COMPLEMENT MEDIATED PREVENTION OF IMMUNE PRECIPITATION IN RHEUMATIC DISEASE

by

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A thesis submitted to fulfil the requirements

for the Degree of Doctor of Medicine

in the University of Glasgow, Faculty of Medicine

July 1988

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ACKNOWLEDGEMENTS

I would like to take this opportunity to thank Professor R.M.M. MacSween, Department of Pathology, Glasgow Western Infirmary in whose department the majority of the work for this thesis was carried out.

Especial thanks to Professor Keith Whaley, Department of Immunopathology whose constant advice, constructive criticism and endless encouragement ensured that this work was completed.

For technical advice and criticism I am indebted particularly to Mrs Jean Veitch but also to the staff of the Immunopathology Department and Laboratory 120. I am also indebted to them all for their continuing support and friendship especially through the difficult patches.

I am grateful to my clinical colleagues, Dr J A Hunter (Gartnavel General Hospital, Glasgow) and Dr Hilary A Capell (Centre for Rheumatic Diseases, Glasgow Royal Infirmary) for permission to study their patients.

I acknowledge the funding of the Arthritis and Rheumatism Council in supporting me during the duration of this research work.

Finally, but not least, I extend my sincerest gratitude to Mrs Pauline Potts for her expert typing of this thesis and her prolonged patience with me.

FOR MAUREEN

SUMMARY

The prevention of immune complex precipitation (PIP) is the phenomenon whereby immune complexes formed at or near equivalence in serum are held in solution and an immune precipitate does not form. PIP is mediated by the complement system via activation of the classical pathway. The formation and deposition of circulating immune complexes is an important cause of immunologically mediated tissue injury. The complement system through the PIP mechanism therefore acts to prevent the formation of these large insoluble complexes which might deposit in tissues and thereby cause disease. My hypothesis was that if PIP was important in inhibiting the formation of these phlogistic complexes then one might expect there to be defects in PIP in patients with immune complex disease. I therefore developed an assay and proceeded to measure PIP in the sera of patients with rheumatic disease in which immune complexes were implicated in disease pathogenesis.

The sera of forty seven per cent of patients with seropositive rheumatoid arthritis (RA) failed to prevent the precipitation of antigen - antibody complexes. This was not due to hypocomplementaemia suggesting that the impairment of PIP in these sera was due to the presence of an inhibitor of PIP.

An assay to measure this inhibitory activity was developed. Inhibitory activity was found in the majority of patients with seropositive RA in both sera and synovial fluid. Sera from patients with seronegative arthritis contained little or no inhibitory activity. In seropositive RA sera inhibitory activity was inversely correlated with PIP. Inhibitory activity in the serum did not correlate with indices of generalised disease activity (articular index, erythrocyte sedimentation rate (ESR), haemoglobin, white cell count or platelets). In the synovial fluid (SF) inhibitory activity did not correlate with SF protein content or white cell count and only weakly with a localised joint activity score. However, the level of inhibitory activity was associated with the presence of

the extra-articular features of RA being highest in those patients with nodular disease and especially in RA patients whose disease was complicated by skin or major organ vasculitis.

Although significant levels of inhibitory activity were only found in sero-positive RA the correlation between inhibitory activity and IgM-RF (as measured by ELISA) was poor. Some patients sera displayed high levels of inhibitory activity and low IgM-RF and vice versa.

The purification of the molecule(s) responsible for inhibitory activity was carried out. Inhibitory RA serum was subjected to IgG Sepharose chromatography and subsequent gel filtration on Sepharyl S300. IgM-RF co-chromatographed with inhibitory activity at all stages of the purification procedure.

However, studies on the mechanism of action of the inhibitor of PIP in RA sera showed that IgM-RF was not alone responsible for the phenomenon in whole RA serum. The inhibitor of PIP reduced C4 consumption by antigen - antibody complexes but purified IgM-RF did not display that capacity; furthermore purified IgM-RF inhibited PIP to a greater extent than the same amount of IgM-RF in RA serum.

To delineate these aspects of inhibitory activity more clearly further purification procedures were developed. Inhibitory activity was shown to exist in the higher molecular weight fractions in both RA and normal serum when these sera were subjected to sucrose density gradient ultracentrifugation. Inhibitory activity was expressed differently in RA and normal serum and this final expression of inhibitory activity in serum was dependent on the modulating influences of various antagonists found in the whole serum. Further purification

using small columns of IgG Sepharose revealed that the majority of inhibitory activity bound to the column and could be eluted with 0.4M NaCl (normal serum) or 2M guanidine hydrochloride (RA serum). SDS-PAGE analysis of the protein bands revealed a constant band representing a protein of MW 60,000 Daltons. I have suggested that this protein was responsible for the inhibition of PIP found in RA and normal serum.

In the final chapter I have discussed the significance of my findings and the role of PIP in immune complex processing in health and disease in the light of recent work by other authors in this field. The failure to prevent immune precipitation in RA serum may have pathogenetic significance as indicated by the close relationship with reduced PIP and the extra-articular manifestations of the disease. Further studies are suggested to address this problem including prospective studies on the natural history of inhibitory activity in the individual RA patient, the effects of disease modifying drugs (eg gold, penicillamine and chloroquine) on the process and the relationship between the inhibitor of PIP and IgM-RF. PIP is only one aspect of a highly complicated physiological phenomenon whereby immune complexes are processed and safely disposed of by the reticuloendothelial system in man. The requirement for continued studies at basic level is stressed in both normal and pathological sera in order to place in perspective the role of inhibitory molecules in the PIP reaction.

CHAPTER ONE

INTRODUCTION

PREFACE

The experimental work contained in this thesis is concerned with the specific interaction of immune complexes with the complement system. I have concentrated on the phenomenon of complement mediated prevention of immune complex precipitation, a consequence of the interaction between immune complexes and complement whereby the complexes remain small and soluble and the formation of large insoluble lattices is prevented (Schifferli, Bartolotti & Peters, 1980). In this introduction I will survey the general background to the studies contained in this thesis. The experimental work contained herein was commenced in 1983, and against this background of previous work in this field I was able to formulate my ideas for further research in this area leading up to my own observations which are set out in the first results section.

INTRODUCTION

When an individual is exposed to an antigen, specific antibodies are synthesised which interact with the inciting antigen, uniting with it non-covalently to form immune complexes. This normal phenomenon is designed to benefit the host by eliminating and/or neutralising the antigens. There are however some circumstances where the formation of immune complexes is detrimental to the host. These harmful effects of immune complexes were first noted by von Pirquet (1911), who, when studying the course of serum sickness, proposed that the interaction of antigen with host antibody in the circulation produced toxic factors. He related the production of these toxic factors to the onset and the course of the serum sickness reaction. Other workers made similar observations (Longcope, 1915; Rich & Gregory, 1943; Hawn & Janeway, 1947).

In the 1950's the definite pathogenetic role of the immune complexes was demonstrated by the experiments of Germuth (1953), Germuth & McKinnon (1957) and Dixon and associates (Dixon et al, 1958; Dixon, Feldman & Vazquez, 1961). The original theory of von Pirquet was confirmed and expanded using the experimental rabbit model of 'one-shot' serum sickness. The onset of generalised vasculitis and glomerulonephritis in this model co-incided with the appearance of soluble immune complexes in the circulation of the rabbits, at the same time serum complement activity decreased and immune complexes were deposited at the sites of tissue injury (Dixon, 1963).

Many factors involved in immune complex formation, removal and localisation have been clarified by in vivo and in vitro experiments.

Formation and Fate of Immune Complexes

Most naturally occurring antigens are multivalent and antibody molecules possess at least two antigen-binding sites. When complexes are formed, extensive cross-linking may occur leading to the formation of large aggregates of reduced solubility. These large aggregates would be difficult to remove if formed in the tissues and, if formed in the circulation, would tend to precipitate in the blood vessels. Insoluble complexes appear to be of central importance in the production of immune complex-mediated tissue injury and the evidence for this comes from several sources outlined below. The complement system plays an important role in the inflammatory reactions induced by these insoluble complexes. In vivo antigen-antibody complexes are formed at three general anatomical sites viz (1) the reaction of antibody with fixed structural antigens eg a cell surface membrane, or basement membrane, (2) the reaction of antibody with antigen which is secreted or injected into the interstitial fluids, (3) the reaction of antibody with soluble antigens found in the circulation.

(1) The basement membrane is one such fixed structural antigen against which immune responses may be directed. An example of antibasement membrane antibody-induced disease is Goodpasture's syndrome characterised clinically by glomerulonephritis and pulmonary haemorrhage, another is anti-tubular basement membrane antibody tubulo-interstitial nephritis (Wilson & Dixon, 1976). In these situations where the antigen is insoluble (basement membrane) one ends up with an insoluble complex.

(2) The Arthus reaction is the classical experimental form of the reaction between antibody and antigen secreted or injected into the interstital fluids (Arthus, 1903; Cochrane & Janoff, 1974). In this reaction antigen is injected intradermally into immunised animals and reacts with antibody in and around

the blood vessels which carry the circulating antibody to the site of the injected antigen. The reaction is a vasculitis which is due to the formation of immune complexes in the region of the vessel wall. The ensuing inflammatory response occurs secondary to the activation of mediator systems which are activated by the complexes (Cochrane & Janoff, 1974). The Arthus reaction is produced much more effectively by precipitating antibody as opposed to non-precipitating antibody (Cochrane & Weigle, 1958). Sequential immunofluorescence studies have demonstrated that following the deposition of immune complexes and complement components in the vessel wall there is a cellular influx of neutrophils initially but after 8 hours mononuclear cells appear and are predominant by 24 - 48 hours.

(3) Circulating soluble antigens can react with antibody to form immune complexes which themselves continue to circulate. The eventual fate of these circulating complexes dictates whether the complexes become pathogenic or not. It can be envisaged that any animal must at all times be constantly exposed to exogenous and/or endogenous antigens which on most occasions must gain access to the circulation. Antibody reacting with these circulating antigens forms immune complexes and in the vast majority of instances these antigen containing immune complexes are disposed of harmlessly by normal physiological mechanisms. In some cases, however, circulating immune complexes become trapped in one or more vascular or filtering structures of the body and induce inflammatory reactions and subsequent immune complex disease (Dixon, 1963; Cochrane & Dixon, 1978). Vascular filtering systems include the glomerulus, choroid plexus, synovium, skin and uveal tract and subsequently they all have a high degree of blood flow per unit mass of tissue. Thus their potential to trap large quantities of immune complexes in their vascular walls is great. Immune complexes may therefore cause disease by virtue of their blocking of these filtering mechanisms and the ensuing

inflammatory response can be seen as a protective mechanism to remove the complexes from that organ thus restoring its function. However, on the other hand excessive or persisting inflammation by itself will further damage the organ. I will now briefly discuss the reaons why this might occur and the factors which determine the fate of circulating immune complexes.

Factors which influence the fate and biological activities of immune complexes include the nature of the antibodies and antigens involved and on the molar ratio of the two reactants. The immunoglobulin class of the antibody determines the antibody's valency for a specific antigen as well as its ability to interact with, and bind to, cellular Fc receptors. The immunoglobulin class also determines the immune complex's ability to activate the complement system. The avidity of the antibody for the union of specific antibody and antigen is also important in influencing the fate of the immune complex. The production of high amounts of antibody of high avidity or affinity in mice allows for accelerated clearance of the antigen from the circulation compared with mice which produce low amounts of low affinity antibodies (Alpers, Steward & Soothill, 1972).

The importance of the nature of the antigen in influencing immune complex disposal is illustrated by experiments using univalent and multivalent antigens. When univalent antigens are injected into the circulation of an animal which contains specific antibodies small complexes are formed, where each antigen forms a separate antigen-antibody bond with a binding site on an antibody molecule. No lattice is formed and these complexes remain in the circulation for long periods without tissue deposition (Schmidt, Kaufman & Butler, 1974). On the other hand multivalent antigens such as proteins when combined with their specific antibodies do form large lattices, the varying composition of the resultant immune complex depending on the molar ratio of the reactants

(Haakenstad & Mannik, 1974).

The most important factor determining the fate and biological activities of immune complexes is the ratio of antigen to antibody in the immune complex. Larger immune complexes are in general rapidly removed from the circulation (Mannik et al, 1971; Haakenstad & Mannik, 1974). Immune complexes formed at large antigen excess are typically very small containing antigen and antibody in the ratio Ag_2Ab_1. They often circulate for long periods but because of their size do not fix complement well and generally are unable to initiate inflammatory processes. Immune complexes formed with a large excess of antibody over antigen are very large and insoluble. Although being very capable of fixing complement and having phlogistic potential their rapid phagocytosis ensures that their phlogistic potential is limited. Immune complexes of intermediate size lying between these two extremes, formed in modest antigen excess are soluble but not rapidly phagocytosed. These complexes can fix complement and thus their phlogistic potential is considerable. This important role of the size of the immune complex and its phlogistic potential was clearly shown by Dixon (1963) in the experimental model of chronic serum sickness in the rabbit where progression of disease was only seen when the immune complexes were soluble. The size of the immune complexes was varied by changing the dose of antigen. Progression of the disease could be arrested by either lowering the dose of antigen to give antibody excess complexes or raising the dose to give antigen excess complexes (Dixon, 1963). However it should be noted that immune complex glomerulonephritis only occurred in those rabbits which made precipitating antibodies. Renal disease only occurred in those rabbits in which immune complexes were formed at equivalence or in antibody excess (Dixon et al, 1961). If the rabbits were maintained in permanent antigen excess renal disease did not develop.

Immune Complex Deposition and Tissue Injury

The development of an immune complex disease is also dependent on the magnitude and duration of antigen exposure. The potential exists for chronic immune complex formation in situations where there is long term antigen exposure such as in chronic infections or with autoantigens. The nearest experimental model to this situation in humans is probably chronic serum sickness (Cochrane & Dixon, 1978). At any given time the concentration of immune complexes in the circulation depends on the rate of immune complex formation and on the rate of immune complex removal. Complexes formed at equivalence are usually large and if injected intravenously are rapidly removed from the circulation by the liver (Haakenstad, Case & Mannik, 1975). Thus one can postulate that such complexes can only produce tissue damage in vivo if they are allowed to circulate, ie if their removal from the circulation by the reticuloendothelial system is impaired.

It is conceivable that large insoluble complexes may be formed within the microcirculation and deposited locally. In experiments where rabbits or pigs were pre-immunised with protein antigens, the injection of small amounts of antigen directly into the renal artery resulted in acute glomerulonephritis (Gabbiani, Badonnel & Vassalli, 1975; Shigematsu et al, 1979) However, in another study rabbits maintained in permanent antigen excess over prolonged periods never developed glomerulonephritis (Boyns & Hardwicke, 1968). Therefore it would appear that large immune complexes formed at or near equivalence by precipitating antibodies are incriminated in the pathogenesis of some types of experimental glomerulonephritis or vasculitis.

It has been demonstrated in experimental animals that release of vasoactive amines and increased vascular permeability are prerequisites for immune complex deposition (Cochrane, 1963; Kniker & Cochrane, 1968) and that large

immune complexes of 19S or greater are necessary for induction of vasculitis (Cochrane & Hawkins, 1968; Wilson & Dixon, 1971). Henson & Cochrane (1971) described a mechanism in the model of acute experimental serum sickness in the rabbit whereby immune complex deposition was mediated by a complement independent, leukocyte dependent process. The mechanism involved the degranulation of basophils sensitised with IgE antibody and antigen with subsequent release of a platelet aggregating factor (PAF). This PAF subsequently caused aggregation of and release of vasoactive amines from platelets (Benveniste, Henson & Cochrane, 1972). PAF is present in human basophils (Benveniste, 1974) and similar mechanisms may operate in man. The anaphylatoxins C3a and C5a produced after complement activation by immune complexes also increase permeability. Vasoactive amines are released from platelets after the direct interaction of immune complexes with the platelet Fc or complement receptors in experimental animals as well as after direct or indirect lysis of platelets by immune complex induced complement activation (Henson & Cochrane, 1971). It is of interest to note that depletion of platelets, the principle reservoir of vasoactive amines, prevents the development of immune complex disease in the rabbit (Kniker & Cochrane, 1968).

When immune complexes are deposited along vascular basement membranes the inflammatory response which is evoked is largely dependent on the biological activities produced by complement activation. These biological activities are generated and mediated by either the immune complex fixed complement components or by fluid-phase activated complement components and their fragments. The biological activities produced secondary to complement activation includes increased vascular permeability, chemotaxis, immune adherence and lysis of cells. The cleavage product of C5 (C5a) is the major chemotaxin which promotes an influx of inflammatory cells to the site

of immune complex deposition. The anaphylatoxin activity occurs via the binding of C3a and C5a to mast cells and basophils with the subsequent release of vasoactive amines (Hugli & Muller-Eberhard, 1978). Immune adherence is the process in which immune complexes containing C3b bind to specific receptors on various cell types, including neutrophils, resulting in phagocytosis. Neutrophils may release lysosomal enzymes thus increasing tissue injury (Cochrane & Dixon, 1978). This sequence of events operates as a general mechanism of immune complex mediated tissue injury in experimental animals. It should be noted that depletion of complement by cobra venom factor or depletion of neutrophils can prevent the development of necrotising arteritis, but the glomerulonephritis can still occur (Henson & Cochrane, 1971). Although complement may not always be necessary for the production of the lesions of immune complex disease, the results of the cumulated studies of immune complex induced tissue lesions in experimental animals implicated complement activation as playing a major role in the pathogenesis of immune complex mediated tissue injury (Cochrane & Koffler, 1973).

The Complement System

At this point I would like to briefly review the complement system as this will allow for easier understanding of the complement-immune complex interactions described below.

Complement activity is the result of the sequential interaction of a number of plasma and cell membrane associated proteins. There are two pathways for complement activation, the classical and the alternative. The classical pathway is activated by antigen-antibody complexes (immune complexes) of the IgG and IgM classes, while the alternative pathway is activated in the absence of antibody by a variety of agents including bacterial lipopolysaccharides, virus infected cells, protozoa, aggregated immunoglobulins,

cobra venom factor and C3 nephritic factor. Activation of either pathway leads to the generation of multi-molecular enzymes which will activate the third (C3) and the C5 components of complement. These enzymes are called C3 convertases and C5 convertases respectively.

The classical pathway is activated by the binding of C1 to antigen-antibody complexes (containing IgM or IgG_1, IgG_2 or IgG_3 antibody molecules). C1 is a macromolecular complex composed of three proteins C1q, C1r and C1s (Cooper, 1985). C1q is the recognition unit which binds to immunoglobulin and undergoes a conformational change leading to activation of C1r to its enzymatically active form $\overline{C1r}$, which then enzymatically activates C1s to become an active enzyme $\overline{C1s}$. $\overline{C1s}$ is the active site of macro-molecular $\overline{C1}$ and has two natural substrates C4 and C2. This active $\overline{C1}$ then cleaves the next two components, C4 and C2, in sequence. Active $\overline{C1}$ produces a limited proteolytic cleavage in the α -chain of C4, separating a 6000 molecular weight peptide, C4a (Schreiber & Muller-Eberhard, 1974). The remainder of the molecule, C4b, is able transiently to bind to antigen-antibody complexes via a labile binding site. This ability of C4b to bind to surfaces is due to the presence of an internal thiolester group in C4. C4b binds to surfaces (eg immune complexes) by covalent bond formation and thus serves to target the rest of the complement sequence to that surface. C4b also possesses a Mg^{++} dependent binding site for C2 and this C2 is cleaved by activated $\overline{C1}$ to yield two fragments, C2a and C2b. C2 binds to C4b via the C2b fragment which remains bound to C2a by non-covalent bonds. The enzymatic site of this molecule is contained in the C2a (Nagasawa & Stroud, 1977). C4b2a is the C3 cleaving enzyme (C3 convertase) generated by classical pathway activation.

The alternative pathway C3 convertase is formed when C3b binds to factor B, in the presence of Mg^{++} ions, to form C3bB. B is then cleaved by D to form C3bBb which is the unstable alternative pathway C3 convertase. This enzyme is unstable because Bb rapidly decays from it. However the enzyme is stabilised by the binding of P to C3b which prevents the decay of Bb from the complex (Fearon & Austen, 1975) This complex C3bBbP is called the properdin stabilised C3 convertase (C3bBbP). Thus two C3 convertases are formed one from classical pathway activation (C4b2a) the other from alternative pathway activation (C3bBbP).

C3 is cleaved to release a small peptide C3a. The remainder of the molecule C3b contains a labile binding site which like C4b allows C3b to bind to surfaces (eg antigen-antibody complexes or polysaccharides). The mechanism of binding of C3b to surfaces is the same as that for C4b. C3 contains an internal thiolester bond (Tack et al, 1980) and the cleavage of C3 by the C3 convertase results in the appearance of a single thiol group (Janatova et al, 1980). The transfer of an acyl group from the thiol to an hydroxyl group in the acceptor molecule results in covalent bond formation (Pangburn & Muller-Eberhard, 1980; Tack et al, 1980). Again it will be evident that this ability of C3b to bind to surfaces allows for the subsequent complement activity to be localised close to the site of activation.

C3b is a product of the enzymatic action of C3 convertases on C3 while at the same time being a constituent of the alternative pathway C3 convertase. Thus a positive feedback mechanism exists which would continue to cleave C3 until the supply of C3 or B became exhausted. This does not occur because of the presence of the control protein factor I and its co-factor H. I in the presence of H will cleave the α chain of C3b to form C3bi which can no longer bind B and thus form a C3 convertase.

The terminal sequence is activated by the cleavage of C3 by C4b2a and C3bBbP. As stated above C3b binds to its target (eg immune-complex) by its labile binding site. Close binding of the C3b to the C3 convertase changes the specificity of the enzyme to that of a C5 convertase. C5 is cleaved to release C5a (MW 12,000) and the C5b which is the remainder of the molecule binds to cell membranes via a labile binding site, however, this binding of C5b is not covalent (Law, Lichtenberg & Levine, 1980) but binding to C6 helps to stabilise the binding of C5b in its active state. C7 binds to C5b6 forming C5b67 which is inserted into lipid membranes. Reaction with C8 to form the C5b-8 complex allows for partial penetration of the lipid membrane. C9 then binds to C8 to form the cytolytic C5b-9 complex which results in osmotic lysis of the cell as proposed by Mayer (1972).

The Biological Activities of Complement

Apart from the cytolytic effect of the complement cascade many other biological activities are generated during complement activation. These activities are important in the expression of the inflammatory events which take place following complement immune complex interactions.

Increased Vascular Permeability

The breakdown products C4a, C3a and C5a are anaphylatoxins. C5a which is the most potent acts, as does C3a, to release histamine from mast cells and basophils. C3a releases serotonin from platelets (Johnson, Hugli & Muller-Eberhard, 1975). C3a and C5a can induce smooth muscle contraction in the absence of histamine. The anaphylatoxic activities of C3a and C5a are inhibited by carboxypeptidase N.

Chemotaxis

C5a and C5a which has been degraded by carboxypeptidase N ($C5a_{desArg}$) are powerful chemotaxins. They will affect neutrophils, eosinophils and monocytes (Fernandez et al, 1978) C3bBb is chemotactic for polymorphonuclear leukocytes (Ruddy, Austen & Goetzl, 1975).

Leukocytosis

The peptide C3e, derived from the α chain of C3, causes an initial leukopenia and then a leukocytosis. The mechanism is thought to be by C3e mediated mobilisation of neutrophils from the bone marrow (Rother, 1972; McCall et al, 1974).

Solubilisation of Immune Complexes and Prevention of Immune Precipitation

These two aspects of complement function will be discussed in a later section.

Opsonisation

One of the most important biological functions of complement is opsonisation, the process whereby antigen-antibody complexes or other particles are coated with C3b. These opsonised particles can bind to receptors for C3b (CR1) found on a number of cell surfaces including polymorphonuclear leukocytes, mononuclear phagocytes and B lymphocytes. There are also receptors for C3bi and C3dg (α_2D) which are further breakdown products of C3b. C3bi binds to CR3 and CR1 and C3dg (α_2D) bind to CR2. When the particle is coated with C3b alone adherence is promoted, however, when IgG antibody in addition coats the particle ingestion is enhanced. C3b acting on C3b receptors on macrophages results in the enhanced intracellular killing of bacteria (Leijh et al, 1979).

Up until the early 1970's complement had always been thought of as being detrimental and playing a significant pathogenetic role in the production of tissue injury in immune complex diseases. In human immune complex disease much evidence has been accrued to support this concept of complement being the major effector mechanism of immune complex mediated tissue damage. The finding of hypocomplementaemia in patients with immune complex disease (Franco & Schur, 1971; Schur, 1975; Whaley & El-Ghobarey, 1981) and the detection of complement components in the involved tissues go a long way to support this idea (Rodman et al, 1967; Koffler et al, 1969; Verroust et al, 1974).

However in the past 15 years or so our views on the role of complement in immune complex disease have been altered by the observations that patients with inherited complement deficiency syndromes have a high incidence of immune complex disease.

Immune Complex Clearing Mechanisms

I have discussed the reasons and possible mechanisms whereby circulating immune complexes are deposited in tissues thereby causing disease. This must only happen when there is a breakdown of the normal mechanisms whereby immune complexes are cleared from the circulation. In experimental animals immune complexes containing IgG antibodies, when injected intravenously are cleared from the circulation mainly by the non-parenchymal cells of the liver (Mannik & Arend, 1971). IgG immune complex entrapment in the liver is mediated mainly by interaction with cellular Fc receptors (van Es, Daha & Kijlstra, 1979). It can be envisaged that if the reticuloendothelial system became overloaded with immune complexes the complexes may then deposit in the tissues (Haakenstad & Mannik, 1974) and initiate active disease.

Studies on the clearance of IgM coated erythrocytes from the circulation have emphasised the importance of complement in the clearance process. When erythrocytes are sensitised with IgM antibody their fate in the circulation is quite different from that of erythrocytes sensitised with IgG. Unlike IgG sensitised cells, in vivo complement activation is critical for the clearance of the IgM sensitised cells (Frank et al, 1977). The cells are cleared when C3b is deposited on their surface after complement activation and they are then sequestered by the Kuppfer cells in the liver. This hepatic localisation of sequestered erythrocytes is very different from the splenic clearance seen in the removal of IgG sensitised erythrocytes from the circulation. Following the hepatic sequestration of IgM coated erythrocytes, they are then released back into the circulation because of the action of factor I and H on the cell bound C3b which is cleaved to C3bi and then subsequently to C3c and C3dg(α_2D). The C3dg coated erythrocytes then are released back into the circulation where they continue to circulate with a half-life equal to that of unsensitised cells.

Complement appears important in the clearance of particulate antigens from the circulation (Brown, Lachmann & Dacie, 1970) but whether it affects the elimination of circulating immune complexes is somewhat controversial. Large immune complexes greater than 30S are more readily endocytosed by adherent macrophages if complement is present (van Snick & Masson, 1978). The degradation of soluble immune complexes is enhanced by complement (Kijlstra, van Es & Daha, 1981). However complement is not essential for the attachment of soluble immune complexes to macrophages in suspension (Arend & Mannik, 1972) nor does complement appear to mediate the blood clearance of IgG containing soluble immune complexes when these are injected intravenously (Bockow & Mannik, 1981). Skogh & Stendahl, (1983) when

studying the blood clearance of small IgG immune complexes found that the clearance of these immune complexes from the circulation was actually retarded by fresh normal mouse serum and not by complement depleted mouse serum.

Although these above studies cast some doubt on the role of complement in the elimination of circulating immune complexes a body of evidence has grown in recent years which has established complement as having a central role in immune complex processing and the subsequent safe disposal of potentially phlogistic immune complexes in the circulation. Central to this has been the recognition of inherited deficiences of the complement components. It is only in recent years that the widespread availability of reagents with which individual complement components can be measured has allowed these complement component deficiencies to be recognised. Individuals deficient in complement have a predilection to development of disease and certain syndromes appear associated with particular complement component deficiencies (Agnello, 1978; Rynes, 1982). In Table 1.1 I have listed the cases reported up to 1983, this table being taken from that of Schifferli & Peters (1983(a)). Ascertainment bias is of course inevitable especially when dealing with small numbers, complement being measured in people presenting with immunological disease much more frequently than in the general population. However even allowing for this, scrutiny of Table 1.1 allows some general conclusions to be drawn. Infection is much more common and widespread in patients with complement deficiencies thus emphasising the major antimicrobial role of complement. However, patients with deficiencies of the early acting components of the classical pathway seem prone to the development of immune complex mediated disease, this seems to be particularly the case for deficiencies of C4 and the C1 subcomponents.

TABLE 1.1

Component	Number with homozygous deficiency	Associated Disease	
		IC disease	Infection
Classical pathway			
C1q	15	14	)
C1r or C1s	8	6	) many
C4	16	14	) pyogenic
C2	66	38	) infections
C1-inhibitor	7500	2%	)
C3 and alternative pathway			
C3	11	8	10 pyogenic (+ Neisseria)
B/D	-	-	
Properdin	3	-	2 (+ 3 died of fulminant infections)
Fac I	5	1	4 pyogenic
H	2 ¶	1 (HUS)	
Membrane attack complex			
C5	12	1	9)
C6	17	2	10)
C7	14	1	6) Neisseria
C8	14	2	8)
C9	Many	no disease associated	

TABLE 1.1
Reported cases of complement deficiencies and associated diseases
¶ partial deficiency $<10\%$
IC disease = SLE, SLE-like syndromes, glomerulonephritis, vasculitis
HUS = Haemolytic-uraemic syndrome

(taken from Schifferli & Peters, Lancet, 1983)

I have stated previously that complement activation is a major effector mechanism of inflammation in immune complex mediated tissue injury. Although this concept still holds true the evidence gleaned from studies of complement deficiency syndromes has led us to reappraise the role of complement in immune complex disease and also puts forward the idea that normal complement function protects against the development of immune complex associated disease.

The Role of Complement in Immune Complex Clearance

(1) The Interaction of Opsonised Immune Complexes with Complement Receptors

Opsonisation is the coating of antigen-antibody complexes or activators of the alternative pathway with large numbers of C3b molecules and is a major biological function of complement. The process of opsonisation permits more efficient clearance of complexes by the fixed macrophage system. The fate of opsonised complexes is dependent on their interaction with specific receptors for C3b found on the surfaces of various cells.

The complement receptor (CR1) is a glycoprotein with four major allotypic forms, molecular weights being 160, 190, 220 and 250 kD (Fearon, 1980; Fearon, 1984; Ross & Medof, 1985). The major ligand for CR1 is C3b and it binds especially well when the C3b is present in clusters. CR1 is found on polymorphonuclear leukocytes, macrophages, B lymphocytes, some T lymphocytes, dendritic reticular cells in germinal centres and glomerular podocytes. In primates CR1 is also found on erythrocytes and it would appear that 90-95 per cent of CR1 is found on the surface of erythrocytes in the circulation of humans (Siegel, Liu & Gleicher, 1981). In vitro complement reacted immune complexes attach mainly to the CR1 found on erythrocytes (Schifferli & Peters, 1983(b)). In vivo immune complexes bind to erythrocytes when injected into the aortas of baboons and monkeys. The binding of opsonised immune complexes to erythrocyte CR1 is a transport mechanism whereby immune complexes are carried safely to the fixed macrophage system (Cornacoff et al, 1983). Immune complexes formed in the extra vascular spaces are probably removed via the lymphatics and then trapped in the regional lymph nodes (Pepys, 1976).

In studies in monkeys Cornacoff et al,(1983) showed that under normal circumstances immune complexes were bound to erythrocytes by a complement dependent mechanism, transported to the liver and then stripped from these erythrocytes. Failure of this mechanism occurred in animals which had been depleted of complement and instead of being cleared by the liver the complexes were found widely distributed in the body (Waxman et al, 1984). Immune complex binding to erythrocytes is therefore thought to be a protective mechanism which transports immune complexes to sites of safe disposal.

The mechanism whereby complexes are transferred from erythrocytes to macrophages is obscure. It may involve enzymatic removal or it may be related to the high density of Fc receptors and CR1 on macrophages. This will provide stronger binding for the complex than the relatively low density CR1 on the erythrocytes and may allow for preferential uptake of the complex by the macrophage. It should also be remembered that the binding of immune complexes to erythrocytes is a relatively transient phenomenon in as much that CR1 also has a potent inhibitory function in the complement cascade being a co-factor for factor I in the cleavage of C3b to C3bi and subsequently to C3dg (Medof et al, 1982; Medof & Prince, 1983). C3dg is thus left attached covalently to the immune complex but C3dg does not bind to CR1 and the immune complex is then released from the receptor.

The range of the numbers of CR1's on the erythrocytes of normal individuals is large (200 to 1,000 per cell) (Ross & Medof, 1985). Reduced numbers of CR1 have been found on the erythrocytes of patients with SLE; initially thought to be an inherited defect (Wilson et al, 1982) it has now been shown that low CR1 numbers on erythrocytes occurs secondarily to the disease process (Walport et al, 1985; Holme et al, 1986). Reduced binding of immune

complexes to CR1 has been observed in various diseases including rheumatoid arthritis, SLE, haematological neoplasia and cold agglutinin disease (Miyakawa et al, 1981; Taylor et al, 1983).

(2) The Interaction of Complement with Immune Complexes

In recent years it has become clear that complement can interact with immune complexes and cause profound changes in the properties of the immune complex. These changes occur in the solubility, composition and aggregation of the complexes (Miller & Nussenzweig, 1975; Casali & Lambert, 1979; Schifferli et al, 1980). Insoluble antigen-antibody complexes become soluble when incubated with serum (Miller & Nussenzweig, 1975) and immune complexes formed at equivalence or in antibody excess do not precipitate but are held in solution (Schifferli et al, 1980). Both these phenomena termed immune complex solubilisation and the prevention of immune precipitation respectively are brought about by complement activation. I will now describe these phenomena in turn and discuss their possible relevance to immune complex disease.

Solubilisation of Immune Complexes

In his classical paper of 1941 Heidelberger when studying complement incorporation into precipitates formed from pneumococcal polysaccharides and rabbit antibody noted that, somewhat unexpectedly, the amount of bound complement per complexed antibody actually decreased as the amount of added serum increased. The explanation of this phenomenon was not forthcoming until 1975 when Miller and Nussenzweig described solubilisation of antigen-antibody aggregates. Since this observation several papers have appeared which have defined the mechanism of solubilisation of immune precipitates and demonstrated the radical alterations which take place in the physical and biological properties of immune complexes on interaction with

complement.

The solubilisation phenomenon is a general phenomenon and many kinds of Ag-Ab complexes formed with soluble antigen can participate. Antibodies of IgG, IgA and IgM from several mammalian species can be involved although the efficiency of the serums show some species differences. Complement can solubilise precipitates formed with the $F(ab')_2$ fragment but less efficiently than with the whole immunoglobulin molecule (Miller & Nussenzweig, 1975). The rate of solubilisation depends on the amount of immune complexes, the properties of antigen and antibody, the composition of the complexes and the conditions of incubation. Precipitates formed at or near equivalence can be solubilised most efficiently (Miller & Nussezweig, 1975) and the affinity of the complexed Ab for Ag is very important suggesting that the primary antigen-antibody bond is implicated in the solubilisation process (Czop & Nussenzweig, 1976). Solubilisation is a complement dependent phenomenon, heat-inactivation of serum (56°C for 30 minutes) or treatment of serum with EDTA, zymosan or cobra venom factor will abrogate the solubilising activity of that serum (Miller & Nussenzweig, 1975).

The solubilisation phenomenon has an absolute requirement for the alternative pathway of complement. Sera which had been depleted of the alternative pathway components, D, B or P were unable to support solubilisation. Replacement of the missing component resulted in the solubilising capacity of the serum becoming normal (Miller & Nussezweig, 1975). In similar experiments using C3-deficient human serum (Czop & Nussenzweig, 1976) or factor D-depleted or properdin-depleted serum (Takahashi et al, 1978) all of these components of the alternative pathway were found to be essential. More recent work using purified alternative pathway components (C3, B, D, P, H and I) has re-emphasised the essential role of the alternative pathway in this

process. Omission of the control proteins either H or I resulted in inappropriate fluid phase turnover of C3 and solubilisation did not occur (Fujita, Takata & Tamura, 1981).

The classical pathway is not essential for solubilisation, sera deficient in C4 or C2 were able to support solubilisation but the onset was delayed and the extent of the reaction was reduced (Czop & Nussenzweig, 1976; Takahashi et al, 1978). C3-deficient serum does not support solubilisation (Czop & Nussenzweig, 1976) but sera deficient in C5 or C6 does (Takahashi et al, 1976). This suggests that the complement components beyond C3 are not involved in the reaction. Up to 95% of the solubilised complexes can be precipitated by antiserum to C3 showing that C3 is bound to the complex (Takahashi, Takahashi & Hirose, 1980).

The kinetics of the solubilisation process have been studied. Deposition of C3b on the surface of the immune complex occurs first. This can occur rapidly if the immune complex can fix C1, the initial C3b deposition taking place due to classical pathway activation (Takahashi et al, 1976). The phase of amplification begins once the first few molecules of C3b are deposited on the complexes with the assembly of the alternative pathway C3 convertase (C3bBbP) on the complex. More molecules of C3 are cleaved and increased deposition of C3b on the complexes ensues. Massive deposition of C3b leads to disruption of the immune complex lattice and solubilisation occurs (Takahashi, Tack & Nussenzweig, 1977). C3b deposition by classical pathway alone is insufficient to produce solubilisation. It has been suggested that the interaction of C3 with antigen-antibody complexes during solubilisation results in the formation of an ester bond, the C3b binding sites possibly being located on amino acid residues in the Fd region of the IgG molecules and the C3b binding to antigen via ester bonds (Takata, Tamura & Fujita, 1984).

When large quantities of C3b have been deposited on the complex the final phase of the solubilisation process begins. Sucrose density gradient studies on the precipitation of radio-labelled antigen with ammonium sulphate (final conc $^{n.}$ 50%) show that most of the soluble antigen is present in the form of immune complexes (Takahashi et al, 1980) C3, C4 and P are found in solubilised complexes (Takahashi et al, 1978) and the molar ratio of C3b to antibody is approximately 1:1 (Takahashi et al, 1977). The size of solubilised complexes is heterogeneous, a large part of the complexes showing sedimentation co-efficients between 11S to greater than 19S.

The mechanism of solubilisation seems best explained by the intercalation hypothesis advanced by Miller & Nussenzweig (1975). Briefly it states that during the interaction of immune complexes with excess complement a large amount of C3 is incorporated into the immune complex lattice, combining with antibody and thus tending to decrease the affinity of antigen combining sites on the antibody molecule. There is breakage of a few antigen-antibody bonds and a reorganisation of the lattice takes place favouring production of smaller aggregates. In favour of this hypothetical mechanism is the fact that degradation products of antigen and antibody have not been found (Czop & Nussenzweig, 1976). Solubilisation does not occur in the absence of C3 and C3 levels in sera correlate well with solubilisation in individual sera (Czop & Nussenzweig, 1976). Incorporation of large quantities of C3b into the antigen-antibody lattice occurs during the solubilisation process and the degree of solubilisation appears dependent on the amount of C3b available (Takahashi et al, 1977). Incubation of complexes with the monovalent Fab fragments of antibodies to the immunoglobulin in the complex can mimic solubilisation; in contrast 7S antibody and $F(ab')_2$ fragments to the antibody in the complex prevented solubilisation. This process would favour cross-linking of the antibody and thus promote precipitation (Czop &

Nussenzweig, 1976).

Some evidence can be presented to explain the mechanism whereby C3b effects solubilisation. The result of the early experiments of Miller (1977) support the mechanism of the reduction of the affinity of antibody for antigen with subsequent dissociation of the complex as a possible means of C3b induced solubilisation. Indeed insoluble complexes formed with high affinity antibody are solubilised more slowly than complexes containing antibody with low affinity for the antigen (Takahashi et al, 1978) suggesting a role for primary antigen-antibody bond disruption. Fc-Fc interactions are important in immune precipitation (Moller, 1979) and C3b fragments may interfere with lattice formation by inhibiting these Fc-Fc interactions. The overall solubility of the immune complex may be modified by the sheer numbers of C3b molecules which bind to the immune complex tending to favour dissociation of the immune complex.

