DISORDERS OF SERUM PROTEINS IN HUMAN DISEASE

A thesis submitted for the degree of

Doctor of Philosophy

of the University of Oxford

by

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Oxford

Michaelmas Term

1975

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ACKNOWLEDGEMENTS

To my supervisor, Dr I.C.M. MacLennan, I would express my sincere thanks for his constant guidance and enthusiasm during my period of research in his laboratory. I would also like to thank Professor P.B. Beeson and his successor Professor D.J. Weatherall for their interest and willingness in allowing me bench space in their Department.

Many fellow laboratory members provided constant and encouraging stimulation, not to mention their serum! These included Peter Hollingsworth, Diana Gale, Leslie Tupchong, Frances Gotch, Julian Clarke, Brian Harding, Danielle Chassoux and Janet McBurney. To them I owe my gratitude.

The members of the Medical Illustrations Department provided many of the diagrams used in this thesis - their help was much appreciated. Miss G. Peat of. Medical Records was of great assistance in retrieving case notes and Mrs A. Houghton provided a very efficient secretarial service.

I am much indebted to Brasenose College for supporting me financially during my period in Oxford and allowing me to share a little in the college life.

Finally I owe much to my new wife who continually supported and encouraged me during the ups and downs of these studies. To Lesley I say a loving thank you.

ABSTRACT

The assessment of serum proteins has become of great importance in the diagnosis and management of many human diseases. Two methods currently used for this purpose are electrophoresis and immunoelectrophoresis both of which separate serum proteins according to their charge. However, in recent years two simple techniques have become available which separate proteins principally according to their molecular size. These methods are sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDSPAGE) and gel filtration chromatography. The aim of this thesis was to examine these methods and to see if they could be profitably applied to the analysis of serum proteins. In particular, emphasis was placed on the assessment of the molecular size of the serum immunoglobulins in certain diseases.

In Chapter one a general introduction was given about the serum proteins and several specific protein disorders were considered.

In Chapter two the methods of SDSPAGE and gel filtration were described. In addition, these methods were used in an analysis of various pathological sera. The sera of eight patients with multiple myeloma were found to contain paraproteins of atypical molecular size. These were considered to be paraproteins with heavy chain deletions or light chain dimers.

In Chapter three a study was undertaken of the clinical and immunological features of 25 patients with IgA myeloma. These patients were divided into two groups on the basis of the degree of paraprotein polymerization. The first group comprised those patients in whom the IgA paraprotein was greater than 50% polymerized, whilst in the second group the paraprotein was

predominantly monomeric. No clinical or pathological differences were seen between the 'polymeric' and 'monomeric' groups of myeloma apart from that directly attributable to the physicochemical effect of the paraprotein polymerization. Thus, five patients out of 11 of the 'polymeric' group developed the hyperviscosity syndrome, whilst no patients in the 'monomeric' group developed this complication. The concentration of the paraprotein during the course of the disease was comparable in both groups.

Chapters four, five and six were concerned with the detection, biological properties and clinical significance of circulating immune complexes containing IgG. To detect these complexes the method of gel filtration was used. The rationale for using this method was based upon the fact that complexed IgG has a larger molecular size than non complexed or monomeric IgG and thus elutes in earlier fractions of the chromatogram. However, SDSPAGE is of no use in detecting complexed IgG as SDS (an anionic detergent) dissociates immune complexes.

In Chapter four a general review was undertaken of the implications of circulating immune complexes and the methods used for their detection. It was concluded that while immune complexes have been implicated in the pathogenesis of a large number of human diseases, there is no simple and sensitive method currently available to detect all forms of complexes.

In Chapter five IgG containing complexes were detected in the majority of the sera of 35 patients with rheumatoid arthritis. These complexes were small, eluting between IgG and IgM, and were not seen in the sera of healthy subjects. A monomer to complexed IgG ratio was used for quantitation of the complexes. This ratio was derived from the IgG profile obtained by

gel filtration of the serum. The quantity of complexes correlated significantly with inhibition obtained by the rheumatoid sera of cytolysis in vitro of IgG sensitised target cells by K cells from human peripheral blood. A significant inverse correlation was observed between the quantity of serum complexes and the chemotactic index of the circulating polymorphonuclear leucocytes obtained from the same rheumatoid patient. This suggests that these complexes may be implicated in the suppression of polymorphonuclear leucocyte chemotaxis observed in patients with rheumatoid arthritis.

