. (1977) ^{125}I-C1q binding test for soluble immune complexes. Ann.Rheum.Dis. 36, suppl.1, 27.

ZUBLER,R.H. and LAMBERT,P.H. (1978) Detection of immune complexes in human diseases. Prog.Allergy 24,1.

ZUBLER,R.H., LANGE,G., LAMBERT,P.H. and MIESCHER,P.A. (1976) Detection of immune complexes in unheated sera by a modified ^{125}I-C1q binding test. J.Immunol. 116,232.

ZUBLER,R.H., PERRIN,L.H., CREIGHTON,W.D. and LAMBERT,P.H. (1977) Use of polyethylene glycol (PEG) to concentrate immune complexes from serum or plasma samples. Ann.Rheum.Dis. 36, suppl.1, 23.

ABBREVIATIONS

BUFFERS

CFD	complement fixation diluent
EDTA	ethylenediaminetetra acetic acid
GBT	glycine barbital tris
LIS	lithium diiodosalicylic acid
PBS	phosphate buffered saline

CHEMICALS

Frigen	trichlorofluroethane
Iodogen	1,3,4,6-tetrachloro-3 ,6 -diphenyl glycouril
$(NH_4)_2SO_4$	Ammonium sulphate
PEG	polyethylene glycol
PMSF	phenyl methyl sulphonyl fluoride
TEMED	N.N.N.N.-tetramethyl-ethylene diamine

COMPLEMENT COMPONENTS

D	Factor D
P	Properdin
C3bINA	C3b inactivator (Factor I)
CRP	C-Reactive protein
β1H	Factor H

DISEASES

HD	Hodgkin's disease
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus

REAGENTS

BSA	bovine serum albumin
anti-BSA	antibody to BSA
E	sheep erythrocytes
EA	antibody coated E
EA 143	antibody coated E with complement components 1,4 and 3
IC	immune complex
IK	immunoconglutinin
K	conglutinin
Kt	conglutinin titre
NHS	normal human serum
SDS	sodium dodecyl sulphate
TCA	Tri-chloroacetic acid

PUBLICATIONS IN THIS THESIS

KILGALLON, W., AMLOT, P.L. and WILLIAMS, B.D. (1982) Anti-C1q column: ligand specific purification of immune complexes from human serum or plasma. Analysis of the interaction between C1q and immune complexes. Clin.exp.Immunol. 48,705.

KILGALLON, W., AMLOT, P.L. and WILLIAMS, B.D. (1983) Immune complexes in Hodgkin's disease: isolation, immunochemical and physico-chemical analysis. Clin.exp.Immunol. 53,308.

Clin exp Immunol (1983) **53**, 308–318

Immune complexes in Hodgkin's disease: isolation, immunochemical and physico-chemical analysis

W KILGALLON, P L AMLOT & B. D WILLIAMS *Department of Immunology, Royal Postgraduate Medical School and Department of Medicine, Guy's Hospital Medical School, London, UK*

(Accepted for publication 28 March 1983)

SUMMARY

Immune complexes (IC) present in sera from patients with Hodgkin's disease (HD) were isolated using three different affinity columns C1q-degalan, anti-C1q sepharose and conglutinin (K)-degalan The isolated IC were analysed by immunoprecipitation, SDS-PAGE and sucrose density gradients and compared with IC similarly isolated from patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and *in vitro* prepared BSA–anti-BSA complexes Isolated material from each disease, and BSA–anti-BSA complexes contained proteins compatible with true immune complexes—IgM, IgG, C1q and C3 breakdown components Albumin, fibronectin and CRP, whose affinity for IgM, C1q and C3 are known, were co-isolated along with IC material The size of isolated IC in HD ranged from 8–40S on sucrose density gradients Despite the operational difference in detecting and isolating HD complexes via the C1q ligand (C1q-degalan or anti-C1q column) and C3bi (K-degalan), material purified by both methods showed remarkable similarity on SDS-PAGE and immunoprecipitation analysis Although IC isolated from different diseases showed disparate banding patterns on SDS-PAGE this was attributed to a variation in the relative concentrations of constituent proteins—IgM, IgG and C3 breakdown products. IgM, IgG and C3 bind loosely, and non-specifically, to macromolecular aggregates formed around immune complexes Using the anti-C1q column, most of this material could be eluted using 0 02M EDTA. Least protein, yet the most specific for antigen and antibody was eluted at pH 3 0

Keywords Hodgkin's disease immune complexes isolation components

INTRODUCTION

The presence of circulating immune complexes (CIC) has now been reported in a wide variety of malignant diseases (reviewed by Williams, 1980) One of the early reports described CIC in Hodgkin's disease, a lymphorecticular malignancy (Amlot, Slaney & Williams, 1976), in which a correlation was made between the presence of immune complexes and the occurence of symptoms such as fever, night sweats and weight loss (symptoms suggesting dissemination of the disease) Furthermore immune complexes were more often found in the histological types of Hodgkin's disease carrying a poor prognosis (Amlot *et al.*, 1978). Pursuing the rationale that an association between immune complexes (IC) and disease manifestation might reveal information about

Correspondence Dr P L Amlot, Department of Medicine, Guy's Hospital Medical School, London SE1 9RT, UK

aetiological factors in the disease or pathogenetic mechanisms involved in symptom production, we decided to isolate and identify the molecular components of these complexes from serum and plasma samples of patients with Hodgkin's disease.

The search for specific antigens in IC has been assisted over recent years by the development of preparative isolation techniques for complexed material (reviewed by Jones & Orlans, 1981). Antigens have been successfully isolated from immune complexes in which the nature of the antigen was already known. Thus in one study DNA in immune complexes from patients with SLE (Theofilopoulos & Dixon, 1979) and in another the antigenic glycoprotein, gp 70, from a murine leukaemia virus induced lymphoma (Tucker, Begent & Hogg, 1978) were detected using a combination of gel filtration and protein A chromatography. Unfortunately this approach will isolate other large molecular weight proteins such as IgM, IgA and aggregated IgG along with immune complexes containing IgG which have affinity for protein A (Vidal & Conde, 1980). Casali & Lambert (1979) employed the use of polyethylene glycol (PEG) precipitation followed by affinity chromatography on bovine conglutinin (K) column to purify the putative antigen, IgG and specific antibody from the immune complexes of a patient with Kala-Azar. This method has the advantage of using a more specific ligand, C3bi, for isolating immune complexes than those described hitherto

This paper describes our experience with three different ligand affinity columns–C1q and K-degalan columns (Casali & Lambert, 1979) and an anti-C1q column (Kilgallon, Amlot & Williams, 1982) in the isolation and characterization of immune complexes from patients with Hodgkin's disease. The composition of these complexes are compared with those from patients with rheumatoid arthritis (RA), SLE and artificially prepared bovine serum albumin (BSA)–anti-BSA complexes.

MATERIALS AND METHODS

Bovine conglutinin (K). K was purified by the method of Lachmann & Hobart (1978).

Human C1q. C1q was purified by the method of Volonakis & Stroud (1972) with minor modifications (Zubler *et al.*, 1976).

Radiolabelling with ^{125}I BSA, BSA–anti-BSA and complexes from patients with Hodgkin's disease were labelled as described previously (Kilgallon *et al.*, 1982) using the Iodogen TM reagent (Pierce Chemical Co)

Isolation and purification of IC Soluble BSA–anti-BSA complexes were prepared in fresh normal human serum as described previously (Kilgallon *et al* , 1982) Briefly the procedure was: (a) Immune complex containing sera or plasma were treated with trichlorofluorethane (Frigen 113 TR-T, Hoechst, West Germany) to remove lipids (b) Immune complexes were precipitated along with other high molecular weight proteins by 4% PEG-6000. The precipitate was redissolved in complement fixation diluent (CFD) and then applied to specific affinity columns as described.

C1q and K columns. These were prepared according to the method of Casali & Lambert (1979). Briefly 20 mg of K or human C1q were incubated with 5 g polymethylmetacrylate (PMMA) beads (Degalan V26, Degussa, West Germany) in 0 05M bicarbonate buffer pH 9 0 at 37°C for 3 h. Efficiency of linkage was between 1–5% which meant 40–200 μg of protein bound per gram of PMMA beads. The beads were washed with 0 5M NaCl to remove unbound protein and were stored in CFD containing 0 01M sodium azide at 4°C.

Redissolved PEG precipitates containing complexes were incubated with these columns at 4°C for 3 h and bound material was eluted with CFD containing 0 02M EDTA (K column) and 0 02M EDTA followed by 0 5M NaCl (C1q columns).

Anti-C1q column. This was prepared and used as described previously (Kilgallon *et al.*, 1982)

Analysis of purified IC Molecular components of purified IC were examined by SDS-PAGE (Laemmli, 1970). Standards were obtained commercially from Pharmacia Ltd., Uppsala, Sweden and included thyroglobulin 330K, ferritin, 220K, albumin 67K, catalase 60K, lactic dehydrogenase 36K and ferritin subunit 18·5K Individual components of IC were identified by immunoprecipitation (Ouchterlony, 1958) or immunoelectrophoresis using specific antisera. Molecular weight profiles of IC were determined by sucrose density gradient ultracentrifugation using radiolabelled BSA, human IgG and IgM as 4S, 7S and 19S molecular weight markers respectively.