Solubilisation of preformed immune complexes is a phenomenon studied in vitro. Whether this phenomenon occurs in vivo is open to debate. If indeed it does how important is it in health or disease? Solubilisation occurs as a general phenomenon under physiological conditions and with a variety of antigen-antibody systems both from human and animal sera (Takahashi et al, 1980). In vivo solubilisation must be important in clearing large antigen-antibody complexes which are formed or found deposited in the tissues and this concept is supported by the observation of the delayed

clearance of complexes from the glomeruli of decomplemented rabbits with acute serum sickness (Bartolotti & Peters, 1978). It has been suggested that solubilisation of complexes by C3 may be important for the generation of B-memory cells based on experimental evidence showing a small proportion of solubilised complexes taken up and retained in the germinal centres of the spleen (Malasit, Bartolotti &Humphrey, 1983). Solubilisation is one of the protective mechanisms against the occurence of large aggregates in the body which would almost certainly cause tissue damage. For this to be important it must be assumed that antigen-antibody immune precipitates are formed and/or deposited and then are acted upon by complement and thus solubilised. This seems an unlikely occurence and it is difficult to conceive of many situations where antigen-antibody immune precipitates are formed in the presence of an intact complement system. In the vast majority of instances antigen-antibody complexes must be formed in the presence of complement (Schifferli et al, 1980).

Schifferli and his colleagues (1980) argued that the action of complement on immune complexes at the time of their formation was likely to be more important than the action of complement on preformed complexes. They carried out studies on immune complex formation in the presence of fresh serum and were able to show that complement prevented the formation of immune precipitates. In this study complexes formed at equivalence or in antibody excess in the presence of human serum did not precipitate but remained soluble for up to 2 hours.

This phenomenon of prevention of immune precipitation had been encountered by several workers previously. Heidelberger noted that "particulation was greatly delayed in the tubes containing active complement" in his studies of complement fixation (Heidelberger, 1941). Later workers, during attempts to

develop double radio-immunoassay techniques, noted inhibitory activity in normal serum and attributed it to the complement system (Morgan, Sorenson & Lazarow, 1964; Grant, 1968) It was also noted that BSA anti-BSA immune complexes did not precipitate when formed at equivalence in the presence of fresh serum at 37°C. Following isolation these immune complexes were found to contain C1q, C1r, C1s and C3 in addition to antigen and antibody (Casali & Lambert, 1979).

Schifferli and his colleagues called prevention of immune precipitation inhibition of immune precipitation (IIP). I have preferred to use the term prevention of immune precipitation (PIP) because this thesis contains work on an inhibitor(s) of this process. It is far easier to discuss an inhibitor of prevention of immune precipitation rather than an inhibitor of inhibition of immune precipitation.

Complement mediated prevention of immune precipitation

Immune precipitation was prevented by fresh normal serum via activation of the complement system. This effect of human serum was blocked by prior treatment of the serum with EDTA or by heating to 56°C for 30 minutes, procedures which would abrogate complement function (Schifferli et al, 1980). Serum which had been depleted of B or P, thus being able only to sustain classical pathway activation, was however able to prevent immune precipitation. Immune complexes formed in Mg^{2+} containing EGTA-treated serum displayed an early phase of precipitation which was then followed by a phase of resolubilisation (Schifferli, Woo & Peters, 1982). The importance of the classical pathway in the prevention of immune precipitation phenomenon is further emphasised by the observation that excessive immune precipitation is known to occur in sera which are deficient in C1q, C4, C2 and C3

(Schifferli et al, 1980; Schifferli et al, 1982) but not in sera which are deficient in C5 or C7 (Naama et al, 1983). These findings again underline the importance of the classical pathway in this phenomenon and show that the terminal components beyond C3 (C5-C9) are not required for the process. As in the complement mediated solubilisation of preformed precipitates C3 activation and binding is the important step in the prevention of immune precipitation phenomenon.

The role of C1 in prevention of immune precipitation is interesting. Purified C1 is able to block the early aggregation of immune complexes and appears to be the first line of defence against immune precipitation (Schifferli et al, 1982; Naama et al, 1984). The C1 molecule is bound to the Fc portion of immunoglobulins and probably interferes with Fc-Fc interactions by steric hindrance. These Fc-Fc interactions are probably the main mechanism by which aggregation of immune complexes occurs (Moller, 1979). However when the C1 molecule was dissociated into its subcomponents by the removal of calcium an immediate aggregation of the immune complexes was seen (Schifferli et al, 1982). This was due to the effect of C1q which remained fixed to the immunoglobulins enhancing the interactions between the complexes (Agnello, Winchester & Kunkel, 1970). These opposite effects of C1 and C1q have been reported in other situations including the C1q induced agglutination of IgG coated latex particles and enhanced agglutination by rheumatoid factor (Hallgren, Stalenheim & Venge, 1979; Hallgren, 1979). Both of these reactions were abrogated by purified whole C1.

The use of individual purified complement components have allowed for precise dissection of the roles of the classical and alternative pathways in the prevention of immune precipitation reaction (Naama et al, 1984; Naama et al, 1985). The anti-aggregational properties of C1 were confirmed. No effect

on immune precipitation was noted with C4 or C2 alone, but when present with C3 at the same concentrations as in normal serum, immune precipitation did not occur. When the six alternative pathway components (C3, B, D, P, H & I) were present immune precipitation occured but this was followed by a phase of resolubilisation. Addition of the alternative pathway components to the components of the classical pathway did not allow for more effective prevention of immune precipitation than the classical pathway components by themselves (Naama et al, 1985). This study indicates that the alternative pathway is of secondary importance to the classical pathway in the prevention of immune precipitation reaction in contrast to the situation with solubilisation of preformed immune precipitates.

At this point it is worth making other comparisons of these two complement mediated phenomena (viz solubilisation and prevention of immune precipitation). Solubilisation is more efficient when complexes are formed in antigen-excess whereas prevention of immune precipitation is more efficient for antibody-excess complexes (Schifferli et al, 1980). The size of the complexes produced after the two reactions were similar. Sucrose gradient ultracentrifugation revealed the size to be approximately 25S which is similar to the size of the complement solubilised immune complexes (Takahashi et al, 1980). Normal serum could maintain about twenty times more complexes in solution by the process of prevention of immune precipitation compared to solubilisation (Schifferli et al, 1980). This may have relevance to the greater biological importance of prevention of immune precipitation compared to solubilisation in the processing of immune complexes <u>in vivo</u>.

The mechanism of prevention of immune precipitation involves the attachment of C3b to immune complexes. Radio-labelled C3 studies have shown that the molar ratio of C3b to IgG in the soluble complexes is 2:5

(Naama et al, 1985), and immune precipitation experiments have shown that over 90% of immune complexes formed in serum are preciptated by anti-C3 antiserum. Antisera to C1q, C4 and C2 preciptated much smaller proportions of immune complexes (Naama et al, 1985). Further work in this field has shown that fragments of C3 and C4 are incorporated into the soluble immune complexes (the C3 fragments which were incorporated were C3b, iC3b, C3c and C3d). Some of these fragments were bound covalently with heavy chains of IgG antibody molecules. It was considered by these workers (Hong et al, 1984) that the formation of covalent bonds between IgG and C3 and probably C4 was essential for the prevention of immune precipitation process. The covalent linkage between IgG and C3 did not appear to be an ester bond (Hong et al, 1984) although this type of bond was reported to be the linkage between IgG and C3 when preformed precipitates were dissolved by complement (Takata et al, 1984).

It is clear therefore that the immune complex lattice is greatly modified following the interaction of antigen and complement fixing antibody in body fluids. A large number of C3b molecules are bound to an immune complex through activation of the classical pathway followed by rapid engagement of the alternative pathway (via the C3b feedback loop) with the result that large lattices are not formed and immune precipitation is prevented.

Hypothesis and proposed investigation

If the phenomenon of prevention of immune precipitation is important in the handling and safe disposal of these circulating immune complexes then a failure of this mechanism would be expected to be associated with immune complex diseases. The most extreme example of this would be hereditary deficiency of one or more of the classical pathway complement components, and indeed these are associated with a high incidence of immune complex like diseases (see Table 1.1). However complement deficiency states are rare in the general population, Hassig and colleagues (1964) found 14 individuals from 41,083 military recruits with persistently undetectable total haemolytic complement, a frequency of 0.03%. Torisu and his colleagues (1970) found only 37 of 42,000 normals to have low CH50 levels. Since genetic deficiencies of complement proteins are therefore probably quite rare in the general population, studies have tended to concentrate on patient populations. Of 545 patients with rheumatic disease Glass et al (1976) found one C2 deficient homozygote and 10 definite, 5 probable and 4 possible C2 deficient heterozygotes. Among 509 controls without rheumatic disease only 6 possible heterozygotes were found. This gave an estimated frequency of 1.2% for the null gene in the general population. Patients with systemic lupus erythematosus had the highest frequency (5.9%) of C2 deficiency.

It is clear that hereditary complement deficiency states are rare even when patient populations, with their considerable ascertainment bias, are screened.

Up until the work contained in this thesis was started in 1983 no studies had been undertaken to measure prevention of immune precipitation in disease. The work of Schifferli and his colleagues (Schifferli et al, 1980; Schifferli et al, 1982; Schifferli & Peters, 1982; Schifferli & Peters, 1983(b)) had concentrated on elucidating the detailed mechanisms of the reaction but no

work on the clinical relevance, if any, of prevention of immune precipitation had been undertaken. The aim therefore of my initial work was to measure prevention of immune precipitation in diseases associated with immune complex formation.

If prevention of immune precipitation (PIP) was important in this respect and as inherited defects of complement are rare one might expect there to be other defects in PIP in patients with immune complex disease. If indeed this was the case I planned to examine the mechanism whereby this occurred and also to study the detailed clinical features of each disease to ascertain which clinical features might be most strongly associated with the failure of prevention of immune precipitation. The sera originally chosen were from patients with systemic lupus erythematosus (SLE) and glomerulonephritis (GN). I also included osteoarthrosis as a disease control being an example of a non-inflammatory rheumatic disease. However in routine clinical practice these diseases (SLE and GN) are relatively rare and I therefore decided to look more closely at sera from patients with rheumatoid arthritis, which is a common, serious rheumatic disorder in which immune complexes are implicated in disease pathogenesis.

Although there is no doubt that cell mediated immune responses play an important role in the pathogenesis of rheumatoid synovitis, humoral immune mechanisms are also intimately involved in the disease process. The histopathological features of the rheumatoid synovium with proliferation of lining cells and intense cellular infiltrate which includes (in established rheumatoid synovitis) large numbers of lymphocytes, plasma cells, macrophages and occasional germinal centre formation testifies to the importance of cellular immune mechanisms in the rheumatoid process (Sokoloff, 1979). In rheumatoid synovitis initially there is congestion and

oedema most marked at the internal surface of the synovium and these changes are associated with the development of effusion into the joint space. Small numbers of polymorphs emigrate with the oedema fluid. In early lesions especially, the principal infiltrating cell is a small lymphocyte. Many of the lymphocytes in the synovial infiltrates are T cells (Van Boxel & Paget, 1975). Studies using OKT4 and OKT8 antibodies have shown two types of T cells, namely helper-inducer and suppressor-cytotoxic, in the synovium (Duke et al, 1982; Kurosaka & Ziff, 1983). In late lesions the majority of infiltrating cells are plasma cells (Piatier et al, 1976) which synthesise rheumatoid factor (Nowoslawski & Brzosko, 1967).

The late lesions are marked by gross synovial thickening, proliferation of synovial fibroblasts and synovial lining cells. Multi-nucleated giant cells may be seen (Grimley & Sokoloff, 1966). This chronic inflammatory tissue which extends over the articular cartilage is called pannus and is accompanied by cartilage destruction. Chondrolysis may occur because of pannus induced disturbance of normal cartilage nutrition or because of the elaboration of proteolytic enzymes from polymorphs and other cells in the chronic inflammatory tissue.

In the synovial fluid however, there is evidence of an acute inflammatory reaction with a predominantly neutrophil accumulation and plasma exudation. Humoral immune mechanisms therefore, especially involving immune complexes, are also considered to be important in rheumatoid arthritis pathological processes. The concept that antigen-antibody complexes might mediate rheumatoid synovitis is supported by the demonstration of phagocytic synovial lining cells and polymorphonuclear leukocytes showing cytoplasmic granules containing immunoglobulins, anti-IgGs (rheumatoid factors) and complement components (Hollander et al, 1965; Kinsella, Baum & Ziff, 1969;

Kinsella, Baum & Ziff, 1970; Vaughan et al, 1968). Further evidence for this notion came from the demonstrations of decreased haemolytic complement levels in rheumatoid synovial fluids (Hedberg, 1963; Pekin & Zvaifler, 1964). The immune complex nature of the molecules responsible for this decreased synovial fluid complement was recognised and rheumatoid factor was also shown to be a part of the synovial fluid immune complex (Winchester, Agnello & Kunkel, 1970; Winchester, 1975).

The experimental model of antigen-induced arthritis originally described by Dumonde and Glynn (1962), where chronic synovitis in rabbits was induced by intradermal immunisation with fibrin in Freund's complete adjuvant followed in 2 to 3 weeks by an intra-articular injection of fibrin, showed that immune complexes could mediate a rheumatoid-like synovitis. An identical chronic synovitis could be induced by antigens such as ovalbumin and bovine serum albumin injected into the joints of previously sensitised animals (Consden et al, 1971; Cook & Jasin, 1972). The histological features described by Dumonde and Glynn included hyperplasia of synovial lining cells, infiltration of synovium with plasma cells and lymphocytes, pannus formation and bone erosions and were similar in many respects to rheumatoid arthritis synovitis. Subsequent studies on antigen-induced arthritis have demonstrated the retention of radio-labelled antigen in the menisci of arthritic rabbits with the immunofluorescent demonstration of antibody and C3 in the same location as the antigen (Jasin et al, 1973). Indeed immune complexes have been found in the hyaline articular cartilage in a majority of patients with rheumatoid arthritis (Cooke et al, 1975; Ishikawa, Smiley & Ziff, 1975).

Circulating immune complexes are also found in rheumatoid arthritis patients (Luthra et al, 1975; Zubler et al, 1976; Hay et al, 1979) and the potential pathogenicity of circulating complexes has been discussed above. The presence

of systemic hypocomplementaemia in some patients with rheumatoid arthritis (Hunder & McDuffie, 1973) and the hypercatabolism of complement components in many others (Kaplan et al, 1980; Krick et al, 1978) suggest that circulating immune complexes may play a role in RA with and without the extra-articular manifestations of the disease. The self association of IgG and IgM rheumatoid factors is well described (Pope, Teller & Mannik, 1974) and may be responsible for a proportion of circulating immune complexes in patients with rheumatoid arthritis (Pope, Yoshinoya & McDuffy, 1981). Circulating immune complexes in patients with rheumatoid arthritis may form cryoglobulins. IgM rheumatoid factor and less frequently IgG rheumatoid factor containing cryoglobulins appear to be associated with, and may indeed mediate rheumatoid vasculitis (Weisman & Zvaifler, 1975). Using the polyethylene glycol precipitation technique over half of the sera from patients with rheumatoid arthritis (32 of 63) contained immune complexes. This compared with only 2 of 52 normal controls who had immune complexes detectable in their sera (Barnett et al, 1979). Using a platelet aggregation assay circulating immune complexes were found in approximately 25% of seropositive rheumatoid arthritis sera (Norberg, 1974). Apart from these studies which were carried out on patients with established disease other studies have shown that circulating immune complexes can be detected in patients with very early disease and even many months before the actual diagnosis of rheumatoid arthritis can be made (Jones et al, 1981). The importance of this study is that there are obviously abnormal circulating immune complexes right at the very inception of disease. From the foregoing I therefore felt justified in looking at rheumatoid arthritis as an immune complex disease although I have taken cognisance of the evidence that there is an ongoing cell-mediated reaction in the rheumatoid synovium along side the type 3 reaction.

The presence of circulating immune complexes in rheumatoid arthritis, often early in the disease, taken with the evidence of systemic hypercatabolism of complement, raises the question of whether there is a failure of clearance of these complexes. Defective reticulo-endothelial function in rheumatoid arthritis has been reported by Williams and colleagues (1979). In this study only 13 patients were studied but the authors could relate the levels of circulating immune complexes and splenic function to disease activity; there was also an inverse relationship between levels of circulating immune complexes and splenic function suggesting a causal relationship. However other studies have not been able to substantiate these findings (Gordon et al, 1981; Henderson et al, 1981). Interestingly, in vitro studies (Hurst & Nuki, 1981) have shown a selective impairment of complement-mediated phagocytosis in patients with rheumatoid arthritis with vasculitis but not in those without vasculitis. Fc receptor function was normal in all patients. Elkon and associates have shown, using heat-damaged erythrocytes, defects in clearance in SLE and various vasculitides (Elkon et al, 1980). Indirect methods of assessing the function of the reticulo-endothelial system by studying its capacity to clear erythrocytes coated with IgG (Rh-D) antibody (splenic Fc mediated clearance) and erythrocytes sensitised with IgM antibody (usually human IgM anti-blood group substance A) and C3 (hepatic clearance) have shown defective reticulo-endothelial function in patients with various immune complex diseases (Frank et al, 1983) but in rheumatoid arthritis the role of reticulo-endothelial blockade in pathogenesis remains unclear. As discussed above complement reactions with immune complexes are important in the processing and delivery of immune complexes to the reticulo-endothelial system for safe removal. Complement mediated prevention of immune precipitation by maintaining complexes small and soluble and by initiating opsonisation whereby the complexes are coated with C3b, is central to this clearance process. It is clear that tissue damaging immune complexes are

made when precipitating antibodies are formed (Dixon et al, 1961). Therefore the ability of complement to keep complexes soluble is of critical importance.

Certainly, at least some of the manifestations of rheumatoid arthritis may be mediated by immune complexes (eg vasculitis) and the fact that immune complexes in serum can be detected very early on in the disease suggests that immune complexes may be important in mediating the early features of the disease. The presence of insoluble immune complexes in the circulation and the synovial fluid may in part be due to the failure of normal clearance mechanisms.

The work in this thesis concerns studies which I carried out between 1983-1986 concerning the investigation of complement mediated prevention of immune precipitation (PIP) in rheumatic diseases. For the reasons discussed above I focussed on rheumatoid arthritis and attempted to relate the findings in serum and synovial fluid to the clinical and laboratory features of disease. If, as has been postulated, a defect of PIP did exist in RA serum or synovial fluid what was its nature and its mechanism of action? If a failure of PIP did exist in RA was it related to any specific clinical or laboratory aspect of the disease? These were the questions which I set out to answer at the beginning of this work.

CHAPTER TWO

MATERIALS & METHODS

I have arranged this chapter in three parts, namely:

(1) Clinical methods

(2) Experimental methods

(3) Statistical methods.

The chapter is arranged in sections and subsections and enumerated as follows:

2.1, 2.2, 2.3, the figure after the point denoting the section.

The figure after the second point indicates the subsection eg 2.1.1, 2.1.2, 2.1.3 etc.

Reagents Obtained Commercially

Gelatin (Merck)
Purified agarose (BDH)
Sheep erythrocytes (Gibco)
Bovine serum albumin (Sigma)
Freund's complete adjuvant] (Difco)
Freund's incomplete adjuvant]
Tyrosine (Sigma)
Phenylmethylsulphonyl fluoride (PMSF) (Sigma)
Soyabean trypsin inhibitor (SBTI) (Sigma)
Pepsin (Sigma)
Trypsin (Sigma)
RA latex test (Wellco test, Wellcome)
^{125}-I (Radiochemical Centre, Amersham)

Tween-20 (Sigma)
o-phenylene-diamine (OPD) (Sigma)
TEMED (BDH)
Acrylamide] (BDH)
Bisacrylamide]

Antibodies

Peroxidase labelled goat antihuman antibodies (Cappell Laboratories, Pennsylvania).

(1) $F(ab')_2$ anti-human μ chain] (Cappell
(2) $F(ab')_2$ anti-human α chain] Laboratories
(3) $F(ab')_2$ anti-human IgG] Pennsylvania)

Sheep antihuman IgM] (Scottish Antibody
Sheep antihuman IgG] Production Unit)

Chromatography Reagents

DEAE cellulose (DE52) (Whatman)
Dowex 1x8-200 (Aldrich Chemical Co)
Sepharose 4B]
Sephadex G-200] Pharmacia
Sephacryl S300]

All other general laboratory reagents used for solutions and buffers were Analar grade and were obtained from BDH Chemicals.

2.1 CLINICAL METHODS

2.1.1 Collection and preparation of biological materials

Serum samples

Blood was collected by venepuncture and allowed to clot at 37°C for 30 minutes and for one hour on ice for clot retraction to occur. After centrifugation at 2000g for 10 minutes the serum was separated and stored in aliquots at -70°C.

Synovial fluid samples

Synovial fluid (SF) was collected from the knee joint of patients undergoing therapeutic arthrocentesis. This was carried out under local anaesthesia and a strict aseptic procedure was employed. The volume of SF aspirated was recorded.

Samples were centrifuged at 2000g for 10 minutes to remove any cells and particulate debris. The supernatants were divided into aliquots and stored at -70°C.

Clinical studies - patient details

2.1.2 Measurement of PIP in patients' sera (see Chapter 3, Section 2)
In this study blood was collected from 32 patients with systemic lupus erythematosus (SLE) (Cohen et al, 1971), 75 patients with classical or definite rheumatoid arthritis (RA) (Ropes et al, 1958) who were seropostive for rheumatoid factor as measured by haemagglutination and 11 with RA who were seronegative for rheumatoid factor. Also included were 9 patients with psoriatic arthritis, 12 with ankylosing spondylitis and 8 with degenerative joint disease (osteoarthritis). All these patients attended a routine rheumatology out-patient clinic. Blood was also collected from a group of patients with glomerulonephritis:-14 with membranous glomerulonephritis (GN), 2 with focal GN and 1 with acute post-streptococcal GN. The diagnosis of GN was based

on renal biopsy findings. Blood samples were also obtained from 25 healthy laboratory personnel, 13 male and 12 female. These served as normal controls.

2.1.3 Measurement of inhibition of PIP in sera of patients suffering from rheumatic disease (See Chapter 3, Section 4)

In this study sera was obtained from 25 normal laboratory personnel (see above). Serum was prepared from venous blood obtained from 130 patients with seropositive RA (Ropes et al, 1958), 25 with seronegative RA, 5 with psoriatic arthritis and 6 with ankylosing spondylitis. Synovial fluid (SF) was obtained from 13 patients with RA as described above. Venous blood was obtained from these patients at the time of arthrocentesis.

2.1.4 The relationship between inhibition of PIP (inhibitory activity) and clinical features of disease.

Study A

2.1.4(a) Twenty-nine patients who underwent therapeutic arthrocentesis of the knee joint, were divided into the following three groups: group A-I included 15 patients with RA positive for IgM-RF, group A-II included 8 RA patients who were seronegative for IgM-RF and group A-III included 6 patients with miscellaneous arthritides (2 psoriatic arthritis, 2 reactive arthritis, 1 sarcoid arthropathy and 1 pyrophosphate arthropathy). Clinical evidence of generalised disease activity and activity within the joint to be aspirated were assessed prior to arthrocentesis.

Clinical assessment of disease activity

Generalised disease activity was assessed by recording the duration of morning stiffness (minutes), pain score (scale 0-3) where 0 = no pain and 3 = severe pain and articular index of joint tenderness (Ritchie et al, 1968).

The degree of inflammation in an aspirated knee joint was assessed clinically by using a cumulative index (scale 0 - 12). This index comprised assessments of pain, synovial hypertrophy, tenderness and effusion (each scored on a scale 0 - 3).

Laboratory assessment of disease activity included the measurement of haemoglobin (Hb), white cell count (WCC), platelet count and erythrocyte sedimentation rate (mm in first hour, Westergren). Total haemolytic complement (CH50) was measured by haemolytic assay (see 2.2.14(b) and C3, C4 and Factor B by nephelometry (see 2.2.14(a) IgM-RF, IgA-RF and IgG-RF was measured in serum by ELISA (see 2.2.7(d).

Synovial fluid analysis included measurement of volume aspirated (mls), WCC, total protein, C3, C4, Factor B, CH50 and rheumatoid factor (IgM, IgA and IgG-RFs) by ELISA (see 2.2.7(d).

Study B

2.1.4(b) Fifty-eight consecutive patients with definite or classical RA (Ropes et al, 1958) were divided into the following three groups according to the presence or absence of extra-articular features of RA. Group B-I included 20 RA patients with articular disease alone; group B-II included 18 RA patients with subcutaneous nodules, and group B-III included 20 patients with RA complicated by systemic vasculitis.

2.2 EXPERIMENTAL METHODS

2.2.1 Assays to measure the complement mediated prevention of immune precipitation (PIP) and inhibition of PIP (see Chapter 3, Sections 1-4)

Reagents

1. GVB^{2+} pH 7.4. This was made as described (2.2.14(b).
2. Phosphate buffered saline (PBS) pH 7.4
3. ^{125}I-BSA
4. anti-BSA antiserum
5. Trichloroacetic acid (TCA) 20%
6. BSA (5mg/ml)

Phosphate buffered saline (PBS) pH 7.4

8g of sodium chloride, 1.21g of potassium phosphate and 0.34g of potassium hydrogen phosphate was dissolved in 500mls water and made up to a final volume of 1 litre.

Trichloroacetic Acid (TCA, 20% W/V)

20g of trichloroacetate was dissolved in water to a final volume of 100mls.

BSA (5mg/ml)

100mg of BSA (Sigma) was weighed out and dissolved in 20mls of PBS pH 7.4. Final concentration of BSA was 5mg/ml.

^{125}I-BSA

BSA was radiolabelled with ^{125}I by a modification of the chloramine T method described by McConahey & Dixon (1966). The procedure was as follows:

Reagents

2mg BSA (in 1ml solution)

^{125}I (1mCi)

PBS pH 7.4

10% BSA in PBS

Chloramine-T 5mg/ml.

0.5mg/ml Tyrosine dissolved in 0.01M NaOH.

10% TCA

Dowex 1 x 8 - 200 Anion exchange resin.

Method

A Dowex slurry was prepared by soaking Dowex beads 1:1 v/v in deionised water overnight.

A 10ml graduated pipette was used as a column. After insertion of a small plug of glass wool the column was filled with the Dowex slurry to approximtely 6mls. The column was equilibrated with PBS, pH 7.4.

Reaction

2mg of BSA (in 1ml solution) was added to vial containing 1mCi of ^{125}I. 10µl of chloramine-T (50µg) was then added. The vial was mixed for 40-50 seconds and the reaction stopped by the addition of 50µl (25µg) tyrosine. The reaction mixture was applied to the Dowex column and the reaction vial washed out with a small volume of PBS. These washings were also applied to the column. 350µl fractions were collected in small tubes from the bottom of the column and the distribution of the radioactivity determined by removing 5µl aliquot for counting. The tubes containing the peak of the radioactivity were pooled.

To determine the ratio of protein bound to free ^{125}I.

10µl of labelled protein pool was added to each of two capped microfuge tubes to which had been added 200µl of 10% BSA, 1ml of 20% TCA was then added to each tube. After centrifugation in a microfuge at 10,000 rpm for 10 mins, the supernatants were separated from the protein pellets. Supernatants and pellets were counted in a γ counter (Packard Model 800C) and the percentage of acid precipitable radioactivity was calculated as follows.

$$\frac{\text{counts in pellet - background}}{\text{(counts in pellet - background) + (counts in supernatant-background}} \times 100$$

Acid-precipitable radioactivity was usually 95-97%.

The concentration of BSA in the labelled BSA pool was measured by reading OD_{280} of the sample in a spectrophotometer. The concentration of BSA in the sample was calculated using the extinction co-efficient for BSA (0.667 for a 1mg/ml solution of BSA at 280nm). The volume of the labelled BSA pool was usually 1.7 to 1.8mls.

Using the above described labelling procedure the recovery of labelled BSA was usually 0.98 to 1.1 mg/ml.

Specific Activity of ^{125}I-labelled BSA

The amount of radioactivity which was incorporated into the ^{125}I-labelled BSA was calculated by dividing the amount of radioactivity in 1ml of the ^{125}I-BSA solution by the BSA concentration. The results were expressed as cpm/µg protein, or µCi/µg protein as µCi = 1.5×10^6 cpm.

Anti-BSA antiserum

Antiserum to BSA was prepared by the immunisation of rabbits. For primary immunisation 1mg of antigen was emulsified in Freund's complete adjuvant and injected subcutaneously and intramuscularly into several sites. After 6, 8 and 10 weeks the immune response was boosted by further intramuscular injections of 1mg of antigen emulsified in Freund's incomplete adjuvant. Ten days after the final injection the animals were bled and the antiserum was heat-inactivated (56°C for 30 min) prior to storage in aliquots at -20°C.

Testing of antiserum

The antiserum was tested using double-diffusion in agarose gel (Ouchterlony, 1958). When antigen and antiserum are placed in adjacent wells cut in agarose they will diffuse towards each other. A line of precipitate is seen when the antigen and antibody meet at equivalence.

Equipment and Reagents

Glass plates 80mm x 80mm

Horizontal table

Gel punch

Vacuum pump

Drawn out capillary tubing

Moist chamber

Agarose

Isotonic veronal buffered saline (pH 7.4) containing EDTA (10mmol/l) and sodium azide (0.02%)

Boiling water bath

Method

A glass plate was carefully cleaned with detergent and rinsed thoroughly in deionised water and then 95% alcohol. It was allowed to dry at room temperature. 1.5g agarose was dissolved in 100mls of veronal buffer by boiling in a bottle in the water bath. The agarose was dispensed in 10ml aliquots and stored at 4°C until used. A 10ml aliquot was melted by boiling and the agarose pipetted onto the glass plate on a level table. The gel was allowed to set at room temperature.

Seven wells each 3mm in diameter were punched into the agar using a plastic template and a gel punch. The arrangement of the wells was one central well surrounded by six peripheral wells. The distance between the centre of the central well and the centre of the peripheral wells was 10mm.

The wells were carefully loaded to the brim using a drawn out capillary tube. The central well contained BSA (1mg/ml). The peripheral wells were filled with serial doubling dilutions of antiserum (from undiluted to 1:32).

The loaded plate was placed in a moist chamber at room temperature to allow diffusion to occur over 24-48 hours. The gel was viewed using incident light against a dark background. A line of precipitation between the central well and the peripheral wells was noted. This was most marked with the undiluted antiserum and became fainter as the antiserum was diluted. A single line was noted confirming that the antiserum was monospecific. The dilution of antiserum which produced a still visible line of precipitation with the antigen in the central well was used as an indication of the amount of specific antibody which was present in the antiserum.

Quantitative precipitin curve for immune complexes

BSA anti-BSA immune complexes formed at equivalence were used in the PIP assay. In order to determine the quantities of BSA and anti-BSA required a quantitative precipitin curve was prepared. Serial doubling dilutions of BSA (2mg - 5μg in 250μl PBS) were set up in a row of nine test tubes. An equal volume of antiserum (250μl) was added to each tube. The tube contents were mixed and then incubated at 37°C for 1 hour, and overnight at 4°C. The immune precipitates were separated by centrifugation (2000g for 30mins at 2°C), washed three times in ice cold PBS and redissolved in 100μl of sodium hydroxide (100mmol/l). The amount of protein in each tube was determined by the Folin phenol method (see on).

The results were plotted as shown in Fig 2.1. The tube containing the greatest amount of precipitate was the equivalence point. This tube contained 350μg of BSA. Subtracting this from the total precipitate value (μg of protein) gave me the amount of antibody in the complex which at equivalence should be all the antibody. In this experiment 805μg of antibody had precipitated in this tube. This gave a molar antigen antibody ratio at equivalence to be 1 to 2.3. This ratio of BSA to anti-BSA was used in my assays to measure PIP and inhibition of PIP.

2.2.1(a) Assay to measure the prevention of immune precipitation (PIP)

The assay used was a modification of that described by Schifferli and colleagues (1980).

Equal volumes 50μl of serum and GVB^{2+} were mixed in a test tube, to which 5μl ^{125}I-BSA (1.75 μg) was added. The mixture was warmed to 37°C in a water bath, after which 10μl anti-BSA (4μg antibody) were added, the contents mixed well and the incubation continued. In the initial kinetic

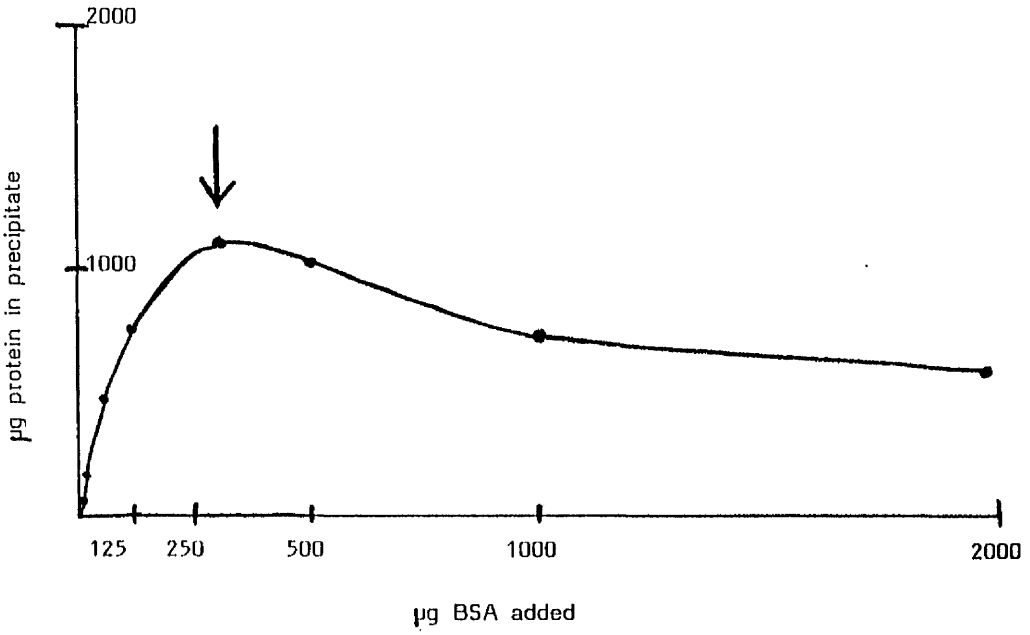


Figure 2.1

Quantitative precipitin curve of BSA and anti-BSA antibody. The arrow denotes the equivalence point, the tube of maximum precipitation. To the right of this point immune complexes are in antigen excess and to the left they are found in antibody excess.

studies, 25µl aliquots of the mixture were removed after 15, 30 and 60 minutes. Once the conditions of the assay had been established, a single aliquot (50µl) was removed after 60 minutes. The aliquots were added to Eppendorf tubes containing 1ml ice cold phosphate-buffered saline (PBS). The tubes were centrifuged (Beckman Microfuge) for 5 minutes and 250µl of the supernatant removed for counting radioactivity.

Control tubes included (1) total radioactivity precipitated by antibody in the absence of serum (100µl GVB^{2+} 5µl ^{125}I-BSA, 10µl anti-BSA. (2) ^{125}I input (115µl GVB^{2+} , 5µl ^{125}I-BSA) and (3) TCA precipitable counts (50µl BSA (5mg/ml), 5µl ^{125}I-BSA, 60µl 20% TCA). The percentage of complexes which remained soluble was calculated as follows:

$$\% \text{ soluble} = \frac{\text{c.p.m. in serum supernatant - c.p.m. in buffer supernatant}}{\text{Total c.p.m./tube - c.p.m. in buffer supernatant}} \times 100$$

All assays were performed in duplicate.

2.2.1(b) Assay to measure the inhibition of prevention of immune precipitation (inhibition of PIP). (see Chapter 3, Section 3).

Preparation of pool of normal serum

10ml blood was collected by venepuncture from 20 normal healthy volunteers. Each sample was allowed to clot at room temperature for 30 minutes and was then placed on ice for 1 hour for clot retraction to occur. After centrifugation (200g for 10 minutes) the serum was separated from each sample and pooled. The pool was made into small aliquots and stored at -70°C until needed.

A quantitative precipitin curve as previously described was constructed in order to determine the equivalence point. From this curve 0.5µg of ^{125}I-BSA in 5µl and 10µl anti-BSA diluted 1:6 were the quantities used in this assay.

The normal serum was titrated in order to determine the dilution which would inhibit precipitation of complexes (0.5µg ^{125}I-BSA in 5µl and 10µl anti-BSA diluted 1:6), by between 70 and 90%. The procedure adopted for the assay was to mix 50µl of diluted normal serum (usually 1:3 in GVB^{2+}) with 50µl of test serum (either fresh or following heat-inactivation for 30 min at 56°C) and ^{125}I-BSA (0.5µg in 5µl) and then to warm the mixture to 37°C. Following the addition of anti-BSA (10µl 1:6 dilution) the incubation was continued at 37°C for 1 hour, after which an aliquot(50µl) was removed and transferred to an Eppendorf tube containing ice cold PBS (1ml). Following centrifugation for 5 min (Beckman Microfuge), 250µl of the supernatant were removed and the radioactivity counted using an automatic γ counter. Control tubes included (1) radioactivity precipitated by antibody in the absence of serum (100µl GVB^{2+} , 5µl ^{125}I-BSA, 10µl anti-BSA), (2) maximum possible PIP by serum alone (50µl diluted normal serum, 50µl GVB^{2+} , 5µl ^{125}I-BSA 10µl anti-BSA) and (3) total ^{125}I-BSA input (110µl GVB^{2+} , 5µl ^{125}I-BSA). The control tubes were processed in an identical fashion to the assay tubes.

Each assay was performed in duplicate, and when the results of replicate tubes varied by more than 5% of the mean the assay was repeated. Results were calculated using the formula:

$$\% \text{ Inhibition} = 1 - \frac{\text{cpm test supernatant - cpm buffer supernatant}}{\text{cpm serum control supernatant - cpm buffer supernatant}} \times 100$$

The day to day variation in the assay was between 1 and 11% (mean 5.5%).

2.2.2 Purification of inhibitory activity from RA Serum

IgG Sepharose chromatography

RA serum (15mls) was diluted in an equal volume of EDTA (20 mmol/l, pH 7.5) and passed over a column (1cm x 40cm) of IgG Sepharose. The IgG Sepharose column was previously equilibrated with veronal buffered saline diluted 1:1 with dextrose solution (5% w/v) pH 7.5. Following application of the sample the column was washed with equilibration buffer until no further protein was eluted.

The bound material was eluted in 2 stages. Firstly with VBS containing 400 mmol/l NaCl pH 7.5 and secondly with VBS containing guanidine hydrochloride (2 mmol/l) pH 7.5.

The column fractions were screened for total protein (OD_{280}), inhibition of PIP (inhibitory activity) and rheumatoid factor activity by haemagglutination and radio-immunoassay for IgM-RF.

Sephacryl S300 gel filtration chromatography

The fractions containing the inhibitory activity were pooled and concentrated using vacuum dialysis. This concentrated pool was dialysed against acetate buffer (100 mmol/l pH 3.5). 1 ml of this concentrate was applied to a Sephacryl S-300 column (1.6 cm x 90 cm) which had been equilibrated with the acetate buffer. The column fractions were collected and neutralised with

Tris-base 1 mmol/l. The fractions were screened for total protein, inhibition of PIP (inhibitory activity) and rheumatoid factor by haemagglutination and radio-immunoassay for IgM rheumatoid factor.

2.2.3 Isolation of inhibitory activity from serum by sucrose gradient ultra-centrifugation of RA serum and normal serum and subsequent affinity chromatography.