There was no correlation between the quantity of the complexes in the sera and other clinical, haematological and biochemical measurements. In addition to these rheumatoid patients the presence of complexes in the sera of patients with other joint diseases was also assessed. In some of these latter patients complexed IgG was found.

In Chapter six four groups of patients were studied for the presence of circulating immune complexes. The first group comprised 19 patients having newly diagnosed acute myeloid leukaemia. The IgG in the majority of these patients' sera eluted in a normal position and complexed IgG was not detected.

The second group was comprised of 11 patients with cutaneous vasculitis. Serum from these patients was examined for cryoglobulin and for complexed IgG. It was observed that complexed IgG was present in six of these sera and was generally associated with the presence of cryoglobulin and with a significant inhibition of K cell mediated cytotoxicity but not always so. In addition, in two patients active vasculitis was seen without the presence of either a cryoglobulin or complexed IgG. In the analysis of the cryoglobulins it was concluded that SDSPAGE might be a rewarding method.

The third group consisted of 26 patients with multiple sclerosis. The percentage inhibition of K cell mediated cytotoxicity by the serum of these patients was similar to that obtained by serum from patients with other neurological disorders. In addition no significant inhibition of cytotoxicity was obtained with any of the cerebrospinal fluid of these patients. Immune complexes are unlikely to be present in the serum or cerebrospinal fluid of these patients.

The fourth group consisted of 7 patients with a variety of diseases. One serum from a woman suffering from severe pre eclampsia was found to have complexed IgG.

In Chapter seven SDSPAGE was used to analyse the blood/CSF barrier in 27 patients with non demyelinating or non infectious neurological diseases. It was concluded that this barrier acts as an efficient molecular sieve to plasma proteins and that SDSPAGE of CSF was a simple means of assessing this barrier.

From the examples given above it was generally concluded that the assessment of the molecular size of serum proteins can provide information supplementary to that obtained using the current electrophoretic methods. It is hoped that now simple techniques are available, the assessment of protein molecular size will be more frequently performed in routine clinical practice.

The topic of Chapter eight deviated from the central theme. In this chapter an examination was undertaken of the cerebrospinal fluid immunoglobulins in various neurological diseases. An increase in the CSF IgG/albumin quotient was observed in 19/36 (53%) cases of definite multiple sclerosis, in 13/47 (28%) cases of probable or possible multiple sclerosis, in

6/9 cases of proven herpes simplex encephalitis, in 3/4 cases of neurosyphylis, in 1/1 case of subacute sclerosing panencephalitis, in 2/9 cases of other neurological infections and in 2/12 cases of polyneuritis when compared with a group of 236 patients having other neurological diseases. In many of these patients a comparison was also made between the IgG/albumin quotient and the CSF kappa/lambda ratio and the CSF and serum viral antibody titre. It was concluded that the measurement of CSF IgG/albumin quotient is a valuable adjunct in neurological diagnosis and can be supplemented by the measurement of the CSF kappa/lambda ratio or antibody titres to certain viruses.

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CHAPTER ONE

INTRODUCTION TO SERUM PROTEINS

Historical introduction

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CHAPTER ONE

INTRODUCTION TO SERUM PROTEINS

Serum proteins are an immensely complex family of macromolecules whose composition is remarkably sensitive to changes in the milieu of the body. As these sympathetic changes reflect many of the disturbances occurring in disease, measurement of serum proteins provides an aid to the clinical diagnosis and management of many human disorders.

The serum proteins are readily available for study by simple venipuncture. But it is only in recent years that technical advances have made their routine study either financially or technically feasible at a clinical level. This, therefore, explains the lack of information about many of these proteins and also the uncertainty of the clinical significance of known protein disorders. With these points in mind the subject of this thesis 'disorders of serum proteins in human disease' can be immediately presented. It is hoped that the conclusions reached in this thesis will further contribute towards our understanding of these proteins both in health and disease.