Rocket immunoelectrophoresis. This was used to measure levels of fibronectin in IC material by the method of Laurell (1965) One per cent agarose was prepared in 0 01M EDTA barbitone buffer containing anti-human fibronectin.

Specific antisera Goat anti-human IgM and sheep anti-human C3 were raised in the Department of Immunology, RPMS and each gave a single precipitin line against whole human serum with complete identity against purified human IgM and C3. Purified C3 was a gift of Dr D Scott, RPMS Commercial antisera were also used goat anti-human IgA, anti-human albumin, anti-α_2-macroglobulin and anti-human transferrin from Burroughs-Wellcome, Beckenham, Kent, anti-human C1q, anti-human CRP, and anti-human ferritin (Seward, Birmingham, UK), and anti-fibronectin (Cappel, Sussex, UK)

RESULTS

Yields of IC material from C1q-degalan, anti-C1q and K-degalan columns

The C1q-degalan and anti-C1q columns both used C1q as the ligand for isolating IC. Although the two columns had different binding capacities the binding capacity in each case vastly exceeded the amount of IC material loaded, so that the figures in Table 1 represent total extraction of IC binding to the column concerned Two- to seven-fold more material was extracted by the anti-C1q column than the C1q-degalan column. There was only rough agreement between the amount of IC material extracted by each column with each other or with the estimate of IC in the original sample by the C1q binding assay Using pre-formed BSA–anti-BSA complexes (400 μg) dissolved in fresh normal human serum the final complexed material probably exceeded twice that weight. If we assume that there was at least 1mg of complexed material after the BSA–anti-BSA IC had been incubated with fresh NHS then approximately 8% was isolated by the C1q-degalan ligand and 20% by the anti-C1q column This agreed with previous estimates based on radiolabelled antigen (Kilgallon *et al.*, 1982)

In Table 2 similar figures are shown for the K-degalan column. Generally smaller amounts of IC were isolated from pathological sera and this accords with lower values of IC's measured by the conglutinin binding assay expressed as HAGG equivalents However, when BSA–anti-BSA complexes were used a greater proportion of IC was retrieved on the K-degalan column than with either anti-C1q or C1q-degalan. Using the previous estimates of total complexed material, probably some 50% of IC was isolated with the K-degalan column

When sera or plasma samples not containing IC were processed, whether from normal subjects or IC negative HD patients, no detectable protein bound to any of the columns

Table 1. Extraction of C1q binding complexes in Hodgkin's disease by the anti-C1q and the C1q-degalan columns

Patient	Anti-C1q column	C1q-degalan column	HAGG equivalents by C1q BA*
C D	116†	64	165
P B	201	47	80
Y M	26	0	60
R L	243	86	120
A McQ	390	51	140
BSA–anti-BSA	193	78	400‡

* Heat aggregated IgG (HAGG) in C1q binding assay (C1q BA) expressed as μg/ml

† Micrograms of protein eluted per ml of original serum or plasma sample by 0 5M NaCl (measured by the method of Lowry *et al*, 1951)

‡ Calculated as amount of BSA and anti-BSA in IC

Table 2. Extraction of K binding complexes in Hodgkin's disease by the K column

Patient	K-degalan column	HAGG equivalent by Kg BA*
K K	43†	5
J S	32	6
M S	72	10
C T	53	18
F M	86	40
BSA–anti-BSA	120	100‡

* HAGG in the K binding assay (KgBA) expressed as μg/ml

† Micrograms of protein eluted per ml of original serum or plasma by 0 02M EDTA

‡ Calculated as amount of BSA and anti-BSA in IC

Components of isolated IC material identified by immunodiffusion

Table 3 lists the components identified in eluates from the anti-C1q column The eluting fluids shown were used sequentially to elute material from IC containing sera which bound to the anti-C1q column Two of the samples included in the RA group were synovial fluid In interpreting the results it should be remembered that most protein eluted in the EDTA buffer and least in the acid eluate whereas the proportion of specific antigen or antibody was the reverse (Kilgallon *et al*, 1982)

IgM was regularly detected in IC from RA but not at all in patients with SLE. IgM occurred predominantly in the EDTA and NaCl eluates of BSA–anti-BSA and HD complexes IgG was identified in all cases of HD, BSA–anti-BSA and SLE complexes IgG was detected in most of the RA complexes, and in most of the acid eluted complexes IgA was not detected in any of the IC material

The presence of human serum albumin in the IC isolates was probably explained by its affinity for IgM and IgG (Mannick, 1967) and its detection correlated well with that of IgM

C3 components were regularly detected but it was interesting that they were not detected in any of the acid elutes from the pathological sera In the SLE patients this was not surprising for sera were obtained during acute bouts of SLE with markedly low C3 levels (10–30%) so that the small amounts of C3 bound may not have been detected by the assay method. C3 and C3d were regularly detected in the BSA–anti-BSA acid eluates.

The large molecular weight protein α_2-macroglobulin did not bind to the anti-C1q column and C4, transferrin and ferritin were rarely found

C reactive protein (CRP) also bound to the column in a calcium dependent fashion for it was readily eluted with EDTA but it was only identified in those serum or heparinized plasma samples which were not treated with EDTA before extraction by PEG. Fibronectin was frequently bound in complexed material

Table 4 shows the identified proteins which bound to K-degalan, C1q-degalan or anti-C1q column from HD serum samples IC containing material was eluted from the K-degalan column with EDTA and from the C1q-degalan and anti-C1q column with NaCl. IgG was the major immunoglubulin present in IC from patients with HD and it was identified in eluates from each of the affinity columns IgA was only occasionally detected and IgM appeared to be isolated only by the anti-C1q column This raised the question as to whether rheumatoid factor reactive with rabbit IgG on the anti-C1q column was not being co-purified along with immune complexes However, none of the IC's from patients with HD showed rheumatoid factor activity The molecular association between IgM and albumin (Mannick, 1967) was emphasised because albumin was only identified in eluates from the anti-C1q column in which IgM was also isolated

Table 3. Proteins identified in eluates of immune complex material binding to the anti-C1q column

Human proteins identified	Immune complex material purified from: BSA–anti-BSA in NHS (*n* = 5)* EDTA†	NaCl†	Acid†	Hodgkin's disease (*n* = 9)* EDTA	NaCl	Acid	RA (*n* = 6)* EDTA	NaCl	Acid	SLE (*n* = 3)* EDTA	NaCl	Acid
IgM	5‡	5	—	4	7	2	6	4	4	—	—	—
IgG	5	5	5	9	9	9	2	4	4	3	3	3
IgA	—	—	—	—	—	—	—	—	—	—	—	—
Albumin	5	—	3	6	6	—	6	4	4	—	—	3
C3	5	5	3	7	7	—	2	—	—	—	—	—
C3c	5	5	—	7	5	—	4	2	—	—	—	—
C3d	5	5	5	1	3	—	4	2	—	—	—	—
C4	3	—	—	—	—	—	—	—	—	—	—	—
C1q	5	5	5	6	6	3	6	6	4	—	—	3
Transferrin	—	—	—	(2)§	—	—	—	—	—	—	—	—
CRP	—	—	—	(2)	—	—	(2)	—	—	(2)	—	—
α_2-macrogl	—	—	—	—	—	—	—	—	—	—	—	—
Fibronectin	2	2	2	4	4	—	2	3	4	—	—	1
Ferritin	—	—	1	—	—	1	—	—	—	—	—	—

* No of patient samples
† Eluting fluid used sequentially on column bound material
‡ No of samples positive of the samples applied
§ Figures in brackets are samples not EDTA treated before application to an anti-C1q column

Table 4. Proteins identified from HD immune complex material binding to K-degalan, C1q-degalan or anti-C1q column

Human proteins identified	Immune complex material purified on: K-degalan column (*n* = 7)*	C1q-degalan column (*n* = 6)*	Anti-C1q column (*n* = 9)*
IgM	—	—	7
IgG	7	6	9
IgA	1	2	—
Albumin	—	—	6
C3	7	6	7
C3c	7	6	5
C3d	1	4	3
C4	2	2	—
C1q	—	—	6
Transferrin	2	2	−(2)†
CRP	5	4	−(2)†
α_2-macrog	—	—	—
Fibronectin	5	2	4
Ferritin	—	—	1

* No of different patient samples applied
† Only detected where EDTA not used in preparation

C1q was only identified in fractions eluted from the anti-C1q column and not from the K- or C1q-degalan columns. C3 and its breakdown components were regularly isolated and C4 was identified in a minority of the eluates from the degalan columns.