Discontinuous sucrose gradients (4ml; 20% - 40%) were prepared in polyallomer ultracentrifuge tubes and the serum sample (0.5ml) was layered on top. The tubes were centrifuged at 40,000 rpm in an ultracentrifuge (Beckman Model L6) for 16 hours at 4°C (155,000 x g). Following centrifugation six fractions of equal volumes (750µl) were collected through a hole pierced in the bottom of the tube. The fractions were extensively dialysed at 4°C in a glass beaker containing 2 litres phosphate buffered saline, (PBS, pH 7.4). The dialysis was repeated with three changes of PBS. This ensured that each fraction was free from sucrose prior to screening using the following assays:

1 Protein content using the Folin phenol method

2 IgM and IgG content by radial immunodiffusion

3 Inhibitory activity as previously described

4 IgM rheumatoid factor activity by Rose-Waaler assay and by enzyme linked immuno-assay (ELISA).

IgG - Sepharose chromatography

Similar sucrose fractions were pooled and subjected to further protein fractionation on small (2ml) columns of IgG sepharose. 1ml fractions were collected. Bound material was eluted in two steps.

1. 0.4M NaCl

2 2M guanidine hydrochloride. Fractions were assayed for inhibitory activity and IgM rheumatoid factor by Rose-Waaler titration and ELISA.

2.2.4 Effect of trypsin and SBTI on inhibitory activity.

This experiment was designed to assess whether (1) the inhibitory activity was sensitive to the enzyme trypsin and (2) whether the inhibitory activity was a trypsin-like enzyme. One hundred microlitres of serum was incubated at 37°C for 1 hour with 100μg trypsin. At the end of the incubation period 200μg soybean trypsin inhibitor was added to arrest proteolysis. Controls included serum with SBTI (200μg) and trypsin (100μg), added in that order prior to incubation, serum incubated with SBTI (200μg) alone, and serum alone. The samples were tested for inhibitory activity at the end of the incubation period.

2.2.5 The effect of PMSF on inhibitory activity

PMSF is a serine protease inhibitor and this experiment was designed to assess whether the inhibitor of PIP was a serine protease. PMSF (Sigma) dissolved in dimethyl formamide (1mol/l), was added to inhibitory serum to give a final concentration of 1 mmol/l. Controls included serum treated with dimethyl formamide alone, and serum alone. Following incubation for 1 hour at 37°C, inhibitory activity was assayed.

2.2.6 Kinetics of inhibitory action

This assay was used to study the kinetics of the inhibitory action of RA serum, RA synovial fluid or purified IgM-RF on PIP. Aliquots (50μl) of normal serum (diluted 1:3) in GVB^{2+}) were added to a series of test tubes. To the

first tube 50µl of inhibitory serum (or synovial fluid or IgM-RF) were added at time 0. To all the tubes ^{125}I-BSA (5µl containing 0.5µg ^{125}I-BSA) was added and the tubes were warmed to 37°C. At time 0 anti-BSA (10µl diluted 1/6) was added to all six tubes and the incubation continued at 37°C. Inhibitory serum (or synovial fluid or IgM-RF 50µl) was added to tube 2 after 5 minutes, and to tubes 3, 4, 5 and 6 after intervals of 15, 30 and 60 minutes. Each tube was incubated for 60 minutes after the addition of the inhibitory material. At the end of the incubation an aliquot of 50µl of the mixture in each tube was removed and transferred to an Eppendorf tube containing 1ml ice cold PBS.

The Eppendorf tubes were centrifuged for 5 minutes (Beckman Microfuge) and 250µl of the supernatant was removed and the radioactivity counted in an automatic γ counter. The control tubes which were processed in an identical fashion were (1) radioactivity precipitated by antibody in the absence of serum (100µl GVB^{2+}, 10µl anti-BSA, 5µl ^{125}I-BSA) (2) maximum possible PIP by serum alone (50µl diluted normal serum, 50µl GVB^{2+}, 10µl anti-BSA and 5µl ^{125}I-BSA) (3) total radioactivity input (110µl GVB^{2+} and 5µl ^{125}I-BSA).

Results were calculated thus:

$$\text{Inhibitory activity (\%)} = 1 - \frac{\text{cpm test supernatant} - \text{cpm buffer supernatant}}{\text{cpm serum control supernatant} - \text{cpm buffer supernatant}} \times 100$$

2.2.7 Measurement of rheumatoid factor

Rheumatoid factor was measured by several different techniques. For some assays the Rose-Waaler test was used, for others a radio-immunoassay and later I developed an ELISA assay to measure rheumatoid factor classes (IgM, IgG and IgA).

2.2.7(a) RA-Latex test

This screening test uses latex particles coated with human IgG and comes available in kit form (Wellcotest: Wellcome). Rheumatoid factor containing serum or synovial fluid was mixed directly with the serum (or recommended serum dilution) on a clean glass slide. Gentle mixing was carried out using a small wooden stick. If rheumatoid factor was present macroscopic agglutination were observed at 2 minutes.

2.2.7(b) Rose-Waaler test

This test is more time consuming but does give a quantitative value for RF in terms of serum titre.

Materials

Sheep red blood cells in Alsever's solution.

Antisheep red blood cell antiserum. The dilution used is titrated and is one third the minimum agglutinating dose.

Plastic round bottom microtitre plates

Phosphate buffered saline (PBS, pH 7.4)

56°C water bath

37°C waterbath

37°C incubator

Procedure

1. The sheep red cells were washed three times in PBS and a 2% suspension in PBS was made. A 1% suspension of control unsensitised cells was made by mixing equal volumes of the 2% suspension and PBS.

2. The antibody-sensitised cell suspension was made by mixing equal volumes of 2% suspension and PBS containing the lowest subagglutinating dilution of rabbit anti-sheep cell antiserum. The mixture was incubated for 30 minutes in a shaking 37°C water bath.

3. The test sera and test samples were heat-inactivated (56°C for 30 minutes) in a water bath. Two drops of concentrated washed sheep red cells were added to 0.5ml of each test sample. This was done to absorb out any anti-sheep red cell activity. After incubation for 1 hour at 37°C the tubes were spun and the supernatant was taken for testing.

4. The test and control sera were titrated by doubling dilutions from 1 in 2 to 1 in 2048 in microtitre plates.

5. Control (unsensitised) cells were added to the first two rows of wells, sensitised cells were added to the remaining wells.

6 The plates were mixed well on a mechanical plate shaker, left at room temperature for 30 minutes and the sedimentation pattern was then read.

Results

A confluent layer of red cells over the bottom of the well indicated agglutination ie, the presence of rheumatoid factor. The titre was taken as the highest dilution of test serum which showed definite agglutination. The first two rows of wells (controls) showed a negative reaction ie, no agglutination (red cell button at bottom of well). The inherent error in any doubling dilution assay is one dilution on either side of the recorded titre.

2.2.7(c) Radioimmunoassay for IgM-RF

Reagents PBS (pH 7.5)

PBS containing 0.05% Tween 20 (PBS-Tween)

Gelatin (0.1%) in PBS

^{125}I-F(ab')$_2$ anti-human IgM

Human IgG (5µg/ml) in PBS

Radio-immunoassay wells were coated with human IgG and then washed in PBS containing 0.05% Tween 20; (PBS-Tween, pH 7.5). The unreacted sites were 'blocked' with gelatin (0.1%) and the wells rewashed. After the test material (100µl) was added, the wells were incubated overnight at room temperature (RT). The wells were then washed three times with PBS-Tween and ^{125}I-labelled F(ab')$_2$ fragments of anti-human IgM added. After a further incubation period (4 hours at RT) the wells were rewashed and the bound counts measured using an automatic gamma-counter. A standard curve using dilutions of a sample of RA serum containing a known concentration ofIgM-RF was included in each series of assays. Each assay was performed in duplicate; only results varying by less than 5% of the mean were accepted. Results were expressed as units/ml.

2.2.7(d) Measurement of rheumatoid factors by enzyme linked immunoassay (ELISA) (This was a modification of the method described by Faith et al, 1982)

IgM, IgG and IgA-RF were measured using a micro-ELISA technique. For the IgM-RF and IgA-RF assays, Dynatech plates were coated with human IgG, but for IgG-RF assay plates were coated with rabbit IgG. Prior to the measurement of IgG-RF, sera and synovial fluids were digested with pepsin (see below). Peroxidase labelled goat anti-human μ chain, anti-human α chain and anti-human IgG, $F(ab')_2$ antibodies (Cappell, Pennsylvania) were used to detect the presence of RF of the different classes.

ELISA for IgM-RF

A Dynatech plate with flat bottomed wells was used. The wells were coated with human IgG (5µg/ml), 100µl per well and incubated overnight at room temperature (RT). The plate was washed 3 times with PBS-Tween. The unreacted sites were blocked by adding 200µl of 0.1%BSA in PBS to each well. The plate was left at RT for at least 1 hour before being washed again with PBS-Tween. The test sample (eg serum) was loaded on to the plate 100µl/well in various dilutions. The plate was then left at RT overnight. After washing 3 times with PBS-Tween, 100µl peroxidase labelled conjugate $F(ab')_2$ anti-IgM diluted 1/4000 in PBS-Tween) was added to each well and the plate incubated at RT for 1 hour. The plate was further washed with PBS-Tween.

The substrate (o-phenylene diamine; (OPD); 17mg) was dissolved in 50mls citrate phosphate buffer, (pH 5.4). Just prior to addition to the washed wells, the substrate was activated by the addition of 20µl of hydrogen peroxide (H_2O_2). 100µl of the activated substrate was added to each well and the plate was incubated in the dark for 10 minutes. The colour reaction was stopped by the addition of 25µl 4N sulphuric acid to each well. The optical density at 492

nm was read using an ELISA reader.

A rheumatoid factor (5000 units/ml) containing RA serum was used as a standard. The level of IgM-RF had been previously determined as 5000 units/ml. This standard was doubly diluted with PBS-Tween from 1/100 to a 1 in 51,200 dilution. A standard curve was plotted of OD_{492} for each dilution of the standard (y axis) against the logarithm of IgM-RF units/ml (x axis). A sigmoid curve was obtained. Values of the test samples were then read off OD_{421} against the x axis (IgM-RF units/ml) utilising the straight line part of the curve. Samples were suitably diluted so that OD_{421} readings fell on the straight line part of the curve.

A normal range was established using sera from 25 normal controls.

Seronegativity of sera was established using sheep cell agglutination test (titre 1/16 or less) in 50 patient sera samples. These sera were assayed for IgM-RF in the above described ELISA. The mean value in units/ml ± 2 standard deviations was calculated for these seronegative sera. On the basis of this, seronegativity was taken as less than 800 units/ml which was 2 standard deviations above the mean for these seronegative sera.

ELISA for IgG-RF

To measure IgG-RF the serum or SF sample must first be digested by pepsin so that IgG is reduced to its $F(ab')_2$ fragments.

Pepsin digestion of serum and SF samples to $F(ab')_2$ fragments.

Pepsin acetate buffer

A stock acetate buffer 100mM pH 3.5. was prepared. This was diluted 1:10 with water and pH checked at 3.5 (10mM acetate buffer pH 3.5). 1mg pepsin was added to 100mls of the acetate buffer.

250µl of pepsin acetate buffer was pipetted into a series of labelled Bijou bottles. 5µl of test sample (RA serum or synovial fluid) was then added to the appropriate bottle. After mixing the bottles were incubated for 20 hours at 37°C.

After this time the digests were neutralised by the addition of 250µl of disodium hydrogen phosphate in PBS-Tween/80mg/ml) to each bottle. 100µl of each digest was loaded on to the ELISA plate.

Measurement of IgG-RF in serum and synovial fluid (SF)

Method

The measurement of IgG-RF by ELISA was similar to that described above for the measurement of IgM-RF. However, for IgG-RF measurement the wells were coated with normal rabbit serum 100µl (5µg/ml). The samples were digested with pepsin as described above prior to application. Peroxidase-labelled goat anti-human IgG $F(ab')_2$ was used as the conjugate (diluted 1:5000 in PBS). The results were calculated as described above for IgM-RF ELISA. A normal range was established using sera from 25 normal volunteers. The normal range of the IgG-RF assay was calculated as less than 30 units/ml.

ELISA for IgA-RF

The assay to measure IgA-RF was a modification of that described above for IgM-RF. The assay was similar in all respects to that described for IgM-RF except that the conjugate used was peroxidase labelled $F(ab')_2$ anti-IgA.

The results were calculated as described for IgM-RF ELISA. Using sera from 25 normal controls the normal range for IgA-RF as measured by this assay was < 20 units/ml.

2.2.8 Measurement of protein

OD_{280}

Protein was detected by measuring its absorbance of ultra-violet light at 280nm (OD_{280}). This is the simplest method for measuring protein concentration and was used in my experiments to give an approximate estimate of total protein concentration. (OD_{280} = mg protein/ml).

Folin phenol method

This method was a modification of the method described by (Lowry et al, 1951). It is a more accurate method for protein estimation than UV absorbance.

Materials	Folin solution A	2g Sodium carbonate
		20mg Sodium potassium tartrate
		Make up to 100mls with 0.1N sodium hydroxide.
	Folin solution B	0.15% Copper sulphate pentahydrate
	Folin solution C	5mls A + 0.1ml B

This is made up fresh each time.

Folin solution D Commercial Folin and Ciocalteu's Reagent.

Standard: BSA 1mg/ml in PBS.

Method

A standard curve was constructed by adding different (25, 50, 100, 150µl) volumes of stock BSA solution to a series of test tubes. To all tubes were added 2mls of Folin solution C followed by 20µl of Folin solution D. To a further series of tubes 100µl of protein sample to be measured was added to each tube. 2mls Folin C and 200µl Folin D were then added to each tube.

The negative control consisted of a tube containing 2mls of Folin solution C, 100µl of buffer and 200µl of Folin solution D.

The Folin reaction is time dependent, so after mixing on a vortex the tubes were allowed to stand for 30 minutes and the OD_{700} in each tube read in the order in which the reaction was started. The standard curve (OD_{700} v protein concentration) was plotted and the concentration of protein in the test sample was calculated.

2.2.9 Preparation of IgG from normal human serum

Reagents

(1) Saturated ammonium sulphate. This was made by dissolving 767g ammonium sulphate to a final volume of 1 litre deionised water, temperature 25°C, (4.1 Molar solution).

(2) 40% ammonium sulphate was prepared by adding 4 parts of saturated ammonium sulphate to 6 parts deionised water.

(3) 10mmol/l potassium phosphate buffer pH 8.0

(4) DE52 equilibrated in the above buffer.

(5) Phosphate buffered saline (PBS) pH 7.2

Method

40mls of saturated ammonium sulphate was added with stirring to 60mls of human serum. The mixture was left overnight at 4°C and then spun at 10,000g for 30 minutes at 4°C. The pellet was resuspended in 100mls of 40% ammonium sulphate, recentrifuged, and the pellet redissolved in 20mls of 500mmol/l sodium chloride in 10mmol/l phosphate buffer, pH 8.0. The sample was dialysed twice, firstly against 2 litres of 10mmol/l phosphate pH 8.0 at 4°C for 2 hours and secondly against another 2 litres for 16 hours at 4°C. After removal of any precipitate by centrifugation at 10,000g for 30 minutes at 4°C, the sample was applied to a 14x5cm column of DE52 equilibrated in the same buffer. The column was washed with 10mmol/l phosphate buffer pH 8.0 and 10ml fractions collected. The exclusion peak containing the IgG was pooled and concentrated by precipitation with an equal volume of saturated ammonium sulphate. The material was centrifuged and the pellet dissolved in 10mls of 500 mmol/l NaCl in PBS. It was further dialysed against PBS and stored at -20°C till used.

The purity of the IgG preparation was tested by SDS-PAGE under reducing and non-reducing conditions. A single band MW 150kD was seen on the unreduced gel. After reduction with 5-mercaptoethanol two bands corresponding to the heavy and light chains of IgG were noted.

2.2.10 Activation of Sepharose 4B by cyanogen bromide (CNBr) and coupling of Sepharose 4B with human IgG

This procedure was a modification of the one described by Goetzl & Metzger (1970).

(1) Buffers -3 litres of 0.1mol/l $NaHCO_3$ pH 8.0
-2 litres of 4M NaOH

(2) Sepharose 4B (100mls)

(3) Human IgG - dialysed against $NaHCO_3$ 0.1 mol/l pH 8.0

(4) Scintered glass funnel and Buchner flask.

Method

Sepharose 4B was washed five times in deionised water and resuspended in 100mls water.

100g CNBr was dissolved in 1 litre of deionised water. Sepharose 4B was added and the mixture stirred slowly with a magnetic stirrer. The pH was brought up to 11 and maintained there by adding 4M sodium hydroxide. The pH eventually stabilised at 11. The reaction took approximately 5 minutes to reach completion.

The cyanogen-bromide activated Sepharose was poured into a Buchner funnel which was packed with crushed ice and attached to a vacuum flask. After extensive washing with 3 litres of ice-cold sodium bicarbonate (0.1mol/l; pH8.0), 100ml of human IgG (20mg/ml) in sodium bicarbonate pH 8.0 were mixed with the Sepharose beads in a sealed beaker and the slurry rotated gently overnight at 4°C. After centrifugation 1000g at 4°C for 10 minutes the supernatant was taken for protein estimation and it was calculated that 93%

of the IgG was bound to the beads. The IgG Sepharose was resuspended in an equal volume of monoethanolamine (1mol/l pH 8.0) and the slurry was rotated at room temperature for 1 hour to block the unreacted protein binding sites.

Further washes were carried out, three times in each of the following; acetate buffer (100mmol/l, pH 3.0) containing sodium chloride 1mol/l; phosphate buffer (10mmol/l, pH 7.5) containing 1mol/l sodium chloride. The beads were stored at 4°C in PBS containing 0.01% sodium azide.

2.2.11 Preparation of $F(ab')_2$ fragments of normal human IgG, and the IgG fractions of anti-IgM antiserum

Materials

0.1M sodium acetate buffer pH 4.5

Human IgG (20mg/ml in acetate buffer)

2M tris

Tris buffered saline (TBS) 10mM tris-HCl pH 7.3 containing 0.15M sodium chloride.

Pepsin (10mg/ml in acetate buffer)

Sephadex G-200 (Pharmacia) equilibrated in TBS and packed into a 1.6 cm x 100 cm column.

Method

Pepsin solution (100 µl) was added to 2.5ml IgG solution. After gentle mixing the solution was incubated overnight (16 hours) at 37°C. 300µl tris (2M) was added to neutralise the solution which was then centrifuged at 2000g for 10 minutes to remove any precipitate. The supernatant was applied to the Sephadex G-200 column and eluted with TBS. 2.5ml fractions were collected and the protein concentration monitored by the absorbance at 280nm.

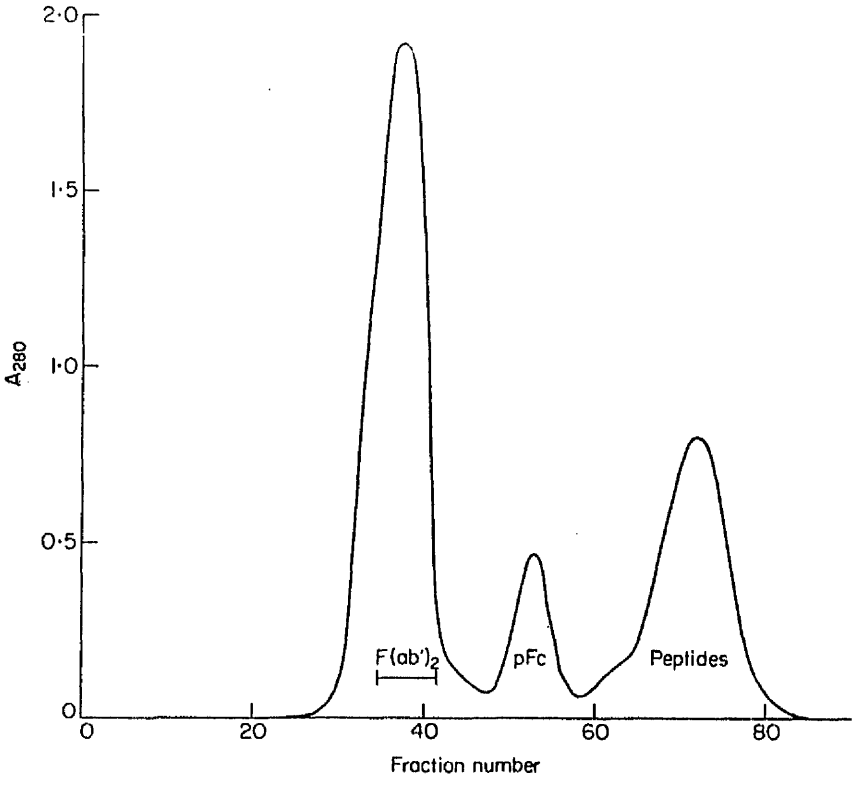


Figure2.2

Chromatography of pepsin digested human IgG on a Sephadex G-200 column in tris buffered saline (TSB).

Undigested IgG will be eluted just prior to the major $F(ab')_2$ peak (Fig 2.2) Every third fraction was tested by SDS-PAGE and only those containing $F(ab')_2$ were used. The fractions containing $F(ab')_2$ were pooled and concentrated to 5mls using vacuum dialysis.

2.2.12 Sodium docecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis of proteins described in this thesis was based on the method of Weber & Osborn, 1975. SDS binds to proteins and because of its strong negative charge, the mobility of SDS-protein complexes on SDS-PAGE is determined by the molecular weight of the protein. The reducing agent 2-mercaptoethanol was used to dissociate polypeptide chains linked by disulphide bridges. Therefore, SDS-PAGE, when run under reducing and non-reducing conditions reveals the molecular weight of the protein and its polypeptide chain structure.

Equipment

Power pack

Glass tubes

Electrophoresis tank

Buffers:

Solution A

1mol/l sodium phosphate buffer, pH 6.5,

219.6g/l sodium hydrogen phosphate dodecahydrate.

Solution B

10% (100g) sodium lauryl sulphate in 1 litre of water.

Solution C

Acrylamide/bisacrylamide mixture. 30g acrylamide and 800mg N,N'-methylene-bisacrylamide made up to 100mls with water. Stored at 4°C.

Method

After sealing the bottom ends of the gel tubes with Parafilm they were placed vertically in a rack. 5% gels were used for most protein analysis work in this thesis. To make 5% acrylamide gels 10mls of solution C was added to 43.4mls of water. 6mls of solution A and 60µl of solution B were then added sequentially. Finally 30µl of TEMED (BDH) [N, N, N' N' tetramethylene-diamine) and 60mg of ammonium persulphate was added, total final volume being 60mls. The mixture was slowly stirred throughout. The gel mixture was added to the tubes with a Pasteur pipette to within 10mm of the top. The gel solution was carefully overlayed with water and the gel allowed to polymerise at room temperature for 1 hour.

The well buffer was made up by adding 100mls of solution A to 10mls of solution B and making up to 1 litre with water.

The protein sample buffer contained the following reagents:

	Reducing	Non-Reducing
Solution B	0.5 ml	0.5 ml

2mercaptoethanol	0.5 ml	-
Glycerol	1.0 ml	1.0 ml
Solution A	0.05 ml	0.05 ml
0.5% bromophenol blue	0.1 ml	0.1 ml
water	3.3 ml	3.35 ml

The protein sample and sample buffer were mixed, 1:1v/v. The total volume applied to the gel was usually between 50-75µl. The mixture was boiled at 100°C (water bath) for 2 minutes prior to loading. The samples were carefully applied to the top of the gel, the water overlay having been removed. One gel containing a molecular weight standard was run as a control.

The electrophoresis was run at a constant current of 6mA/tube until the bromophenol blue marker had migrated approximately 80% down the tube.

The gels were removed from the glass tubes and a piece of stainles steel wire inserted into the gel to mark the buffer front (bromophenol blue band).

The gels were fixed and stained in test tubes containing 0.1% Coomassie brilliant blue in a mixture containing 5 parts methanol, 1 part glacial acetic acid and 4 parts water. After incubation 40°C for 2-6 hours the gels were destained in several changes of destaining solution (1 part methanol, 1 part glacial acetic acid and 8 parts water).

The distance between the protein band and the buffer front is proportional to the logarithm of the molecular weight of the protein. Using the known molecular weight standards in the control gel the molecular weight of the sample protein under study can be determined.

2.2.13 Estimation of IgM and IgG concentration using single radial immunodiffusion

When an antigen solution is placed in a well cut in an antibody containing gel a circular immune precipitate is formed around the well. The area contained within the ring is proportional to the concentration of the antigen being measured. This method was described by Mancini and colleagues in 1965. However Fahey and McKelvey (1965) introduced a modification of the method which allowed for the reading of the plates after a much shorter time.

Equipment and reagents

1. Petri dishes 35mm in diameter
2. The equipment for gel preparation and the procedure for preparing the agarose were the same as that described previously (see Section 2.2.1)

Method

1. A 10ml aliquot of agarose (1.5%) was melted in boiling water and cooled to 56°C in a 56° water bath.

2. An appropriate volume of antiserum IgM or IgG was prewarmed to 56°C and carefully mixed with the agarose to give a final dilution of 1 in 20 of antibody in agarose. A 1 in 20 dilution was chosen for practical purposes because this gave a satisfactory size of precipitin ring (diam 5-7mm) round a well to which undiluted serum had been added.

3. A standard template was placed under the gel and the well was made using a 2mm punch. By this method each Petri dish could accommodate 19 equidistant wells.

4. The test material (eg column fractions) was loaded into the wells to the brim using a drawn out capillary tube. A new tube was used for each sample.

5. The gels were placed in a moist chamber at room temperature on a horizontal surface and diffusion allowed to proceed for 24 or 48 hours.

6. After that time the plates were read inverted using incident light against a dark background. A measuring magnifier was used to measure the diameter of the rings.

7. The presence of IgG or IgM was shown by a ring of precipitation round the well. The diameter of the ring was measured in mm and the plates were read at 24 hours for IgG and 48 hours for IgM.

This technique was used to detect the presence of IgM and IgG in column fractions and sucrose gradient fractions, semi-quantitatively. No attempt was made to accurately quantify IgG or IgM concentration using control standards.

2.2.14 Measurement of Complement

2.2.14(a) Immunochemical Assays C3, C4 and Factor B.

Serum levels of C3, C4 and B were measured by nephelometry using a Beckman ICS Analyser II. This method is an immunochemical means of measuring serum complement components. Monospecific precipitating antisera to individual complement components are available commercially. The basic principle of nephelometry is that a light beam is passed through a glass cuvette containing a solution. The light beam passes straight through the cuvette if the solution is clear. If there is particulate material in the solution then some of the light is deflected as a result of impinging on the surfaces of the particulate material. The deflected light is measured at right angles to the incident beam. The principles of nephelometry were applied to the antigen-antibody reaction by Boyden, Bolton & Gemeroy (1947). However, it was only with the development of continuous flow nephelometry by Larson, Orenstein & Ritchie, (1971) that this technique gained wider popularity. Monospecific antisera of high avidity and titre are used. The antibody is added at a correct proportion to the antigen in the serum and an immune precipitate is formed. It is this precipitate which deflects the light. The machine measures the peak rate of precipitation, measured as the rate of change of light scattering from the time at which the antigen and antibody are mixed. Nephelometry can detect protein concentrations between 1-20 µg/ml and can give answers within a few minutes.

The patterns of complement activation in individual patients can provide the physician with useful diagnostic information. Consumption of C1, C4 and C2 follows activation of the classical pathway whereas factor B, D and properdin are utilised when alternative pathway activation is occurring. Cleavage of C3, C5 and the incorporation of C6, C7, C8 and C9 into the C5b-9 membrane attack complex occurs on activation of either pathway.

Therefore from a clinical point of view it is important to measure serum levels of at least one classical, one alternative and one terminal component in order to determine the pathway of complement activation in disease. In practice this is accomplished by measuring C3, C4 and Factor B.

2.2.14(b) Measurement of Complement - Functional Assays

Buffers used in assays

Stock veronal buffered saline (5 x VBS)

85g of sodium chloride, 3.75g Sodium-5,5-diethyl barbiturate was dissolved in 1 litre of deionised water (solution 1). 5.75g Na5, 5-diethyl barbituric acid was dissolved in 600 mls of water by vigorous stirring on a heated magnetic stirrer (solution 2). Solutions 1 & 2 were mixed and made up to 2 litres with water. pH should be 7.4-7.6

Isotonic EDTA (86 mmol/l)

89.338g of ethylene diamine N, N, N', N' tetra acetic acid (Sodium salt) was dissolved in 1500 mls of water and then made up to 2 litres. pH was approximately 4.5 (solution 1). 24g of sodium hydroxide pellets were dissolved in 1500 mls of water and then made up to 2 litres (solution 2). Solution 2 was added to solution 1 until the pH was betwen 7.2 and 7.6.

The final molarity of the EDTA was calculated as follows:

$$\frac{\text{Starting volume EDTA}}{\text{Starting volume EDTA + volume of NaOH used}} \times 0.12$$

The concentration should be 86 mmol/l.

EGTA Ethyleneglycol-bis (β-aminoethyl ether) N, N, N', N'- tetra acetic acid (Sodium Salt) 100 mmol/l)

38.04g EGTA was added to 500 mls of water stirring constantly with a magnetic stirrer. NaOH (10 mol/l) was slowly added until the EGTA was dissolved and the pH was 7.4. The solution was made up to 1 litre with water.

10% gelatin

10g gelatin (Merck) were dissolved in 100 mls of water with continuous stirring on a heated magnetic stirrer. The gelatin solution was made into 10 mls aliquots in sterile glass universal containers and stored at 2-4°C. When required for use in buffers the universal container was heated in boiling water until the gelatin had melted.

Calcium Chloride (30 mmol/l)

3.286g $CaCl_2 . 6H_2O$ was dissolved in water and made up to a final volume of 500mls.

Magnesium Chloride (100 mmol/l)

10.165 g of $MgCl_2 . 6H_2O$ was dissolved in water and made up to a final volume of 500mls.

Isotonic veronal buffered saline containing gelatin and cations (GVB^{2+}).

200mls. 5 x VBS

10mls $MgCl_2$ (100 mmol/l)

5mls $CaCl_2$ (30 mmol/l)

10mls 10% gelatin

The melted gelatin was thoroughly mixed with 100mls of water at room temperature to prevent it resolidifying when added to the other agents which were kept on ice. The solution was made up to 1 litre with water.

Isotonic veronal-buffered saline containing gelatin but without cations ($GVB^{=}$)

200mls 5 x VBS

10mls 10% gelatin

The gelatin was melted in boiling water and added to 100mls of water before mixing with 5 x VBS. It was made up to a final volume of 1 litre with water.

EDTA (40mmol/l) $GVB^{=}$

232.5mls of EDTA (86 mmol/l) and 267.5mls $GVB^{=}$ were mixed together. Final volume was 500mls.

EDTA (10 mmol/l) $GVB^{=}$

58mls 0.086 mol/l EDTA was mixed with 442mls $GVB^{=}$ Final volume was 500mls.

Isotonic dextrose without cations ($D5W^{=}$)

50g of D-glucose was dissolved in water and made up to a final volume of 1 litre.

Isotonic dextrose containing cations ($D5W^{2+}$)

50g D-glucose was dissolved in 500mls of water. To this was added 5ml $CaCl_2$ (30mmol/l) and 10ml $MgCl_2$ (100 mmol/l) and the solution was made up to a final volume of 1 litre. This buffer is isotonic and is used to reduce the ionic strength of the buffers without making them hypotonic.

Dextrose gelatin veronal buffered saline containing cations ($DGVB^{2+}$)

Equal parts of GVB^{2+} and $D5W^{2+}$ were mixed together producing a low ionic strength buffer (μ=0.075) containing cations. Protein-protein interactions are enhanced by the low ionic strength and as such increases the sensitivity of haemolytic complement assays.

Mg-EGTA

10mls of EGTA (100mmol/l)

5mls $MgCl_2$ (100mmol/l)

83mls $DGVB^{=}$ (3 parts $D5W^{=}$: 1 part $GVB^{=}$)

pH 7.4-7.6. Final volume 100mls.

Preparation of Haemolytic Intermediates

Antibody sensitised sheep erythrocytes (EA)

Antibody to sheep erythrocytes (E) was prepared by the immunisation of rabbits with intact erythrocytes or their plasma membranes. The buffy coat was removed carefully from sheep E stored in Alsever's solution. The cells were washed three times in VBS and 1ml of a 10% suspension (V/V) of E in VBS was emulsified in an equal volume of Freund's complete adjuvant. The immunisation procedure and schedule was the same as that described for the preparation of antibody to bovine serum albumin (BSA) as described earlier in this section. As a result of this immunisation schedule the antiserum contained antibody which was predominantly IgG. The antiserum was heat-inactivated (56°C for 30 mins) prior to use.

Preparation of EA

Sufficient sheep E was withdrawn from the stock Alsever's solution and centrifuged at 2000g for 10mins at 2°C. After removal of the supernatant plasma and the buffy coat the cells were washed three times in 10 volumes of EDTA (10mmol/l) $GVB^{=}$ to give approximately a 5% suspension. 1ml of the suspension was added to 29mls of deionised water and the optical density (OD) of the lysate measured using a spectrophotometer at wavelength 541nm (OD_{541}). An OD_{541} of 0.385 corresponds to a sheep E concentration of $1X10^{9}$ cells/ml. The volume of the erythrocyte suspension should be adjusted to $1X10^{9}$ml as follows:

$$\text{Volume E required} = \text{Initial volume} \times \frac{\text{OD measured}}{0.385}$$

The sheep E in a glass conical flask was warmed to 37°C in a shaking water bath. Antibody to sheep E was diluted in an equal volume of EDTA (10mmol/l) $GVB^{=}$ and prewarmed to 37°C. The dilution of antiserum to be used in the preparation of EA is the minimum dilution which does not agglutinate an equal volume of sheep E at a concentration of $1X10^{9}$/ml. This titration is carried out on microtitre plates.

The antiserum was added to the sheep E suspension while being constantly shaken. The mixture was incubated for a further 30 mins in the shaking water bath at 37°C.

The EA were then centrifuged (2000g for 5 mins at 2°C) washed once in EDTA (10mmol/l) $GVB^{=}$, twice in GVB^{2+}, and finally resuspended to their original volume in GVB^{2+}. EA can be stored at 0°C for up to 1 week.

Preparation of rabbit erythrocytes (E^{rab})

Blood was collected from the marginal ear vein of the rabbit into an equal volume of Alsever's solution and stored at 0°C until needed. Rabbit E were separated from the plasma and buffy coat by centrifugation as described for sheep E above. The cells were washed three times in EDTA (10mmol/l) $GVB^{=}$ resuspended in the same buffer and incubated at 37°C for 15 mins. The cells were then washed three times in Mg-EGTA and stored in this buffer at 0°C for up to 2 weeks or until used.

Total Haemolytic Complement Activity (CH50)

The method used was a modification of that described by Kent & Fife (1963). EA when added to serum are lysed as a result of activation of the classical pathway and the formation and insertion of the cytolytic C5b-9 membrane attack complex into the cell membranes. A standard suspension of EA is used and the ability of serum to lyse the cells can be titrated.

Reagents

GVB^{2+}

EA ($5x10^{8}$/ml) in GVB^{2+}

Test serum

Method

1. Test sera were diluted 1 in 30 in ice-cold GVB^{2+} and then kept on ice.

2. A row of test tubes was set up and to these were added increasing volumes of diluted serum, starting with 50µl and increasing in 50µl increments to 450µl. The volumes were adjusted in each tube to 450µl by the

addition of GVB^{2+}. Two control tubes each contained 450µl. GVB^{2+} (cell blank and 100% lysis).

3. 300µl of EA was added to each tube.

4. The tubes were removed from the ice and transferred to a shaking water bath at 37°C for 1 hour.

5. 2mls of isotonic saline was added to all tubes except the 100% lysis control. To this tube 2ml of water was added.

6. The tubes were centrifuged at 1000g for 5 mins at 2°C.

7. The optical density of the supernatants at 541nm (OD_{541}) was read using a spectrophotometer.

Calculation of Results

The proportion of cells which were lysed (y) in each tube was calculated using the formula:

$$y = \frac{OD_{541}\ \text{test} - OD_{541}\ \text{cell blank}}{OD_{541}\ 100\% - OD_{541}\ \text{cell blank}}$$

The percentage of cells which were lysed in each tube is obtained by multiplying y by 100

Some serum samples can contain large amounts of haemoglobin and the colour of the serum influences the OD reading. To compensate for this a parallel series of serum dilutions should be made in EDTA (40 mmol/l) $GVB^{=}$. Lysis will not occur in these tubes and OD of their supernatants should be substracted from the readings of the corresponding tubes in which lysis has occurred.

A plot of y (or percent lysis) against the volume of serum in each tube produces a sigmoid curve (Fig 2.3(a)). The central part of the curve (between 20% and 80% lysis) is steep and the proportion of cells lysed is sensitive to small changes in the amount of complement in the serum. For this reason the end point of the titration is taken as 50% haemolysis.

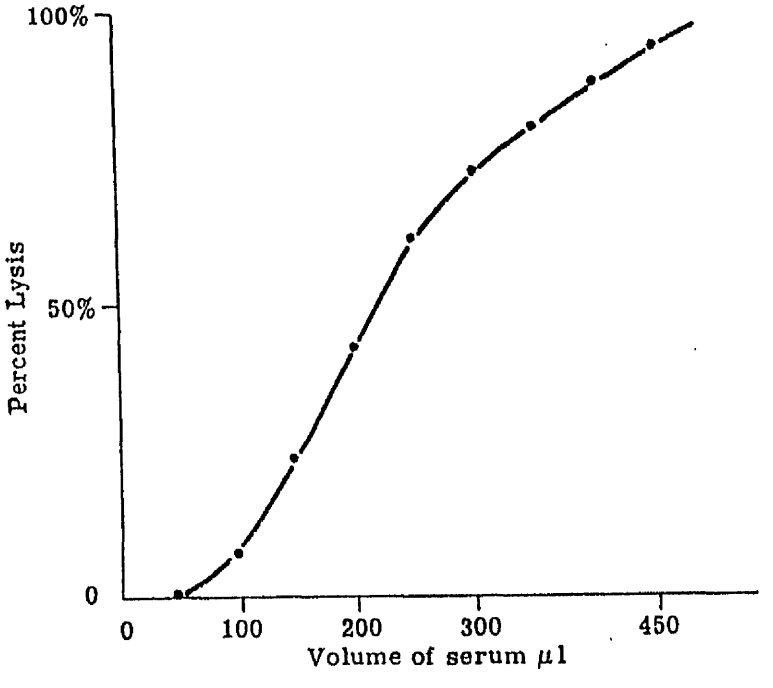
The sigmoid curve may be mathematically described using the von Krogh equation (von Krogh, 1916).

$$x = K\left(\frac{y}{1-y}\right)^{1/n}$$

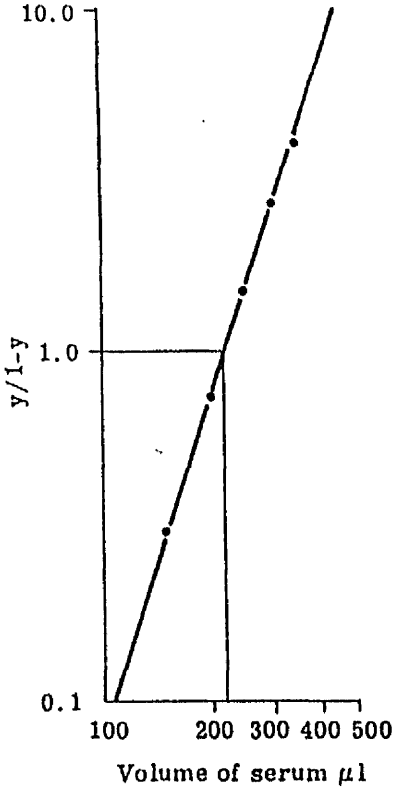
x = the amount of serum added to the tube (µl) and K is a constant which is the 50% unit of complement. At the point of 50% haemolysis 1-y=1, hence x = K

The value of K can therefore be obtained by plotting log x against log y/1-y (Fig. 2.3(b))

The number of CH50 units/ml = initial dilution x 100. The normal range established in the laboratory in which this work was performed was 150-250 units/ml.