In subsequent chapters of this thesis, several illustrations will be given of specific disorders of serum proteins and the clinical significance of these disorders will be considered. In each of these chapters the disorder will be introduced and a detailed review will be undertaken of the relevant literature. However the purpose of this introductory chapter is to present some general background information about the serum proteins.

The general topic relating to serum proteins is vast and for additional

information and a comprehensive literature review the reader is referred to the excellent text book by Schultze and Heremans (1966) and to the texts by Allison (1974), Turner and Hulme (1971) and Sandor (1966).

Historical introduction (see Sandor (1966), Chapter one)

Emil Fisher in the early part of this century provided a fresh and invigorating stimulus to the study of serum proteins when he demonstrated the importance of the peptide bond in the structure of these proteins and emphasized their heterogeneity.

The next major advances were dependent on the technological achievements of the Swedish researchers, Theodor Svedberg and Arne Tiselius. These workers were the first to apply two analytical methods which had fundamental implications in the characterization of individual serum proteins. Svedberg showed that serum proteins sedimented in gravitational fields (produced experimentally in the analytical ultracentrifuge) at rates which were related to the size and conformation of the protein. Thus for the first time it was possible to give a precise physical measurement to a particular serum protein. For example Svedberg found the sedimentation coefficient of albumin was 4.21 S (where S = unit of sedimentation) and he was able to calculate the molecular weight from this value as being 66,740 daltons. This was indeed a very accurate estimation.

Tiselius, a former pupil of Svedberg, provided a further impetus to the study of serum proteins when, in 1937, he separated serum proteins by means of an electric field (moving boundary electrophoresis). In this procedure serum proteins were separated in a phosphate buffer into the classic A, α, β and γ

regions; albumin (the weak acid soluble protein) being found in the A region, and the globulins (weak acid insoluble) in the other regions. Antibody was found in the γ region. Subsequent investigators quickly modified this technique when they used paper, cellulose acetate or agar gel as the electrophoretic medium.

Thus by the mid 1940's specific serum proteins could be characterized both by their size and their electrophoretic mobility.

The third major stimulus to protein research was provided in the early 1950's by the immunochemical analytic methods of Elek, Oudin, Ouchterlony (immunodiffusion) and Grabar and Williams (immunoelectrophoresis). In both these techniques antiserum produced in experimental animals was allowed to precipitate with its specific serum protein in an agar gel medium. By using these immunochemical techniques it was possible to characterize serum proteins by their antigenicity (antigenic determinants) and further studies indicated the complex relationship between various serum proteins (e.g. lipoproteins and immunoglobulins) and the large variety of different proteins.

The next 15 years witnessed a number of refinements in these three basic methods together with the development of several new techniques. By using these latter techniques it was possible to isolate many serum proteins with high degrees of purity. These techniques included ion exchange chromatography pioneered by Sober and Peterson (1954), gel filtration chromatography (Porath and Flodin, 1959), electrophoresis in high resolution media such as polyacrylamide (Davis, 1964), isoelectric focussing and affinity chromatography. With the preparation of purified serum proteins it was then a relatively easy task to study these proteins in some depth.

Of recent years it has been the objective of several research groups to analyse the chemical structure of many of the serum proteins using the method of amino acid sequencing developed by Sanger and his colleagues. This knowledge gave a valuable clue to the genotype of the host and contributed greatly to the genetic theory. Concurrent with these structural studies animal experiments and studies in vivo were performed to assess the function of these proteins and to give information concerning their synthesis, catabolism and distribution. In addition X-ray crystallography and electron-microscopy were also contributing to the understanding of the biological properties of these proteins.

Thus at the present time a considerable body of knowledge has accumulated for many of the serum proteins concerning their structure, function, synthesis, catabolism and distribution. With this knowledge has come the realization that frequent abnormalities of these properties occur in various diseases. Detection and characterization of these abnormalities is a major concern for many of those who are interested in serum proteins and has provided a further stimulus for research in this field. However from this brief historical review it is evident that many details concerning serum proteins come from recent research and it is equally evident that an enormous amount remains to be discovered.