CRP was frequently identified and eluted with EDTA from the C1q-degalan and K-degalan columns

SDS-PAGE analysis of isolated complexes

Examples of the banding patterns of isolated IC from HD and RA are seen in Figs. 1 & 2. Fig. 1 shows material eluted with 0 02M EDTA, and Fig. 2 material subsequently eluted with 0·5M NaCl (except for the K column where the EDTA eluate was used both times). Acid eluates are not shown as the only bands seen even in highly concentrated material were IgG heavy and light chains.

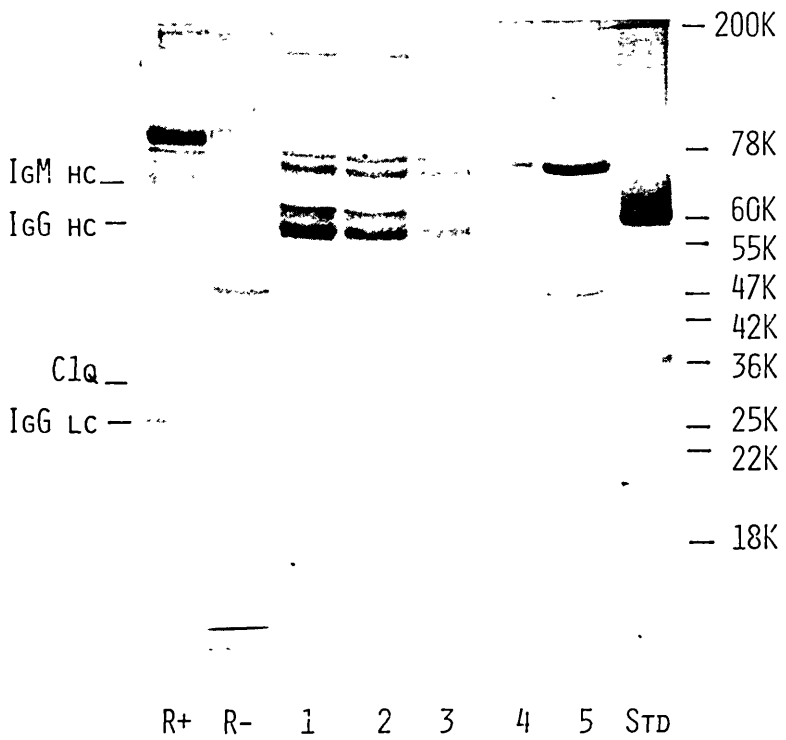


Fig. 1. EDTA eluted IC run on 10% PAGE with 0 1% SDS IC material eluted with 0 02M EDTA then reduced and alkylated Subscript abbreviations stand for R+ = seropositive RA off anti-C1q column, R− = seronegative RA off anti-C1q column, 1 = HD (MS) off K column, 2 = HD (MS) off C1q-degalan column, 3 = HD (RL) off C1q-degalan column, 4 = HD (RL) off anti-C1q column, 5 = HD (PB) off anti-C1q column, STD = Standards Patients MS had both KgB and C1qB IC in his serum, while RL and PB only had C1qB IC

The protein banding pattern was different in HD, RA and BSA–anti-BSA complexes (not shown). The banding pattern was very similar between IC isolated from different patients with HD whether the IC were isolated from K-columns, C1q-degalan columns or anti-C1q columns. Identification of protein bands on SDS-PAGE gels was achieved by combining immunoprecipitation data on the isolated complexes with the known molecular weights of the identified components and their breakdown products The most common components were immunoglobulin heavy and light chains and complement proteins.

The clearer and larger number of bands seen on SDS-PAGE and EDTA eluates corresponded to the greater amount of protein found in EDTA eluates Almost all of the bands could be accounted

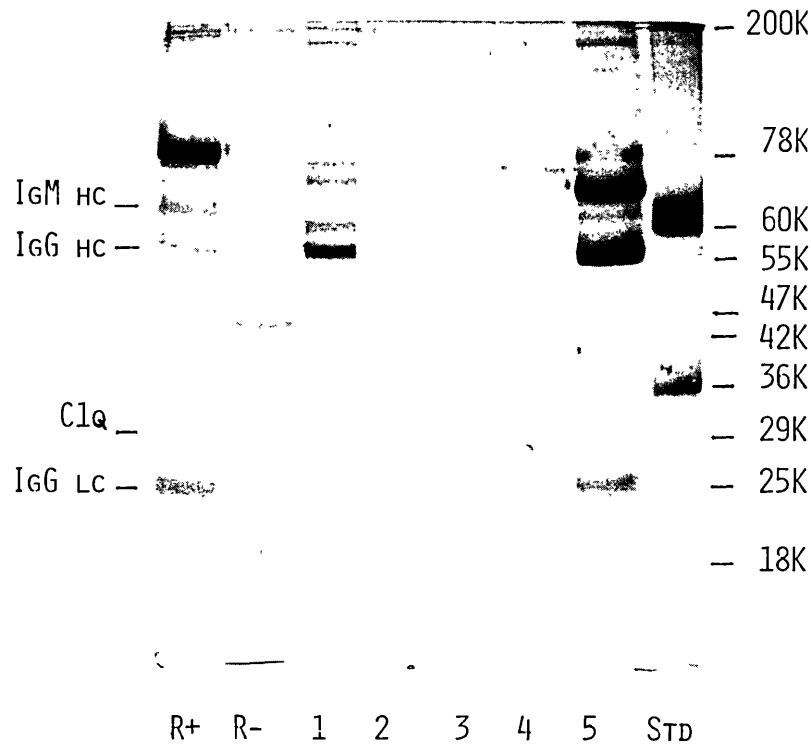


Fig. 2. NaCl eluted IC run on 10% PAGE with 0 1% SDS IC material eluted with 0 5M NaCl then reduced and alkylated Subscript as in Fig 1

for by IgM, IgG and the breakdown products of C3. The RA patient's synovial fluid sample (R+) isolated on an anti-C1q column contained IgM, IgG heavy and light chains as well as probable C3 breakdown products of the α chain (mol wt approximately 78, 70, 60, 47 and 42K) and the β chain at 75K (Harrison & Lachmann, 1980) By way of contrast the seronegative serum sample (R−) from an RA patient had no IgM, only IgG heavy and light chains with a restricted range of probably C3 breakdown products (α chain at approximately 70 and 47K) with no identifiable β chain C1q (29K) was detected and two other protein lines at approximately 80K and 48K which were not identified The R+ and R− isolated immune complexes were respectively positive and negative for rheumatoid factor by latex agglutination

Although the banding pattern in the HD samples differed from the rheumatoid samples similar components were identified. IgG was the major immunoglobulin with IgM only identified off the anti-C1q column (samples 4 and 5). Probable C3 breakdown components of the α chain mol. wt 78, 70, 60, 47, and 42K were detected along with the β chain (75K) in all cases. C1q was not detected in any EDTA eluate Of particular note is the small mol wt band (Col 5) from an HD patient It has an approximate mol wt of 22K and corresponds to the band described by Begent, Tucker & Keen (1980) It was only seen in EDTA eluates This band corresponds to monomeric CRP

Comparison of Col. 1 and 2 showed a remarkable similarity in bands seen on SDS-PAGE between those isolated from the K column and the C1q degalan column, (despite the fact that sera positive by the C1q BA did not bind to the K column and vice versa) Only sera positive by both C1q BA and KgBA bound to both columns. In none of the isolated HD complexes was a band identified corresponding to the mol wt of ferritin.

Sucrose density gradient profiles of IC

IC material isolated from K-degalan, C1q-degalan and anti-C1q columns ranged in size from 8S to 40S There was no notable size difference in complexes eluted from one as opposed to another