(a)



(b)

Figure 2.3

CH50 assay (a) plot of percent lysis against volume of diluted serum. The shape of the curve is sigmoidal. (b) Log-log plot of y/1-y against volume of diluted serum. 50% lysis has occurred when y/1-y=1. By drawing a vertical line to the x axis this gave the volume of diluted serum which had produced this amount of lysis.

Alternative Pathway CH50 (APH50)

When rabbit erythrocytes are added to human serum lysis occurs because they activate the alternative pathway to generate the lytic C5b-9 complex. (Platts-Mills & Ishizaka, 1974).

Method

1. Test serum was diluted 1/25 in Mg-EGTA.

2. Increasing volumes of diluted test serum were added to a series of test tubes on ice (10µl, 20µl, 40µl, 60µl, 80µl, 100µl). The volume in each tube was adjusted to 100µl by adding Mg-EGTA.

3. 100µl from each tube was transferred to another series of test tubes. 100µl of buffer was added to each of two control tubes (cell blank and 100% lysis).

4. 100µl of E^{rab} was then added to each tube and the tubes were incubated in a shaking water bath at 37°C for one hour.

5. 2ml of isotonic saline was added to each tube except the 100% lysis tube. This tube received 2ml of water.

6. The tubes were centrifuged at 1000g for 5 mins at 2°C. The optical density of the supernatants was read at 414nm (OD_{414}).

7. The calculation of the results was similar to that described for the classical pathway CH50 assay.

The normal range established in the laboratory in which this work was performed was 185-327 units/ml

Complement Deficient Sera

Sera from patients who were genetically deficient for C2, C5, C7 and C1-inhibitor were obtained. These sera were stored in aliquots at -70°C until used.

EDTA-treated serum

Whole serum was diluted with EDTA (40mmol/l) $GVB^{=}$ pH 7.4. The final pH of the mixture was 7.4. Calcium and magnesium ions are chelated by this treatment and as such the serum is unable to form the classical or alternative pathway C3 or C5 convertases. The EDTA-treated serum was unable to lyse EA or rabbit erythrocytes.

EGTA-treated serum

Serum was diluted in Mg-EGTA (10mmol/l EGTA: 5mmol/l $MgC1_2$) pH 7.4-7.6 to give a final concentration of EGTA of 10mmol/l. The final concentration of Mg^{2+} ions was 5mmol/l. EGTA selectively chelates Ca^{2+} ions thus preventing C1 activation and therefore classical pathway activation. The presence of the Mg^{2+} ions permits activation of the alternative pathway as it is required for the formation of C3bBb. The sensitivity of the assays is enhanced by the low ionic strength of the solutions. Magnesium EGTA-treated serum failed to lyse EA.

Factor B-depleted serum (RB)

A glass conical flask containing fresh serum was incubated in a shaking water bath at 50°C for 20 mins. Immediately after this the flask was cooled by plunging it into iced water. When cooled the RB was divided into aliquots

and stored at -70°C. The factor B-depleted serum was titrated and shown to be able to lyse EA, in order to demonstrate that the classical pathway was intact. RB failed to lyse rabbit erythrocytes.

2.2.15 Measurement of C4 using C4- deficient guinea pig serum

This was based on the method described by Gaither, Alling & Frank (1974)

Reagents

1. $DGVB^{2+}$
2. C4-deficient guinea pig serum diluted 1/75 in $DGVB^{2+}$ C4-deficient guinea pig serum was prepared from the blood of guinea pigs which were genetically deficient in C4.
3. EA ($1x10^8$/ml) in $DGVB^{2+}$

Method

1. The test serum was diluted in $DGVB^{2+}$ (1/25,000, 1/50,000, 1/100,000, 1/200,000, 1/400,000, 1/800,000, 1/1600,000)

2. 100µl of each serum dilution was transferred to a series of tubes on ice.

3. 100µl of C4 deficient guinea pig serum was added to each tube followed by 100µl of EA. The varying additions of reagents to the control tubes is shown below. The tubes were incubated at 37°C for 1 hour.

Controls	$DGVB^{2+}$	C4 def. serum	EA
Cell blank	200µl	-	100 µl
Reagent blank	100 µl	100 µl	100 µl
100% lysis	100 µl	100 µl	100 µl

2ml saline was added to all tubes except the 100% lysis control tube to which 2ml of water was added. After centrifugation (1000g for 5 min at 2°C) the optical densities of the supernatants were read at 414nm (OD_{414})

Calculation of Results

$$y = \frac{\text{OD sample} - \text{OD reagent blank}}{\text{OD 100\%} - \text{OD reagent blank}}$$

% lysis = 100 x y

When y is plotted against the serum dilution the curve is concave to the x axis. Based on the shape of the curve Mayer and his colleagues (Borsos, Rapp & Mayer, 1961) postulated that complement-mediated lysis was due to a single effective hit by a complement molecule (one-hit theory) rather than to cumulative hits. It follows therefore, that if the total number of effective molecules offered per cell = z then the proportion of unlysed cells(e) (cells with nil hits) will be e^{z}.

$$\text{Thus } -z = \ln(1-y)$$

$$\text{or } z = -\ln(1-y)$$

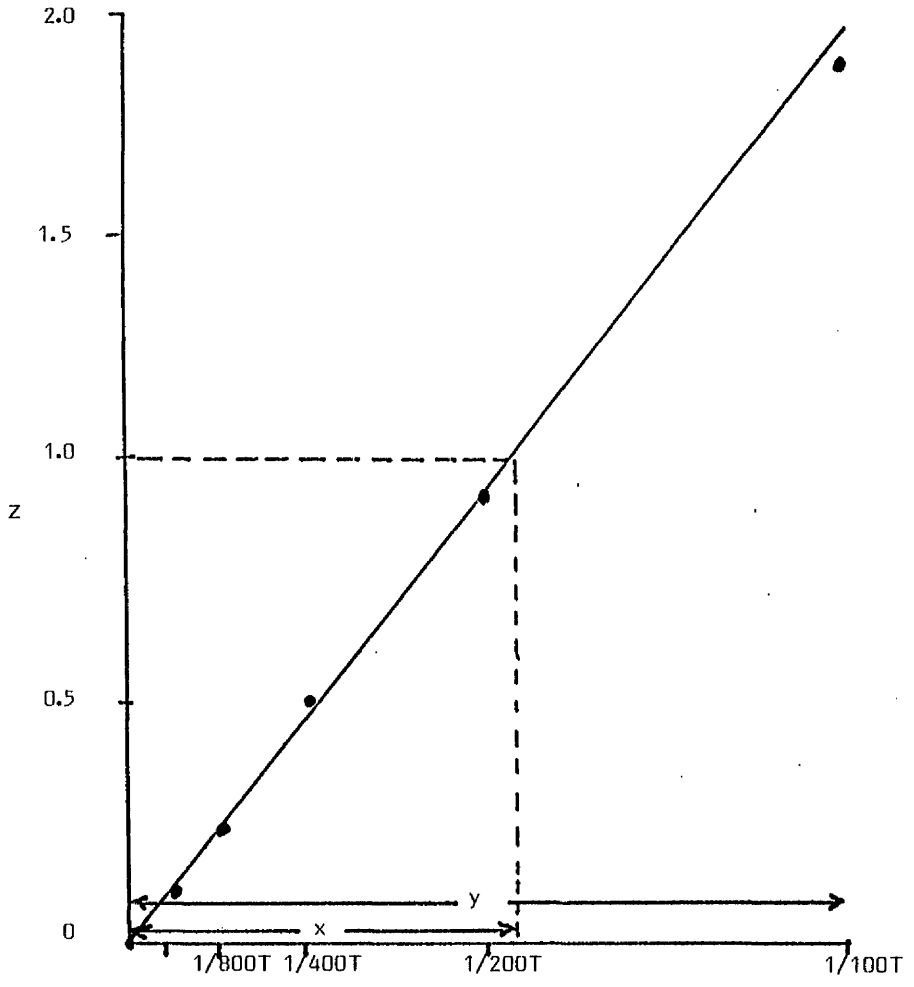


Figure 2.4

Plot of haemolytic titration for C4. Z was plotted against dilution of C4 used. Dilution of serum along x axis, T=thousand, therefore 1/100T=1/100,000 etc.
E.g. If initial dilution of serum was 1/100T, C4 $\text{conc}^{n} = \frac{y}{x} \times 100T$

When z is plotted against the concentration of the component offered, a straight line, passing through the origin is obtained (Fig 2.4). When z=1 then -1n (1-y) and y = 0.623. That is 62.3% of the cells have been lysed. The number of effective molecules of component in a given volume is found by drawing a vertical line from the point on the graph which passes through z=1, to the x axis.

The number of units of component were calculated thus:

$$\text{Initial dilution } x \quad \frac{\text{Distance along x axis to initial dilution}}{\text{Distance along the x axis to z=1}}$$

In all component assays 100µl of cell suspensions were used (EA 1×10^8/ml) ie 10^7 cells/tube and 100µl of component of each dilution.

The concentration of effective molecules = units x 10^7/ml. The results of haemolytic assays are usually expressed as units/ml.

2.2.16 C4 Consumption assay

Reagents

1. GVB^{2+}
2. BSA
3. Anti-BSA antiserum (1:2 dilution). This antiserum was raised in rabbits.
4. Serum or synovial fluid from patients with RA.

Method

A row of test tubes was set up in a rack, labelled and set on ice. 50µl of test-serum was mixed with an equal volume of GVB^{2+} and 5µl (1.75µg) of BSA was added. The mixture was warmed to 37°C in a water bath. Immune

complexes were formed at equivalence by the addition of 10µl of anti-BSA (diluted 1:2 in GVB^{2+}).

The tubes were incubated for one hour at 37°C, after which complement activation was stopped by the addition of 1ml ice-cold EDTA (10mmol/l) $GVB^{=}$. Residual C4 content was measured haemolytically. Controls included two serum containing tubes without immune complexes. One was incubated at 37°C and the other at 0°C. The total volume in each tube was kept constant by the addition of buffer.

2.2.17 The effect of RA serum and purified rheumatoid factor on the consumption of C4 by immune complexes

This assay was designed to examine the effect of the test material (fraction from chromatography columns, purified IgM-RF, heat-inactivated RA serum from which the purified RF had been prepared) on the ability of immune complexes to activate the complement system (measured as C4 consumption) in normal human serum.

The C4 consumption assay was modified in these experiments. Fifty microlitres of normal serum (undiluted), 50µl of test material (column fractions, purified RF or heat-inactivated (30 mins at 56°C) RA serum) BSA (0.5µg in 5µl GVB^{2+}) and anti-BSA(10µl 1:6 diluted in GVB^{2+}) were mixed at 0°C and then incubated at 37°C for one hour. The reaction was stopped by the addition of 1ml ice cold EDTA (10mmol/l) $GVB^{=}$. Residual C4 content was measured using C4-deficient guinea pig serum as described above.

The control for C4 consumption by complexes in the presence of RA serum was:- 50µl normal serum, 50µl heat-inactivated normal serum, BSA and anti-BSA. The control for the effect of purified RF consisted of 50µl normal

serum, 50µl GVB^{2+}, BSA and anti-BSA. Other controls included identical reaction mixtures to which 15µl GVB^{2+} had been added in place of BSA and anti-BSA.

2.3 STATISTICAL METHODS

The results were analysed using the statistical procedures listed below.

Chapter 3,Section 4

The comparison of inhibition of PIP in SF and serum was carried out using the Wilcoxon signed rank test. Although the sample size in this comparison (Fig 3.7) is fairly small, this non-parametric test is the most appropriate for analysis.

Chapter 3, Section 5

The comparison of C4 levels in serum samples from RA patients with normal PIP and subnormal PIP was carried out using the grouped t-test (Students t-test). This test was also used to compare the percentage C4 consumed in the sera of RA patients with normal and abnormal PIP and controls (see Table 3.2).

Grouped t-tests were used for comparing the means of different groups, since non-parametric tests have low power for small sample sizes.

Although the t-test is in fact comparatively robust against non-normality, the data were checked for approximate normality and equality of variance between the two groups compared.

To quote "....whereas tests on population means (ie. "Students" t-test for the mean of a normal population, and for the difference between the means of two normal populations with the same variance) are rather insensitive to departures from normality, tests on variances...." are very sensitive to such departures. Tests on means are robust; by comparison tests on variances can only be described as frail". (Kendal & Stuart, The Advanced Theory of Statistics, Vol 2, Ch 31, pp 483-484).

The correlation between C4 consumption and inhibitory activity and between C4 consumption and PIP was carried out using Spearman's rank correlation co-efficient (see Fig 3.8). This analysis was also used in comparing C4 consumption with inhibitory activity and PIP in synovial fluid.

The comparison between the mean C4 consumption in serum and SF was carried out using the grouped t-test (see Table 3.2).

Chapter 3, Section 7

The significance of the difference in consumption of C4 when complexes were formed in the presence of RA serum or IgM-RF was tested using the grouped t-test (see Table 3.3)

Chapter 3, Section 8

Statistical analyses were carried out using the University of Glasgow Computer Service. The software was the Statistical Package for the Social Sciences (McGraw-Hill).

Computer entries:

(1) Clinical indices

Diagnosis, age, sex, disease duration, morning stiffness duration, pain score. Joint pain, joint tenderness, synovial hypertrophy, cumulative index.

(2) Synovial fluid

Volume, protein, white cell count, CH50, C3, C4, Fac B, C1-inhibitor, PIP, inhibition of PIP, RF latex, IgM-RF, IgG-RF, IgA-RF.

(3) Blood

Haemoglobin, ESR, platelets

(4) Serum

CH50, C3, C4, FacB, PIP, inhibition of PIP, RF latex, IgM-RF, IgG-RF, IgA-RF.

STUDY A

PIP and inhibitory activity in sera and synovial fluids.

Correlations with clinicaland laboratory indices of disease activity.

All patients had blood and SF data available.

STUDY B

Study of PIP and inhibition of PIP in RA patients with and without extra-articular features of disease.

In this study measurements were carried out on blood only.

Statistical Methods

(a) Non-parametric tests were used for all analyses.

(b) Comparison of parameters between different groups was carried out using the Mann-Whitney U test, significance level < 0.05, two tailed.

(c) Comparison between different parameters was carried out using the Spearman Rank correlation co-efficient expressed as $r(\sqrt{r^2})$ with positive or negative direction. Significance level was taken as $\ll 0.05$, two tailed.

Because of the large numbers of tests used in the statistical analyses of results of this clinical study, the Bonferroni inequality demands that a correction be applied to the significance values obtained. (Snedecor & Cochran, 1980). 'True' significance therefore can be found by correcting the P value according to the formula $0.05/_{n}$ where n = the number of tests applied. For example, if 10 correlations have been used P would have to equal 5/10% or p = 0.005 for significance to be reached. (For further discussion of the statistical significance of the results obtained in this clinical study see Chapter 4, Section 7).

CHAPTER THREE

RESULTS

The chapter is arranged in nine sections and at the end of each section a small paragraph is used to summarise the experimental data.

SECTION 1

The establishment of an assay to measure complement-mediated prevention of immune precipitation (PIP) in human serum.

The assay to measure PIP in serum is described in Materials and Methods, section (2.2.1(a)).

Kinetics and complement requirements for the prevention of immune precipitation

In the absence of serum approximately 95% of the complexes had precipitated by the time the first sample was taken at 15 minutes (Fig 3.1(a)). In the presence of normal human serum (50µl undiluted) over 80% of the complexes remained in solution even after 1 hour (Fig 3.1(a)). Treatment of serum with EDTA completely abrogated its ability to keep complexes in solution (Fig 3.1(a)). In C2 deficient serum and C1-inhibitor deficient serum, most of the formed complexes precipitated (Fig 3.1(a)). Likewise the importance of the alternative pathway was illustrated by partial precipitation in the Mg-EGTA treated serum and in B depleted serum (Fig 3.1(a)). The lack of requirement of the terminal components was demonstrated by the failure of complexes to precipitate in C5 deficient and C7 deficient sera (Fig 3.1(a)).

Formation of complexes in normal serum

An experiment was carried out to establish the optimum amount of serum which was used in the assay to measure PIP. The optimum amount of serum would be that quantity which would hold the maximum amount of immune complexes soluble. This dilution of serum was established by titration.

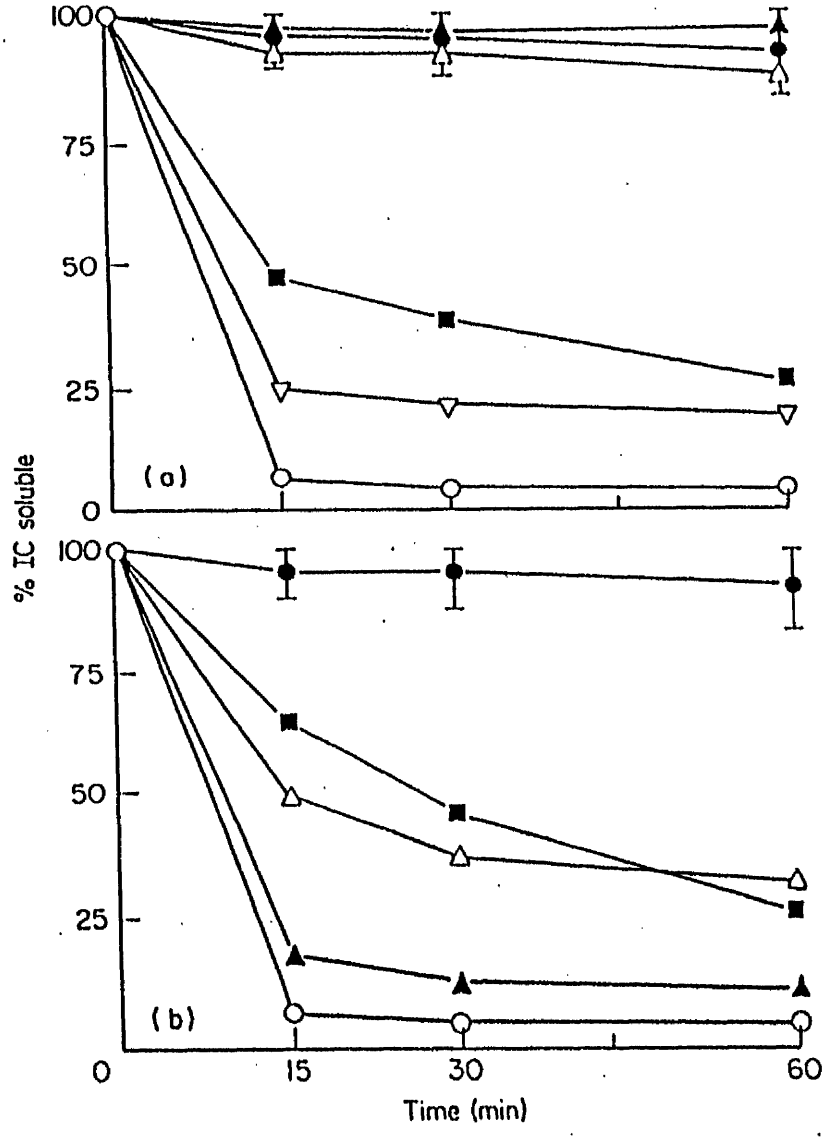


Figure 3.1(a)

Kinetics of immune precipitation in serum (●——●) and in buffer (0——0). The vertical bars show the range (±2 s.d. of the mean) at each time point for 25 serum samples from normal individuals.

(a) shows the ability of C2 (■——■), C5 (△——△), C7 (▲——▲), and C1-inhibitor (▽——▽) deficient sera to prevent immune precipitation.

(b) shows the ability of B depleted serum (■——■), EDTA treated serum (▲——▲) and Mg-EGTA treated serum (△——△) to prevent immune precipitation.

A row of plastic Eppendorf tubes was set up. To the first tube was added 200µl of normal human serum. Doubling dilutions were made of this serum into GVB^{2+} (100µl) which had been added to subsequent tubes. The dilutions of serum were therefore neat, 1/2 to 1/16. Immune complexes were formed in each tube by the addition of 5µl ^{125}I-BSA (350 µg/ml) and subsequently 10µl of anti-BSA antiserum (diluted 1/2). Complexes were thus formed at equivalence in each dilution of normal serum.

Control tubes were (1) TCA precipitable counts (this tube contained 50µl BSA (5mg/ml) and 60µl of TCA (20%) and 5µl of ^{125}I-BSA (2) total radioactivity, this tube contained 110µl GVB^{2+} and 5µl ^{125}I-BSA.

After mixing, all the tubes were incubated for 1 hour at 37°C. A 50µl aliquot of the mixture was taken and transferred to an Eppendorf tube containing 1ml of ice-cold PBS. The tubes were centrifuged (Beckman Microfuge) for 5 minutes and 250µl of supernatant was taken for counting in a γ counter.

The cpm in the supernatant divided by cpm in the supernatant of the total radioactivity control tube, multiplied by 100, gave the % immune complexes soluble, or %PIP. This was plotted on a graph against dilutions of normal serum in the assay tube (Fig 3.1(b)).

The dilution of serum was associated with a rapid decline in its ability to prevent immune precipitation. As a result of this observation it was decided to use 50µl undiluted serum (final dilution approximately 1/2 in reaction mixture) in routine assays.

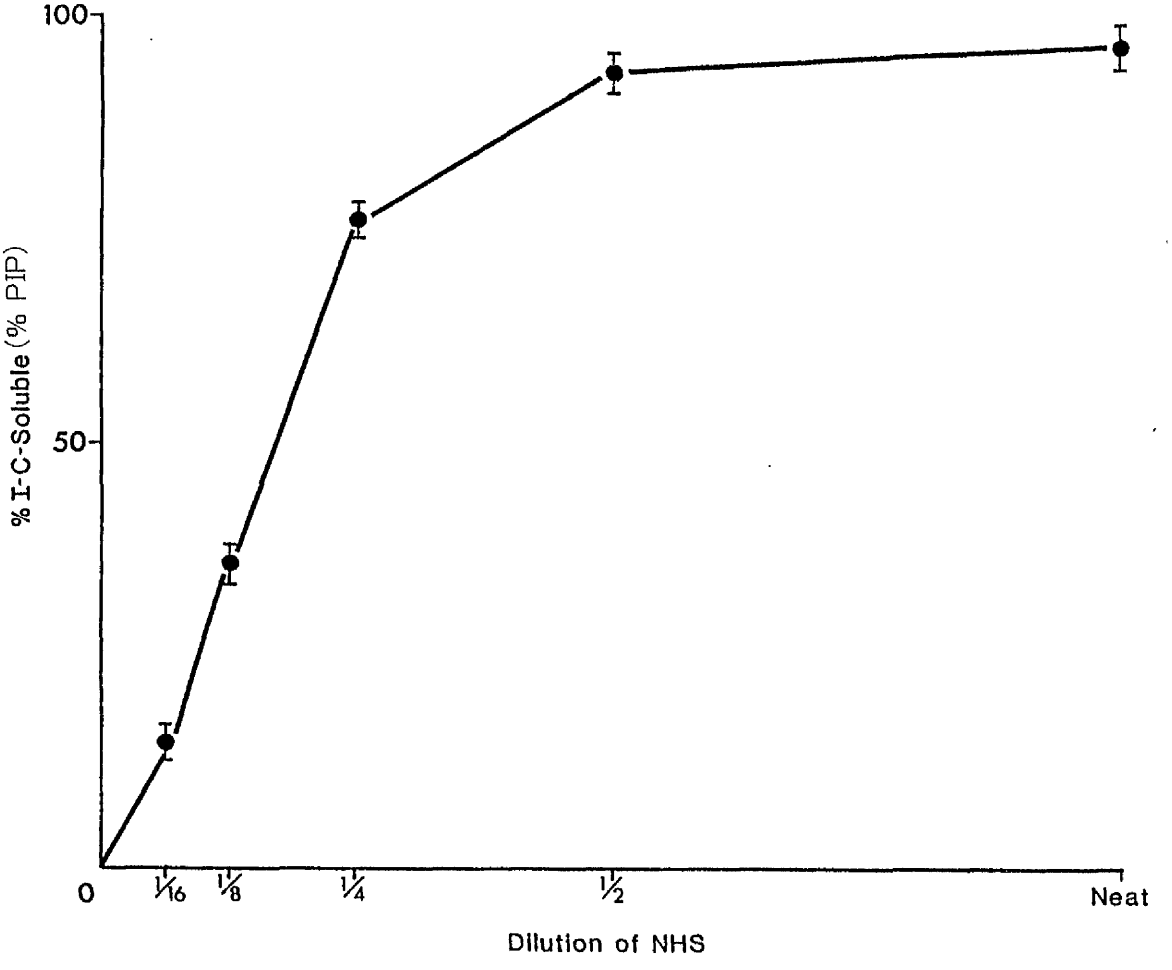


Figure 3.1(b)
Graph showing percent of immune complexes remaining soluble (% PIP) in various dilutions of normal human serum (NHS).

In 25 normal sera the mean percent PIP at 15, 30 and 60 minutes was 95.9 (±s.d. 3.44), 95.6 (±3.89) and 92.1 (±4.55) respectively. By subtracting 2 standard deviations from the mean the lower limit of the normal ranges (% PIP) in my system at the three time intervals studied was 89.0%, 87.8% and 83.0% respectively (Fig 3.2). Thus, in my routine assay, in which a single incubation time of 1 hour was used, and when % PIP was less than 83% it was reduced.

In the normal sera studied the distribution of % PIP of different sera was symmetrical about the mean at each of the times (15, 30 and 60 minutes) and the standard deviations reflected the small scatter about the means.

For routine purposes assays on individual samples were repeated if the results performed on duplicate determinations differed by 10% or greater.

The reproducibility of the assay was tested by repeating the assay on six serum samples on two separate days. The variation between assays on individual sera was between 1% and 10% the mean being 6%

Storage of serum samples at 4°C for 24 hours, -20°C for 3 days and repeated freezing and thawing of samples (up to five cycles) did not result in a significant loss of serum PIP activity.

SUMMARY

An assay to measure PIP was established and its dependency upon the complement system confirmed. Its reproducibility was acceptable and was therefore able to be used on a routine basis. The first study was to measure PIP in the sera of patients suffering from a variety of diseases. In some of these conditions humoral immune mechanisms are considered to play an

important pathogenetic role (SLE, GN, RA).

SECTION 2

Measurement of prevention of immune precipitation in patients' sera

The clinical details of the patient groups in this study are described in Materials and Methods (2.1.2). Blood was collected and serum prepared as described (2.1.1). C3, C4, Factor B, total haemolytic complement and alternative pathway haemolytic activity were measured as described in section (2.2.14(a)(b)).

Rheumatoid arthritis and other arthropathies

Thirty-six of 75 sera (48%) from patients with seropositive RA showed reduced PIP whereas sera from patients with seronegative RA, ankylosing spondylitis, psoriatic arthritis and degenerative joint disease showed normal PIP (Fig 3.2). Eleven of the RA sera showing a reduced PIP were hypocomplementaemic as judged by the APH50. Of these 11, 5 had a reduced CH50 level, one of whom had a low C4 level. The haemolytic complement levels in the remaining sera which showed reduced PIP were either normal or elevated (Fig 3.2).

The relationship between the capacity of seropositive RA sera to prevent precipitation of complexes and the CH50 and APH50 levels is shown in Fig 3.3. Although there was a tendency for the lowest levels of PIP to be associated with reduced CH50 and APH 50 there were many patients who had low PIP and normal or elevated CH50 and APH50.

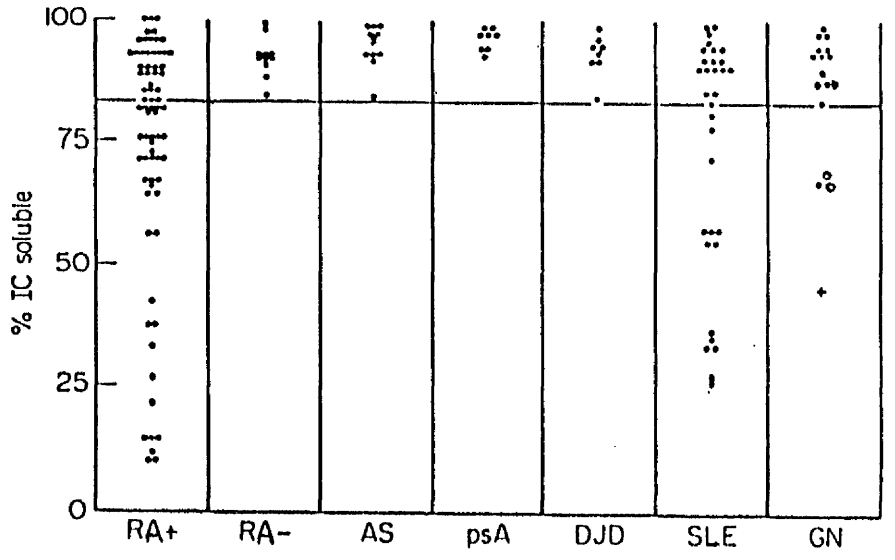


Figure 3.2
Prevention of immune precipitation by sera from patients.
The horizontal line shows the lower limit of the normal range (83%)

RA+ = seropositive RA.
RA- = seronegative RA.
AS = ankylosing spondylitis.
psA = psoriatic arthritis.
DJD = degenerative joint disease.
SLE = systemic lupus erythematosus.
GN = glomerulonephritis.
(o = focal GN; ● = membranous GN; + = acute post streptococcal GN).

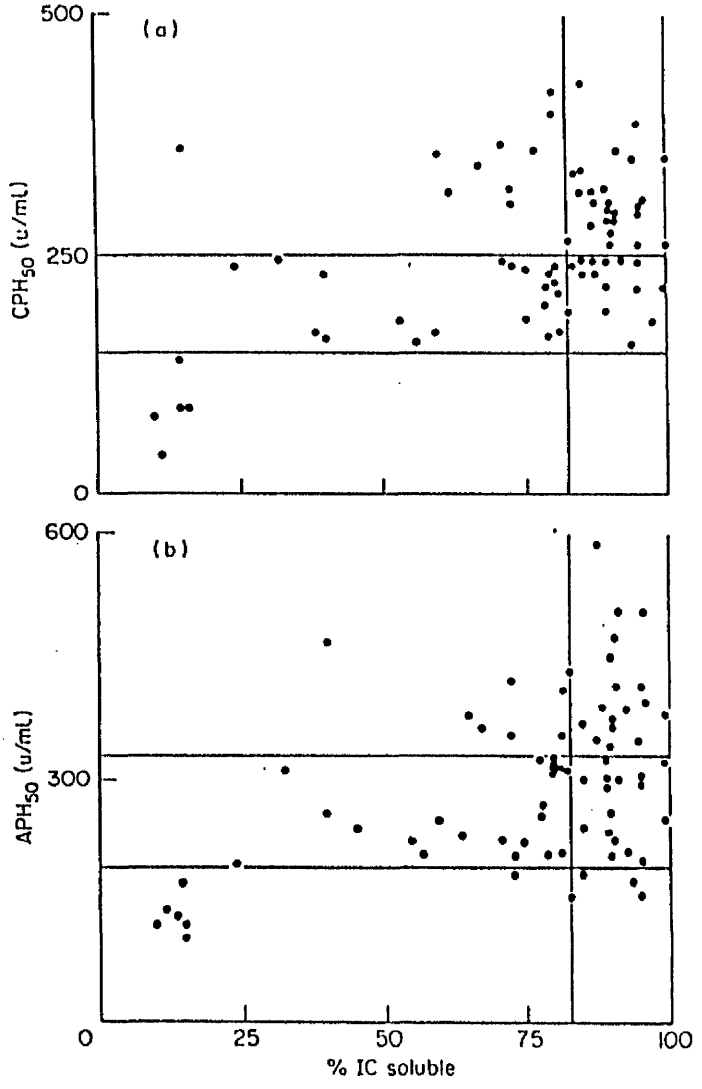


Figure 3.3

Relationship between % of complexes remaining soluble and (a) classical pathway (CPH50) and (b) alternative pathway (APH50) haemolytic activity in sera from patients with seropositive rheumatoid arthritis. The horizontal and vertical lines show the limits of the normal ranges. (%IC soluble 83%-100%; CPH50 150-250 units/ml; APH50 185-327 units/ml).

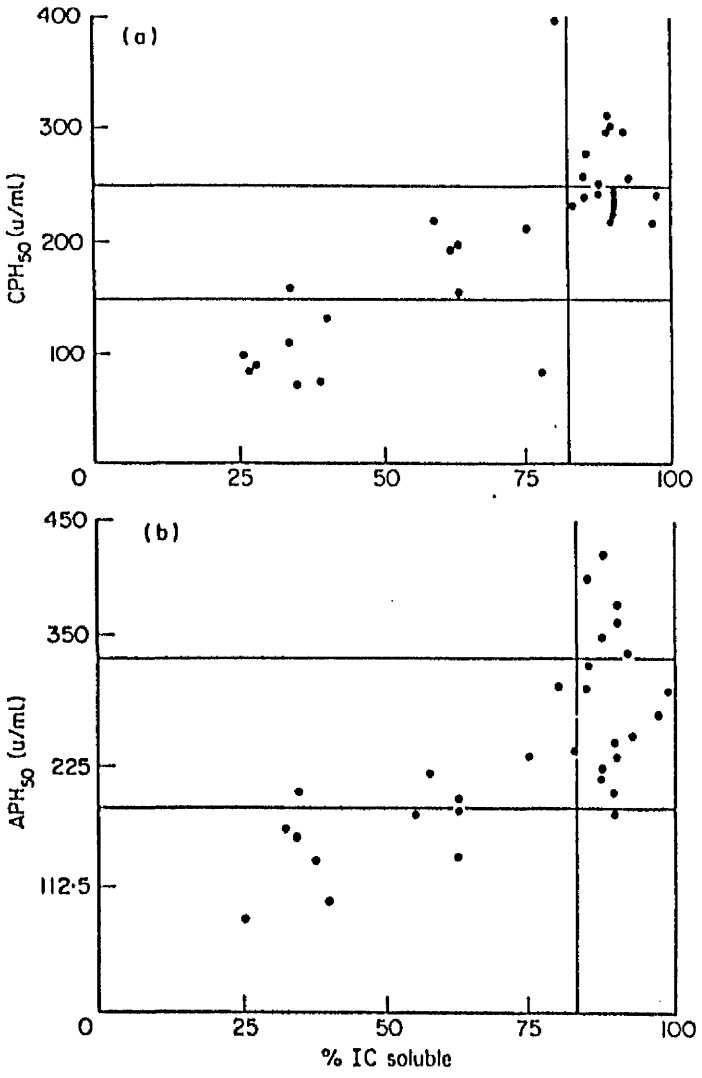


Figure 3.4

Relationship between % of complexes remaining soluble and (a) classical pathway (CPH50) and (b) alternative pathway (APH50) haemolytic activity in sera from patients with SLE. The horizontal and vertical lines show the limits of the normal ranges. (%I.C. soluble 83%-100%; CPH50 150-250 units/ml; APH50 185-327 units/ml).

Systemic lupus erythematosus(SLE)

Fourteen of 32 (43.4%) SLE sera had reduced PIP (Fig 3.4). Of these 14 sera, 8 had subnormal CH50 levels and 8 had reduced APH50 levels. Low levels of PIP tended to be associated with reduced CH50 and APH50 but again there were several patients who displayed low levels of PIP but had normal CH50 and APH50 (Fig 3.4)

Glomerulonephritis (GN)

Four of 17 sera (23.5%) had reduced PIP (Fig 3.2). One sample had been taken from a patient with acute post-streptococcal GN who was severely hypocomplementaemic. None of the remaining three samples were from hypocomplementaemic patients; 2 patients had focal GN and one had membranous GN.

SUMMARY

The most striking finding from this study was the impairment of PIP found in 48% of patients with sero-positive RA. This proved even more interesting when it was noted that this impairment of PIP in RA could not be attributed to hypocomplementaemia; indeed the vast majority of these RA sera had normal or often elevated serum complement levels. This suggested that the reduced PIP in RA patients' sera was due to the presence of a serum inhibitor of PIP.

SECTION 3

The establishment of an assay to measure the inhibition of the prevention of immune complex precipitation (inhibitory activity) in serum

The assay used to measure the inhibition of PIP was as described in Materials and Methods (2.2.1(b)).

Demonstration of inhibitory activity in RA serum

A series of Eppendorf tubes was set up in a rack and placed on ice. To each tube 50µl of neat normal human serum was added. Another row of tubes was set up and to these 100µl of RA serum, with known markedly impaired PIP, was added to tube one, 100µl of GVB^{2+} was added to the other tubes. Doubling dilutions were carried out in these tubes, the RA serum being undiluted or doubly diluted to 1:32 in GVB^{2+} (pH 7.4). 50µl of each dilution was added to the tubes containing 50µl of undiluted normal serum. Immune complexes were formed by adding ^{125}I-BSA 5µl (350µg/ml) and then 10µl of anti-BSA (diluted 1:2) to each tube.

Control tubes included:

(1) Total radioactivity input, 5µl ^{125}I-BSA and 100µl of GVB^{2+}

(2) TCA precipitable counts, 50µl of BSA (5mg/ml), 60 µl of 20% TCA and 5µl ^{125}I-BSA

(3) Radioactivity precipitated by antibody alone 100µl GVB^{2+}, 5µl ^{125}I-BSA and 10µl anti-BSA

(4) Maximum possible PIP by serum alone, 50µl normal serum, 50µl GVB^{2+}, ^{125}I-BSA 5µl and 10µl anti-BSA.

After mixing and incubating for 1 hour at 37°C, a 50µl aliquot was removed from each tube and added to 1ml ice cold PBS (pH 7.4) in another Eppendorf tube. Following centrifugation in a microfuge a 250µl aliquot was taken for counting in a γ counter. The per cent immune complexes which remained soluble (% PIP) was calculated thus:

$$\% \text{ PIP} = \frac{\text{cpm in serum supernatant - cpm in buffer supernatant}}{\text{Total cpm/tube - cpm in buffer supernatant}} \times 100$$

The control tube containing normal serum alone plus buffer allowed for 93% PIP. Addition of increasing amounts of the RA serum resulted in a dose dependent decrease in % PIP. The % inhibition of PIP was expressed as 100-n where n=% PIP. It can be seen that the inhibitory effect of RA serum on this process becomes less marked as the RA serum is further diluted (Fig 3.5).

The effect of heat-inactivation of RA serum on the inhibition of PIP

3mls of RA serum which displayed reduced PIP was heat inactivated at 56°C for 30 minutes. A row of test tubes was set up and 100µl of the heat inactivated (HI) RA serum was added to the first tube. 100µl of GVB^{2+} was added to the remaining tubes. Doubling dilutions of (HI) RA serum were made ranging from undiluted to 1:32. The experiment was carried out exactly as described above (demonstration of inhibitory activity in RA serum). The only difference was that the RA serum in this experiment was heat-inactivated (56°C for 30 minutes). The control tubes were similar and the results calculated in the same manner. % inhibition = 100-n where n = % PIP.

It can be seen that the degree of inhibition of PIP became more pronounced following heat-inactivation of the RA serum (see Fig 3.5).