General survey of serum proteins

With the application of the more recent analytical and preparative techniques it has been possible to characterize, in detail, the structural features and biological properties of many of the serum proteins. These details will

now be described for the principal serum proteins. A comprehensive literature citation will not be attempted. However, references to recent reviews will be given together with references relating to interesting or controversial topics.

Albumin: (Peters, 1970). Albumin is the most abundant of the serum proteins constituting over 60% of the mass of protein in serum. It is a carbohydrate-free molecule weighing 66,500 daltons and is structurally composed of a long polypeptide chain making up four globular segments each of which is stabilized by multiple disulphide bridges (Franglen, 1974). This structure has overall dimensions of about 40 x 140 Å and a considerable degree of flexibility. These properties together with the low viscosity, high solubility and stability, and negative charge (at the pH of blood) make albumin an ideal molecule to withstand the dynamic stresses of the circulation. Over 70% of albumin contains a reactive thiol group which is naturally blocked as this protein ages (Franglen, 1974). This free thiol group is probably important in the polymerization of albumin which tends to occur when this protein is prepared in a purified form.

Albumin is made in the liver and appears to be catabolized by nearly all the tissues of the body. It is probably used as a source of amino acids. The half life of albumin is about 19 days. The factors controlling synthesis and catabolism are multifactorial and include the plasma albumin level and / or the colloid osmotic pressure, the dietary protein intake and the action of steroid and other hormones.

Albumin has many biological functions (Freeman, 1968). This protein provides about 80% of the colloid osmotic pressure and serves as a transport molecule for fatty acids, bile acids, heavy metals, amino acids and

for various drugs and hormones.

Genetic and acquired abnormalities of serum albumin have been well documented (Peters, 1970). A depressed level is seen in almost all pathological states and is caused by impaired synthesis, increased loss, altered distribution or a combination of these causes. Changes in the size of albumin, its charge, optical rotation, viscosity and binding affinities have been sporadically reported in various diseases but the clinical significance of these findings is obscure. Of particular interest is the rare condition of analbuminaemia which is inherited as an autosomal recessive (Ott, 1974). The affected individual has a hypergammaglobulinaemia (presumably to provide an effective colloid osmotic pressure), a marked hyperlipoproteinaemia and hyper-cholesterolaemia, a low blood pressure and usually has suffered from a mild oedema during infancy. However the affected person is generally well and this suggests that the body can compensate for the absence of serum albumin.

Transferrin: (Dagg et al., 1971). Transferrin is a glycoprotein with an electrophoretic mobility in the β_1 position, a molecular weight of 90,000 daltons and a serum concentration of 200-320 mg%. The protein has two polypeptide chains linked by disulphide bonds and has 6% carbohydrate by weight. Many phenotypes of transferrin are now recognized by their slightly atypical electrophoretic mobility in starch gel and this has led to the identification of at least 14 genetic alleles.

Transferrin is synthesized in the liver and possibly in other sites including the central nervous system (see Chapter seven), spleen, bone marrow and intestinal mucosa, and circulates with a half life of 6-8 days.

The function of transferrin is to act as a carrier for iron and each

molecule is capable of transporting two atoms of ferric iron (Fletcher and Huehns, 1968). The majority of the iron is carried to the erythropoietic bone marrow and there it is taken up only by those red cell precursors actively synthesizing haemoglobin. Attachment of the transferrin / iron complex to specific receptors on the red cell surface probably produces a conformational change in the complex and allows the transfer of iron to the cell. Transferrin with two atoms of iron releases iron more readily than if it has only one. Transferrin may also have antibacterial and antiviral activity although those functions are less well defined.

Transferrin levels are increased in iron deficiency states, hypogammaglobulinaemia and pregnancy. Reduced levels are seen in acute and chronic infection, rheumatoid arthritis, liver disease, protein starvation, protein losing states and in many malignancies (Wintrobe, 1969, p. 134). A congenital deficiency of transferrin is known and this condition is characterized by an iron deficiency anaemia not responsive to therapeutic iron (Goya et al., 1972).