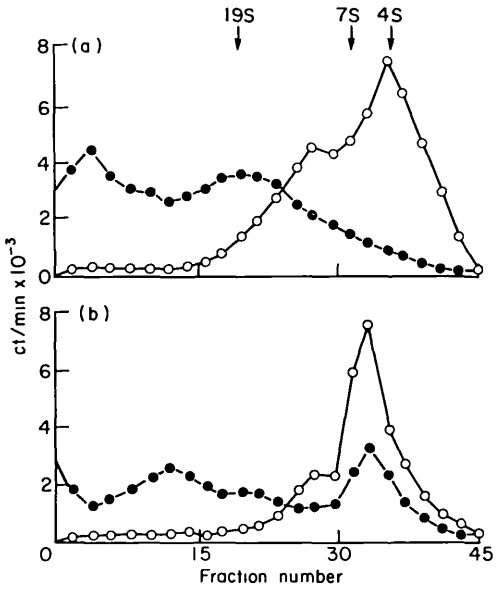


Fig 3

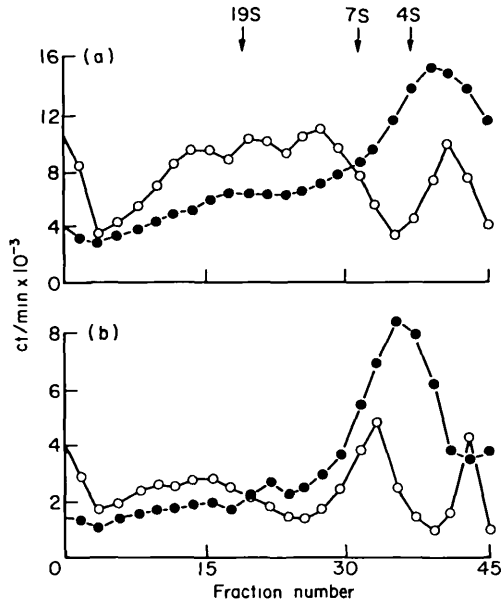
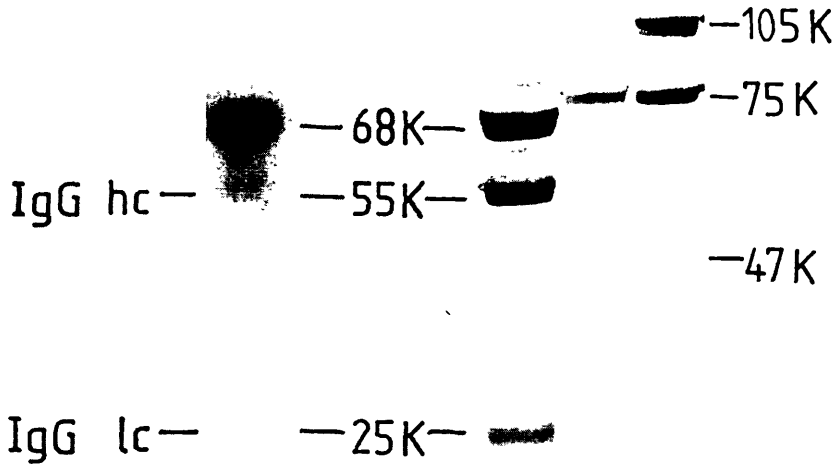


Fig 4

Fig. 3. Sucrose density gradient profiles of IC isolated on the K column (a) ^{125}I-BSA–anti-BSA complexes, (b) BSA–^{125}I-anti-BSA complexes ●——●, pH 7 4 (CFD), ○——○ pH 3 0 (0 15M NaCl, 0 2M acetate)

Fig. 4. Sucrose density profiles of IC isolated on the K column (a) HD complexes iodinated after purification, (b) BSA–anti-BSA complexes iodinated after purification ●——● = pH 7 4, ○——○ = pH 3 0



a b c C3

Fig. 5. Radiolabelled components of IC off anti-C1q column IC material run as in Fig 2 a = autoradiogram of col b, b = HD (PB) off anti-C1q column with 0 5M NaCl, c = HD (PB) off anti-C1q column at pH 3 0 C3 = purified C3

column. Attempts to dissociate ^{125}I-labelled IC from pathological sera using low pH were unsuccessful. It can be seen in Fig 3 that BSA–anti-BSA complexes made with previously labelled ^{125}I-BSA or ^{125}I-anti-BSA show the anticipated dissociation and shift at low pH, but, if purified IC were radiolabelled *in toto* irrespective of whether they were from HD (Fig. 4a) or BSA–anti-BSA complexes (Fig. 4b) then there was a net shift to a higher molecular weight profile at low pH. The reason for the unsuccessful dissociation of the radiolabelled IC was shown by SDS-PAGE analysis (Fig. 5). The most heavily ^{125}I-labelled component was a 68K band probably representing an α chain breakdown component of C3 There was weaker ^{125}I-labelling of IgG (55K and 25K bands). The net effect produced denaturation and aggregation of the heavily labelled protein component.

DISCUSSION

In this study three different affinity columns have been used to isolate IC and the yield and identity of the materials isolated from each column compared. IC could be isolated by both the C1q-degalan and the anti-C1q column, the major difference however between these two methods was one of performance since the yield obtained with the C1q column was very much lower than that of the anti-C1q column. Several reasons may account for the relative inefficiency of the C1q-degalan column to isolate IC. Degalan solid phase has a limited capacity to absorb protein and since human C1q is a labile protein its binding activity was rapidly lost. Also there were differences in the mechanism by which binding between the respective columns and IC occurred. It is thought that interchange between C1q on the solid phase and C1q bound to complexes leads to isolation of IC by C1q-degalan However, we have found no evidence for this and it seems more likely that isolation is by attachment of solid phase C1q to available C1q binding sites on the IC. On the other hand the anti-C1q column isolates IC by binding to accessible C1q present in the complexes. An estimated 20% of the total IC present in sera was isolated by this means and this represented significantly more than that obtained by C1q-degalan column. It should be emphasized however that both extraction procedures only account for a minority of the immune complexes present Despite the differences in yield the isolated material obtained was essentially similar, the only important difference being that IgM, albumin and C1q were found much more frequently in the eluates from the anti-C1q column It was unlikly that the IgM represented naturally occurring rheumatoid factor binding to rabbit IgG since we were unable to demonstrate any rheumatoid factor activity in either the initial sera or in the extracted material. In pathological material containing rheumatoid factor this could prove to be a disadvantage but it could easily be overcome by using $F(ab')_2$ digests of the anti-C1q antibody rather than whole IgG. C1q was frequently found in the eluates from the anti-C1q column but not in the isolates from the C1q-degalan column. In part this may reflect the relative insensitivity of the methods used to detect C1q but also it may imply that the complexes extracted from C1q-degalan column leave C1q behind on the solid phase.

The K-degalan column isolated IC because of the affinity between bovine conglutinin or K and IC bound C3bi. Binding of conglutinin to degalan beads was no more efficient than that of C1q but the bound protein was stable and the K column could be used many times without loss of activity. Using *in vitro* prepared complexes it was more efficient than the C1q based affinity columns and an estimated 50% of IC present in sera bound to this column. The greater efficiency of the K-degalan column presumably reflects the more accessible distribution and the greater quantity of C3bi bound onto the complex. This may also explain why the detection of immune complexes, estimated as HAGG equivalents, is more sensitive using the conglutinin binding assay than the C1q binding assay.

There was however a discrepancy between the efficiency shown by the K column when compared with the C1q columns in isolating IC and the frequency with which the respective K and C1q binding assays detected IC in pathological sera. In Hodgkin's disease the C1q binding assay was positive four times more frequently than the K binding assay (Amlot *et al.*, 1978) and usually one assay was positive to the exclusion of the other suggesting that they detected different types of IC Similar findings have been observed in RA and leprosy (Casali *et al*, 1977) as well as SLE (Eisenberg, Theofilopoulas & Dixon, 1977). In SLE during acute exacerbations the C3 level may fall

dramatically and under these circumstances the complexes may not have sufficient C3 on their surface to react with the K. This explanation is unlikely to account for the discrepancy observed in Hodgkin's disease, RA or leprosy since in these conditions the C3 level is usually normal or elevated. Other explanations have therefore to be considered. It is possible that the C3 coating of the KgB complexes allows for their rapid removal *in vivo* by cells of the RES and therefore they are only detected in the circulation when the RES is saturated and 'blocked'. There are also important differences between the complexes prepared *in vitro* and solubilized by serum and the complexes occurring *in vivo*. These two features may be mutually interdependent for C3 is an important component for solubilizing *in vitro* prepared IC The coating of C3 and its breakdown products on *in vitro* prepared complexes which favour its binding to K may favour their removal *in vivo* through the same C3 ligand.

EDTA elution is the single and essential step in dissociating IC from the K column for binding between C3bi and K is calcium-dependent. The sequential elution, starting with EDTA, for the C1q columns was intended to remove 'contaminant' or 'passenger' molecules such as CRP, which have a calcium-dependent binding. As anticipated CRP was found in the EDTA eluates of both K and C1q columns. However it was not expected that so much Ig and C3 would be eluted and in situations where CRP acting as a link molecule could not be implicated. It was also clear from previous experiments (Kilgallon *et al*, 1982) that Ig isolated in the EDTA eluate was largely non-specific and indeed EDTA eluates of BSA–rabbit anti-BSA complexes contained mostly human immunoglobulin presumably non-specifically acquired during solubilization in human serum. In the presence of divalent cations therefore there is an augmentation of complexed material which is irrelevant to the initial IC. This additional material is however dependent on activation of the complement pathway by IC for it was dependent on the initial presence of IC in the serum samples. It could not be mimicked by activation of the alternative pathway by zymosan.

Elution with 0 5M NaCl disrupts binding between C1q and the CH2 domain of Ig This was the means by which IC binding to the C1q-degalan column was achieved and led to some dissociation of IC from the anti-C1q column. Apart from Ig and C3 components, fibronectin was isolated and this was presumably due to the affinity between fibronectin and collagen like portion of C1q which is exposed when C1q binds to Ig (Menzel *et al.*, 1981)

Lastly acid elution is an essential step using the anti-C1q column and although it yields the least protein it contains most of the specific antigen and antibody constituting the IC. Acid eluates from HD complexes contained so little material that apart from stating that they contained IgG and complement components little more can be said More sensitive techniques than those applied in this study will have to be used in order to examine the acid eluted material in which, from the evidence of this study, the specific antigen and antibody is most likely to be found.