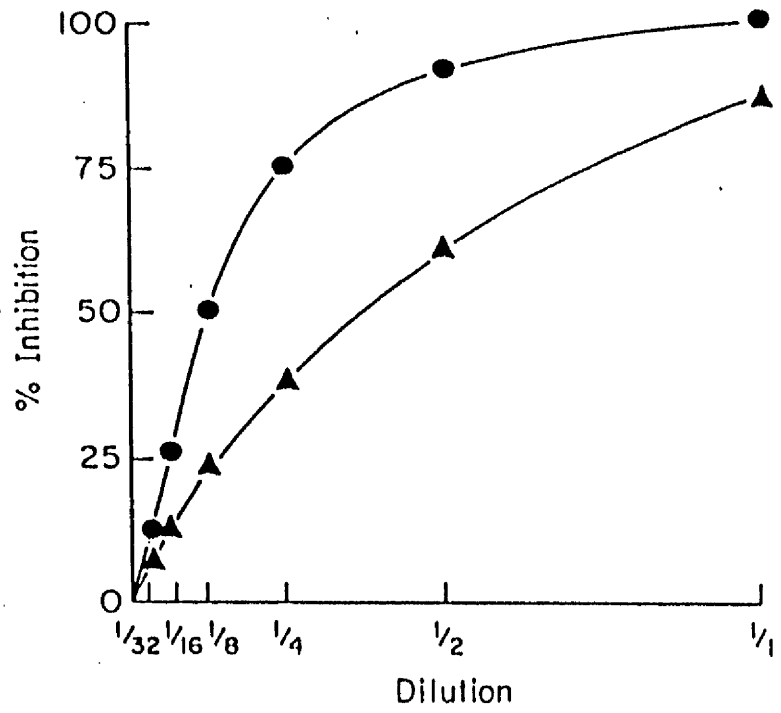


Figure 3.5

Effect of RA serum on prevention of immune precipitation by normal serum. Dose-response curves for RA serum prior to (▲——▲) and after (●——●) heat-inactivation. % Inhibition on the y axis refers to the degree of inhibitory activity.

Measurement of inhibition of PIP in sera

Because heat-inactivation increased the amount of inhibition of PIP observed in the experiment performed above (Fig 3.5) it was decided to measure inhibitory activity in 40 sera (10 normal, 10 seropositive RA, 10 seronegative RA, 5 ankylosing spondylitis and 5 psoriatic arthritis) before and after heat-inactivation. The results are summarised in Table 3.1. Following heat inactivation a small degree of inhibitory activity ($< 5\%$) was observed in normal sera. On the basis of this finding it was decided that inhibitory activity was present if it was present in excess of 5%. The proportion of sera which inhibited PIP increased dramatically following heat-inactivation (Table 3.1). Thus for all further studies I decided to use heat-inactivated sera exclusively.

All assays were performed in duplicate. Assays were repeated if the results differed by more than ±5% of the mean of the duplicate determinations.

The assay was carried out on 10 serum samples on two separate days. The variation between assays on individual sera was ±5% mean 7%.

SUMMARY

A reproducable assay to measure inhibition of PIP was established. This assay was used to measure inhibition of PIP in patients' sera.

Table 3.1

Effect of heat-inactivation of serum on measurement of inhibitory activity.

Group	Number in group	% Inhibition of PIP *	
		Fresh Serum	Heat Inactivated
Control	10	0	1.3 ± 0.57
RA seropositive	10	*19.9 ± 8.1	59.2 ± 8.1
RA seronegative	10	0	3.7 ± 2.1
Ankylosing spondylitis	5	2.3 ± 1.2	13.6 ± 1.2
Psoriatic arthritis	5	0	0

* % inhibition of PIP recorded as mean ± standard error of mean

SECTION 4

Measurement of inhibition of PIP (inhibitory activity) in sera of patients suffering from rheumatic disease.

Inhibition of PIP was measured as described in Materials and Methods (2.2.1(b)). Clinical aspects of patient groups were as described in Materials and Methods (2.1.3).

Prevalence of inhibitory activity in different diseases

Inhibitory activity did not exceed 5% in 25 sera from normal individuals. Ninety-two of the 130 sera (71%) from patients with seropositive RA were inhibitory, the mean level being 33.2% (± standard error of mean 2.7%). In contrast only 6 of 25 (24%) from patients with seronegative RA were inhibitory, but the level of inhibitory activity was low (mean 13.3 ± 1.0%). Six of 7 sera (85.7%) from patients with ankylosing spondylitis were inhibitory (mean 13.1 ± 2.1%) whereas none of 5 sera from patients with psoriatic arthritis were inhibitory (Fig 3.6).

Inhibition of PIP in synovial fluid

Synovial fluid was obtained from 13 patients and prepared for assay as described in chapter (2.1.1). The method of measuring inhibition of PIP in SF was identical to that described for serum samples above. SF was used instead of test serum (eg RA serum). As with patients' serum samples the SF was heat-inactivated at 56°C for 30 minutes prior to being tested in the assay.

Synovial fluid inhibited PIP. The mean level of inhibitory activity in synovial fluid (58 ± 6.8%) did not differ significantly from that in paired serum samples (53 ± 11.2%) Fig 3.7. (Wilcoxon signed rank test).

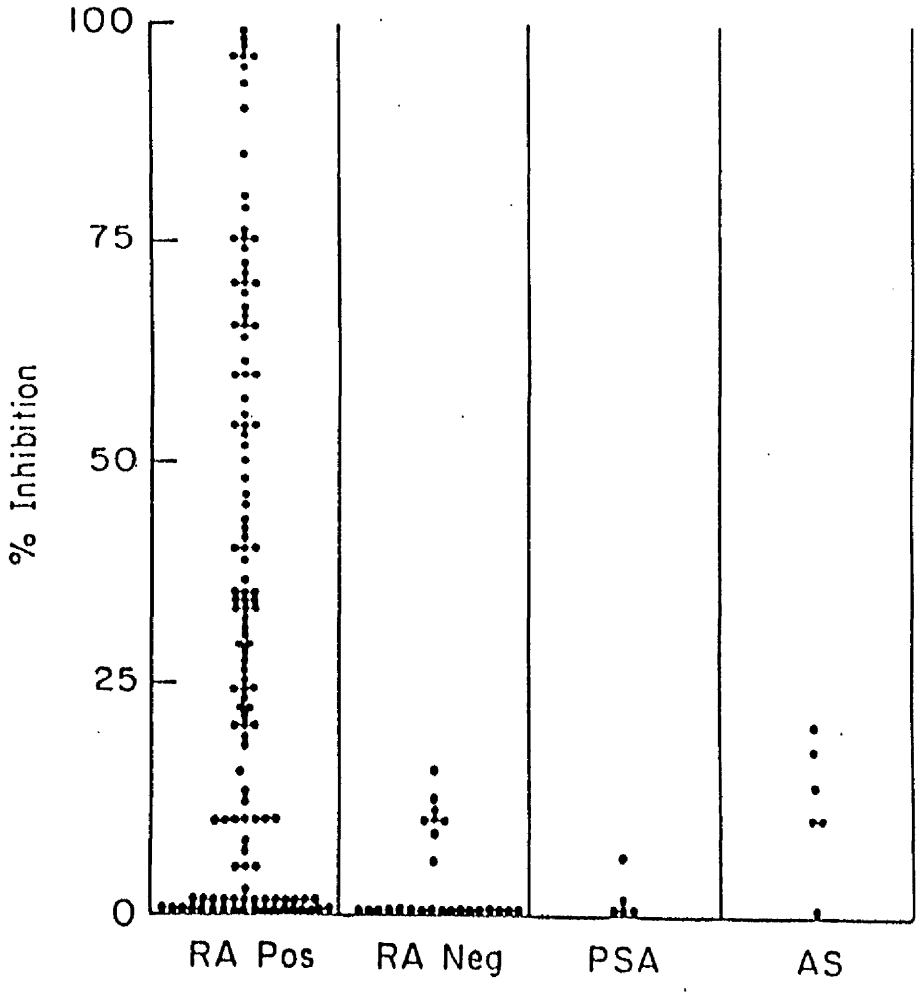


Figure 3.6

Level of inhibitory activity (% Inhibition) in heat-inactivated sera from patients with seropositive RA (RA Pos), seronegative RA (RA Neg) psoriatic arthritis (PSA) and ankylosing spondylitis (AS).

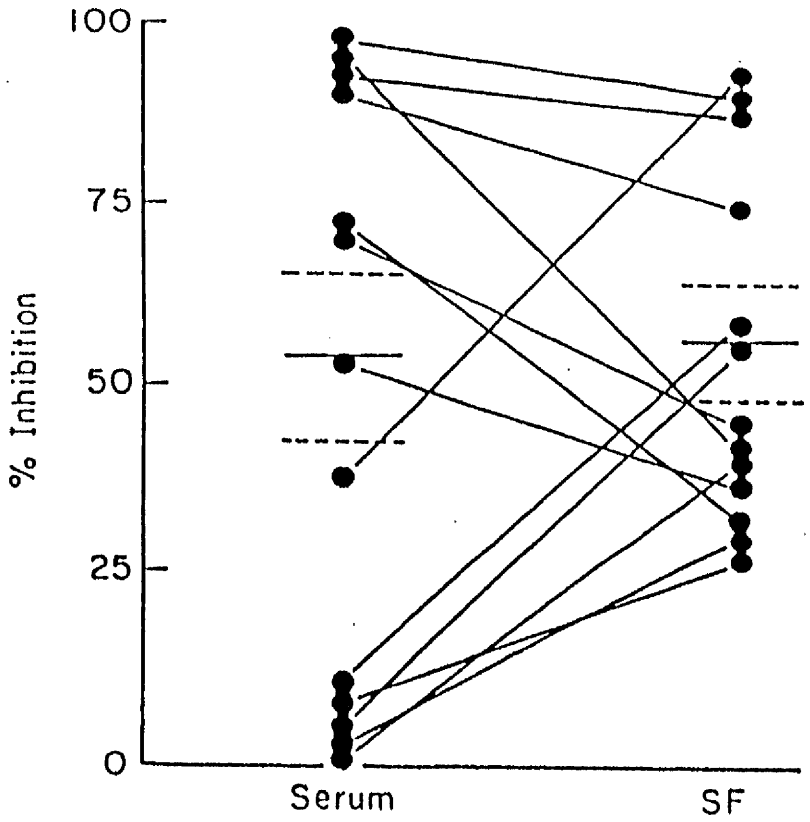


Figure 3.7

Inhibitory activity in paired serum and synovial fluid (SF) samples. The horizontal bars represent the mean (———) and the standard error of the mean (------).

SUMMARY

Inhibitory activity has been demonstrated in RA serum and to a lesser extent in sera from patients with seronegative arthritis. It was therefore decided to undertake studies which would help to clarify its mode of action as these might shed light on the nature of the inhibitory molecule in whole serum and perhaps indicate a method for the isolation of the inhibitory activity from RA serum.

SECTION 5

Studies on the mechanism of action of an inhibitor of PIP in serum and synovial fluid (SF)

C4 and C4 consumption were measured in serum and SF as described in Materials and Methods (2.2.15 and 2.2.16).

C4 levels and C4 consumption in serum and synovial fluid

C4 levels were measured in sera and synovial fluids before and after the addition of antigen-antibody complexes (Table 3.2). The C4 levels in serum samples from RA patients with normal PIP (mean 317, 309 units/ml ± SE 18,152), were significantly higher than the levels in those with subnormal PIP (212,566 ± 15,572; $P < 0.001$). (Grouped t-test).

Following the formation of antigen-antibody complexes in the serum C4 consumption occurred. The percentage of C4 consumed in the sera from RA patients with abnormal PIP (39 ± 5.0%) was significantly lower ($P < 0.001$) than in the sera from patients with normal PIP (68 ± 2.3%) and control sera (64 ± 3.1%). (Grouped t-test).

There was an inverse correlation between C4 consumption and inhibitory activity and a direct correlation between C4 consumption and PIP in RA sera (Fig 3.8). (Spearman's Rank correlation). ($P < 0.01$. in both cases).

C4 consumption in synovial fluid (39 ± 4.0%) was lower than that in normal serum (64 ± 3.1%; $P < 0.001$) (Grouped t-test) and was almost identical with C4 consumption in RA serum with reduced PIP (Table 3.2).

Table 3.2

C4 levels and C4 consumption in serum and synovial fluid

Group	No	PIP (%)	Inhibition of PIP %	C4 (units/ml)			C4 Consumption*
				Pre-incubation	Post-incubation	Consumption	(%)
Control	6	89 ± 2.8	0	307,200 ± 29,123	110,400 ± 15,768	196,800 ± 17,779	64 ± 3.1
RA Normal †	8	90 ± 1.3	2.5 ± 1.6	317,309 ± 18,152	99,176 ± 4,302	218,133 ± 17,449	68 ± 2.3
RA subnormal§	19	55 ± 3.8	61.3 ± 5.0	212,566 ± 15,572	129,016 ± 14,869	83,550 ± 12,706	39 ± 5.0
Synovial Fluid	12	13.2 ± 3.1	51 ± 5.8	38,276 ± 14,168	26,603 ± 11,534	11,664 ± 4,678	39 ± 4.0

* C4 level measured after 1 hour incubation with antigen-antibody complex

All figures represent the mean ± standard error of the mean

† RA with normal PIP

§ RA with subnormal PIP

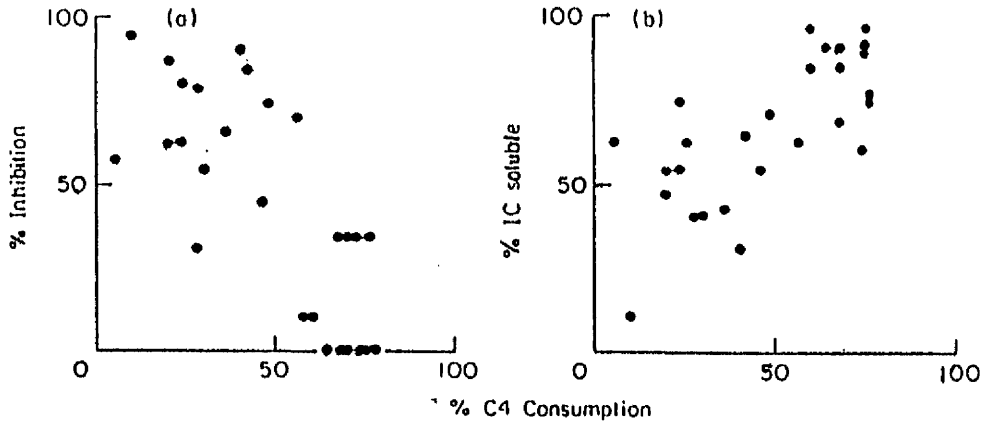


Figure 3.8

Correlation between (a) C4 consumption and serum inhibitory activity (% Inhibition) $r = -0.72$; $P < 0.01$
(b) C4 consumption and PIP (% IC soluble) $r = 0.67$; $P < 0.01$
(Spearman's rank correlation)

In order to exclude the possibility that the proportion of C4 consumed was related to the concentration of C4 available at the onset of the experiment, C4 consumption was measured in serum samples (undiluted and diluted 1/2, 1/4, 1/8) from normal controls and RA patients. The proportion of C4 consumed in the diluted samples was similar to that in the undiluted samples in normal sera and inhibitory and non-inhibitory sera from patients with RA.

Kinetics of inhibition of PIP (see Materials and Methods (2.2.6).

When RA serum or synovial fluid was added to normal serum prior to the addition of antigen and antibody (times 0) the inhibitory effect was maximal. When added after antigen and antibody the inhibitory effect was less marked. The magnitude of the effect diminished as the time after the addition of antigen and antibody increased (Fig 3.9)

Sensitivity of inhibitory activity to trypsin, SBTI and PMSF

(See Materials and Methods 2.2.4 and 2.2.5)

Incubation with trypsin destroyed over 90% of the inhibitory activity. Trypsin + SBTI and SBTI alone had no effect. Exposure to PMSF did not reduce the inhibitory activity.

SUMMARY

C4 levels and C4 consumption was measured in serum and SF and the relationship to inhibitory activity noted. The inhibitor of PIP was sensitive to trypsin but not to PMSF. Kinetic studies showed that inhibition of PIP was maximal when RA serum or SF was added prior to immune complex formation.

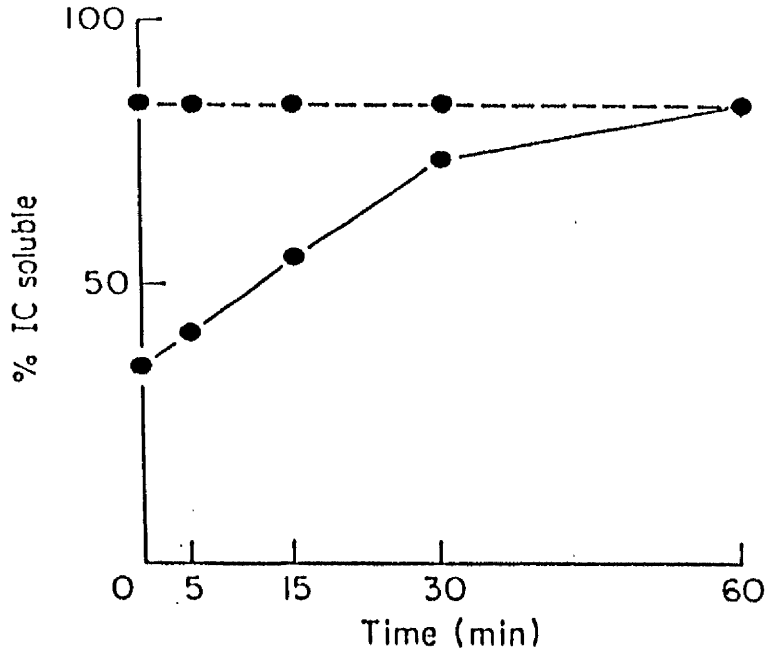


Figure 3.9

Kinetics of inhibitory activity of RA serum. Inhibitory RA serum (unbroken line) was added to normal serum at time 0, or 5, 15, 30 and 60 minutes after the addition of antigen and antibody. The inhibitory effect was maximal at time 0. Synovial fluid gave a similar result.

Normal control without addition of RA serum (broken line).

SECTION 6

The isolation and purification of inhibitory activity from RA serum.

The methods used in this section are described in Materials and Methods (2.2.2)

IgG Sepharose chromatography of RA serum

Reagents

Veronal buffered saline (VBS pH 7.5, 0.15M)

EDTA (20 mmol/l, pH 7.5)

Dextrose solution 5% W/V

Veronal buffered saline containing 400 mmol/l NaCl pH 7.5

Veronal buffered saline containing guanidine hydrochloride 2 mol/l pH 7.5

IgG Sepharose, prepared as previously described (see 2.2.10).

RA serum. This had been tested previously to confirm that it was able to inhibit PIP.

Method

This procedure was carried out in a walkin 4°C cold room. RA serum (15mls) was diluted in an equal volume of the EDTA (20 mmol/l pH 7.5) and applied to a column (1cm x 40cm) of IgG Sepharose. This IgG Sepharose column had previously been equilibrated with VBS diluted 1:1 with dextrose solution (equilibrating buffer).

The starting material was slowly passed through the column (rate 5ml/hour) using a pump. Following application of the sample the column was washed extensively with equilibrating buffer (5 column volumes) until no further protein was detected in the eluate. 3ml fractions were collected using an automatic fraction collector.

The bound material was eluted in two stages. Firstly with VBS containing 400 mmol/l NaCl (3 column volumes) and secondly with VBS containing guanidine hydrochloride (2 mol/l) (3 column volumes). Alternate fractions were screened for protein concentration (OD_{280}), inhibitory activity (% inhibition of PIP) and rheumatoid factor by Rose-Waaler titration and radioimmunoassay (Fig 3.13a). Prior to screening, each fraction including the RA serum starting material was dialysed extensively against VBS pH 7.5 0.15M at 4°C. 50µl samples of each fraction were taken and measured for inhibition of PIP (inhibitory activity) using the assay previously described.

Three protein pools were made, pool (a) represented the exclusion peak, pool (b) comprised the protein peak eluted with 0.4M NaCl and pool (c) the protein peak eluted with 2M guanidine hydrochloride (see Fig 3.10). These protein pools were further dialysed against VBS 0.15M pH 7.5.

The pools were tested for inhibitory activity and it was found that pool (a) was non-inhibitory, pool (b) was slightly inhibitory and pool (c) which contained rheumatoid factor activity, was extremely inhibitory. (Fig 3.11). Therefore inhibitory activity bound to IgG Sepharose and the majority of it was eluted with guanidine hydrochloride.

Mixing Experiments

This experiment was carried out to see if the inhibitory activity of the starting material could be reconstituted by mixing together pools (a), (b) or (c) in various combinations. The volumes of the various pools which were tested for inhibitory activity (in the assay to measure inhibition of PIP) and the various combinations used are detailed in the legend to Figure 3.12. The inhibitory activity of the starting material could be reconstituted fully by mixing the exclusion peak pool (a) with pool (c) or pools (b) and (c), but not

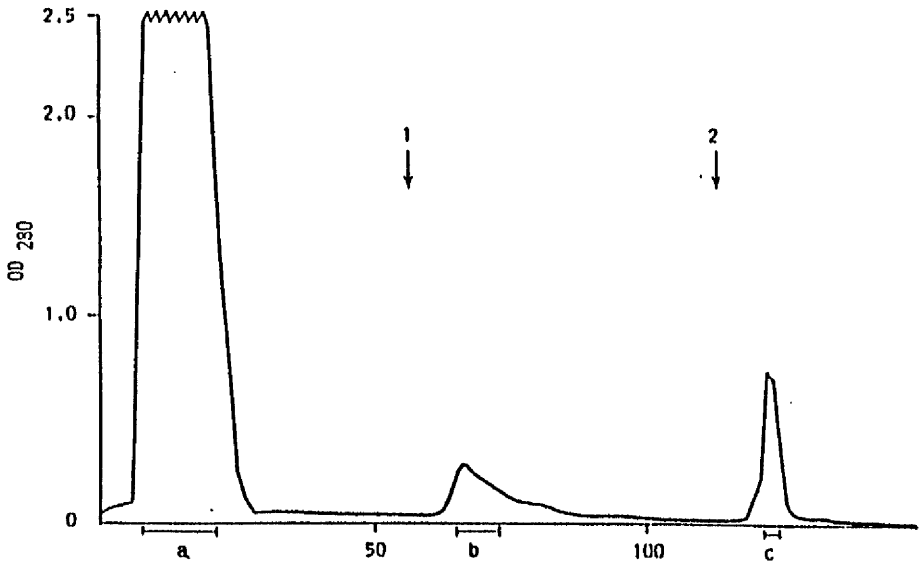


Figure 3.10

Chromatography of RA serum (15 mls)
on Sepharose-IgG column (1.0 x 40 cms)
Protein concentration (OD_{280} ——).
Pool a represents the exclusion peak;
pool b consists of protein eluted with NaC1 (400 mmol.l^{-1}) and
pool c is the protein eluted with guanidine-HC1 (2mol.l^{-1}).
The arrows 1 and 2, represent the points of application of the NaC1 and guanidine-HC1 respectively.
The scale of the abscissa represents the number of the individual column fractions.

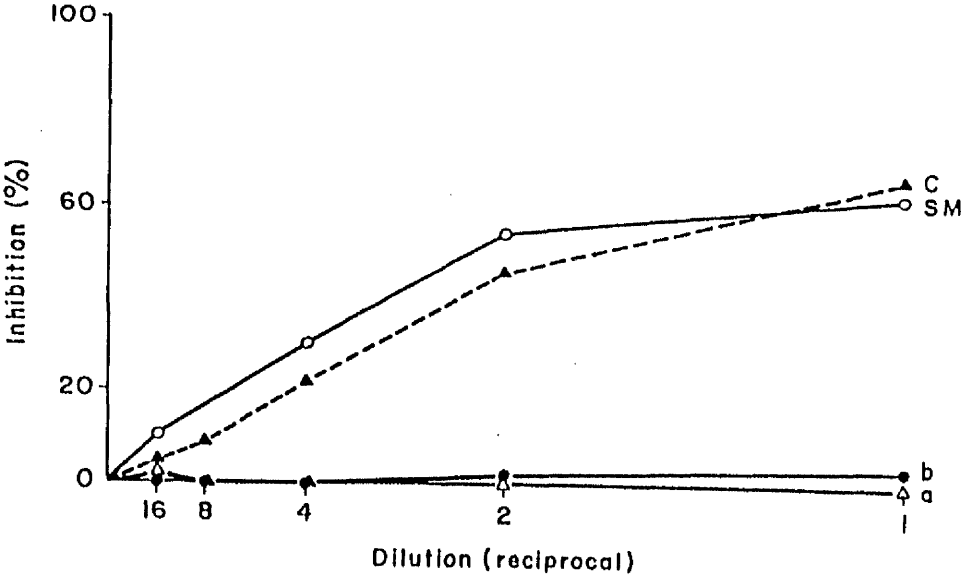


Figure 3.11

Titration of the inhibitory activity in pools

a (△—△) b (●——●) c (▲--▲) (See Fig. 3.10)

and the RA serum which was the starting material (sm o—o).

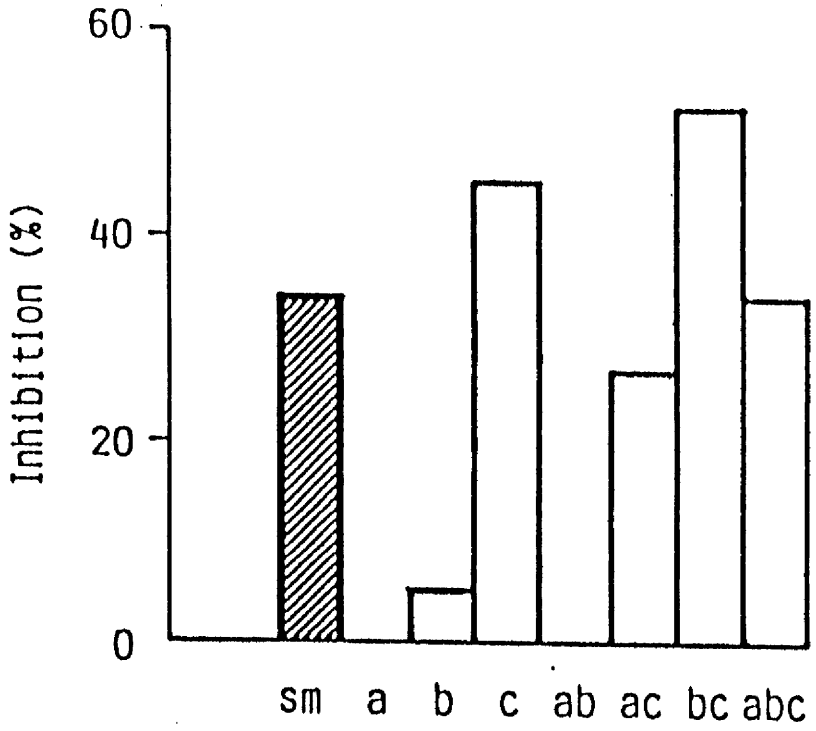


Figure 3.12

Reconstitution of inhibitory activity of RA serum.

The inhibitory activities of 25 µl of RA serum starting material (sm ▨); 100 µl of pool a (exclusion peak) 25 µl of pool b and 25 µl of pool c are shown above.
Combinations of the various protein pools 100 µl pool a + 25 µl pool b; 100 µl pool a + 25 µl pool c;
25 µl pool b + 25 µl pool c, and 100 µl pool a + 25 µl pool b + 25 µl pool c were also tested for inhibitory activity.
When pool c or pools b and c together were added to the exclusion peak (pool a), inhibition of PIP was restored.

(Protein pools as in Figure 3.10)

with pool (b) alone (Fig 3.12).

Gel filtration chromatography on Sephacryl S300

Reagents

Acetate buffer 100 mmol/l pH 3.5

Tris base 1 mol/l

Sephacryl S300 column (1.6 x 90cm)

Method

The inhibitory pool (c) from the IgG-Sepharose column was concentrated by vacuum dialysis and then dialysed against acetate buffer. One ml of the concentrated pool (OD_{280} = 15.1) was applied to the Sephacryl S300 column which had been equilibrated with the same buffer.

The column was eluted at a flow rate of 16mls/hour and 1.5ml fractions were collected into tubes containing 20µl of tris (1mol/l). to restore the pH of the fractions to neutrality (pH 6.5-7.5).

Alternative fractions were screened for total protein (OD_{280}), inhibitory activity (% inhibition of PIP) and rheumatoid factor by radio-immunoassay. Two protein peaks were obtained; the first, which was eluted synchronously with IgM (elution volume 112.5 mls) contained IgM-RF and the majority of the inhibitory activity. The second peak which contained small amounts of inhibitory activity was eluted with the same buffer volume (135 mls) as IgG. (Fig 3.13(b)).

These two protein peaks were pooled separately, concentrated by vacuum dialysis and analysed by SDS-PAGE (see Materials and Methods (2.2.12). The gel electrophoretic patterns are shown in (Fig 3.14). The first inhibitory pool

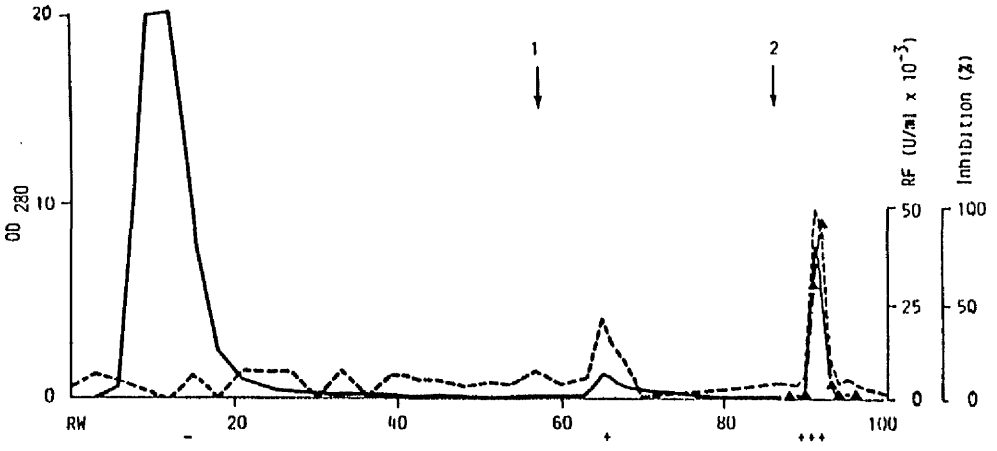


Figure 3.13(a)

Purification of inhibitory activity

IgG Sepharose chromatography. 15 mls of RA serum were applied to a (1.0 x 40 cm) column which was eluted as described in the text. Protein (OD_{280} ——); inhibitory activity (%------); Rheumatoid factor by radio-immunoassay (units/ml, ▲------▲) and haemoglutination (Rose-Waaler test; RW +/-).
The arrows, 1 and 2 represent the points of application of NaC1 and guanidine-HCl respectively.
The scale of the abscissa represents the number of the individual column fractions.

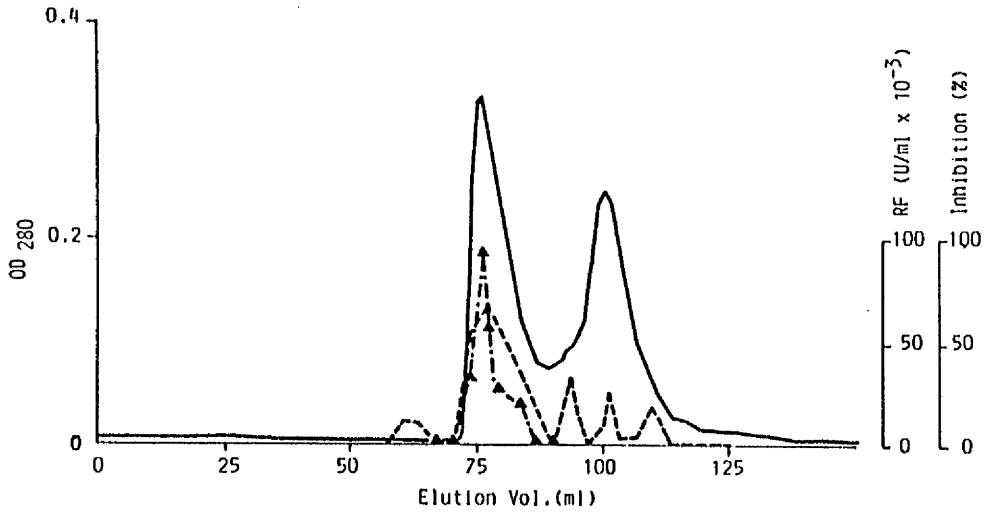


Figure 3.13(b)

Sephacryl S300 chromatography. 1ml of pool c was applied to a 1.6 x 90cm) column which had been equilibrated at pH 3.5 as described in the text. Symbols as in Figure 3.13(a). The elution volume shown on the abscissa. Two major protein peaks were eluted, representing IgM and IgG rheumatoid factor respectively.

(Pool c was a guanidine eluting peak, see Figs 3.10 and 3.13(a))

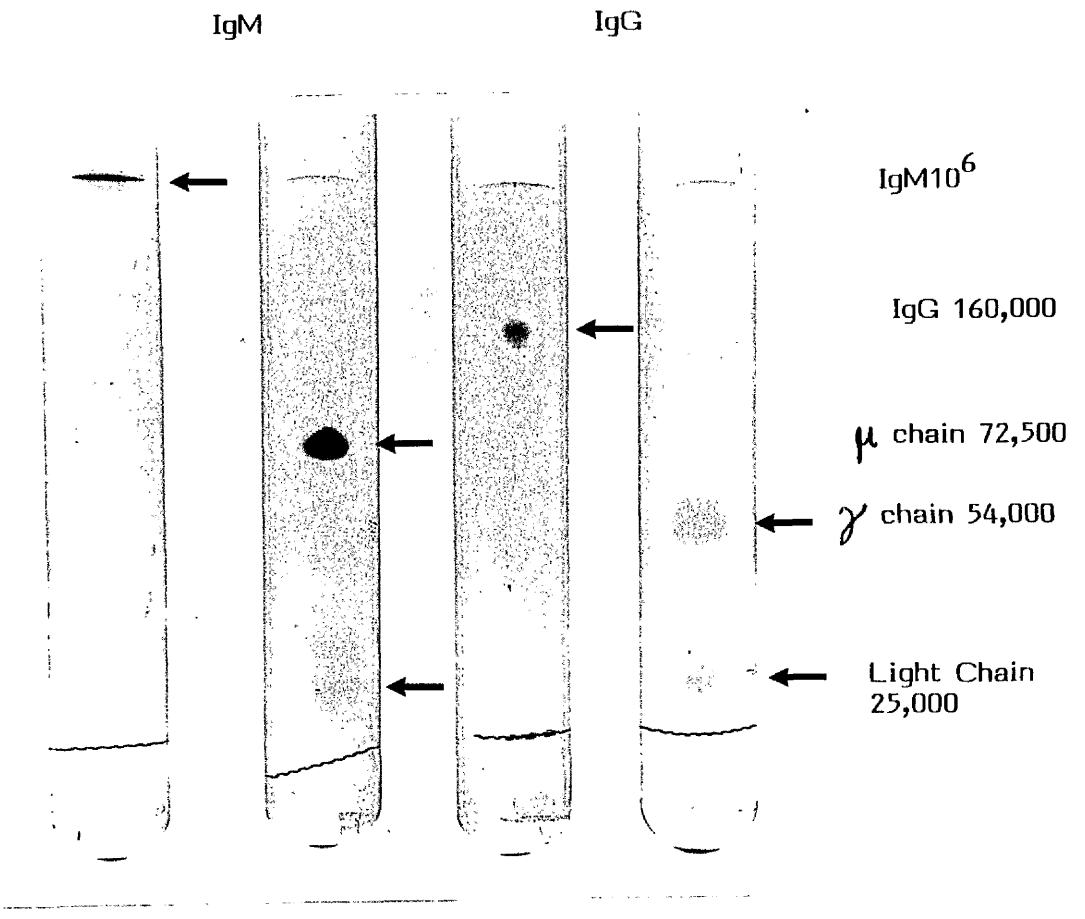


Figure 3.14

SDS-PAGE analysis (5% gels) of protein pools from Sephacryl S300 columns (Fig.3.13(b)). Electrophoretic analysis of the first pool is shown on the left (non-reduced and reduced), and the analysis of the second pool is shown on the right. The estimated molecular weights of the bands are shown. The band above the light chain of IgM has not been identified.

contained IgM, and the second IgG. $F(ab')_2$ fragments of the IgG pool bound to EA and inhibited the binding of IgM-RF. Thus pools 1 and 2 were IgM and IgG-RF respectively.

SUMMARY

Inhibitory activity was isolated from RA serum and shown to be a property of IgM-RF. Although IgM-RF accounted for the majority of inhibitory activity in RA serum small amounts of inhibitory activity were found associated with IgG-RF.

SECTION 7

The following section describes studies in which the effects of purified IgM rheumatoid factor and RA sera on PIP were compared. These studies were undertaken in an attempt to demonstrate that the inhibitory activity present in RA sera could be explained on the basis of their content of rheumatoid factor.

Studies evaluating the role of rheumatoid factor on the inhibition of PIP

The experimental methods used in the section can be found in Materials and Methods (2.2.6, 2.2.15, 2.2.16).

Comparison of the effects of purified IgM-RF with whole RA serum on PIP

Purified IgM-RFs and the RA sera from which they were prepared were suitably diluted so that they contained identical concentrations of IgM-RF. When tested for their ability to inhibit PIP, purified IgM-RF was more potent than the same amount of IgM-RF contained in RA serum (Fig 3.15). Normal IgM did not inhibit PIP.

Effect of IgG-RF on PIP

Normal monomeric IgG did not inhibit PIP. IgG-RF (1mg/ml) exhibited a moderate capacity (35.5% ± 2.5 SEM) to inhibit PIP, as did the same concentration of $F(ab')_2$ fragments (26.7% ± 5.2 SEM).

Kinetics of inhibitory action (see 2.2.6).

When RA serum or IgM-RF (both containing 5000u RF/ml) were added to normal serum prior to the addition of antigen and antibody (time 0) the inhibitory effect was maximal. When added after antigen and antibody the inhibitory effect was less marked. The magnitude of the effect diminished with increasing time after the addition of antigen and antibody (Fig 3.16)

Joyce

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of Freezer :-

324 µg | ml

Equiv pr citrate 190 µg/ml

2.07%

Dilute 1:2 before use

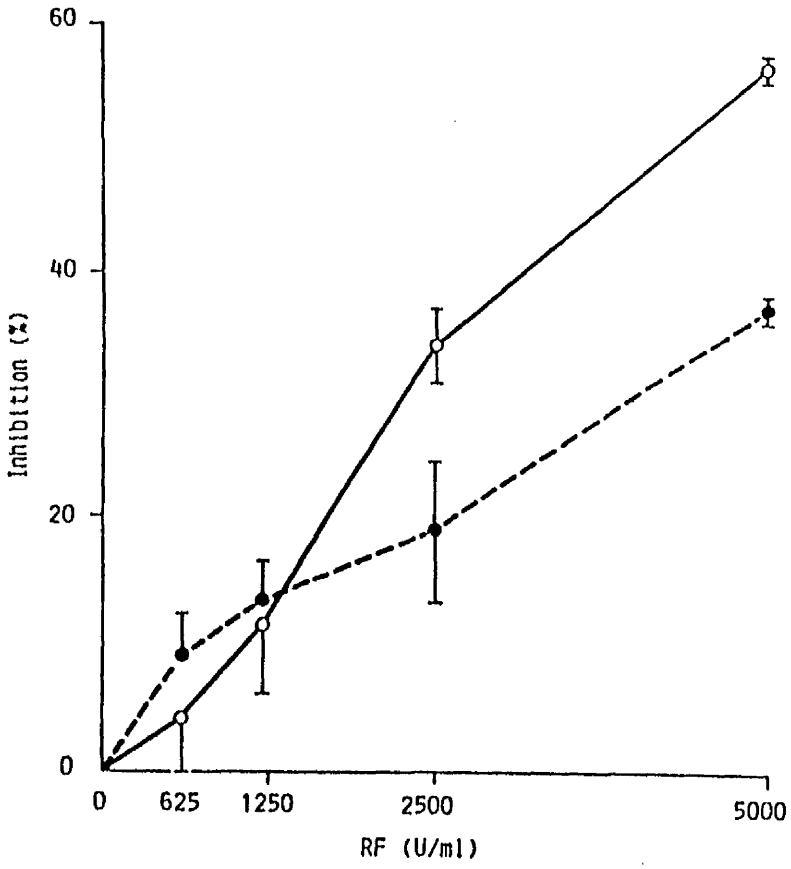

Figure 3.15

The effect of purified IgM-RF and whole RA serum on PIP.