Hemopexin: (Müller-Eberhard and Liem, 1974). Hemopexin is a glycoprotein of molecular weight 57,000 daltons. It migrates at the β position, has 22% carbohydrate by weight and is present in the serum with a concentration varying between 80-100 mg%. This protein is synthesized by the hepatocytes and a variety of drugs and hormones are known to be potent inducers of synthesis. The half life of hemopexin is about seven days but the site of catabolism is unknown.

The main function of hemopexin is its ability to bind haem and other metallo-porphyrins. Thus hemopexin levels are decreased in states associated

with gross haemolysis and usually in proportion to the severity of the haemolysis. Low levels may also be observed in liver disease, states of undernutrition, porphyria cutanea tardia, erythropoietic protoporphyria and chronic haemolytic states. Genetic deficiencies of hemopexin have not been reported.

Haptoglobin: (Giblett, 1969; Pintera, 1971). Haptoglobin is a term for a series of closely related α_2 glycoproteins which can bind free circulating haemoglobin. Three basic haptoglobin structures are recognized and these are derived from two alleles which are inherited without dominance. The structural heterogeneity and the genetic polymorphism of this protein will be described in more detail in Chapter two. Haptoglobin has a large amount of attached carbohydrate (18%) and is present in the serum in moderate quantities (30-190 mg%). Haptoglobin is synthesized in the liver and is destroyed, both in its free state and in the haptoglobin / haemoglobin complex, in the hepatocytes and probably in the phagocytic cells of the reticulo-endothelial system. The half life of the haptoglobin / haemoglobin complex is about nine minutes while the half life of the free protein is four days.

Two main functions have been ascribed to this protein. Firstly, haptoglobin combines specifically, rapidly and stoichiometrically with the $\alpha\beta$ dimer of the globulin moiety of haemoglobin to form a highly stable complex. This complex formation is considered to be a mechanism for haemoglobin (and thus haem) retention under normal and pathological conditions because in this form the complexed haemoglobin is too large to pass the glomerular membrane i.e. haptoglobin serves as a continual blockade to potential iron loss. However the precise significance of this iron-conservation property of haptoglobin is unknown because patients who have little or no haptoglobin do not

necessarily become iron deficient, even if chronic haemolysis is occurring. Moreover it should be emphasized that haptoglobin, in combining with haemoglobin, does not act as a true transport or 'biophilic' protein. This is because both components of the haptoglobin/haemoglobin complex are rapidly removed from the circulation, i.e. the complex formation with haemoglobin leads to the 'suicide' of haptoglobin (Freeman, 1968).

The second function of haptoglobin is that it belongs to the group of proteins constituting the acute phase reactants (vide infra). In addition, some authors believe that haptoglobin may have a role in neutralizing enzymes released in tissue destruction (Pintera, 1971).

Acquired and genetic abnormalities of haptoglobin are relatively common and genetic studies have been particularly rewarding. In the clinical context depressed levels of haptoglobin are seen in intravascular haemolysis and severe liver disorders while elevated levels are found in active inflammatory states. Hereditary deficiencies of haptoglobin are known, the individual being unaffected.

Acute phase reactants: (Koj, 1974). Acute phase reactants are plasma glycoproteins produced in the liver which increase in the acute phase of an inflammatory process. These proteins include fibrinogen, haptoglobin, α_1-antitrypsin, the third component of complement C_3, α_1-acid glycoprotein, ceruloplasmin and C reactive protein. The biological function of this response is teleologically to equip the body with a group of proteins which are potentially useful e.g. fibrinogen in the clotting pathway; haptoglobin for haemolysis, α_1 antitrypsin for enzyme neutralization etc. However the complete details of this reaction are still to be elucidated.

The degree of elevation in response to injury and the time/response curve vary for each of these proteins and, in addition, the response is dependent on hormonal and neural activity. Haptoglobin has been mentioned previously. The remaining proteins of this group will now be mentioned.

Ceruloplasmin is an α_2 glycoprotein of molecular weight of 160,000 daltons and a serum concentration of 27-63 mg%. This protein accounts for the major portion of copper in the serum and also has oxidase activity against a number of substrates including the ferrous iron. The depression of ceruloplasmin in Wilson's disease suggests a function for this protein in copper metabolism. Elevation of ceruloplasmin is seen in pregnancy and hyperoestrogenic states besides inflammatory conditions.