This study was supported by a grant from the Cancer Research Campaign

REFERENCES

AMLOT, P L, SLANEY, J M & WILLIAMS, B D (1976) Circulating immune complexes and symptoms in Hodgkin's disease *Lancet*, **i**, 449

AMLOT, P L, PUSSELL, B, SLANEY, J M & WILLIAMS, B D (1978) Correlation between immune complexes and prognostic factors in Hodgkin's disease *Clin exp Immunol* **31**, 166.

BEGENT, R H J, TUCKER, D F & KEEN. J (1980) A serum factor with potential as a tumour marker in malignant lymphoma *Br J Cancer*, **41**, 481

CASALI, P, BOSSUS, A, CARPENTIER, N A & LAMBERT, P-H (1977) Solid phase enzyme immunoassay or radioimmuno-assay for the detection of immune complexes based on their recognition by conglutinin conglutinin binding test *Clin exp Immunol* **29**, 342

CASALI, P & LAMBERT, P-H (1979) Purification of soluble immune complexes from serum using polymethylmetacrylate beads coated with conglutinin or C1q. *Clin exp Immunol* **37**, 295

EISENBERG, R A, THEOFILOPOULOS, A N & DIXON, F J (1977) Use of bovine conglutinin for the assay of immune complexes *J Immunol* **118**, 1428

HARRISON, R A & LACHMANN, P J (1980) Novel cleavage products of the third component of human complement *Mol Immunol* **17**, 219

JONES, V E & ORLANS, E (1981) Isolation of immune complexes and characterization of their constituent antigens and antibodies in some human diseases a review *J. Immunol Meth* **44**, 249

KILGALLON, W, AMLOT, P L. & WILLIAMS, B D (1982) Anti-C1q column lig and specific purification of immune complexes from human serum or plasma. Analysis of the interaction between C1q

and immune complexes *Clin exp Immunol* **48,** 705

LACHMANN, P J & HOBART, M (1978) Complement technology In *Handbook of Experimental Immunology* (ed by D M Weir) Vol 1 p 5A 7 Blackwell Scientific Publications, Oxford

LAEMMLI, U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4 *Nature*, **227,** 680

LAURELL, C B (1965) Antigen–antibody cross immunoelectrophoresis *Anal Biochem* **10,** 358

LOWRY, O H, ROSEBOROUGH, N J, FARR, A L & RANDALL, J (1951) Protein measurement with the folin phenol reagent *J biol Chem* **193,** 165

MANNIK, M (1967) Binding of albumin to γ A-myeloma proteins and Waldenstrom macroglobulins by disulphide bonds *J Immunol* **99,** 899

MENZEL, E J, SMOLEN, J S, LIOTTA, L & REID, K B M (1981) Interaction of fibronectin with C1q and its collagen-like fragment (CLF) *FEBS Lett* **129,** 188

OUCHTERLONY, O (1958) Diffusion in gel methods for immunological analysis *Prog Allergy*, **5,** 1

THEOFILOPOULOS, A N & DIXON, F J (1979) Immune complexes in human diseases *Am J Pathol* **100,** 531

TUCKER, D F, BEGENT, R H & HOGG, N M (1978) Characterization of immune complexes in serum by adsorption on staphylococcal protein A model studies and application to sera of rats bearing a gross virus-induced lymphoma *J Immunol* **121,** 1644

VIDAL, M A & CONDE, F P (1980) Studies of the IgM and IgA contamination obtained by eluting from a protein A sepharose column with pH steps *J Immunol Meth* **35,** 169

VOLANAKIS, J E & STROUD, R M (1972) Rabbit C1q purification, functional and structural studies *J Immunol Meth* **2,** 25

WILLIAMS, R C (1980) *Immune complexes in clinical and experimental medicine* Harvard University Press, Massachusetts

ZUBLER, R H, LANGE, G, LAMBERT, P-H & MIESCHER, P A (1976) Detection of immune complexes in unheated sera by the modified ^{125}I-C1q-binding test *J Immunol* **116,** 232

Clin exp Immunol (1982) **48**, 705–714

Anti-C1q column: ligand specific purification of immune complexes from human serum or plasma. Analysis of the interaction between C1q and immune complexes

W KILGALLON, P L AMLOT & B D WILLIAMS *Department of Immunology, Royal Postgraduate Medical School and Department of Medicine, Guy's Hospital Medical School, London*

(Accepted for publication 18 December 1981)

SUMMARY

An efficient and reproducible procedure has been developed for the specific isolation of immune complexes PEG precipitation of EDTA serum or plasma was an essential preliminary step to separate complex-bound from free C1q PEG had no discernible effect on the molecular weight size of the extracted complexes Redissolved complexes were incubated with a Sepharose-4B column coated with anti-human C1q antibodies and following removal of unbound material the bound complexes were sequentially eluted with 0 02 M EDTA, 0 5 M NaCl and 1 M propionic acid Characteristics of the affinity column were established by the purification of ^{125}I-labelled BSA–anti-BSA complexes and heat-aggregated IgG (HAGG) incubated in normal human serum (NHS) EDTA and NaCl eluted complexes were of similar molecular size and contained antigen, specific antibody, as well as human IgM, IgG, albumin, C3, C3c, C3d and C1q Acid eluted complexes contained the highest yield of specific antigen and antibody and comprised in addition human C1q and C3d Activation of complement components after C1q made the bond between C1q and immune complexes resistant to 0 5 M NaCl and interfered with the binding between solid phase anti-C1q and complex bound C1q Using BSA–anti-BSA complexes and HAGG activated in NHS it was apparent that only a minority of the complexed material was isolated via the C1q ligand and this probably applies to the C1q binding assay Most complexed material could be isolated using an anti-C3 affinity column

INTRODUCTION

In a large number of diseases 'immune complexes' have been detected in either patients' plasma or sera using a number of different non-specific techniques (WHO Bulletin, 1977, Lambert *et al*, 1978) Whereas in some of these diseases a pathogenetic role for the immune complexes seems established, e g in immune complex-mediated nephritis, in the majority of diseases at most only a tentative pathogenetic role can be suggested Since in all cases the 'immune complexes' are detected by non-specific means it is possible that the material detected does not represent true antigen–antibody complexes Non-specific binding between immunoglobulin, complement, acute phase proteins and other serum proteins in many of the chronic inflammatory diseases in which 'immune complexes' are found may mimic true immune complexes as a result of the test used to detect them Efforts therefore have been made to resolve this problem by isolating immune complexes from pathological

Correspondence P L Amlot, Department of Medicine, Guy's Hospital Medical School, London SE1 9RT, UK

0009-9104/82/0600-0705$02 00

sera and attempting to identify the individual components within them Evidently the most attractive viewpoint would be that the immune complexes are true antigen–antibody complexes and isolation of either antigen or antibody might help to elucidate the aetiology of some of the poorly understood diseases in which they are found

For the enrichment of immune complexes in sera several methods have used polyethylene glycol (PEG) which preferentially precipitates molecules of high molecular weight from serum or plasma (Creighton, Lambert & Miescher, 1973, Chia *et al*, 1979, Male & Roitt, 1979) Although this is a relatively simple procedure further fractionation of the sample is necessary to achieve better separation of immune complexes from coprecipitated high mol. wt material. One such further step has used the affinity of protein A for immunoglobulin, a *sine qua non* of immune complexes (Chenais *et al.*, 1977) However protein A does not bind all subclasses of IgG and will bind any high mol wt fraction of IgG such as aggregated IgG.

Logically the most appropriate method for isolating 'immune complexes' from serum or plasma would use the same ligand for extraction as utilised in the assay to detect them The use of C1q as a biological receptor for the isolation of 'immune complexes' was first described by Svehag & Burger (1976) Casali & Lambert (1979) improved this technique by attaching C1q to degalan beads and using the interchange of complexed material between solid and fluid phase C1q to isolate immune complexes This paper examines the use of anti-human C1q antibody molecules bound to a solid phase support as an alternative method for isolating C1q binding complexes.