Purified IgM-RFs (o—o) and the heat-inactivated RA sera (●----●) from which they were prepared were diluted so that they contained identical quantities of IgM-RF. The level of inhibition produced by each is shown. Each point represents the mean and standard error of the mean of three preparations.

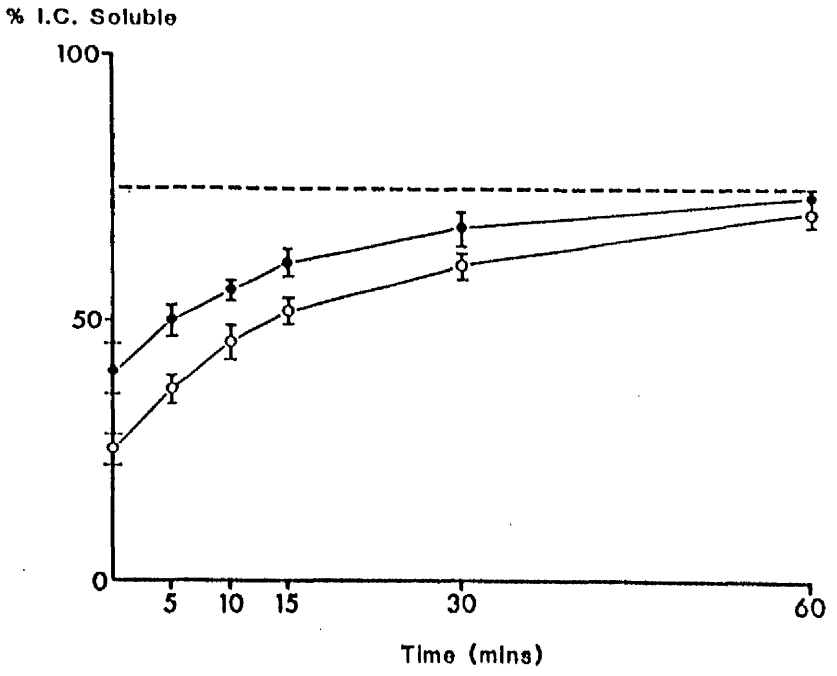

Figure 3.16

Kinetics of precipitation of antigen-antibody complexes in the presence of serum and IgM-RF

The effect of adding heat-inactivated (HI) RA serum (●—●) or IgM-RF (o—o) at different times after the mixing of antigen and antibody at equivalence. The horizontal line (-----) shows the lower limit of the normal range. Each point represents the mean ± SEM of 3 experiments.

IgM-RF produced more inhibition than RA serum.

Kinetics of precipitation of antigen-antibody complexes in the absence of serum.

When RF or heat-inactivated RA serum (both at 5000u/ml) were present during the formation of complexes at equivalence, the rate of precipitation was increased (Fig 3.17).

C4 consumption by complexes formed in the presence of RA serum and IgM-RF (see 2.2.15, 2.2.16).

Following the formation of antigen-antibody complexes in normal serum, C4 consumption occurred (Table 3.3). Consumption of C4 was reduced when complexes were formed in the presence of RA serum, but not in the presence of IgM-RF.

The relationship between IgM-RF and inhibitory activity (%)

The sera of one hundred and fifty seven patients with rheumatoid arthritis were tested for inhibitory activity. It can be seen (Fig 3.18) that the level of inhibitory activity (% inhibition of PIP) showed a poor correlation with serum levels of IgM-RF. Many patients with very high levels of IgM-RF in their sera have low levels of inhibitory activity and vice versa.

SUMMARY

Purified IgM-RF was found to behave somewhat differently from RA serum containing the same amount of IgM-RF in relation to expression of inhibition of PIP. This difference was noted in kinetic studies and in C4 consumption studies. Purified IgM-RF was more inhibitory than the same amount of IgM-RF present in RA serum. IgM-RF and inhibitory activity were only poorly correlated when measured in a large group of patient sera.

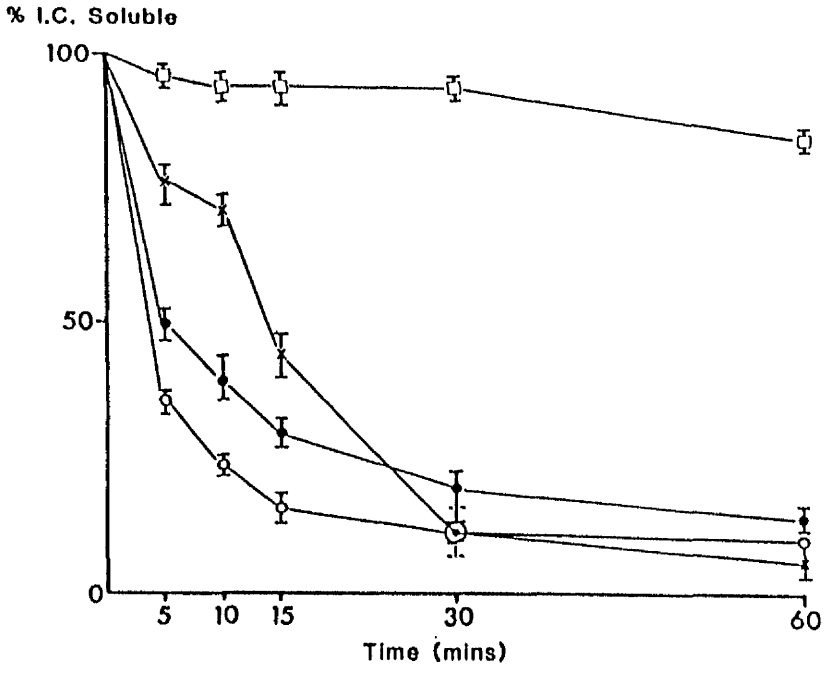

Figure 3.17

Kinetics of precipitation of antigen-antibody complexes in the presence of normal serum, heat-inactivated RA serum and purified IgM-RF

Antigen-antibody complexes were formed at equivalence in the presence of
IgM-RF (o—o);
heat-inactivated RA serum (●—●);
normal serum (□—□) and
buffer alone (×—×).
The rate of precipitation of complexes is shown. Each point represents the mean (+SEM) of 3 experiments.

Table 3.3

The effect of IgM-RF and RA serum on the consumption of C4 by immune complexes

	Units C4* pre I.C.	Units C4 post I.C.	Units of C4 consumed	% C4 Consumption
NHS	24160±2445	5660±141	18500±2340	76.1±2.11
NHS+IgM-RF	24000±2788	5120±152	18880±2646	78.2±1.88
NHS+(HI)NHS	36053±1499	8830±537	28348± 837	76.2±1.64
NHS+(HI)RAS	30577± 510	14709±252	15867± 740	51.6±1.62

HI = Heat inactivated
NHS = Normal human serum
RF = Rheumatoid factor
(HI)RAS= (HI) Rheumatoid arthritis serum
* = Results represent mean ± SEM of three experiments

The amount of C4 consumed by immune complexes was measured by comparing the quantity of C4 in the reaction mixture before and after incubation (1 hour at 37°C) with immune complexes formed at equivalence. C4 consumption is expressed as a percentage of the starting value. Immune complexes consumed C4 in comparable amounts in the presence of NHS, NHS + RF, NHS + (HI) NHS. C4 consumption was reduced (51.6% ± 1.62) in the presence of (HI) RA serum.

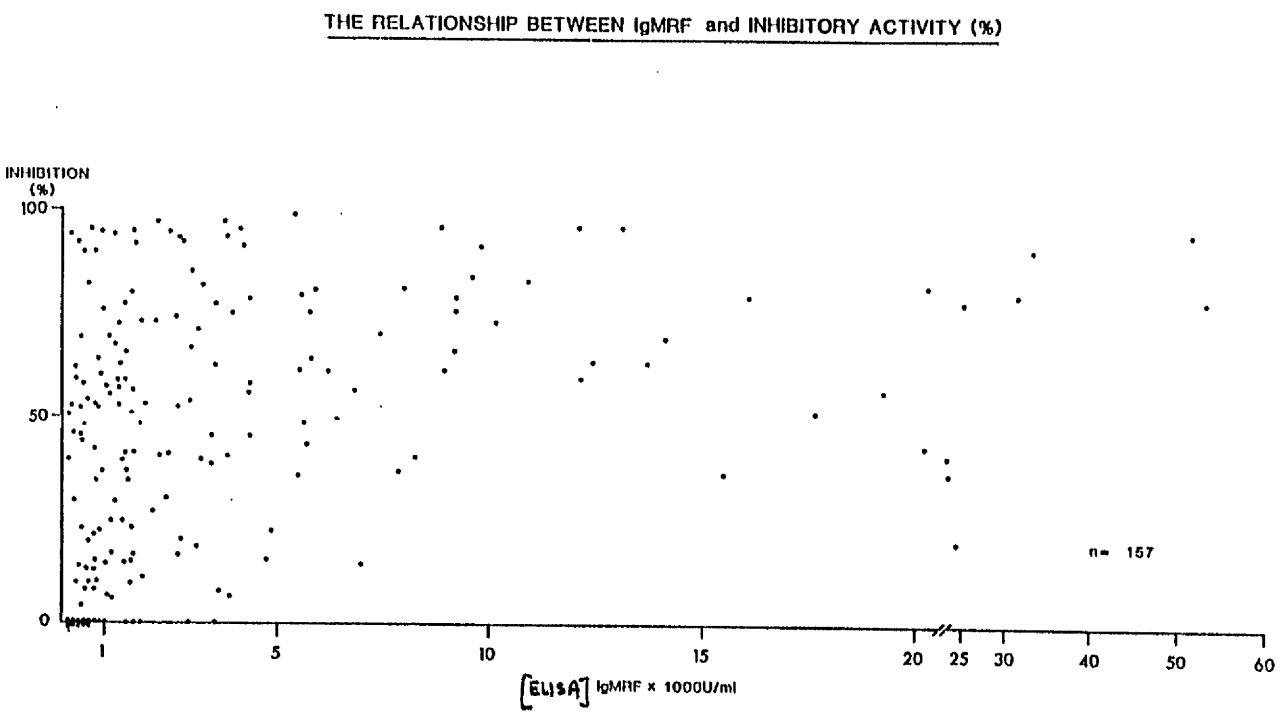

Figure 3.18

The relationship between IgM-RF and inhibitory activity (%)

SECTION 8

The relationship between inhibition of PIP and clinical aspects of disease

The patient groups studied in this section are detailed in Materials and Methods (2.1.4(a)(b)). PIP and PIP inhibitor levels were measured in serum and SF as previously described. Statistical methods used are detailed in Materials and Methods (2.3).

STUDY A

PIP and inhibition of PIP in sera and synovial fluids

Patients in group A-I comprised 15 patients with seropositive RA, group A-II comprised 8 patients with RA who were seronegative for IgM-RF and group A-III comprised 6 patients with miscellaneous arthritides. PIP (%) in group A-I sera was reduced markedly in 60% (9/15) of patients, whereas only minor reductions were observed in 2 of 7 patients in group A-II (Fig 3.19). Likewise inhibitory activity was markedly elevated in group A-I patients with 87% (13/15) having increased inhibitory activity. Of the 8 patients in group A-II only one had a significant level of inhibitory activity in her serum and one patient in group A-III with psoriatic arthritis had a marginally elevated level of serum inhibitory activity at 12% (Fig 3.19). PIP was reduced in the synovial fluids from all 3 groups. The mean level of IC which remained soluble in SF of patients in group A-I was significantly lower ($p < 0.01$) than the mean level in group A-III. The mean level in group A-II did not differ from the mean of either group A-I or group A-III (Fig 3.20). Levels of inhibitory activity in SF from group A-I were raised in all but one patient. The mean level (48% ± 7% SEM) was significantly higher than the mean level of inhibitory activity in group A-II and A-III (20% ± 7% SEM $p < 0.02$) and 12% ± 6% SEM $p < 0.01$) respectively. Inhibitory activity was also seen in SF of 6 of 8 patients in group A-II and 2 of 6 patients in group A-III, however, there was no significant difference between the mean levels of inhibitory

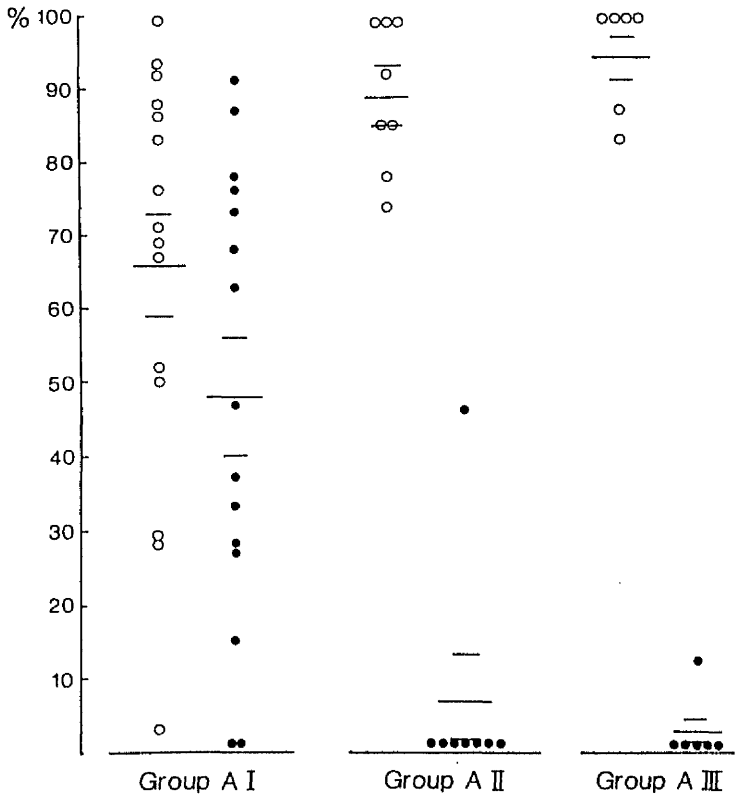

Figure 3.19

Study A: Serum (PIP) % (O) and inhibition of PIP (%) (●) (inhibitory activity) on y axis; in seropositive RA (group A-I), seronegative RA (group A-II) and miscellaneous arthritides (group A-III).

The horizontal line in each group represent the mean.
The short horizontal lines above and below each mean represents the standard error of the mean (SEM)

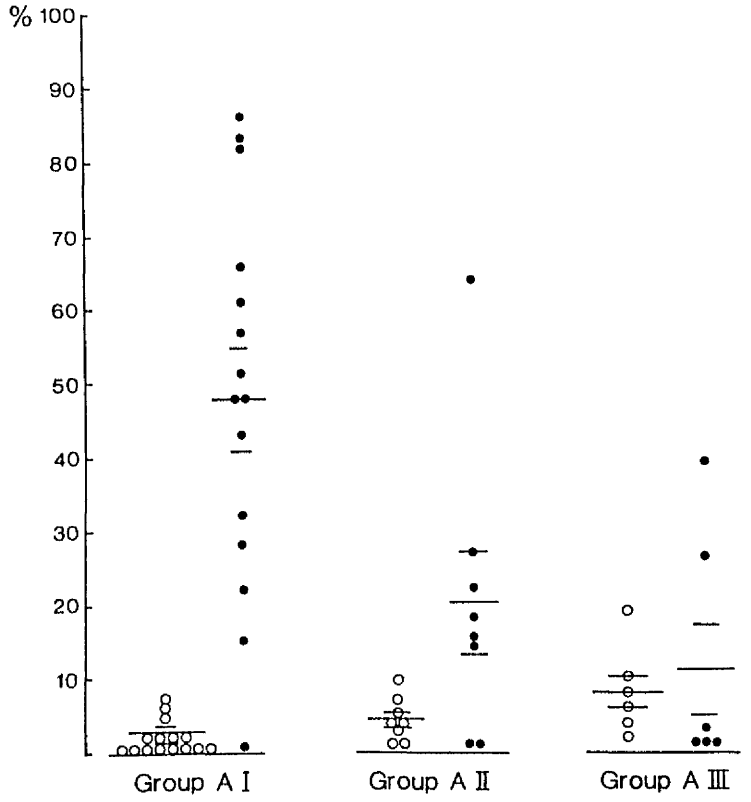

Figure 3.20

Study A: Synovial fluid PIP(%)(o) and inhibition of PIP (%)(●) (inhibitory activity) on y axis; in seropositive RA (Group A-I), seronegative RA (Group A-II) and miscellaneous arthritides (Group A-III).

The horizontal line in each group represents the mean. The short horizontal lines above and below each mean represents the standard error of the mean (SEM).

activity in the two groups (Fig.3.20).

Correlations with clinical indices of disease activity

In group A-I the level of PIP in the serum did not correlate with any of the clinical or laboratory parameters of systemic disease activity which were examined. Furthermore there was no correlation between serum PIP and any of the indices of local disease activity within the aspirated joints. The serum levels of inhibitory activity of group A-I patients did not correlate with any of the clinical or laboratory indices of disease activity.

The level of synovial fluid PIP in group A-I patients showed a negative correlation with joint hypertrophy ($r= -0.52$, $p < 0.05$), and the level of inhibitory activity in SF correlated with joint tenderness ($r= 0.54$, $p < 0.04$). In group A-II, there were no correlations between SF, PIP or inhibitory activity and the clinical parameters of disease activity. In group A-III however patients' SF PIP showed a negative correlation with joint hypertrophy ($r= -0.88$, $p < 0.02$). No other correlations were observed.

Correlations with laboratory indices of disease activity

The only significant correlations observed were in group A-I. The serum level of PIP correlated directly with CH50 ($r= 0.72$, $p < 0.002$), and indirectly with the level of IgM-RF in serum ($r= -0.64$, $p < 0.01$) and the level of inhibitory activity in serum ($r= -0.77$, $p < 0.002$). The levels of serum PIP and inhibitory activity in any of the three groups did not correlate with the levels in SF. (%) inhibition of PIP did not correlate with IgM-RF levels.

Study B

Patients with RA were divided into 3 groups. Group B-I comprised patients with purely articular RA. Group B-II comprised patients with RA and nodules and Group B-III comprised patients with RA and vasculitis (see Materials and Methods (2.1.4(b)).

PIP and inhibition of PIP (inhibitory activity) in RA sera

Reduced PIP occurred in 7 of 20 (35%) of sera in group B-I, 15 of 18 (82%) of group B-II and 18 of 20 (90%) of group B-III (Fig 3.21). Likewise inhibitory activity was markedly elevated in group B-III patients with 100% (20/20) having increased levels of inhibitory activity. In addition all the patients in group B-II had increased levels of inhibitory activity which was also present in 15 of 20 (75%) of patients in group B-I (Fig 3.22).

The mean level of PIP in group B-III sera was significantly lower than the mean level in group B-II ($p < 0.05$) and group B-I patients ($p < 0.0001$). The mean level in group B-II was also significantly lower than group B-I ($p < 0.005$). The mean level of inhibitory activity in sera from group B-III (74% ± 5 SEM) was significantly higher than the mean level of inhibitory activity in group B-II (60% ± 6 SEM; $p < 0.05$) and group B-I (38 ± 6 SEM; $p < 0.0005$). A significant difference was also observed between inhibitory levels in groups B-I and B-II ($p < 0.05$).

In group B-I the levels of PIP in the serum correlated inversely with the IgM-RF ($r = -0.58$ $p < 0.01$) and the inhibitory activity ($r = -0.74$, $p < 0.001$). In the same group serum levels of C3 and B showed a positive correlation with the levels of inhibitory activity in the serum ($r = 0.46$, and $r = 0.61$ respectively ($p < 0.002$ in both cases).

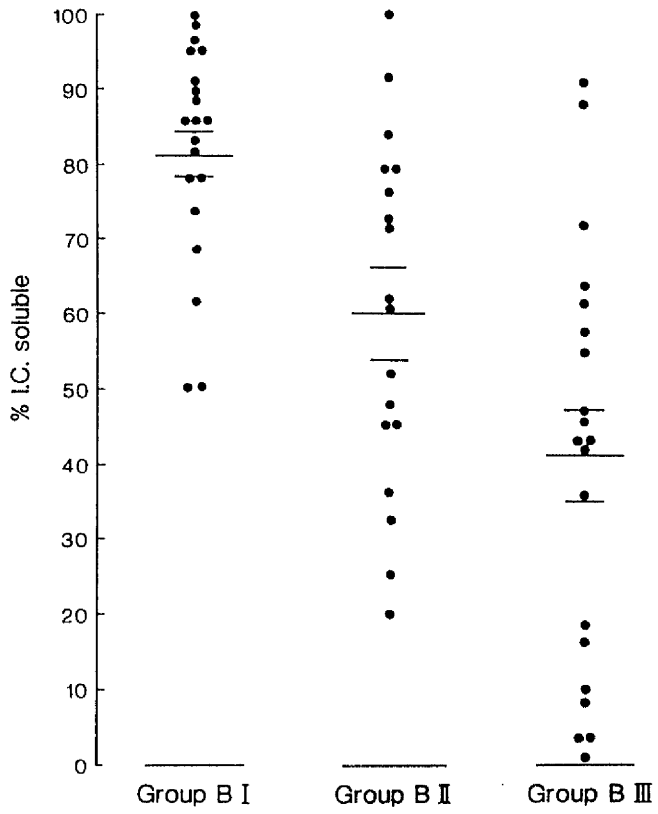

Figure 3.21

Study B: Serum PIP (% IC soluble) in RA without nodules (Group B-I), with nodules (Group B-II), and with vasculitis (Group B-III)

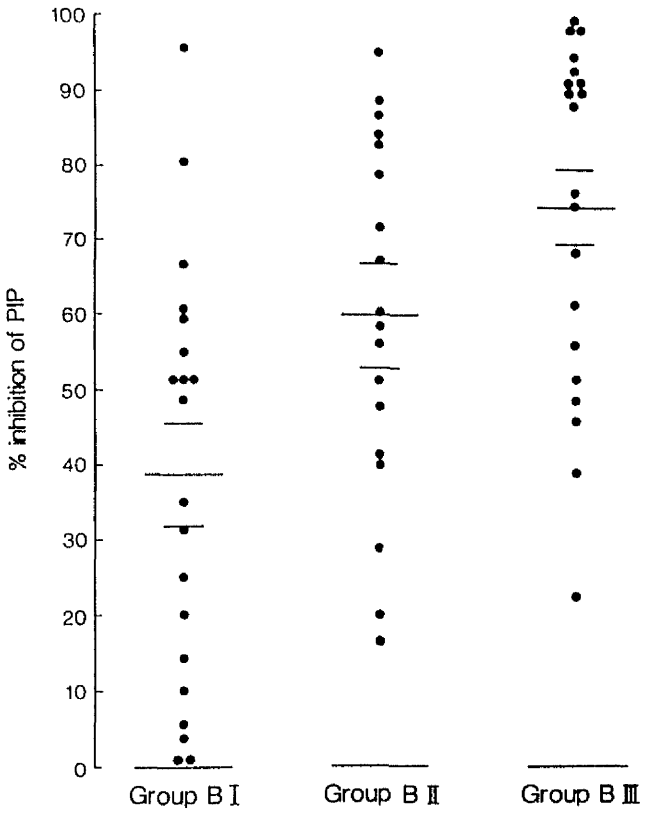

Figure 3.22

Study B: Serum inhibitory activity (% inhibition of PIP) in RA without nodules (Group B-I), with nodules (Group B-II), and with vasculitis (Group B-III)

The only significant correlation in group B-II was an inverse relation between PIP levels and the IgM-RF concentration ($r= -0.60$, $p<0.01$).

In group B-III the levels of PIP correlated with C4 levels ($r= 0.73$, $p<0.002$) (Fig 3.23) and inversely with IgG-RF concentrations ($r= -0.63$, $p<0.005$) (Fig 3.24) and levels of inhibitory activity ($r= -0.80$, $p < 0.001$). No correlation was found between the inhibitory activity levels and the concentrations of the IgM, IgG and IgA RFs.

SUMMARY

No correlations were noted for inhibitory activity and the rheumatoid factor isotypes IgM-RF, IgG-RF and IgA-RF. Although it was shown that purified IgM-RF does inhibit PIP in vitro (see Results, Section 6) other factors present in whole serum appear also to be important in modulating this process. (see Results, Section 7 and Discussion, Section 7). It was therefore decided to attempt the isolation of inhibitory activity by different procedures, from RA serum. Normal serum was used also as the starting material as it displayed a small but significant amount of inhibitory activity (see Results, Section 3).

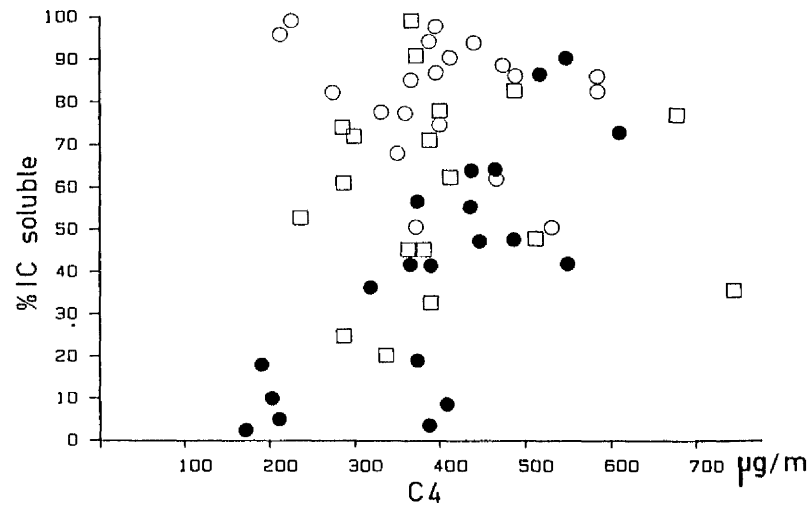

Figure 3.23

Study B: Correlation of C4 levels with PIP in Groups B-I (o), B-II (□), and B-III (●).

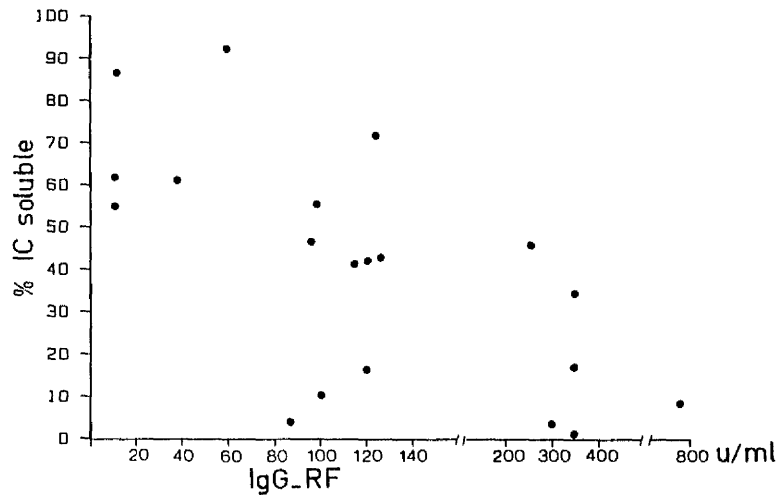

Figure 3.24

Study B: Correlation of serum PIP with IgG-RF levels in Group B-III

SECTION 9

Isolation of inhibitory activity by sucrose gradient ultracentrifugation and IgG Sepharose chromatography from normal serum and RA serum.

Sucrose density gradient fractionation of normal serum and RA serum.

The method used was that described in detail in Material and Methods (2.2.3).

The level of inhibitory activity in the various fractions is shown in Fig 3.25. In this experiment neither of the starting sera (SM) (normal serum or RA serum) displayed inhibitory activity. However high levels of inhibitory activity were found in fractions 2 and 3 of both normal and RA serum. The peak inhibitory fraction (No. 2) in both sera co-incided with the IgM peak. A similar protein concentration profile across the fractions and in the starting sera was seen in normal serum and RA serum. Rose-Waaler positivity and IgM-RF activity was most abundant in fractions 2 and 3 of RA serum and in the RA serum starting material. The fractions from the normal serum did not contain IgM-RF.

Sucrose density gradient fractionation of normal and rheumatoid serum - the effect of heat-inactivtion (HI)

RA and normal sera were heat-inactivated at 56°C for 30 minutes prior to sucrose density gradient ultracentrifugation. The gradient profiles for inhibitory activity and IgM and IgG are shown in Fig 3.26. There was marked loss of inhibitory activity in fractions 2 and 3 of normal serum while the inhibitory profile in RA serum was essentially unchanged and indeed appears to be slightly more pronounced with increases in fractions 1 and 3. It is worth noting that inhibitory activity could only be detected in the RA serum following heat-inactivation. The protein concentrations and the rheumatoid factor profiles were both unchanged in the heat-inactivated fractions of RA

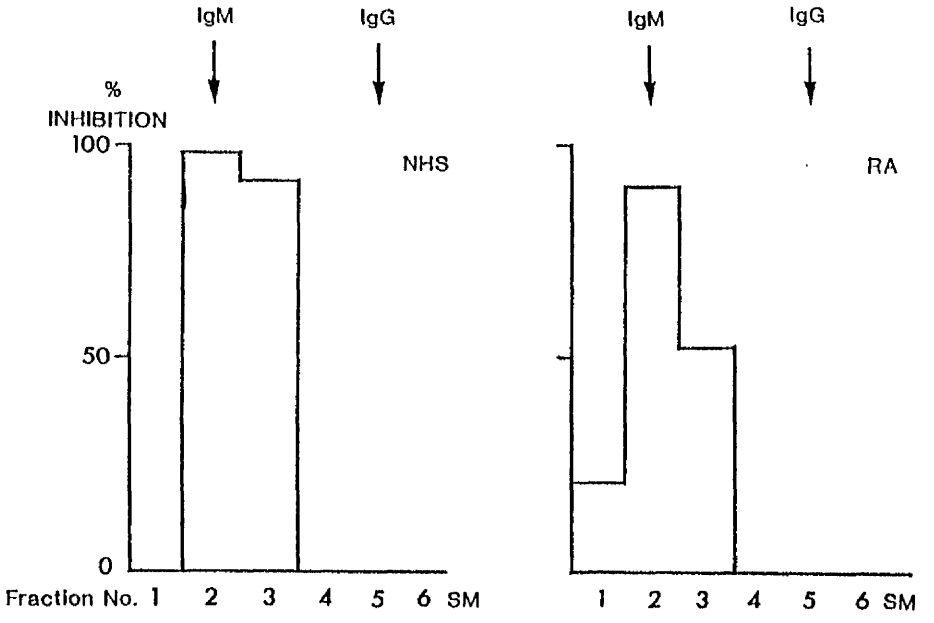

Figure 3.25

Sucrose density gradient ultracentrifugation of normal serum (NHS) and rheumatoid serum (RA).

Inhibitory activity (inhibition of PIP) is measured on the y axis (% inhibition).

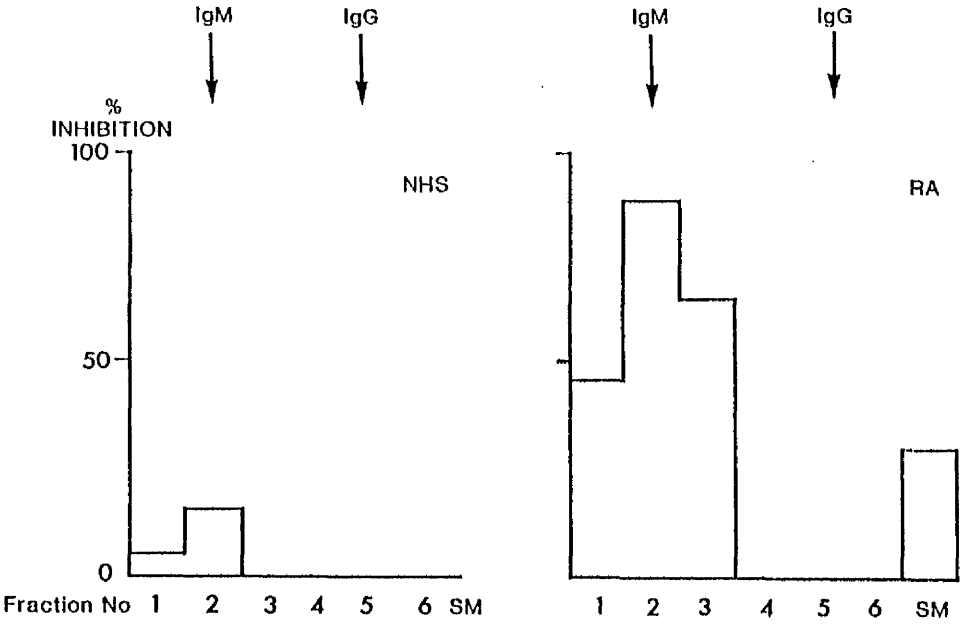

Figure 3.26

Sucrose density gradient ultracentrifugation of heat-inactivated (HI) serum.

SM - starting material
NHS - Normal human serum
RA - Rheumatoid arthritis serum

serum.

The titration of inhibitory activity in fraction 2 from both normal serum and RA serum is shown in Fig 3.27. The starting protein concentration was adjusted so that both were comparable. The nature of the curves suggest that inhibitory activity in normal and RA fraction 2 is found in similar quantities.

Reconstitution of PIP

In these experiments the peak inhibitory fraction from the sucrose density gradients of normal and RA serum was mixed with fractions 1, 3, 4, 5 and 6 and the level of inhibitory activity measured.

The method was as follows:

The peak inhibitory fraction from normal and RA serum was fraction 2. A row of 6 plastic Eppendorf tubes were set up on ice and 50µl of fraction 2 from normal serum was added. Buffer (50µl PBS) was added to tube 1, and 50µl fractions of 1, 3, 4, 5 and 6 were added to tubes 2, 3, 4, 5 and 6 respectively. The procedure was repeated with the gradient fractions of the RA serum.

	Normal Serum	RA Serum
Tube No	Sucrose Fraction No. (50µl)	Sucrose Fraction No. (50µl)
1	2 + buffer (PBS 50µl)	2 + buffer (PBS 50µl)
2	2 + 1	2 + 1
3	2 + 3	2 + 3
4	2 + 4	2 + 4
5	2 + 5	2 + 5
6	2 + 6	2 + 6

All tubes then received 9µl normal serum, then 5µl anti-BSA antiserum (diluted 1/3 in PBS) and 5µl ^{125}I-BSA at (100µg/ml).

The controls were (1) normal serum alone (9µl normal serum, 100µl PBS, 5µl anti-BSA and 5µl ^{125}I-BSA), (2) total radioactivity input (114µl PBS and 5µl ^{125}I-BSA), (3) radioactivity precipitated by antibody in the absence of serum (PBS 109µl, anti-BSA 5µl, 5µl ^{125}I-BSA). The total volume in each tube was 119µl.

After mixing, all tubes were incubated at 37°C for 1 hour. Following this a 50µl aliquot was taken and transferred to an Eppendorf tube containing 1ml ice cold PBS. The tubes were centrifuged (Beckman Microfuge) for 5 minutes and a 250µl supernatant sample was taken from each for counting in the γ counter. The percent inhibitory activity was calculated as described previously. (See section 2.2.1(b)).

It can be seen (Fig 3.28) that when fraction 2 was mixed with fraction 5 inhibition of PIP did not occur.

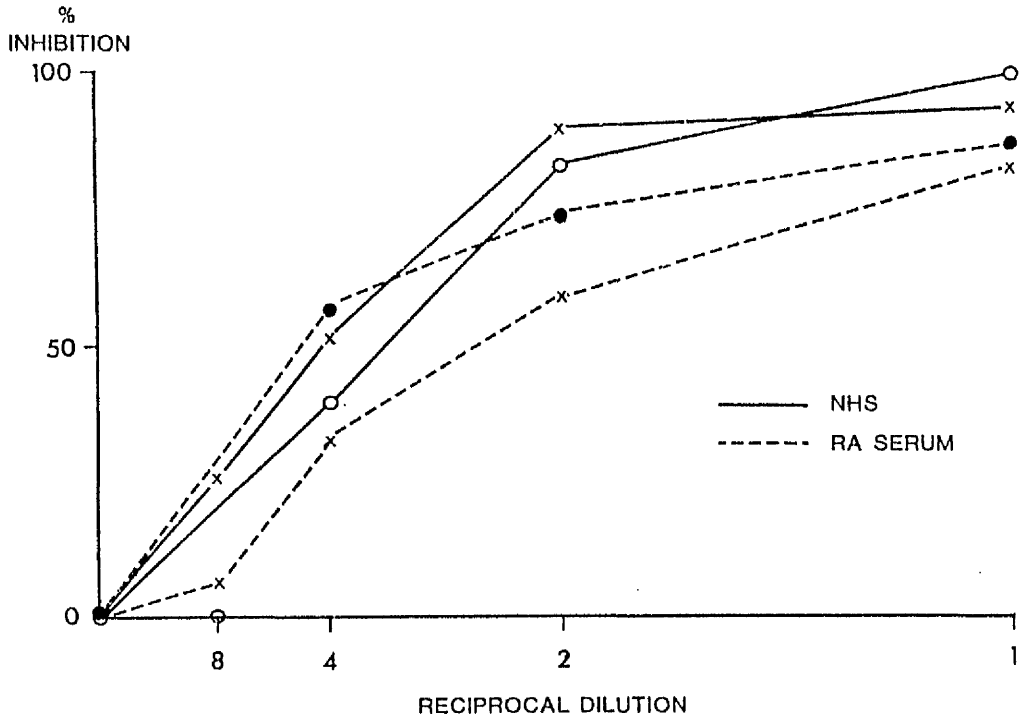

Figure 3.27

Titration of sucrose gradient fraction number 2 from rheumatoid and normal serum for inhibitory activity (% inhibition).

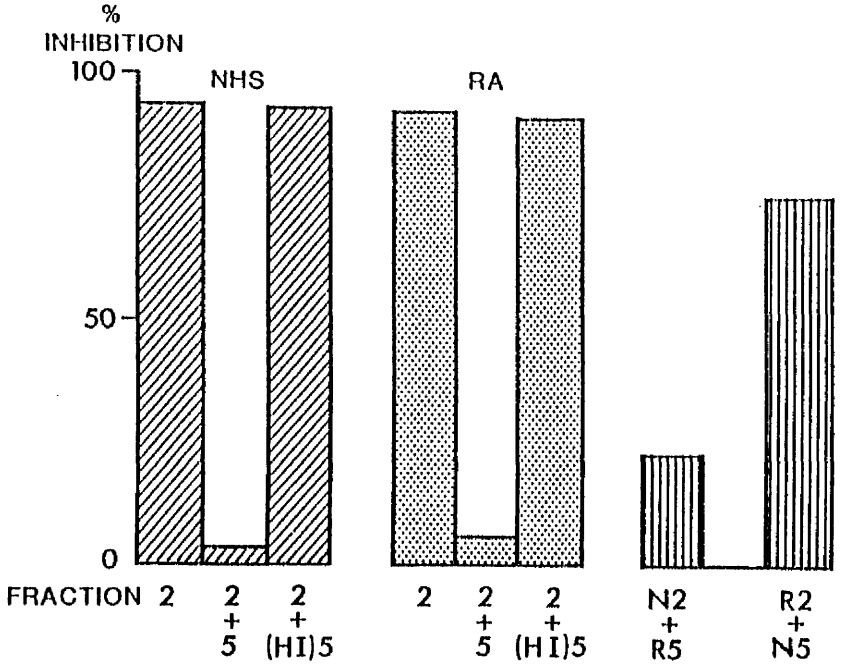

Figure 3.28

Reconstitution of PIP

N = normal serum
R = rheumatoid serum
(HI) = heat-inactivated

(%) inhibition of PIP was measured in sucrose gradient fractions from normal serum (NHS) and RA serum (RA). The numbers under each bar denote the sucrose gradient fraction numbers, showing which fractions were mixed in each sample.