Alpha$_1$ -acid glycoprotein (orosmucoid) has a molecular weight of 44,000 daltons, a serum concentration of 75-100 mg% and a half life of 5-6 days. The serum concentration is depressed in liver disease and protein losing states.

C reactive protein (CRP) is present in normal human serum in very low or undetectable amounts but rapidly rises many fold in inflammatory states. This protein specifically precipitates the C polysaccharide from the pneumococcus and hence its name. The molecule is composed of six identical subunits, each with a molecular weight of 27,000 daltons, and this structure may represent a precursor for the formation of liver haemoproteins (e.g. catalase).

Alpha$_1$ - antitrypsin has a molecular weight of 45,000 daltons and is responsible for 90% of the total trypsin inhibiting capacity of serum; its reaction with trypsin being stoichiometric. This protein also shows affinity for various other proteases such as plasmin, chymotrypsin and enzymes released from

leucocytes and inflamed tissues. Genetic polymorphism of this protein has led to the description of the proteinase inhibitor (Pi) system and a number of alleles are known. Alpha$_1$ - antitrypsin is present in the serum in moderate quantities (210 -500 mg%) and a hereditary deficiency has been associated with severe pulmonary emphysema and juvenile cirrhosis (Alper and Johnson, 1970).

An assessment of the acute phase reactants is of some relevance in diagnosis and management of several human conditions. CRP has been important in this respect since it is measurable within 24 hours of injury and disappears at the onset of convalescence. Persistence of this protein indicates persistence of disease or the commencement of some complication. However experimental studies have shown that the acute phase response is not consistently related to the severity of the tissue injury and thus the assessment of acute phase proteins has some limitations. It can nevertheless be useful in monitoring both the disease and the therapeutic response to anti-inflammatory drugs.

Lipoproteins: (Fredrickson et al., 1967). Lipoproteins are macromolecular complexes consisting of lipid and protein. The protein portion of the molecule (apoprotein) acts as a carrier for the water insoluble lipid and three different types have been described. The resulting lipoprotein complex is remarkable for its heterogeneity but four different classes can be generally recognized varying in charge, density and immunochemical characteristics. The smallest lipoprotein complex, α_1 lipoprotein or high density lipoprotein (HDL) has a molecular weight between 1.7 - 3.6 x 10^5 daltons and consists of 50% protein and the remainder of cholesterol and its esters, phospholipid and triglycerides. Low density lipoprotein (LDL) or β lipoprotein consists of spherical particles with

a molecular weight in the region of 3 - 4 x 10^6 daltons and is composed of 20% protein, and a considerable percentage of cholesterol and its esters and a lesser amount of phospholipid and triglycerides. Pre β lipoprotein or very low density lipoprotein (VLDL) are larger opaque particles of variable size and consist of 8% protein, 50% triglycerides and the remainder of cholesterol and phospholipid. Chylomicra are even larger particles found in lymph and plasma and predominantly consist of triglyceride.

In each of the different lipoprotein classes the apoprotein has both common and specific antigenic determinants while the lipid has a varying proportion of esterified and non esterified cholesterol, phospholipid and triglyceride. The importance of the apoprotein in the lipoprotein complex is now widely acknowledged. It is probable that the apoproteins of VLDL constitute a precursor of the apoproteins of both LDL and HDL.

Chylomicra are synthesized in the intestinal mucosa while the other lipoproteins are made in the liver. Lipoprotein catabolism occurs either in the peripheral tissues or in the liver. The main function of chylomicra and VLDL is to transport triglycerides while the function of LDL and HDL are less well defined. These latter lipoproteins appear to be involved in the esterification of cholesterol in plasma and may be involved in maintaining the integrity of phospholipid cell membranes.

A large number of genetic and acquired diseases are known which have abnormalities in the lipoproteins (Fredrickson et al., 1967).