MATERIALS AND METHODS

Radiolabelling Proteins were radiolabelled with ^{125}I-Na or ^{131}I-Na using the Iodogen™ (Pierce Chemical Co.) reagent (Markwell & Fox, 1978). Twenty-five micrograms of Iodogen in 100 μl chloroform were dried under nitrogen onto the base of a 1 ml glass container. Fifty micrograms protein and 500 μCi ^{125}I-Na or ^{131}I-Na were added and after 30 min incubation at room temperature the protein was removed from the reaction vessel, diluted with complement fixation diluent (CFD) (Oxoid Ltd , London) containing 0 25 M NaI and dialysed against CFD Specific activities were in the range 1–5 μCi/μg

Preparation of immune complexes. Immune complexes were prepared at equivalence by incubating 100 μg bovine serum albumin (BSA) and 360 μg of affinity purified anti-BSA antibody with 3 ml of fresh normal human serum (NHS) at 37°C for 60 min. The anti-BSA antibody was raised in rabbits which had been rendered tolerant to human serum albumin (HSA) (gift of Professor J Humphrey) Where appropriate, trace amounts of labelled antigen or antibody were added to the unlabelled reagents Insoluble material was removed by centrifugation and the soluble immune complexes were extracted with PEG before being applied to the anti-C1q column In some experiments 250 μg heat aggregated human IgG (HAGG) were used instead of BSA–anti-BSA complexes

Heat-aggregated human IgG (HAGG) Human IgG was prepared by DEAE-cellulose chromatography, heated at 63°C for 15 min and processed according to the method of Greenwood *et al* (1971) HAGG so prepared remained soluble in CFD. The sedimentation coefficient, measured by centrifugation through 10–50% sucrose density gradients, was in the range of 30–35S

Purification of human C1q Human C1q was purified by the method of Volonakis & Stroud (1972) with one modification (Zubler *et al*, 1976) Relative salt concentrations of 0 04 and 0 078 were used for the first and second purifications respectively

Preparation of ^{125}I-C1q-BSA anti-BSA and ^{125}I-C1q HAGG complexes ^{125}I-C1q was incubated with 100 μg BSA and 360 μg anti-BSA antibody (equivalence) or 250 μg HAGG in CFD at 37°C for 30 min. Insoluble ^{125}I-C1q-BSA–anti-BSA complexes were recovered by centrifugation and were either studied alone or after incubation with 3 ml of NHS at 37°C for 30 min Complexes were extracted and purified as described later

Extraction of immune complexes. Lipids were removed from immune complex containing serum or plasma by the addition of one volume of trichlorofluorethane (Frigen 113 TR-T, Hoechst, Germany) to two volumes of serum or plasma The mixture was stirred at room temperature for 30

min and then centrifuged at 1500*g* for 15 min at 4°C. Largely lipid-free serum or plasma (EDTA—see results) was mixed with an equal volume of 8% polyethylene glycol 6,000 (PEG) After 2 hr at 4°C the mixture was centrifuged at 1500*g* for 30 min at 4°C and the resulting precipitate was dissolved in CFD

Anti-C1q affinity column The immunoglobulin fraction of a rabbit anti-human C1q antiserum (Seward) was precipitated with 50% $(NH_4)_2SO_4$ and then dialysed against 0 1 M $NaHCO_3$ buffer at pH 8 3 The anti-C1q antiserum gave a single precipitin line against whole serum and this line gave a reaction of complete identity against purified human C1q Fifteen milligrams of protein was reacted with 1 gm (dry wt) of CNBr–Sepharose-4B (Pharmacia, Uppsala, Sweden) by the method of Cuatrecasas & Anfinsen (1971) Ninety-five percent of the protein bound to the sepharose

PEG extracted and redissolved immune complexes were poured onto a 1 5 ml volume column and left for 30 min at room temperature The unbound material was removed by extensive washing with CFD. Subsequent elution of bound material was effected by the sequential application of CFD buffer containing 0 02 M EDTA, 0 5 M NaCl and 1 M propionic acid Propionic acid eluates were immediately neutralized with 2 M NaOH

Other affinity columns CNBr–Sepharose-4B affinity columns of mouse IgG and sheep anti-human C3 were prepared as described for the anti-C1q column Immune complexes or HAGG which had ^{125}I-C1q incorporated in them and which failed to bind to the anti-C1q column, were incubated with the anti-C3 column at room temperature for 30 min Material not bound to the column was removed by extensive washing with CFD and bound material was eluted with 1 M propionic acid and immediately neutralized with 2 M NaOH

Analysis of eluted complexes Eluates from the anti-C1q and anti-C3 columns were analysed by immunodiffusion, immunoelectrophoresis, SDS-PAGE (Laemli, 1970) and sucrose density gradient centrifugation Samples were applied to 10–50% sucrose gradients and centrifuged in a 6 × 5 cm rotor on an MSE centrifuge for 17 hr at 30,000 rpm (100,000*g*) The gradients were calibrated using ^{125}I-radiolabelled BSA (4S), human IgG (7S) and IgM (19S) as molecular weight markers

Specific antisera The antisera used for identifying proteins by Ouchterlony were goat anti-human IgM, goat anti-human IgG and a sheep anti-C3, each of which were raised in the Department of Immunology and gave single precipitin lines against whole serum and purified IgM, IgG and C3 Commercial antisera were obtained from Seward, UK (anti-human C1q), Organon, Teknika, Germany (anti-C3c) and Netherlands Red Cross Blood Transfusion Service (anti-C3d)

RESULTS

Preparative enrichment of immune complexes with PEG

Four percent PEG efficiently precipitated immune complexes over a wide range of antigen/antibody ratios (Fig 1) Although it did not differ in efficiency from 40% $(NH_4)_2SO_4$ it had two important advantages it precipitated very much less extraneous protein and it did not precipitate free C1q from EDTA plasma or EDTA treated sera as demonstrated by Ouchterlony analysis of the precipitate (Fig 2) Thus plasma obtained directly in EDTA (EDTA plasma) and sera to which EDTA was added (EDTA sera) at a final concentration of 0 02 M were used before PEG precipitation to dissociate the C1 molecule into its components Furthermore 4% PEG precipitation of ^{125}I-BSA–anti-BSA complexes formed in NHS showed no evident change in the molecular size of the complexes analysed by sucrose density gradient (Fig 3)

Affinity binding between C1q bound to HAGG and anti-C1q

^{125}I-HAGG was incubated in either NHS or EDTA serum at 37°C for 30 min then extracted with 4% PEG and incubated with the anti-C1q column (Table 1) Conditions in EDTA-serum should only allow C1q to react with the HAGG but inhibit subsequent activation and binding of other complement components and in these conditions 63% of the ^{125}I-HAGG bound to the anti-C1q column and most of the bound material was eluted with 0 5 M NaCl In marked distinction when another sample of the same ^{125}I-HAGG was incubated with NHS only 6% of the added material

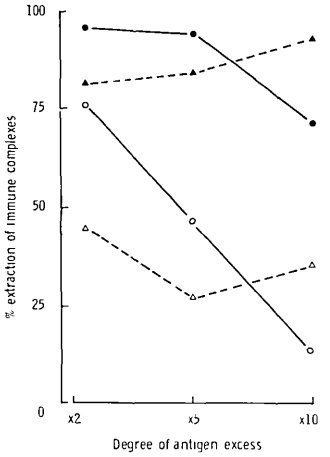


Fig. 1. Extraction of ^{125}I-BSA-anti-BSA complexes from NHS Efficiency of extraction is expressed as a percentage of the total complexed antigen (●——●=4% PEG, ○——○=2% PEG, ▲——▲=40% $(NH_4)_2SO_4$, △——△=20% $(NH_4)_2SO_4$)

bound to the column The maximum binding achieved for ^{125}I-HAGG in NHS (29 2%) was never as high as that in EDTA sera or plasma ^{125}I-HAGG alone did not bind to the anti-C1q column nor did ^{125}I-HAGG or ^{125}I-C1q-HAGG bind to a sepharose column coated with a non-specific immunoglobulin (mouse IgG)

Binding of pre-formed immune complexes to the anti-C1q column

BSA–anti-BSA complexes incubated in NHS bound to the anti-C1q column in similar proportions to HAGG (i e 15–30% of total complexes) BSA–anti-BSA complexes formed as described precipitate in EDTA serum and therefore their binding in the absence of subsequent complement components could not be tested BSA–anti-BSA complexes formed in NHS and processed as described above were predominantly eluted in the NaCl and acid washes from the anti-C1q column (Table 2) A comparison of the amount of specific antigen or antibody isolated relative to the total protein eluted in the various washes expressed as the proportion of specific labelled material showed that the acid eluate had the highest yield (Table 2) Increasing the salt concentration to 1 M did not

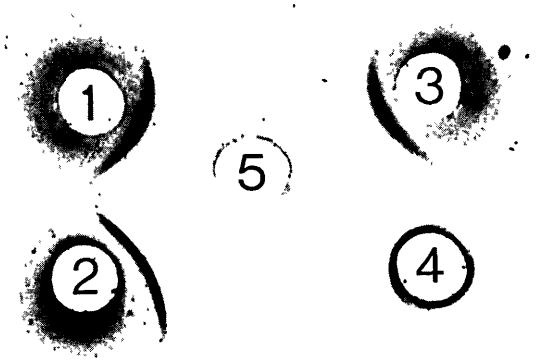


Fig. 2. Ouchterlony demonstration that free C1q is not precipitated by 4% PEG (1) NHS, (2) 4% PEG ppt of NHS (3) EDTA serum, (4) 4% PEG ppt of EDTA serum (5) Rabbit anti-human C1q