This reversal phenomenon was seen with both normal and RA sera. This antagonistic effect of fraction 5 was abolished if fraction 5 was subjected to heat-inactivation. When inhibitory fraction 2 from normal serum was mixed with fraction 5 from RA serum the reconstitution of PIP was much less. However, the antagonist to inhibitory activity appeared to be more potent in fraction 5 from normal serum when acting against the inhibitory activity in RA fraction 2.

IgG Sepharose chromatography of inhibitory material isolated from sucrose density gradients. (see Materials and Methods (2.2.3).

Small (2ml) columns containing IgG Sepharose were prepared and the inhibitory fractions from normal and rheumatoid sucrose gradients (fraction 2) were applied and washed through with starting buffer (phosphate buffered saline 0.15M; pH 7.4). The volume of the starting material was 1.5mls and the protein concentration as measured by OD_{280} was 350µg/ml for normal fraction 2 and 890µg/ml for RA fraction 2. These starting materials were dialysed against the starting buffer (PBS, pH 7.4) prior to application to the column. After application the column was washed through with 5 column volumes (10ml) starting buffer to ensure all unbound protein was washed through. The bound protein was eluted in two steps:

1. 0.4M NaCl (5 column volumes, 10mls)
2. 2M guanidine hydrochloride (5 column volumes, 10mls)

1ml fractions were collected. All fractions were extensively dialysed against PBS pH 7.4 and were then screened individually for inhibitory activity (%) and rheumatoid factor (Rose-Waaler titration) (Fig 3.29(a)(b)). The vast majority of inhibitory activity bound to IgG Sepharose both in normal and rheumatoid serum and was eluted by 2M guanidine hydrochloride or 0.4M NaCl (Fig

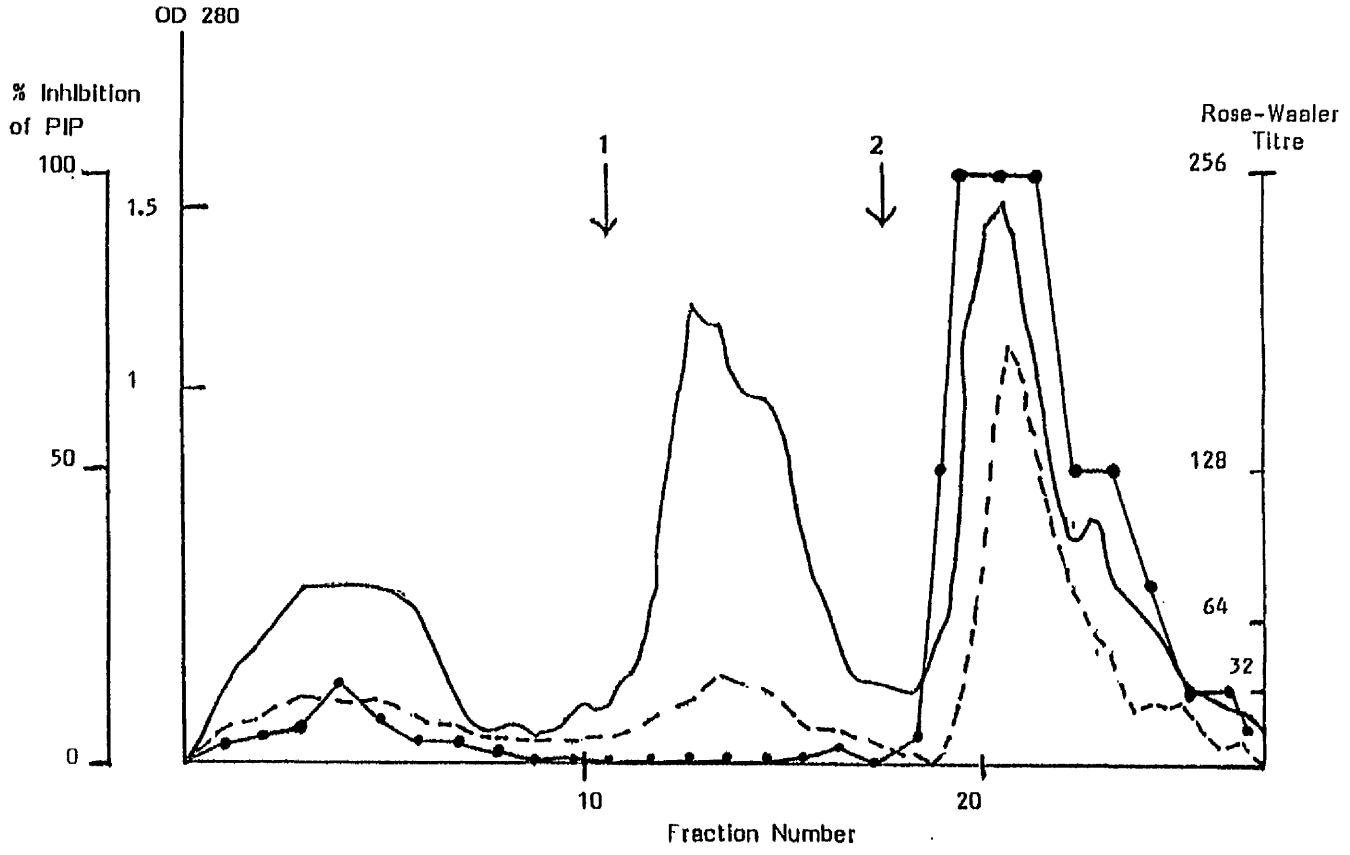

Figure 3.29(a)

Representative chromatogram IgG Sepharose chromatography of sucrose gradient fraction number 2 from RA serum.

1 = 0.4M NaCl elution
2 = 2M guanidine elution

(OD_{280} ——)

% Inhibition of PIP (inhibitory activity) - - - - - -
IgM-RF (Rose-Waaler titre) ●—●

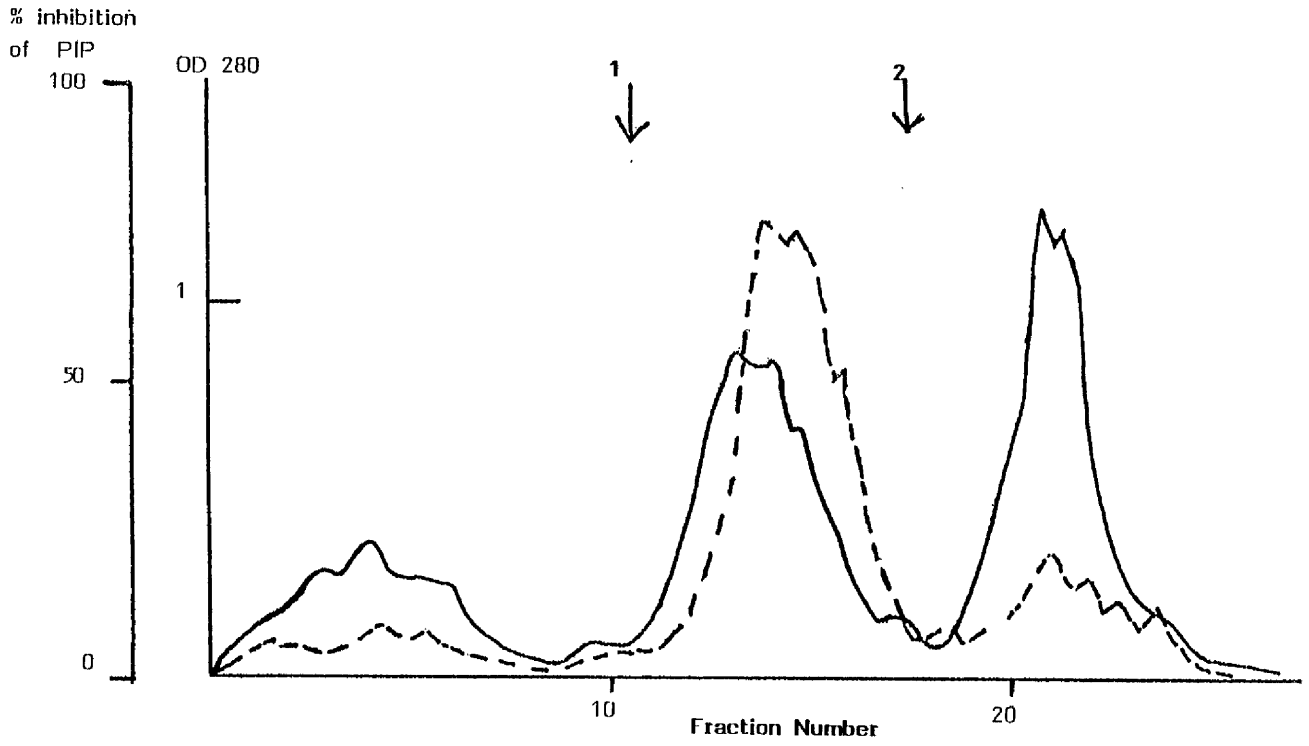

Figure 3.29(b)

Representative chromatogram IgG Sepharose chromatography of sucrose gradient fraction number 2 from normal serum.

(OD 280 ——) 1 = 0.4M NaCl elution
2 = 2M guanidine elution
% Inhibition of PIP (inhibitory activity) --------

3.29(a)(b)). In the rheumatoid column IgM-RF was found in the protein peak eluted with 2M guanidine hydrochloride. In the normal serum column most of the inhibitory activity was eluted with 0.4M salt (Fig 3.29(b)) but in the RA serum column most of the inhibitory activity resided in the 2M guanidine eluted peak.

Protein analysis (see Materials and Methods (2.2.12)

The protein fractions eluted from the IgG Sepharose columns described above were tested individually for inhibitory activity. For RA fraction 2 this inhibitory activity was found in the fractions eluted with 0.4M NaCl and to a much greater extent in the fractions eluted with 2M guanidine hydrochloride. These inhibitory fractions were pooled separately so that two inhibitory pools were made, viz. a 0.4M NaCl eluted inhibitory pool and a 2M guanidine eluted inhibitory pool. Likewise two inhibitory protein pools (0.4M NaCl eluted and 2M guanidine eluted) were made for the normal serum fraction 2 IgG Sepharose column.

The four protein pools were concentrated by freeze-drying and subsequently were reconstituted in a small volume of PBS pH 7.4 prior to being analysed by SDS-PAGE. The method for SDS-PAGE analysis is described in Materials and Methods, Section (2.2.12). The concentrated protein pools were analysed by SDS-PAGE under reducing and non-reducing conditions. The tube gels were stained with Coomassie blue and a molecular weight standard was run under similar conditions. The gel pictures of the protein pools showing the greatest amount of inhibitory activity, RA serum and normal serum, are shown in Figs 3.30 and 3.31. The protein eluted with 0.4M NaCl from IgG Sepharose chromatography of normal serum fraction 2 contained most of the inhibitory activity. It can be seen that there are several high MW bands and a band at approximately 60 kD, which was not reduced with 5-mercaptoethanol.

The intensely inhibitory protein pool eluted with 2M guanidine hydrochloride from IgG Sepharose chromatography of RA serum fraction 2 contained large amounts of IgM-RF as tested by Rose-Waaler and ELISA. The gel picture shown in Fig 3.31 demonstrates the protein constitution of this pool. In the non-reduced gel there are two bands, high and low MW. The high MW band represents IgM-RF and reduces to heavy and light chains of IgM respectively. The lower MW band (approx 60 kD) was not reduced under these conditions.

SUMMARY

Inhibitory activity has been shown to be present in normal serum as well as RA serum. The expression of inhibitory activity appears to be controlled by other (as yet unknown) serum factors which antagonise the inhibitory molecule. Purification of the inhibitor of PIP from normal and RA serum (using sucrose gradient separation and subsequent affinity chromatography) has revealed the presence of a 60kD molecule in addition to IgM-RF on SDS-PAGE.

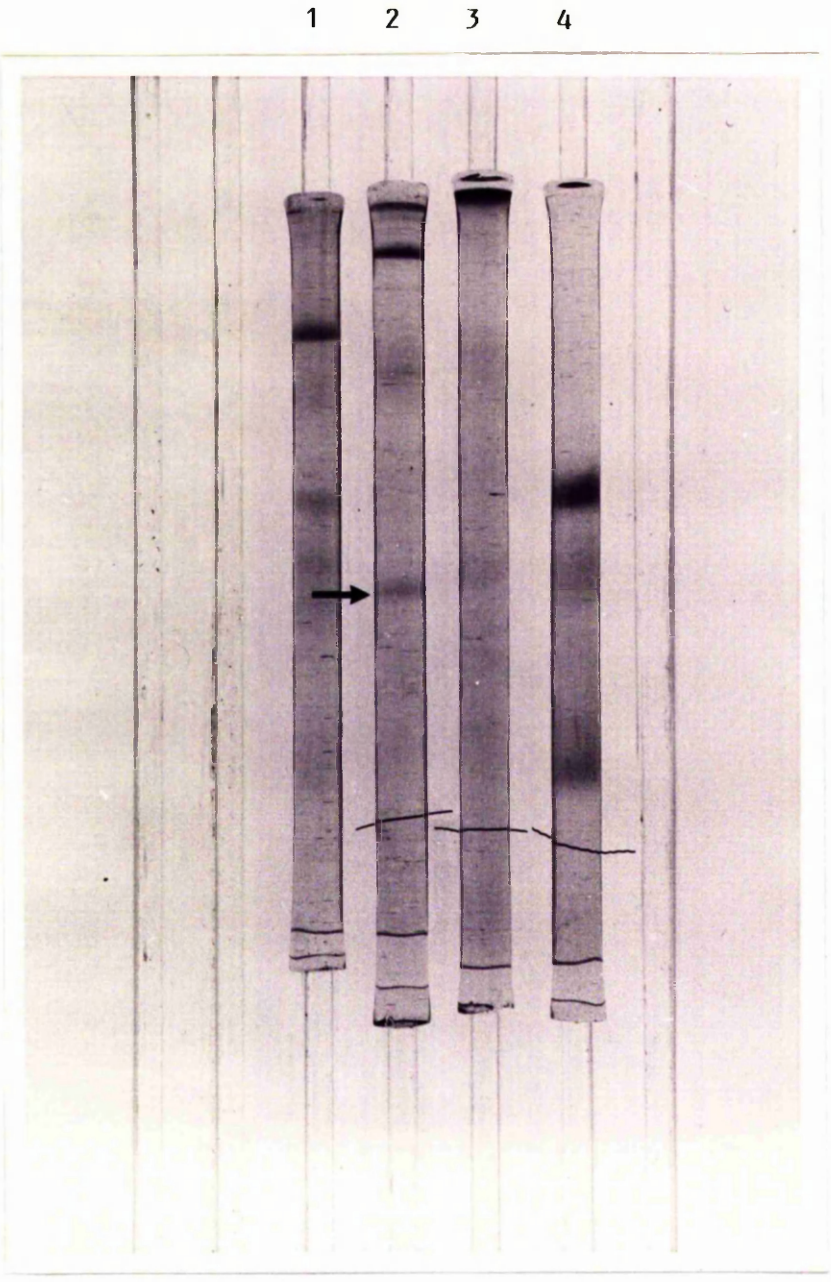

Figure 3.30

SDS-PAGE. Inhibitory protein pools from IgG Sepharose chromatography of sucrose gradient fraction 2. Gels 1 and 2 are from normal serum (reduced and non-reduced respectively). Gels 3 and 4 are from rheumatoid serum (non-reduced and reduced respectively).

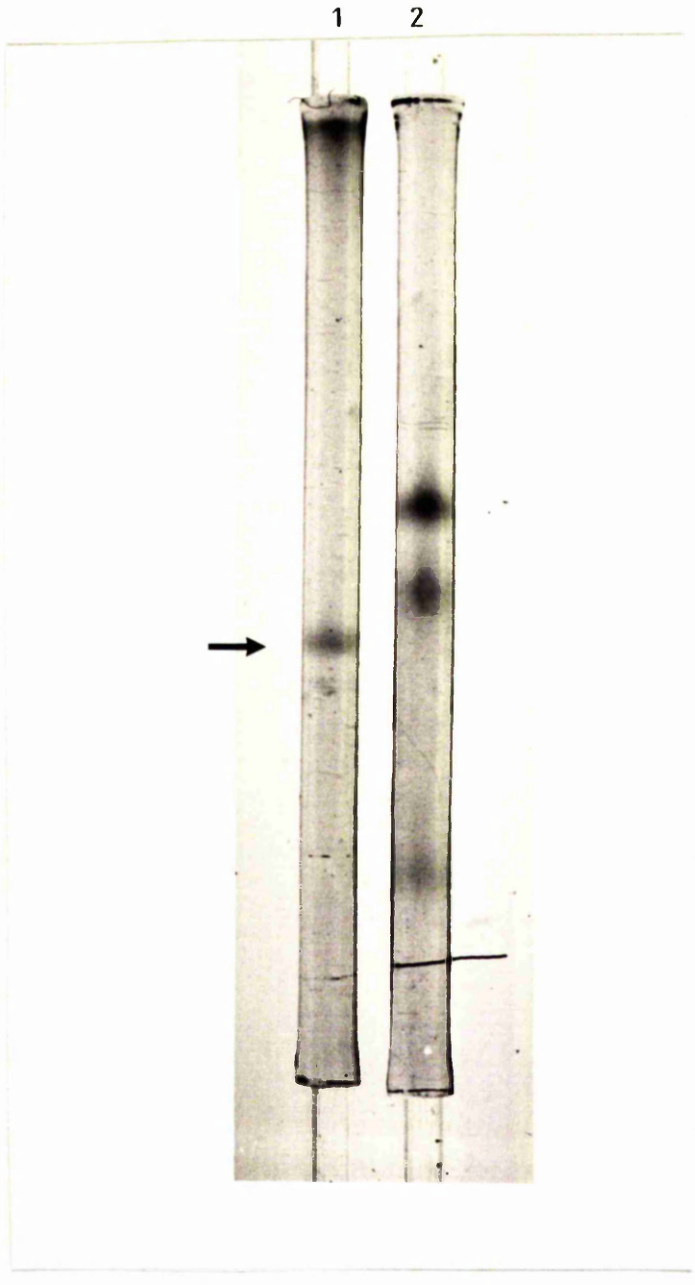

Figure 3.31

SDS-PAGE. IgG Sepharose chromatography of sucrose gradient inhibitory fraction 2 from RA serum. 2M guanidine hydrochloride eluted inhibitory pool.

1 = non reduced
2 = after reduction with 5-mercaptoethanol.

The protein band at MW $\sim$ 60,000 Daltons is clearly seen.

CHAPTER FOUR

DISCUSSION

In this Chapter I will discuss my clinical and experimental work the results of which have been presented in the preceding Chapter (3). Each Results Section will be discussed separately and will follow the same sequence as in Chapter 3.

SECTION 1

The establishment of an assay to measure the complement-mediated prevention of immune precipitation (PIP) in human serum.

The establishment of an assay to measure prevention of immune precipitation has been described. The method was based on the assay described by Schifferli and colleagues (1980). The immune complexes used were formed at equivalence. The equivalence point had previously been determined by a standard quantitative precipitation curve as described in (2.2.1) and Fig 2.1. This was confirmed when complexes which were formed in buffer alone were shown to quickly precipitate, almost completely by 15 minutes. In contrast complexes formed in normal serum remained soluble; over 80% of complexes were retained in solution at 1 hour (Fig 3.1). As expected serial dilution of normal serum was accompanied by a rapid decline in the capacity of serum to retain complexes in solution. The findings that serum treated with EDTA and serum which had been heat-inactivated (56°C for 30 minutes) were unable to prevent immune precipitation support the view that PIP depends upon the complement system.

The importance of the classical pathway in PIP was shown by the observations that in serum deficient in C2 and C1-inhibitor most of the complexes precipitated. The precipitation of complexes in C2 deficient serum was slow and incomplete and was similar to that seen in C1-inhibitor deficient serum. The C1-inhibitor deficient serum was depleted of C4 and C2, leaving C1 intact. It has been shown that purified C1 slows immune precipitation (Schifferli, et al, 1982 ; Naama et al, 1984) so it is probable that intact C1 in these sera was responsible for this effect. In Mg EGTA treated serum, which prevents classical pathway activation but allows alternative pathway

function, there was a considerable impairment of PIP with marked precipitation of complexes. However precipitation was not complete and the degree of precipitation levelled out after 30 minutes incubation. This failure of complete precipitation may be due to solubilisation of the precipitated immune complexes by the alternative pathway.

In C1-inhibitor deficient serum C4 and C2 activation occurs and therefore patients with C1-inhibitor deficiency often have reduced levels of C4. C4 deficiency is strongly associated with an "SLE like syndrome" suggesting that C4 is important in immune complex processing. In the setting of PIP absence of C4 would prevent the formation of a C3 cleaving enzyme, (this is also the case for C2). C1-inhibitor deficient patients although often having reduced C4 levels do not appear to be as prone to the "SLE like" illness which is seen in C4 deficient patients although an increased incidence of immune complex like diseases is seen in C1-inhibitor deficient patients. Therefore it follows that what C4 there is in C1-inhibitor deficiency it is obviously enough to form a C3 cleaving enzyme. In C1-inhibitor deficiency complement activation occurs spontaneously in the fluid phase; the presence of C1-inhibitor allows for the complement activation to occur on the immune complex therefore allowing PIP to proceed normally.

Partial precipitation in the Mg EGTA serum and B-depleted serum showed the importance of the alternative pathway in the PIP process. In Mg EGTA and B depleted serum full precipitation of the complexes was not observed and the complexes did not precipitate fully even after 1 hour of incubation. The initial fast aggregation of complexes seen in the presence of Mg EGTA (Schifferli et al, 1982) was not seen in my experiments but this may have been missed because my first sample was taken at 15 minutes after mixing whereas the initial fast aggregation described by the above workers tended to

occur prior to that time. In B-depleted serum (deprived of alternative pathway function) precipitation is delayed, but slowly over an hour the immune precipitate was seen to form (Fig 3.1).

These results are in agreement with those of (Schifferli et al, 1980) who showed that the early events in PIP are mediated by the classical pathway and the later events by the alternative pathway.

After establishing an assay for the measurement of PIP in serum, a normal range was established using 25 normal sera. In these sera PIP was never below 83%. Sera in which PIP was less than 83% were considered to show reduced PIP activity. The reproducibility of the assay was acceptable with a day to day variation of between 1% - 10%. The ability of the serum to withstand repeated freeze thawing showed that PIP was not likely to be a difficult phenomenon to measure and allowed me to be confident in assaying routine clinical samples for PIP where delays in transport are sometimes inevitable. Quality controls were included in every run of serum samples which strengthened the validity of the assay.

SECTION 2

Measurement of prevention of immune precipitation in patients' sera.

PIP was measured in an unselected group of patients' sera using the previously described assay. The patients were grouped only according to disease. The unexpected finding of reduced PIP in nearly 50% of patients with RA certainly warranted closer scrutiny. Several patients with SLE and GN also had reduced PIP levels. The most obvious reason for this impairment of a

complement dependent function was hypocomplementaemia, a feature which is not uncommon in patients with these conditions. However, when those hypocomplementaemic sera were excluded there still remained 25 RA sera, 3 GN sera and 5 SLE sera which showed reduced PIP. The only idea which would satisfactorily explain these findings would be the presence of inhibitory factors in these patients' sera, which were in some way interfering with this complement mediated function. It is possible that the inhibitory factor could be the same in the three diseases studied (RA, SLE, GN) but on the other hand more than one factor could be involved. The fact that only seropositive RA patients seemed to have reduced PIP argued that IgM-RF may be the responsible factor at least in RA, but high levels of IgM-RF are not frequently found in SLE and are rare in GN. I did not measure IgM-RF in my SLE or GN patients. All these diseases may be associated with circulating immune complexes (Theofilopoulos & Dixon, 1980) but the presence of immune complexes causing complement consumption and thereby reducing PIP would be reflected in reduced complement levels but these sera of course often had normal or even elevated complement levels (Fig 3.3 a and b). There was a correlation between serum PIP, CH50 and APH50 in patients with RA and SLE (Fig 3.3 and 3.4). This implies that levels of CH50 and APH50 regulate PIP, and this may occur through the C3 level common to both these pathways.

Hypocomplementaemic sera (reduced CH50) always had reduced PIP in patients with RA although there were a few patients with reduced APH50 who had normal levels of PIP, thus demonstrating that the alternative pathway, in contrast to the classical pathway, was not essential for normal PIP. In patients with SLE (Fig 3.4) CH50 was more closely related to the level of PIP observed than the APH50 in these patients. Complement studies were not performed on my normal controls and therefore I cannot comment

on the relationship of PIP with haemolytic complement in normal sera.

Studies using the model of complement mediated solubilisation of preformed precipitates have shown reduced solubilisation in the majority of SLE sera (Baatrup et al, 1983, 1984; Aguado et al, 1981; Sakurai et al 1982; Schifferli et al, 1981). In these studies low levels of complement components were generally associated with the reduced solubilisation seen in SLE sera. However the studies of Baatrup and colleagues (1984), pointed to the existence of inhibitory factors in SLE sera which could in part be responsible for the low levels of solubilisation seen. Using an inhibition of solubilisation assay he suggested that incompletely solubilised immune complexes may be the inhibitory factors in SLE sera (Baatrup et al, 1984). Measurement of immune complex levels in RA sera could allow one to assess the relationship between circulating immune complexes and levels of PIP inhibitory activity and IgM-RF but to date such studies have not been performed.

Balestrieri and colleagues (1983) studying PIP in SLE sera noted that 5 out of 9 sera of patients with active SLE had reduced PIP compared with only 1 from 44 patients with inactive disease. He however, related this impairment of PIP to low C4 levels. He did suggest, although based on very small numbers of abnormal SLE sera, that other factors besides reduced C4 may be responsible for the impairment of PIP seen in these sera. He maintained that rheumatoid factor may be important, although no evidence was presented to show that these SLE sera contained rheumatoid factor (Balestrieri, Pagani & Tincani, 1983).

SECTION 3

Establishment of an assay to measure inhibitory activity in serum

The presence of an inhibitor of PIP in RA serum was demonstrated by the finding of a dose dependent reduction of PIP level when RA serum was added to normal serum (Fig 3.5). In this experiment the assay to measure PIP was performed (as detailed in Results, Section 3) in which RA serum was added to normal serum and immune complexes were formed in this mixture. In the tube containing normal serum and undiluted RA serum there was a total abrogation of normal serum PIP. This effect became less marked as the RA serum was diluted out. The only explanation for this finding was that the RA serum contained an inhibitor of PIP. This inhibitory phenomenon was found to a varying degree in other RA sera tested in this way. Inhibitory activity was expressed as the percent reduction of PIP of normal serum when RA serum was added.

In order to optimise the conditions under which inhibitory activity in RA sera could be measured the PIP assay was modified to make it more sensitive to small amounts of inhibitory activity. The basic concept behind the assay to measure inhibition of PIP was to be able to demonstrate that the sample under test could reduce the amount of PIP due to the normal serum in the test tube. If there was too much normal serum in the tube, and therefore very high levels of PIP (eg 100%), then small amounts of inhibitory activity in the test sample would not be detected. PIP in normal serum is dose dependent (Fig 3.1(b)) and therefore small amounts of inhibitory activity in the test sample are more likely to be detected if it is operative on the steep part of the curve (Fig 3.1(b)). This was achieved by (1) reducing the load of immune complexes in the assay (2) by titrating the normal serum to give between 70% - 80% PIP. Therefore the immune complex load was reduced from 1.75 μg

BSA for the PIP assay to 0.5µg BSA for the inhibition of PIP assay. Immune complexes were still prepared at equivalence. Therefore using this immune complex load the normal serum was titrated to find that dilution which gave 70% - 80% PIP and this was used in the assay to measure inhibition of PIP. These manoeuvres meant that it was much easier to demonstrate inhibitory activity than it would have been with a higher immune complex load and less dilute serum.

The inhibitory effect became more pronounced following heat-inactivation of the RA serum showing that the factor was heat stable (56°C for 30 minutes). Heat-inactivation only destroys C1, C2 and factor B of the components which are involved in PIP. Therefore one is in effect adding among others C4 and C3. Therefore one would expect that PIP might be increased in sera with high levels of C3 and C4. However, PIP in RA sera has been shown to correlate poorly with C3 and C4 levels (Naama et al, 1983). Therefore for PIP to occur it appears to be related to complement activation and the generation of C3 convertase and this would only be supplied by the normal serum in the test tube. Therefore one would expect to see increased inhibitory activity in heat-inactivated serum.

Another possible reason why heat-inactivation "released" more inhibitory activity could be that the expression of the inhibitor is modulated by some unknown antagonist. This question, interestingly, will be raised again based on the results recorded in Section 9 of Chapter 3.

When establishing my normal range for inhibition of PIP a small amount of inhibition of PIP was observed in normal serum after heat-inactivation. This can only be explained by the presence of inhibitory material in normal serum being added to the system. It is worth bearing in mind that the amount of

inhibitory activity generated from normal serum $< 5\%$ may be attributed to the inherent error in any biological assay (up to 10%). However the finding of inhibitory activity in sucrose gradient fraction 2 of normal serum (see Chapter 3, Section 9 and Chapter 4, Section 9) provides firm evidence of a normal serum factor which inhibits PIP.

SECTION 4

Measurement of inhibition of PIP in patients' sera

Inhibition of PIP activity (inhibitory activity) was measured in both serum and synovial fluid (SF) of patients with a variety of rheumatic diseases. In 25 normal individuals inhibitory activity did not exceed 5% as noted in the previous study (see Section 3 of this chapter). Sera from patients with seronegative arthritis (seronegative RA, ankylosing spondylitis, psoriatic arthritis) contained little or no inhibitory activity. It is quite clear (Fig 3.6) that the highest levels of inhibitory activity are found in seropositive RA sera.

The synovial fluid was obtained from 13 patients with sero-positive RA. All SFs measured showed evidence of inhibitory activity ranging from 26% to 93%. Inhibitory activity in SF may be due to factors other than those which are present in RA sera. For instance, chondroitin sulphate binds to C1q and inhibits its activity (Silvestri et al, 1981). As cartilage degradation occurs in RA chondroitin sulphate must be released into the synovial fluid. However, subsequent studies showed that the inhibitory factor binds to IgG and not to C1q (Chapter 3, Section 6). Further evidence that inhibition of PIP was not due to a peculiarity of SF was obtained from the experiments described in

Chapter 3, Section 8, which showed that SF for some patients with seropositive RA (1 sample), seronegative RA (2 samples) and miscellaneous arthritides (3 samples) were non-inhibitory (see Fig 3.20).

There was no relationship between the level of inhibitory activity in serum and SF, although the mean levels were comparable. Scrutiny of Fig 3.7 shows that many serum samples displayed less inhibitory activity than their corresponding SF sample but also that there were some serum samples which had greater amounts of inhibitory activity than their corresponding SF sample. It does suggest that serum and SF behave independently as regards the expression of inhibitory activity and that local factors in the joint are probably important in the final expression of inhibitory activity in this environment. If only one type of molecule is responsible for inhibitory activity it does suggest that local production in the joint is occurring or that other factors in the rheumatoid joint eg immune complexes may be modulating the final expression of the inhibitory activity found in SF.

Using the model of complement mediated solubilisation of preformed precipitates Dayer and colleagues (1983) examined RA serum and SF for solubilisation activity. He found immune complex solubilisation capacity to be reduced in RA SFs and related this to low levels of SF alternative pathway haemolytic complement. However, these workers suggested that other factors in SF (eg antiglobulins or immunoconglutinin) might influence the solubilisation process. Their experiments also showed that factors present in SF could interfere with the capacity of fresh normal serum to solubilise immune complexes. A role for IgM-RF in this process was suggested (Dayer et al, 1983). Although the solubilisation of a preformed precipitate is distinct from PIP some similarities do exist (Schifferli et al, 1982). PIP is absolutely dependent on the classical pathway while solubilisation depends on an intact

alternative pathway. However, solubilisation works inefficiently in the absence of an intact classical pathway. Therefore the inhibitory substances in SF (to solubilisation and PIP) may be the same and act through the common features of both processes. In other words, if an inhibitory substance was acting via classical pathway functions this would be apparent as an impairment of PIP (as described above), and solubilisation would be inefficient because of lack of classical pathway contribution to the process.

SECTION 5

Studies on the mechanism of action of an inhibitor of PIP in serum and synovial fluid (SF)

Studies on the kinetics of inhibition of PIP showed that for maximal effect inhibitory activity must be present at the time of formation of the antigen-antibody complex (Fig 3.9) and that the effect diminishes with time after the onset of the formation of the immune complex. When this study was performed using inhibitory synovial fluid a similar pattern was seen, a finding which supports the view that the nature of the inhibitory molecule(s) is the same in serum and SF. This pattern of kinetic behaviour (Fig 3.9) where the maximal inhibitory effect was seen at the onset of the reaction suggests a direct effect of the inhibitory molecule(s) on the antigen-antibody complex at the time of its formation or on the complement system rather than an effect on the complexes containing bound complement components. This tends to exclude a role for immunoconglutinins in inhibition of PIP. If immuno-conglutinins were responsible for inhibition of PIP then one would expect them to work even when the complex has been rendered soluble which clearly was not the case.

Inhibitory activity could be achieved by a number of mechanisms up to the important step of C3 activation and binding to the complex. The inhibitor could prevent C1 binding to antibody, prevent C1 activation or could prevent C1 activating C4 or C2. An inhibitor could also act by inhibiting the binding of C4b to the immune complex, or the binding of C2 to C4b. It could bind to the C3 convertase (C42) and reduce its activity by blocking its active site. An inhibitor could also act by preventing C3 being cleaved by C3 convertase and it could also act by preventing C3b binding to the complex either by binding to C3b itself, or binding to the complex. An inhibitor of PIP could also act by activating the complement system directly and causing inappropriate complement activation in the fluid phase. Inappropriate complement activation would occur when the serum contained circulating antigen-antibody complexes which were still capable of causing complement activation, and sufficient in concentration to activate the majority of the C4 and C3, and thus such sera would probably be hypocomplementaemic. In order to discriminate amongst these possibilities C4 consumption following the addition of complexes to sera and synovial fluids was examined. The finding that sera and synovial fluid from RA patients with subnormal PIP showed reduced C4 consumption (Table 3.2) favours one of the first three explanations advanced above as well as the mechanism of inhibition of C4 cleavage by C1, i.e. C1 binding and/or activation is reduced. The final explanation would require excessive C4 consumption for validation. If any of the other explanations was correct C4 consumption would be normal. The negative correlation between C4 consumption and the degree of inhibition of PIP and the direct correlation between C4 consumption and the degree of PIP (Fig 3.8) support the conclusion that the factor in RA sera which inhibits PIP acts by inhibition of complement activation.

Two more recent reports have produced evidence to support this mechanism. Measuring the uptake of radiolabelled C1q by both aggregated IgG and BSA/anti-BSA complexes in the presence or absence of rheumatoid factor, O'Sullivan and her colleagues were able to show that preincubation of complexes with rheumatoid factors leads to inhibition of radio-labelled C1q uptake (O'Sullivan et al, 1987). They also reported a significant negative correlation between the inhibition of C1q uptake and the binding of IgM-RF. It should be noted that the rheumatoid factors described in this report were no more than crude preparations and it is of course possible that these preparations contained other constituents.

In their paper examining the mechanism of inhibition of PIP in RA sera, Niven and Whaley (1986) described a constituent of RA sera and synovial fluids which reduced the binding of C1q to immune complexes. They were, however, unable to find any correlation between reduction in binding of C1q and inhibition of PIP in the SF samples tested which might suggest that the inhibition of PIP in SF may occur by mechanisms other than the prevention of activation of the classical pathway. The factor which they described could bind to IgG Sepharose, reduce immune complex binding to C1q, and inhibit PIP, suggesting that it was operating via binding to the immune complex rather than to C1q. As regards the possibility that RF itself may be involved these workers stated that purified IgM-RF and $F(ab')_2$ IgG-RF did not reduce the binding of immune complexes to C1q. The data on this point however, were not shown in this paper (Niven & Whaley, 1986).

Thus far the nature of the inhibitory factor has not been ascertained. However, the trypsin sensitivity of the activity suggests that it is a protein, while its resistance to PMSF and SBTI argue that it is not an enzyme of the serine protease class. The observation that inhibition of PIP occurs almost

exclusively in patients with seropositive RA suggests that it may be RF, or some related serum constituent.

Regarding the possibility that the inhibitory activity may be RF, it is of interest that under conditions of high antibody density RF has been shown to inhibit complement-mediated lysis of antibody coated sheep erythrocytes (Schmid, Roitt & Rocha, 1970). Recent studies by Ng, Peters & Walport (1988) have examined the role played by RF in immune complex activation of complement. Monoclonal RF-IgG complexes fix C3 and C4 poorly. However, preformed RF-IgG complexes did activate complement but because of limited fixation of C3 and C4 the complexes did not bind to CR1. These workers argued that poor fixation of opsonic C3 and C4 to RF containing complexes would result in impaired transport of the complexes to the cells of the reticulo-endothelial system for safe disposal. It was postulated that a similar failure of clearance of RF containing complexes might be operative in the rheumatoid joint (Ng et al, 1988). The role of RF in the process of inhibition of PIP in RA will be discussed later in this chapter in the light of my own findings.

SECTION 6

Isolation of inhibitory activity from RA serum by IgG Sepharose and Sephacryl S300 chromatography

In patients with seropositive RA the impaired ability of serum to prevent immune precipitation is due to the presence of an inhibitor of this process (see Chapter 3, Section 3). In the previous section I have shown that sera and synovial fluid from RA patients with subnormal PIP showed reduced C4 consumption. This reduction in C4 consumption correlated with the degree of inhibition of PIP (see Chapter 3, Section 5). The most likely explanation was that C1 binding and/or activation was reduced. Studies on the kinetics of

inhibition of PIP showed that for maximum inhibitory effect the inhibitory substance had to be present at the time of formation of the antigen-antibody complex (see Chapter 3, Section 5).

In order to isolate this inhibitory activity I first assessed its ability to bind to and be eluted from an IgG Sepharose column which is in effect immobilised bound antibody. Initial studies showed that inhibitory activity could bind to and be eluted from an IgG Sepharose column. Most of the inhibitory activity could be eluted with guanidine hydrochloride and mixing of all the eluted protein pools (a, b and c) could fully reconstitute the inhibitory activity of the starting RA serum. This shows that all the inhibitory activity could be eluted from the column under the conditions described. Pool (c) (guanidine eluted pool) displayed more inhibitory activity than the RA serum starting material itself. The exclusion peak (pool a) was devoid of inhibitory activity. However when pool (c) and pool (a) were mixed together the inhibitory activity of the mixture was less than that of pool (c) alone. This suggested that pool (a) contained factors which were antagonising the inhibitory effect of pool (c). When the RA serum starting material and the material eluted with guanidine hydrochloride from the IgG Sepharose column (pool c) were titrated by doubling dilution for inhibitory activity, it was noted that RA serum and pool (c) exhibited similar dose response patterns.

Using sera from 7 individual seropositive RA patients, inhibitory activity was eluted from IgG Sepharose under the conditions described. On subsequent gel filtration (Sephacryl S300) chromatography, the majority of inhibitory activity was eluted as a single peak which on SDS-PAGE was shown to contain 19S IgM.

On the basis of the following observations it was concluded that the inhibitory activity in RA sera was due to IgM-RF.

(1) Significant levels of inhibitory activity are only found in the sera of patients who are seropositive for IgM-RF.

(2) The factor responsible for the inhibitory activity binds to IgG-Sepharose.

(3) Inhibitory activity co-chromatographs with IgM-RF throughout the purification procedure.

Balestrieri and his colleagues described an inhibitory effect of IgM-RF on PIP and immune complex solubilisation (Balestrieri et al, 1984). In contrast to my own approach these workers made the assumption that IgM-RF was the molecule responsible for this inhibitory activity before describing its inhibitory effect. A sounder scientific approach, was to isolate the inhibitory activity from RA serum as it makes no prior assumptions as to the nature of the functional activity which is being sought. More importantly it enables one to detect other substances which might be important in inhibition of PIP. For example the small but definite amount of activity eluted with 0.4M salt (Fig 3.13(a)) and similarly the small amount of activity which could possibly be attributed to IgG-RF (Fig 3.13(b)).