Alpha$_2$ macroglobulin: (Turner and Hulme, 1971, p.64). This glycoprotein has a molecular weight of 820,000 daltons, is composed of 8.4% carbohydrate and has a serum concentration of 220-380 mg%. Few details are known about its structure except that it can be dissociated by 5M urea. Recent evidence (Tunstall and James, 1974) suggests that this protein may be synthesized by a subpopulation of lymphocytes. The protein is an active inhibitor of trypsin, chymotrypsin and plasmin and may also function as a carrier of insulin. The function of α_2 macroglobulin as an immunosuppressent remains speculative. Levels of this protein are increased in the nephrotic syndrome while low levels have been reported in chronic lymphatic leukaemia.

Complement: (Müller-Eberhard, 1975; Alper, 1974 and Lachman, 1975). Complement consists of a number of serum proteins which are intricately related to each other through activating and inactivating steps. This system which is mainly triggered following antigen-antibody interactions provides a direct cytolytic mechanism and causes secondary activation of a number of humoral and cellular effector pathways. Complement may also be indirectly involved in the clotting and fibrinolytic system. The mechanism of interaction between each of the complement components is now known in some detail and is well reviewed by Müller-Eberhard (1975). The third component of complement C_3 appears to be a focal protein in this pathway. This protein is synthesized in the liver, has a molecular weight of 185,000 daltons and has a small amount of carbohydrate (2.7%). It has a serum concentration of about 150 mg% and migrates as a visible band in the β_1 position.

C_3 can be activated by other complement components either through the

'classical' pathway or the 'alternate' pathway. The most common biological activators of the classical pathway are immune complexes containing IgG or IgM while naturally occurring lipopolysaccharide, polysaccharide and aggregated IgA can activate the alternate pathway. During activation C_3 is enzymically cleaved into two fractions, a small C_3a fraction (M.W. 9000) which has chemotactic and anaphylatoxin activity and C_3b which when fixed to membrane surfaces, is active in the biological functions of opsonization, immune adherence, viral neutralization and immunoconglutination. In addition C_3b is concerned with further production of C_3b by an amplification feedback step via the end stages of the alternative pathway. Fixed C_3b in conjunction with earlier activated complement components can also activate the terminal complement components and this may result in membrane lysis. C_3b itself is inactivated by C_3b inactivator.

Opsonization and the activation of the lysosomal systems of phagocytes are probably the most important biological functions of complement. Therefore, it is not surprising that recurrent pyogenic infections have been described in one patient with the rare genetic deficiency of C_3 and in other patients with a secondary deficiency of C_3 due to the absence of C_3b inactivator. This latter defect resulted in a continuous activation and consumption of C_3.

Acquired deficiencies of C_3 are seen in a number of diseases including many of the glomerulonephridities, serum sickness, severe liver disorders, essential cryoglobulinaemia, various diseases of the immune complex type and occasionally in certain viral and bacterial infections (Ruddy et al., 1972). Acquired and genetic deficiencies of other complement components also occur in certain diseases and the assessment of these deficiencies can be helpful in

diagnosis, and in understanding the pathogenesis of these diseases.

Immunoglobulins: (Metzger, 1970; Edelman and Gall, 1969). Five classes of immunoglobulins are recognized in man IgG, IgA, IgM, IgD and IgE and each class differs from the other in structure, function and distribution.

Each immunoglobulin molecule has a common and basic polypeptide structure which consists of two identical light chains (M.W. 22,500 daltons) bound by disulphide bonds to two identical heavy chains (M.W. between $50 - 75 \times 10^3$ daltons). Each light chain can belong to one of two subclasses, kappa or lambda. Each heavy chain is specific and determines the class of the immunoglobulin. Five heavy chains are known; γ (IgG), α (IgA), μ (IgM), δ (IgD) and ϵ (IgE). These classes of heavy chain can also be subdivided into subclasses depending on differences in the primary sequence of the heavy chain. Four subclasses are known for IgG, two for IgA and IgM, and one each for IgD and IgE. The molecular weight of the different classes of heavy chain varies between $50 - 70 \times 10^5$ daltons, and, in addition, slight structural differences occur in each of the subclasses. IgM consists of five members of the basic structure while polymerization of the basic structure commonly occurs for IgA. Both these immunoglobulins require J chain (J = joining) if polymerization is to occur and this additional polypeptide has a molecular weight of about 15,000 daltons.