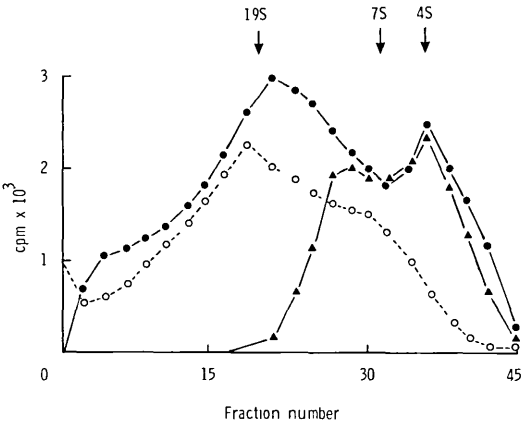


Fig. 3. Sucrose density centrifugation of ^{125}I-BSA–anti-BSA complexes in NHS (●——●), 4% PEG supernatant (▲——▲) and the redissolved PEG precipitate (○——○)

Table 1. Binding of ^{125}I-HAGG to the anti-C1q column

	^{125}I-HAGG incubated* in	
	EDTA serum	NHS
% total c p m bound	63 4	6 1
% total c p m eluted with		
0 02 M EDTA	19 7	38 2
0 5 M NaCl	70 9	37 9
1 M Propionic acid	9 4	23 9

* ^{125}I-HAGG incubated at 37 C for 30 min When incubated in saline <0 3% total c p m bound to the anti-C1q column

Table 2. Distribution of protein and BSA–anti-BSA complexes in the three eluates from the anti-C1q column

Eluting solutions	Antigen labelled complexes		Antibody labelled complexes	
	% BSA eluted*	μg BSA/mg protein†	% anti-BSA*	μg anti-BSA/mg protein†
0 02 M EDTA	9 2	2 3	14 1	3 4
0 5 M NaCl	43 9	16 1	42 1	19 5
1 M Propionic acid	46 9	51 0	43 8	62 0

* % of total label eluted from anti-C1q column

† μg antigen and antibody were calculated from recovery of ^{125}I and total protein was measured by the method of Lowry *et al* (1951)

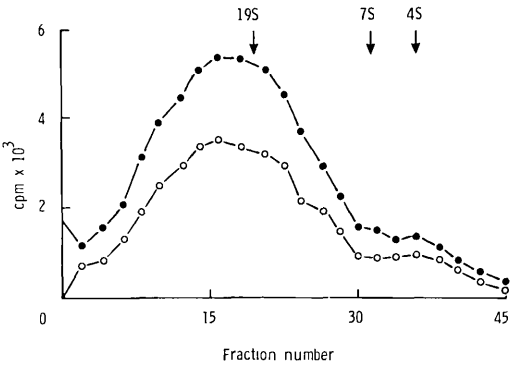


Fig. 4. Sucrose density centrifugation of ^{125}I-BSA–anti-BSA complexes formed at equivalence in NHS and extracted with 4% PEG (●——●) Complexes which did not bind to the anti-C1q column (○——○)

dissociate the acid elutable material from the column Neither free antigen nor antibody, when incubated in NHS, bound to the anti-C1q column ^{125}I-BSA–anti-BSA complexes formed in NHS did not bind to a mouse IgG-sepharose-4B column No protein from NHS activated with zymosan and precipitated with PEG as described bound to the anti-C1q column

Binding of immune complexes to the anti-C1q column

Fig 4 shows the molecular weight profiles of ^{125}I-BSA–anti-BSA complexes prepared at equivalence in NHS, precipitated with 4% PEG and redissolved in CFD When incubated with the anti-C1q column 15–30% of the complexes bound The unbound complexes had a similar molecular weight profile to those which bound to the anti-C1q column The failure of the majority of these complexes to bind to the column was not due to saturation of the column, for they also failed to bind when they were re-applied to either a fresh or a regenerated anti-C1q column

Complexes not binding to the anti-C1q column do bind to an anti-C3 column

^{125}I-C1q was incorporated into BSA–anti-BSA complexes at equivalence and HAGG to further investigate the molecular characteristics of the material which failed to bind to the anti-C1q column Table 3 shows that high percentages of ^{125}I-C1q bound to the anti-C3 column after previously not binding to the anti-C1q column It is self-evident that ^{125}I-C1q was present in complexes that did not bind to an anti-C1q column

Characteristics of anti-C1q column purified immune complexes

By Ouchterlony and SDS-PAGE analysis of the isolated BSA–anti-BSA complexes we were able to demonstrate the expected presence of BSA and rabbit anti-BSA immunoglobulin G (see also Table

Table 3. Binding of ^{125}I-C1q-immune complexes sequentially to the anti-C1q and anti-C3 columns

	% bound c p m /total c p m	
	^{125}I-C1q-BSA–anti-BSA*	^{125}I-C1q-HAGG*
Anti-C1q column	19 6	29 2
Anti-C3 column†	56 3	32 9

* incubated in NHS at 37°C for 30 min

† c p m not binding to anti-C1q column subsequently applied to the C3 column

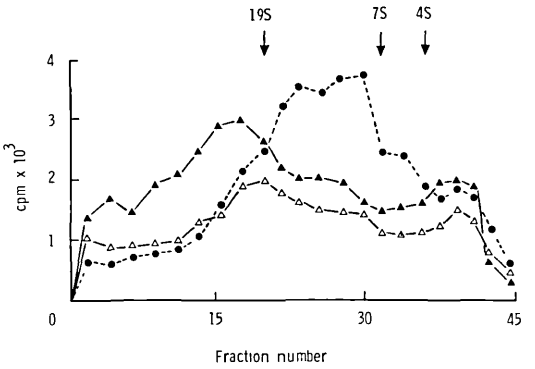


Fig. 5. ^{125}I-BSA–anti-BSA complexes formed at equivalence in NHS, PEG extracted and eluted from the anti-C1q column with 0 02 M EDTA (△——△), 0 5 M NaCl (▲——▲), 1 M propionic acid (●——●)

2) but in addition there were human serum proteins—human IgG, IgM, albumin, C3, C3c, C3d and C1q in both of the EDTA and NaCl eluates Only BSA, anti-BSA and human C1q and C3d were identified in the acid eluates When either stored or fresh NHS, free of immune complexes, was put through the extraction and isolation procedures described, no detectable protein was recovered from the anti-C1q column

Fig 5 demonstrates that the molecular weight profiles of the complexes eluted with EDTA and NaCl were similar to each other and to the complexes precipitated from EDTA-treated sera with PEG Immune complexes eluted with acid were of a smaller molecular size Subsequent experiments have shown that acid treatment and subsequent neutralization of preformed immune complexes of known molecular size does not generate smaller sized complexes

DISCUSSION

C1q binding assays are frequently used to detect immune complexes in pathological sera. When therefore attempts were made to isolate these complexes from sera it was logical to utilize C1q as an affinity ligand on an immunoadsorbent column The first attempts to do this, however, were hindered by the competition between free C1 in serum and the agarose bound C1q for immune complex bound C1q (Svehag & Burger, 1976). In an attempt to overcome this polyethylene glycol was used to precipitate immune complexes from the serum, which were then redissolved and incubated with Degalan beads to which had been attached purified C1q (Casali & Lambert, 1978) Although immune complexes could be purified from pathological sera using this technique the quantities isolated were small An additional disadvantage which we found with this method was the loss of activity of the C1q after two or three isolation procedures

This paper describes the use of an anti-C1q immunoadsorbent column to remove C1q binding immune complexes from serum or plasma An $(NH_4)_2SO_4$ cut of monospecific anti-C1q serum was coupled to the CNBr–Sepharose-4B in order to bind specific anti-C1q antibody molecules randomly on the solid phase with other non-specific Ig to act as spacers The effective light substitution of anti-C1q molecules allowed easier elution of bound immune complexes from the column

An essential preliminary step involved the separation of free C1q from C1q bound to immune complexes and this was achieved by precipitation of bound C1q from EDTA-serum or EDTA-plasma with 4% PEG The resolubilized material was then applied to the anti-C1q column and eluted in three stages. Three eluting solutions were used in order to examine the bonds between the solid phase anti-C1q antibody and immune complex material bound to the column Thus 0 02 M EDTA dissociates bonds dependent on divalent cations, especially calcium ions, 0 5 M NaCl should dissociate binding between C1q and antibody, while 1 M propionic acid should dissociate anti-C1q

antibody binding to the C1q molecule The efficiency of the system has been examined using heat-aggregated IgG (HAGG) and preformed immune complexes of BSA–anti-BSA

HAGG bound to C1q alone by incubation in EDTA serum was soluble and was purified with a high degree of efficiency from the anti-C1q column. As expected the majority dissociated in 0 5 M NaCl (Table 1) When HAGG was incubated with NHS, however, very much less of the aggregate was isolated from the column, the majority passing through the column unbound. In examining the possible explanations for this observation, viz displacement of bound C1q or interference/masking of the C1q by other complement components or proteins, we were able to show that when labelled C1q was pre-incubated with HAGG and the complexed material added to serum, the labelled C1q remained part of the complexes although they failed to bind to the anti-C1q column (Table 3) The non-binding complexes, however, could bind to the anti-C3 affinity column and similar results were obtained when BSA–anti-BSA complexes were used (Table 3) It was likely therefore that after incubation in NHS other proteins, especially C3 and its breakdown products, interfered with binding to the anti-C1q column. In order for BSA–anti-BSA complexes to be solubilized NHS is necessary, and solubilization is dependent upon adequate levels of C3, factor B, factor D and properdin (Czop & Nussenzweig, 1976) It has been shown that solubilization depends on intercalation of C3b onto the immune complex lattice It is likely therefore that molecules such as C3 or C4 and their degradation products stoichometrically interfere with the binding between C1q and anti-C1q on the solid phase column