Thus it would appear that IgM-RF and to a lesser extent IgG-RF could account for the majority of the inhibitory activity found in RA serum. The 5% phosphate gels shown in Fig 3.14 attest to the purity of the IgM and IgG-RF. It can be seen, however, that a protein band above the light chain of IgM was noted in some but not all of the preparations. The nature of this band has not been identified.

SECTION 7

Studies evaluating the role of IgM-RF on the inhibition of PIP of immune complexes.

Purified IgM-RF and the whole RA serum from which it had been prepared (as described in Chapter 3, Section 6) were compared for their capacity to inhibit PIP. The purity of the IgM-RF was confirmed by SDS-PAGE and did not contain any contaminating protein bands. An ELISA was used to measure the quantity of IgM-RF in each preparation. To ensure uniformity the samples were diluted so that they contained equal amounts of IgM-RF. Purified IgM-RF was a more potent inhibitor of PIP than the same amount of IgM-RF in whole RA serum. To ensure that this effect was not just a feature of IgM itself, purified IgM was also tested but did not inhibit PIP. This shows that it was the rheumatoid factor activity of the IgM-RF which was responsible for the observed inhibition of PIP.

There are two reasons why purified IgM-RF might be a more potent inhibitor of PIP than IgM-RF found in RA serum. Firstly the IgM-RF may have been altered by the purification process or secondly, other factors may be operative in reducing the inhibitory activity of IgM-RF in whole RA serum.

The nature of the modulating influences on IgM-RF on the inhibition of PIP reaction which might be present in the whole RA serum are unknown at present. Inhibition of PIP increases with increasing quantities of IgM-RF in a dose dependent fashion as expected (Fig 3.15) but the difference between the inhibitory properties of the purified IgM-RF and the IgM-RF contained in whole RA serum remains much the same except at levels of IgM-RF of 1250 units/ml and less. Whole serum contains C1q and other Fcγ binding proteins

and an inhibitory molecule may exist in serum bound to such proteins. Thus inhibition of PIP in whole serum may be modulated by the degree of binding of an inhibitory molecule to such Fcγ binding proteins.

As the RA serum was heat-inactivated prior to the measurment of inhibition of PIP these modulating influences in whole serum must be heat-labile. Purified IgG-RF was shown to be moderately inhibitory and this was related to its $F(ab')_2$ fragments. IgG-RF or another RF class may be responsible for modulating the inhibitory effect of IgM-RF on PIP. This could be brought about by competition between IgM-RF and IgG-RF for the available binding sites on the immune complex. Prior depletion of serum of the other RF classes by immuno-adsorption would enable this hypothesis to be tested.

The inhibitory effect of RA serum or IgM-RF on PIP was maximal if these reagents were added prior to the addition of antigen and antibody (Fig 3.16). This confirms the results shown in Fig 3.9 where inhibitory RA serum was added to normal serum, the kinetic profile of both these experiments being similar. Again it was noted that purified IgM-RF was a more potent inhibitor of PIP at all times than the same amount of IgM-RF in whole RA serum.

In this section the effects of IgM-RF on the kinetics of precipitation of antigen-antibody complexes in the absence of serum were studied. Purified IgM-RF, or IgM-RF in heat-inactivated RA serum was able to increase the rate of precipitation of immune complexes (Fig 3.17). Again it will be noted that purified IgM-RF was more potent in this respect than IgM-RF contained in whole serum. These findings of an increased rate of precipitation of complexes in the presence of IgM-RF would be consistent with the mechanism of IgM-RF mediated inhibition of PIP suggested above ie. that IgM-RF inhibits PIP by acting on the complexes prior to them being rendered soluble by

complement activation.

There are two other reports of inhibition of PIP by IgM-RF. Using monoclonal and polyclonal rheumatoid factors Balestrieri and his colleagues studied the effects of these RF's on the PIP reaction (termed ICPIC assay in his paper) (Balestrieri et al, 1984). Their assay was similar to that described in this thesis. However the purity of Balestrieri's RFs can be questioned as their preparations were not apparently analysed by SDS-PAGE although the authors stated that IgA-RF and IgG-RF contaminated their IgM-RF preparations. Notwithstanding this an inhibitory effect of IgM-RF on PIP was described which was dose dependent and (although only two doses were used) similar to that described in my own experiments.

Balestrieri and colleagues were able to show that inhibition of PIP by RF was most marked when the RF was added at the time of immune complex formation which is in agreement with my results. They were also able to demonstrate this inhibitory effect of RF on PIP using a human immune complex system (tetanus toxoid and anti-tetanus toxoid antibodies) which was not used in my experiments. Their RF's did not display any anticomplementary activity which is again in keeping with my own results (see Table 3.3). (The amount of C4 in normal serum and in normal serum + IgM-RF was the same in these experiments on C4 consumption thus showing that IgM-RF had no anticomplementary activity by itself.).

In 1985 Miletic & Rodic published their paper on the kinetics of rheumatoid factor influence on PIP (Miletic & Rodic, 1985). In their system ovalbumin anti-ovalbumin immune complexes were used. They made measurements of the extent of the lattice formation of immune complexes using a laser nephelometric technique. These workers assessed the purity of their

monoclonal rheumatoid factor preparation by using double diffusion and immuno-electrophoresis against anti-whole human serum and anti-light chain antisera but not by SDS-PAGE. Measurement of RF concentrations in their preparations was determined using a commercial quantitative nephelometric method and this was approximately quantified in arbitrary units and compared to a latex agglutination titre.

In their kinetic experiments RF in the absence of complement caused a dose dependent enhancement of immune complex lattice formation as measured by their laser nephelometric method. This is in keeping with my findings of an increased rate of precipitation of complexes in the presence of RF (see Fig 3.17).

They also showed that complement was able to inhibit this RF dependent enhancement of precipitation and also that RF induced a dose dependent inhibition of PIP. In their experiments RF was able to inhibit these complement effects in the phase of precipitate formation. This finding is in keeping with my own demonstration of IgM-RF exerting its most potent inhibitory effect on PIP when the RF was present at the begining of the reaction ie. at the time of immune complex formation. Miletic and Rodic also postulated the reverse phenomenon and showed that activation of the classical pathway of complement, inhibited RF effects on precipitation. This reaction had previously been demonstrated in agglutination experiments in which complement activation could inhibit the interaction between RF or C1q and IgG coated latex particles (Hallgren, 1979).

I have shown previously (see Chapter 3, Sections 3 and 5) that whole RA serum not only inhibits PIP but also inhibits the consumption of C4 by complexes. The inverse correlation between inhibition of PIP and C4

consumption suggested that the mechanism of inhibition of PIP was due to inhibition of the binding or activation of C1 by the complexes. However in this set of experiments, examining the effect of IgM-RF and RA serum containing the same amount of IgM-RF on the consumption of C4 by immune complexes, a clear difference was noted (see Table 3.3). In the presence of purified IgM-RF immune complexes were able to activate the complement system normally and thus C4 was consumed in the reaction. However, when IgM-RF was present in the same quantity in heat-inactivated RA serum C4 consumption was reduced. Thus in whole RA serum the inhibition of C4 consumption observed cannot be explained by the presence of IgM-RF. The evidence points to the presence of other factors which influence the interaction of complexes with the complement system. This conclusion is reinforced by the observation that serum levels of IgM-RF (as measured by ELISA) showed only a low degree of correlation with the level of inhibitory activity (%) (Fig 3.18).

SECTION 8

The relationship between inhibition of PIP (inhibitory activity) and clinical aspects of disease.

In this study PIP and inhibition of PIP was measured in serum and SF of patients with rheumatic disease. The patients in this study were well characterised clinically and I attempted to relate PIP and inhibition of PIP to the detailed clinical aspects of their disease. The results of clinical studies detailed earlier in this thesis (see Chapter 3, Sections 2, 3 and 4) were mainly concerned with studying PIP and inhibition of PIP in the sera of unselected patients attending routine out-patient clinics.

The study was divided into two parts, A and B.

In Study A, PIP and inhibition of PIP was measured in the sera and SF of three groups of patients with arthritis.

In Group A-I 60% of the patients' sera displayed impaired PIP and conversely 87% of sera in this group showed increased inhibitory activity. These patients all had seropositive RA. Patients in Groups A-II and A-III suffered from seronegative arthritis and serum PIP and inhibition of PIP was in general normal in these two groups. These findings are in keeping with those described previously in this thesis where significant reduction in PIP and elevation of inhibition of PIP were virtually confined to seropositive RA patients (see Chapter 3, Sections 2, 3 and 4).

All SF's tested showed reduced PIP, the greatest amount of impairment of PIP being found in the seropositive RA group. PIP inhibitory activity was almost invariably present in SF. No patients were found who had normal levels of PIP, all showed marked reduction in PIP levels, the highest levels being of 20% or less. This may be a feature of all SF as no normal SF's were available for testing and thus no normal range was established. It may be that the impairment of PIP in SF is an artefact and may be due to the different physical characteristics of SF compared to serum eg. viscosity. The most likely reason is that low complement levels were found in the SF of these patients. Although SF PIP levels were low and SF complement levels were low there was no significant relationship between the individual SF PIP and SF C3, C4, Factor B or CH50 levels in the patients studied. However in contrast inhibition of PIP was not abnormal in all SF's tested (Fig 3.20). There have been no studies to date by other authors examining PIP in SF with which to compare my own results. In general terms the greatest amount of inhibition of PIP in SF was found in the seropositive RA group although elevated levels

were seen in some patients with seronegative arthritis. The only significant correlation between serum PIP and serum inhibition of PIP was found in group A-I, those patients with seropositive RA ($r = -0.77$ $p < 0.002$).

Interestingly there was no apparent correlation between serum levels of PIP or inhibition of PIP with any of the clinical parameters of systemic disease measured or with any of the laboratory indices of systemic disease activity. Indeed there was also a lack of correlation between serum PIP and serum inhibition of PIP with any of the clinical or laboratory tests of local disease activity in the inflammed knee joints examined. This suggests that PIP and inhibition of PIP may reflect aspects of the underlying rheumatoid process rather than day to day variation of clinical disease. It would be interesting to see if so called disease modifying drugs eg gold or penicillamine would alter these measurements (PIP and inhibition of PIP) and whether normalisation of these parameters mirrored improvement in clinical disease. One might expect that this would occur as disease modifying drugs are known to reduce the concentrations of RF found in RA (Wright & Amos, 1980).

It is important at this point to voice a caveat in the interpretation of results relying so heavily on correlation co-efficients which often occur in clinical/laboratory studies such as the one discussed here. Based on the concept of attempting to assess the importance of a new measurement and parameter (in my case PIP and inhibition of PIP) in clinical disease one often carries out extensive correlations of this new parameter with more standard clinical and laboratory assessments. The significance of these values are often quoted and from that value the importance of the correlate is judged. The inherent flaw in this kind of study is that if many correlations are carried out some of these will occur by chance and that the significance of the values obtained should be corrected for the number of tests applied (see Statistical

Methods, Chapter 2).

In this study no corrections for the numbers of tests applied were carried out and a relatively large number of correlations were done. For the purposes of this discussion I have therefore tried to concentrate on those correlations which were still significant after I had at a later stage applied correcting factors. For example in this study there were no significant correlations observed in the level of SF PIP or inhibition of PIP and the clinical and laboratory parameters measured.

Moving on to the second part of this clinical study which is probably the most important, I examined the relationship between PIP and inhibition of PIP in seropositive RA sera from patients with and without extra-articular features of their disease. The hypothesis is advanced in the introduction (Chapter 1) that failure of immune complex clearance mechanisms via impaired PIP might be responsible for the extra-articular manifestations of RA which are thought to be mediated by immune complexes. This concept can be directly addressed in the clinical studies presented in this section.

The hypothesis was supported by the finding that 82% of patients with nodular RA and 90% of patients displaying the extra-articular features of RA had impaired PIP and conversely inhibitory activity was found in all patients with extra-articular RA. The figure of 35% of sera with reduced PIP, found in purely articular RA, is lower than the 47% of RA sera with impaired PIP previously recorded in this thesis (see Chapter 3, Section 2). However, the number of patients in this study was lower, and in the previous study (see Chapter 3, Section 2) the RA patients were unselected for the presence of nodules or extra-articular features. 75% of patients with uncomplicated RA displayed inhibitory activity which is in keeping with that previously recorded

(see Chapter 3, Section 4). Not unexpectedly the mean level of PIP was lowest in those patients with vasculitis or nodules and this was reflected in the highest levels of inhibitory activity being also found in these two groups.

There was no highly significant correlation between levels of PIP and inhibition of PIP, and IgM-RF. Although the quoted r values do give significant P values these became less so when the correction for the number of tests used was applied (see above). The only strongly significant correlations were between levels of PIP and levels of PIP inhibitory activity (negatively correlated). This is not unexpected as it is probable that reduced PIP in RA serum is due to the presence of inhibitory activity in the serum (see Chapter 3, Section 3).

In the patients with vasculitis, PIP correlated strongly with inhibitory activity (negative correlation) and with levels of C4. It is difficult to explain why this association with C4 was confined to patients with RA and vasculitis but not found in the other two groups (B-I and B-II). It may be that C4 plays an important role in RA with vasculitis. However, C4 levels were not reduced in RA patients with vasculitis. C4 levels were comparable in groups B-I, B-II and B-III (Figure 3.23). Plasma C4 consists of the products of two separate genes C4A and C4B (O'Neill, Yang & Dupont, 1978) each of which binds covalently to surfaces but C4A binds more efficiently to amino groups whereas C4B binds more efficiently to hydroxyl groups (Law, Dodds & Porter, 1984). C4A has been shown to be more effective in PIP (Schifferli et al, 1986) than C4B and thus it would be of interest to measure the relative serum concentrations of C4A and C4B in these three groups of RA patients (Groups B-I, B-II & B-III). In group B-I, those patients with purely articular RA, serum inhibitory activity was found to correlate with serum C3 and factor B levels $r = 0.46$ and $r = 0.61$ respectively ($p < 0.002$ in both cases). I am unable to explain this

association and indeed when corrected for the number of tests applied this association became much less significant.

The most striking feature of this study was the lack of correlation between inhibition of PIP and any of the rheumatoid factor classes (IgM, IgG and IgA) although it was noted that in the vasculitic group PIP was inversely related to IgG-RF concentration. This may indicate that IgG-RF is potentially more important in the development of the vasculitic features of RA as has previously been reported (Scott et al, 1981). This lack of association of PIP inhibitory activity with serum levels of IgM-RF was demonstrated earlier in this thesis (see Chapter 3, Section 7). In a large number of RA patients (157 sera) there was no clear relationship between IgM-RF levels and % PIP inhibitory activity. This argues strongly against IgM-RF having a major role to play in PIP inhibitory activity in RA serum. Thus in this study, although inhibition of PIP is strongly associated with RA complicated by vasculitis which supports the hypothesis proposed in the introduction (see Chapter 1), the evidence suggests that in whole serum the various RF classes have at most only a limited role to play in the expression of inhibition of PIP in RA sera.

SECTION 9

Purification of inhibitory activity by Sucrose gradient ultracentrifugation and IgG Sepharose affinity chromatography.

I have shown previously (Chapter 3, Section 6) that the majority of inhibitory activity in RA serum resides with IgM-RF. The results in this study in general support this concept. Inhibitory activity in RA serum was found in the higher M.W. fractions on the sucrose gradient along with IgM, and these fractions

were strongly RF positive. This RF positive fraction bound to IgG Sepharose and the inhibitory activity could be eluted with 2M guanidine hydrochloride (see also Chapter 3, Section 6). The presence of heat-labile antagonists to the inhibitory molecule operative in the whole RA serum is supported by the finding of greater expression of inhibitory activity in the whole heat inactivated RA serum as has been noted before (see Chapter 3, Section 3 and Table 3.1).

An important finding in this study was that similar treatment of normal serum (sucrose gradient fractionation and IgG Sepharose chromatography) revealed the presence of an inhibitory molecule in normal serum. This molecule appeared to have a similar sedimentation coefficient to that found in RA serum (see Figure 3.27). Fraction 2 from the gradient contained material which inhibited PIP. This inhibitory activity in fraction 2 was completely abrogated by the addition of fraction 5. This must mean that fraction 5 contained an antagonist to the inhibitory activity found in fraction 2 and that under normal circumstances inhibitory activity in normal serum is suppressed by this antagonist. This ability of fraction 5 to antagonise the inhibitory activity in fraction 2 was similar in both normal and RA serum. The mixing experiments (described in Chapter 3, Section 9) were carefully adjusted for final volume of reagents and therefore the effect was not one of dilution. The mechanism whereby fraction 5 was able to antagonise the inhibitory activity of fraction 2 is so far unexplained.

The effect of heat-inactivation (HI) on the final expression of inhibitory activity in both sera is interesting and may provide some clues to this problem. Inhibitory activity in normal serum was heat-labile whereas in RA serum it was heat-stable (Fig 3.26). The antagonists to inhibitory activity were both heat-labile and addition of (HI) fraction 5 did not abrogate

inhibitory activity (Fig 3.28). This was further supported by the finding of increased inhibitory activity expression in heat-inactivated RA sera. If, however, one considers inhibitory activity in normal and RA sera to be due to the presence of the same factor one must postulate that in RA sera (sero-positive for IgM - RF) the factor responsible for inhibitory activity is in some way protected from heat-inactivation by the presence of the IgM-RF. It may be however, that there is a heat-labile factor which is present in normal serum and rheumatoid serum and a heat-stable factor which is only present in rheumatoid serum. This heat-stable factor would of course be IgM-RF.

The results of later studies in this chapter lend some support to this concept. Inhibitory activity (from fraction 2) of normal and RA serum bound to IgG Sepharose but the elution profile was, however, different. RA inhibitory activity, as expected, was almost entirely confined to the guanidine hydrochloride eluted pool where it was found with IgM-RF. In normal serum, however, the majority of inhibitory material was found in the fraction eluted with 0.4M NaCl which did not contain IgM-RF.

The concept that IgM-RF is responsible for the inhibiton of PIP found in RA serum is somewhat refuted by the demonstration of an apparently similar inhibitory activity in normal serum which is quite clearly devoid of IgM-RF activity as measured by Rose-Waaler titration and ELISA. This does not mean that IgM-RF is not responsible for inhibition of PIP in RA serum, as I and others (Balestrieri et al, 1984) have shown that purified IgM-RF does indeed inhibit PIP (see Chapter 3, Section 7) but it does cast doubt on the central role of IgM-RF in this process. However, both the IgM-RF preparations used in this study and those used by other workers (Balestrieri et al, 1984) may not have been pure enough, and other molecules may have been present which had gone undetected by our analytical methods. Indeed it has been reported

(Ahmed & Whaley, 1988) that a 60kD plasma glycoprotein, termed gp60, can account for the inhibition of PIP in normal serum and RA serum.

Recently, O'Sullivan, Amos & Williams (1986) have reported their findings on rheumatoid factor and its influence on complement-mediated inhibition of immune precipitation. In agreement with my own findings and those of Balestrieri et al, (1984), they showed that isolated monoclonal IgM-RF displayed a dose-dependent inhibitory activity. However, with polyconal IgM-RF this inhibitory activity was less marked and was not observed in the whole RA serum. It was also shown that heat-aggregated IgG could inhibit the binding of RF. This study supports the results of my own experiments in which IgM-RF in serum was less potent than purified IgM-RF (see Chapter 3, Section 7). I have not carried out measurements on immune complex levels in my own serum samples and am therefore unable to comment on the role of immune complexes in RA serum as being a major influence on the inhibition of PIP. Further evidence against IgM-RF having a central role in inhibition of PIP in RA serum has also come from this study by O'Sullivan and her colleagues. She found a poor correlation between inhibition of PIP and IgM-RF as measured by ELISA in a large group of patients, thus confirming my own findings (see Chapter 3, Section 7, Fig 3.18).

In view of the above, the demonstration of another protein band in addition to IgM-RF (Fig 3.31) in the purified inhibitory fraction from RA serum eluted from IgG Sepharose is of considerable interest. I have been unable to separate this approximately 60 kD protein from IgM-RF and as such cannot state that this protein is responsible for the inhibition of PIP in RA serum. SDS-PAGE analysis (Fig 3.30) showed that a protein of similar size could also be demonstrated in the purified inhibitory fraction from IgG Sepharose chromatographed normal serum and it is therefore tempting to suggest that

these proteins may play a role in the inhibition of PIP in RA and normal serum.

In support of this notion Ahmed & Whaley (1988) have reported the isolation and purification from normal serum of an inhibitor of PIP which was a 60kD molecular weight glycoprotein (termed gp60). This protein was found in normal serum and, in greater quantities, in RA serum. The serum levels of gp60 correlated very strongly with the ability of that serum to inhibit PIP. Gp60 appears to be a normal plasma protein which inhibits PIP by dose dependent inhibition of C1q binding to IgG, thus preventing complement activation. It appears to bind to the Fc piece of IgG and so inhibit the binding of C1q to the IgG molecule.

The purification method used by Ahmed and Whaley (1988) to isolate their gp60 molecule was in some respects similar (IgG Sepharose affinity chromatography) to that used by me to isolate a 60kD molecule. The strength of their purification scheme was that they attempted their isolation from normal serum which could be obtained in relatively large quantities. This of course avoided the contaminating and sometimes confusing influence of rheumatoid factors which were encountered in my purification procedures performed on RA serum. However, my approach was justified on the grounds that the inhibitor of PIP was first demonstrated in seropositive RA sera and only at a later stage was I able to demonstrate the presence of an inhibitor in normal serum. A further criticism of my studies is that my SDS-PAGE analysis was probably insufficiently sensitive to detect other contaminating proteins. If other gel techniques and staining procedures were used eg Laemmli system with silver staining, my own 'purified' preparations could possibly have been shown to contain other proteins including the 60kD molecule described above. Indeed this criticism could be levelled at other

workers in this field concerning IgM-RF and its actions on PIP and solubilisation (Naylor et al, 1979; Balestrieri et al, 1984 & O'Sullivan et al, 1986). None of these workers' IgM-RF preparations were shown to be pure by the same stringent standards as those adopted by Ahmed and Whaley (Ahmed & Whaley, 1988) and thus all could have contained varying amounts of gp60 which could explain their findings.

The results of Ahmed & Whaley remain to be confirmed but it does appear that their gp60 molecule is an important contribution to our understanding of the mechanism of inhibition of PIP by RA serum. Certainly the presence of this molecule explains the relatively weak correlation between serum levels of IgM-RF and inhibition of PIP.

CONCLUDING DISCUSSION

The studies described in this thesis concern the specific interaction between complement and immune complexes at the time of their formation. Complement, reacting with an immune complex immediately after the formation of an immune complex prevents the formation of insoluble, and therefore potentially tissue-damaging aggregates which may be deposited in vessel walls or remain localised in tissues. This complement-mediated prevention of immune precipitation reaction (PIP) allows for the formation of complement-processed soluble complexes. In Chapter 1 I have outlined the complex physiological mechanisms whereby circulating immune complexes react with complement and are transported to cells of the fixed macrophage system, some via complement receptors, for safe disposal. A major role for complement in this process appears to be opsonisation of immune complexes. The complement reacted immune complexes are coated with C3b in the PIP and solubilisation reaction and can subsequently be taken up by cells bearing CR1. Complexes rendered small and soluble by the PIP process bind poorly to erythrocyte CR1 in contrast to solubilised complexes which bind well. This is probably related to the fact that because the number of CR1 on erythrocytes is low, larger amounts of C3b would be required for stable multipoint binding to occur (Arnaout et al, 1981).

To summarise, antigen-antibody complexes can be formed either in the intravascular or extravascular compartments. Complement is activated at the time of immune complex formation and the complex is coated with C3b. Under normal circumstances a large number of C3b molecules are bound to the complex following activation of the classical and subsequently the alternative pathway (the C3b feedback loop). Immune precipitation and the formation of large lattices is prevented (Schifferli et al, 1980). The next important step is the reaction of opsonised complexes with CR1 on the surfaces of various cells. The binding of opsonised complexes to CR1 (mainly

on red blood cells) prevents the immune complex reacting with nearby structures (eg vascular endothelium) and it also allows for the carriage of complexes to the fixed macrophage system. If formed in the extravascular space the immune complexes are removed by the lymphatics and are trapped in the regional lymph nodes (Pepys, 1976).

Erythrocytes with bound immune complexes then transport the complexes to the cells of the fixed macrophage system. The complexes are transferred to macrophages by as yet unknown mechanisms but probably the high density of Fc receptors and CR1 receptors on the macrophages enable a stronger binding for the complex to the macrophage than to the erythrocyte with its relatively low concentration of CR1. Defects at any level of this physiological immune complex clearance process, summarised above, could lead to immune complex disease. I have studied the earlier stages of this process namely, prevention of immune precipitation by complement at the time of immune complex formation.

This physiological system for handling immune complexes can fail for several reasons, eg a genetic or acquired deficiency of complement, a failure of the various classes of antibody to fix complement, a deficiency of CR1, or a depletion or other occupation of CR1. An impairment of the fixed macrophage system itself either because of disease or because of functional overload can also result in defective immune complex clearance. Under normal circumstances because of the speed of activation of the classical pathway to prevent the formation of precipitating complexes (initially by the binding of macromolecular C1 followed by covalent binding of C3b and finally recruitment of the alternative pathway) immune complexes probably very rarely are able to form large insoluble aggregates which will be easily deposited in tissues and cause disease. If formed in the blood immune

complexes will circulate possibly bound to erythrocyte CR1 (Cornacoff et al, 1983) and be removed from the circulation by the cells of the fixed macrophage system. Thus their tendency to be deposited in the microcirculation and produce disease is reduced. When formed in the tissues their small size allows them to diffuse away from the site of formation and thus the establishment of a focus of persistent inflammation is prevented.

When PIP fails a large lattice is built up with the formation of insoluble immune aggregates. This can be compensated for by the alternative pathway mediated solubilisation process (see Chapter 1). Covalent binding of C3b to the antigen and antibody reduces the various forces (Fc-Fc interactions, antigen-antibody bonds) holding the lattice together (Miller & Nussenzweig, 1975; Takata et al, 1984 & Fujita et al, 1981). Although solubilisation can deal with approximately ten times less immune complexes than PIP and generates a greater local inflammatory reaction (Schifferli, Steiger & Paccaud, 1986) it is nevertheless a potent back up system if PIP fails for any reason. It (solubilisation) is, of course, the only system so far described which limits immune complex size and allows for opsonisation in the situation where immune complexes for whatever reason are unable to fix C1 and initiate classical pathway activation. Thus IgA immune complexes which are unable to activate the classical pathway and are somewhat ineffective in fixing C3 have been shown to bind poorly to erythrocytes, and subsequently are not cleared by the liver (Waxman et al, 1986).

A wide range of erythrocyte CR1 numbers is found in normal individuals (Wilson et al, 1982; Walport et al, 1985; Holme et al, 1986). The number of CR1s on normal erythrocytes has been reported to be controlled by two autosomal co-dominant alleles (Wilson et al, 1982) based on the finding that erythrocyte CR1 numbers were distributed trimodally. This same group of

workers have also shown that a restriction fragment length polymorphism of the CR1 gene correlates with erythrocyte CR1 numbers (Wilson et al, 1986). Other studies have suggested that the inheritance of erythrocyte CR1 in normal individuals was polygenic (Walport et al, 1985; Ross et al, 1985; Holme et al, 1986) based on the demonstration of a continuous distribution curve for erythrocyte CR1. As mentioned above failure of the physiological handling of circulating immune complexes can occur when there is an impairment of the binding of complement reacted complexes with the complement receptor. Reduced numbers of CR1 are found on the erythrocytes of patients with SLE. Initially thought to be an inherited defect (Wilson et al, 1982) it has now been shown quite clearly that the reduction of CR1 on erythrocytes in SLE is due to the disease process (Walport et al, 1985; Holme et al, 1986). In experiments using blood transfusions, transfusing erythrocytes into patients with SLE and low numbers of CR1 on their erythrocytes resulted in the rapid loss of CR1 from the transfused erythrocytes (Walport, Ng & Lachmann, 1985).

In the case of rheumatoid arthritis reduced levels of CR1 are found on RA erythocytes, the reduction being much less however than that found in SLE (Iida, Mornaghi & Nussenzweig 1982; Taylor et al, 1983; Yoshida et al, 1985 & Ross et al, 1985). There is also evidence suggesting a defect in the mononuclear phagocyte system (MPS) in RA; this impairment of MPS function has been suggested by the demonstration of defects in the clearance of radio-labelled IgG anti-Rh (D)-sensitised autologous erythrocytes (Gordon et al, 1981; Fields et al, 1983 & Malaise et al, 1985). A more recent study has examined MPS function in RA using kinetic analysis to allow evaluation of both complement-mediated and Fc-mediated clearance processes. This study of nine RA patients revealed a specific complement-mediated clearance dysfunction which was associated with normal Fc receptor function and

normal complement levels (Kimberly, Meryhew & Runquist, 1987). Therefore it appears that complement-mediated immune complex clearance defects can occur in RA independent of serum complement levels and Fc receptor function.

I will now try and place into perspective my own results in the light of the above. The hypothesis formulated at the outset of my work was related only to the early stages of this intricate physiological system for immune complex handling in the human and addressed the problem of failure to make immune complexes small and soluble by PIP. The question was asked whether a failure of PIP was present in any of the rheumatic diseases in which immune complexes had in some way been implicated in pathogenesis. If this was the case what was the mechanism involved and what was the clinical relevance, if any, of this failure of PIP? I maintained that a failure of PIP was a possible mechanism which might allow the formation of large insoluble complexes in the circulation with subsequent deposition in tissues thus permitting immune complex mediated manifestations of the disease. A failure of PIP would of course not only make the complexes insoluble but because of the reduction of the amount of C3b on the surface of the complex such a complex would clearly be unable to bind to CR1 effectively whether there were normal numbers of CR1 or not. I have shown that failure of PIP was due to the presence of an inhibitor (Chapter 3, Sections 2 and 3). If the inhibitor bound to the Fc piece of the complex, the Fc piece would not then be able to bind to an Fc receptor on the surface of a phagocytic cell. Therefore it can be seen that a failure of PIP with reduced opsonisation and possible Fc blockage could result in a very serious defect in the defence sequence of immune complex handling. Thus defects early on in this sequence would be expected to lead to a much more serious problem than selective defects lower down the defence cascade.

My results in general support this concept. A failure of PIP was found in RA sera and this was shown to be related to the presence of an inhibitor (see Chapter 3, Sections 2 and 3). Failure of PIP and increased inhibitory activity was found in only a proportion of RA sera (47%) but was strikingly associated with the presence of the extra-articular features of RA (see Chapter 3, Section 8). High levels of PIP inhibitory activity were also found in the SF of RA patients. Both these findings support the concept of a failure of PIP in RA. The presence and level of inhibitory activity was most closely related to situations in RA where immune complex levels were high (eg SF and vasculitic disease). Further work on this aspect requires to be performed relating the inhibitory activity to levels of immune complexes in serum and SF. It would be interesting to know whether the level of inhibitory activity was related to the circulating immune complex load or to any specific type of immune complex.

The clinical studies carried out by me were limited. A number of further investigations are necessary to examine the relationship between inhibition of PIP, PIP and disease activity. These would require more long term studies, eg

1. A one or two year prospective study in RA patients examining the changes in PIP and inhibition of PIP in relation to clinical disease activity.

2. A further prospective study comparing the changes in PIP and inhibition of PIP with the clinical and laboratory parameters of disease activity in a group of RA patients undergoing second-line drug therapy with gold, penicillamine or chloroquine.

3. Further examination of the vasculitic RA patients, noting whether impairment of PIP or inhibition of PIP is related to the time of onset of vasculitis and whether treatment of the vasculitis by cytotoxic drugs or corticosteroids will alter PIP and inhibition of PIP in these patients.

In addition to studying the clinical relevance of inhibition of PIP in RA, much of the work in this dissertation was concerned with trying to establish the nature of the molecule(s) responsible for inhibition of PIP in RA sera. I approached this problem by attempting to isolate and purify the molecule responsible for inhibition of PIP from RA sera. Initial results pointed strongly to IgM-RF being the responsible protein and indeed I and others found that purified IgM-RF was able to inhibit PIP (Balestrieri et al, 1984; O'Sullivan et al, 1986) (see Chapter 3, Section 6). However, when I compared purified IgM-RF and IgM-RF in whole RA serum to see if all the inhibitory activity in RA serum could be attributed to the IgM-RF certain discrepancies emerged which argued against IgM-RF being solely responsible for inhibition of PIP in RA serum (see Chapter 3, Section 7).

The development of ELISA techniques for sensitive and specific measurement of RF classes was a major factor in allowing me to dissect the relationship between the various RF classes (IgM, IgG and IgA) and inhibition of PIP. I was able to show quite clearly that IgM-RF could not by itself be completely responsible for the inhibition of PIP in RA sera (see Chapter 3, Section 7; Fig 3.18 and Chapter 3, Section 9). This finding has been confirmed by other workers (O'Sullivan et al, 1986).

In order to try and resolve these problems I undertook further studies on purification of inhibitory activity using sucrose density gradient ultracentrifugation and subsequent affinity chromatography. The results

showed that inhibitory activity could be demonstrated in normal serum as well as RA serum. Normal serum was devoid of IgM-RF as measured by ELISA and Rose-Waaler titration (see Chapter 3, Section 9). My final purification revealed a 60kD molecule which could conceivably be implicated as the molecule responsible for inhibitory activity.

In retrospect the confusing influence of RF could have been removed if I had used normal serum in my purification procedure initially once it became clear that IgM-RF could not account for all of the inhibitory activity in RA serum. Recent work by Ahmed and Whaley (1988), has demonstrated a 60kD glycoprotein (gp60) which has been shown to inhibit PIP. It is probable that the 60kD molecule which I detected is the same as that described by Ahmed and Whaley.

Much work is still needed to unravel the complex relationship of a putative 60kD inhibitor of PIP and IgM-RF and the other classes of RF both in serum and in SF. As regards the mechanism of action of an inhibitor I was able to show, using C4 consumption studies that an inhibitor of PIP in RA serum and SF probably acted by preventing complement activation (see Chapter 3, Sections 5 and 7). This work has recently been extended and three reports have confirmed my findings, namely that an inhibitor of PIP acts by preventing complement activation by inhibiting the binding of C1q to antibody (Niven & Whaley, 1986; Ahmed & Whaley, 1988; O'Sullivan et al, 1987). O'Sullivan and colleagues showed this effect with purified monoclonal and polyclonal IgM-RF (O'Sullivan et al, 1987).

Gp60 is present in normal serum (Ahmed and Whaley, 1988). Certain questions are at once raised eg , what role does it serve in normal physiological control of immune complex processing in man? Does it occur in other species? Where

is it synthesised? Discovering its cell of origin will allow for in vitro cell culture studies to be set up to establish its biosynthetic patterns and to assess the factors which regulate its production.

As outlined at the beginning of this closing discussion, it is clear that the processing of potentially phlogistic immune complexes is an intricate and complicated phenomenon in which complement mediated prevention of immune precipitation is only one aspect. My results support the hypothesis that failure of PIP in RA due to the presence of serum inhibitors may allow for the formation and deposition of potentially damaging immune complexes and this may contribute to some of the pathological features of RA. However, in vitro studies cannot be taken in isolation especially in what appears to be a very complicated in vivo phenomenon. It is tempting to suggest that the clinical correlations between disease and inhibition of PIP are causally related. In this dissertation I have only examined one aspect of human immune complex processing namely prevention of immune precipitation and have not investigated the role played in this process by, for example, the complement receptor (CR1), other immune complex systems especially human immune complexes, and genetic influences.

Whether a raised level of an inhibitor of PIP in RA and possibly other diseases is of pathogenetic importance or is just a consequence of that disease remains to be ascertained. It has been shown that the HLA-DR4 gene is most strongly associated with the presence of extra-articular RA and Felty's syndrome (Westedt et al, 1986). Both of these subgroups of RA are associated with circulating immune complexes (Zubler et al, 1976; Hurd et al, 1979). I have shown that inhibition of PIP is most strongly associated with vasculitic RA (see Chapter 3, Section 8). Careful immunogenetic studies are required to delineate the relationship between the presence and level of the

inhibitor of PIP and HLA-DR4 or an extended haplotype containing DR4 and complement alleles. This would be an important aspect to study as the genes for C2 and C4 are found on the short arm of chromosome number six within the region of the major histocompatibility complex.

C4A null alleles have been shown to be associated with SLE in humans (Fielder et al, 1983; Howard et al, 1986). It is C4A rather than C4B which is important in the complement mediated PIP reaction (Schifferli et al, 1986). However, somewhat paradoxically, Felty's syndrome has recently been shown to be associated with C4B nulls rather than C4A nulls (Thomson et al, 1987), so it would seem that at least in this subgroup of RA patients that partial deficiency of C4A is not contributing to disease pathogenesis through a mechanism involving PIP as measured by the assay described in this thesis. I have not studied PIP in Felty's syndrome as such and it may be that the type of immune complex found in Felty's syndrome is different to that in RA vasculitis without Felty's syndrome. Further studies are required on this point looking for C4A or C4B nulls in a group of RA vasculitis patients without Felty's syndrome.

It is worth remembering, however, that C4A deficient heterozygotes will almost certainly have enough C4A in their serum to enable the PIP reaction to proceed as it has been shown that a very small amount (approximately 1% of normal levels) of purified C4 is all that is required to normalise PIP in human C4 deficient serum (Schifferli et al, 1985). This argues against C4 allotype deficiency, by causing impaired PIP, as being an important factor in the increased susceptibility to immune complex disease noted in these complement deficient patients. The C4 null genes may only be a marker for a disease susceptibility gene. In view of the fact that the genes for C4 and C2 are found within the major histocompatibility complex, the association of

deficiencies of C2 and C4 with immune complex diseases may be explained by the genetic linkage of these abnormalities with aberrant immune response genes which are responsible for the auto-immune disorders. Indeed null alleles are found in extended haplotypes which are known to be associated with auto-immunity (Welch et al, 1985).

Failure or impairment of PIP found in RA, especially complicated by vasculitis, occurs secondary to the prescence of an inhibitor of this process as described in this dissertation. Studies on the genetics of this inhibitor of PIP are needed to delineate its relationship to the other genetic markers in RA. This could be carried out by genetic family studies in RA as well as attempting to clone the gene responsible for the putative 60kD inhibitory molecule, utilising existing DNA technology. It would be interesting to see if the gene coding for the 60kD molecule described above was also to be found within the major histocompatibility complex.

Further studies concerning the nature of the 60kD (or gp60) molecule are required. Radiolabelling will allow for experiments to be set up to measure the metabolic turnover of this molecule in normal and in disease states. The development of radioimmunassays and/or ELISA will ascertain its role in disease by relating the levels measured to the clinical features observed. Work on these metabolic studies of gp60 could be carried out initially in animal models of arthritis. Particularly, what is the relationship between gp60 and rheumatoid factors of the various classes?

1. Does gp60 influence the binding of RF to IgG?
2. Is gp60 made by the same cells as RF? This would require tissue culture experiments to study the production of RF by cells and to look for concomitant production of gp60 under the same conditions as those which

enhance RF production. Also what is the influence of adding gp60 itself to these RF producing cells in culture?

3. In vivo studies could be carried out to study the production of gp60 in the experimental animal under conditions which are known to stimulate RF production (eg lipopolysacchride induced RF production in mice).

To conclude I have demonstrated that serum factors impair PIP in RA and that these are related to the extra-articular manifestations of disease. I have explored the relationship between PIP and rheumatoid factor and studied the mechanism of impairment of PIP in RA serum and SF. I have demonstrated the presence of a new molecule in serum and its possible implication in the impairment of PIP has been discussed in the light of recent findings.

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