Although small quantities of either HAGG or BSA–anti-BSA were added to the serum, much larger amounts of protein were isolated from the affinity column, particularly in the EDTA and NaCl eluates (Table 2) The isolation of this protein, generated by complement activation, was however dependent on the presence of immune complexes since no protein bound or was isolated where a PEG extract of zymozan activated NHS was applied to the column The majority of these serum proteins which were assembled on the immune complexes were susceptible to elution with 0 02 M EDTA even though the mechanisms by which these proteins bound to the complexes and to each other are not known, their EDTA sensitivity suggested that divalent cations were necessary The generation of fragments containing macromolecular C3 following the addition of immune complexes to NHS probably accounts for the high incidence of positive results obtained when these fragments were identified on gel chromatography Of four separate assays used to examine the incidence of immune complexes in Hodgkin's disease, the macromolecular C3 assay was more frequently positive when compared with the C1q binding assay, K binding assay and raised levels of plasma C3d (Amlot *et al*, 1978)

Of the complexes which bound C1q and were available to interact with the anti-C1q antibody a proportion were eluted from the column with 0 5 M NaCl This fraction does however contain many complement components and other non-complement serum proteins such as IgM, IgG and albumin Small amounts of IgM rheumatoid factor or immunoconglutinins (IK) may account for the isolation of IgM and IgG The albumin may be present simply because of its association with IgM and IgG (Mannik, 1967, Hauptman & Sobczak, 1976) The binding between C1q and immunoglobulin is known to be sensitive to 0 5 M NaCl, therefore the inability to dissociate all C1q complexes with 0 5 M NaCl supported previous observations that subsequent binding of other complement components stabilized the attachment of C1q to immunoglobulin (Table 1)

The complexes isolated in the acid eluate were smaller than those found in the EDTA or NaCl eluates The difference was not simply due to an alteration in the ratios of antigen and antibody after extraction, since complexes of known molecular weight size showed no alteration in their molecular weight profiles following PEG extraction, acid treatment and neutralization They may therefore represent complexes which had either a high affinity for C1q or those complexes in which interference of binding by complex bound C3b had been reduced following the cleavage of this molecule into its small fragment C3d Supporting this idea was our finding that anti-sera to C3d reacted with these complexes, whereas anti-sera to C3 or C3c did not

The combination of PEG precipitation of EDTA serum or plasma followed by affinity chromatography on an anti-C1q affinity column is an efficient and reproducible method for isolating immune complexes Only a small percentage ($\leqslant 30\%$) of the complexes present are isolated by this method and the EDTA and NaCl eluates contain relatively large amounts of serum proteins

which bind to the complexes following complement activation Nevertheless the acid eluate is relatively free from these proteins and is clearly the fraction of choice when studies are undertaken to identify the antigen or examine the specificity of the antibody in an immune complex It also offers a considerable advantage in that SDS-PAGE of this eluate is free from co-purified serum proteins and their peptide fragments

Two points of interest arise out of this study on the isolation of C1q binding complexes Firstly, a minority (ranging from 15–30%) of immune complexes having bound C1q have accessible C1q on their surface and yet a larger proportion (50–60%) have C3 or C3 breakdown products on their surface Secondly, the stabilization of the C1q bond to the immune complex by subsequently bound C3 or C4 components is unlikely to allow free interchange between the bound C1q and ^{125}I-C1q as normally performed in a C1q binding assay (C1q-BA) This means that the majority of immune complexes will be undetectable by the C1q-BA and could explain the poor yield obtained with a C1q-degalan column which relies upon interchange of fixed and free C1q (Casali & Lambert, 1979) These observations lend support to the proposal that C3 in a macromolecular form is a good marker and indicator of immune complexes (Amlot, Slaney & Williams, 1976) and has obvious implications in the assessment of immune complexes by assays which recognise C1q (Zubler *et al.*, 1976) as opposed to those that recognise C3 and its activated products (Theofilopoulos, Eisenberg & Dixon, 1978, Casali *et al*, 1978) Thus to obtain a good assessment of immune complexes in pathological sera a combination of assays should be employed in order to detect total immune complexes

This work was financially supported by a grant from the Cancer Research Fund

REFERENCES

AMLOT, P L , SLANEY, J M & WILLIAMS, B D (1976) Circulating immune complexes and symptoms in Hodgkin's disease *Lancet*, **i,** 449

AMLOT, P L , PUSSELL, B , SLANEY, J M & WILLIAMS, B D (1978) Correlation between immune complexes and prognostic factors in Hodgkin's disease *Clin exp Immunol* **31,** 166

CASALI, P , BOSSUS, A , CARPENTIER, N A & LAMBERT, P -H (1977) Solid-phase enzyme immunoassay or radioimmunoassay for the detection of immune complexes based on their recognition by conglutinin conglutinin binding test A comparative study with ^{125}I-labelled C1q binding and Raji cell RIA tests *Clin exp Immunol* **29,** 342

CASALI, P & LAMBERT, P -H (1979) Purification of soluble immune complexes from serum using polymethylmetacrylate beads coated with conglutinin or C1q *Clin exp Immunol* **37,** 295

CHENAIS, F , VIRELLA, G , PATRICK, C C & FUDENBERG, H H (1977) Isolation of soluble immune complexes by affinity chromatography using staphylococcal protein A-sepharose as substrate *J Immunol Meth* **18,** 183

CHIA, D , BARNETT, E V , YAMAGATA, J , KNUTSON, D , RESTIVO, C & FURST, D (1979) Quantitation and characterization of soluble immune complexes precipitated from sera by polyethylene glycol (PEG) *Clin exp Immunol* **37,** 399

CREIGHTON, W D , LAMBERT, P -H & MIESCHER, P A (1973) Detection of antibodies and soluble antigen–antibody complexes by precipitation with polyethylene glycol *J Immunol* **111,** 1219

CUATRECASAS, P & ANFINSEN, C D (1971) Affinity chromatography *Meth Enzym* **22,** 345

CZOP, J & NUSSENZWEIG, V (1976) Studies on the mechanism of solubilisation of immune precipitates by serum *J exp Med* **143,** 615

GREENWOOD, B M , BROWN, J C , DE JESUS, D G & HOLBOROW, E J (1971) Immunosuppression in murine malaria II The effect on reticulo-endothelial and germinal centre function *Clin exp Immunol* **9,** 345

HAUPTMAN, S P & SOBCZAK, G (1976) Origin of immunoglobulin albumin complex *Nature*, **263,** 64

LAEMLI, U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4 *Nature*, **227,** 680

LAMBERT, P -H , DIXON, F J , ZUBLER, R H , AGNELLO, V , CAMBIASO, C , CASALI, P , CLARKE, J , COWDERY, J S , MCDUFFIE, F C , HAY, F C , MACLENNAN, I C M , MASSON, P , MULLER-EBERHARD, H J , PENTTINEN, K , SMITH, M , TAPPEINER, G , THEOFILOPOULOS, A N & VERROUST, P (1978) A collaborative study for the evaluation of eighteen methods for detecting immune complexes in serum *J clin Lab Immunol* **1,** 1

LOWRY, O H , ROSEBOROUGH N J , FARR, A L & RANDALL, J (1951) Protein measurement with the folin phenol reagent *J Biol Chem* **193,** 265

MALE, D & ROITT, I M (1979) Analysis of the components of immune complexes *Mol Immunol* **16,** 197

MANNIK, M (1967) Binding of albumin to γA-myeloma proteins and Waldenstrom macroglobulins by disulfide bonds *J Immunol* **99,** 899

MARKWELL, M A K & FOX, C F (1978) Surface-specific iodination of membrane proteins of viruses and eukaryotic cells using 1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril *Biochemistry*, **17,** 4807

SVEHAG, S E & BURGER, D (1976) Isolation of C1q-binding immune complexes by affinity chromatography and desorption with a diaminoalkyl compound *Acta path Microbiol Scand Sect C* **84,** 45

THEOFILOPOULOS, A N , EISENBERG, R & DIXON, F J (1978) Isolation of circulating immune complexes using Raji cells *J clin Invest* **61,** 1570

VOLONAKIS, J E & STROUD, R M (1972) Rabbit C1q purification, functional and structural studies *J Immunol Meth* **2,** 25

WHO REPORT OF A SCIENTIFIC GROUP (1977) The role of immune complexes in disease *WHO Technical Report Series* No 606

ZUBLER, R H , LANGE, G , LAMBERT, P-H & MIESCHER, P A (1976) Detection of immune complexes in unheated sera by a modified ^{125}I-C1q binding test *J Immunol* **116,** 232