The immunoglobulins contain a variable proportion of carbohydrate (3 - 12%) and their serum concentration ranges from about 1000 mg% for IgG to minute quantities for IgE. Electrophoretically these proteins migrate between the α and the γ position with the majority of IgG being found in the γ region.

The immunoglobulins are produced in the lymphoid tissues (plasma cells) and elevation occurs with antigenic stimulation. A number of factors are known which control this response. The catabolic site for these proteins includes the liver, the intestinal mucosa and lumen and probably other sites. IgG has a prolonged half life between 13 - 21 days while the half life for the other immunoglobulins varies between 2 - 6 days.

The functions of the immunoglobulins are complex and are generally concerned with the recognition of foreign antigens. This involves the Fab or fragment antigen binding portion of the molecule which specifically binds with antigen. However it is not known if all the immunoglobulin proteins have immunological (or antigenic) specificity. The C terminal end of the immunoglobulin molecule (Fc or fragment crystallizable) interacts with a wide range of immunological effector mechanisms such as the complement system and the cells involved in phagocytosis and cytotoxicity. Activation of the mechanism occurs spontaneously when the immunoglobulin molecule is complexed to its antigen and it is generally postulated that in this act the molecule undergoes a conformational change which opens up new determinants in the Fc region which can then react with the various effector mechanisms.

IgM and IgG are important in antigen recognition in the blood and tissue fluids while IgA, being secreted onto mucosal surfaces, performs this function at this site. IgE is the reaginic molecule concerned in anaphylaxis while the function of IgD is unknown.

A large body of knowledge has accumulated concerning acquired and genetic abnormalities of the serum immunoglobulins (Hobbs, 1971). This has greatly increased our understanding on the structure and function of these

molecules and has provided a great impetus in the diagnosis and management of many human diseases. Further examples of the usefulness of assessing these proteins in clinical practice will be described in this thesis.

Other serum proteins: At least 100 separate proteins have been defined in serum. Some of these proteins are present in only minute quantities and little is known about their structure and biological properties. However, other proteins, especially those concerned in the clotting and fibrinolytic system, the complement system, the kinin system and the renin — angiotensin system have been characterized in more detail and the measurement of these proteins (usually by radioimmunoassay or by a functional assay) has emphasized their importance in health and disease. It is predicted that future studies will concentrate on the further characterization of these less plentiful serum proteins in the hope that they will provide answers to many clinical problems.

Aim of the thesis

An examination of the serum proteins has become of some importance in the diagnosis and management of many human diseases. Simple measures used for this task include the inspection of serum for its colour and opacity (e.g. for lipoprotein abnormalities), the measurement of the erythrocyte sedimentation rate (e.g. for elevation of the acute phase reactants or immunoglobulin abnormalities) and the detection of cryoglobulins (a hallmark of an immunological disturbance, see Chapter five). For a more definitive examination of the serum proteins the two routine clinical techniques used are electrophoresis and immunoelectrophoresis, which either gives basic

information about the charge or the immunochemical nature of these proteins. Quantitation of specific proteins is commonly performed by single radial immunodiffusion or by a nephelometric method. However, until recently, the assessment of the molecular size of the serum proteins has been considered a more difficult task and has required an analytical ultracentrifuge; a costly machine with a poor resolving capacity when applied to serum. Consequently this examination has been infrequently used and its clinical relevance is poorly defined.

In the last decade two simple methods have become available which separate proteins according to their molecular size. These methods are gel filtration chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDSPAGE). The aim of this thesis has been to examine these methods and to determine if they can be profitably applied to the clinical analysis of serum proteins. In Chapters 3-6, these methods have been used to study the size heterogeneity of certain serum proteins and the results from these studies have been compared with the relevant clinical findings. In Chapter seven the method of SDSPAGE has been used in an examination of the sieving effect of the blood/cerebrospinal fluid barrier while in Chapter eight the information derived from this study has been utilized in a study of the cerebrospinal fluid proteins in various neurological disorders.

CHAPTER TWO

METHODS AND MATERIALS

Gel filtration chromatography

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CHAPTER TWO

METHODS AND MATERIALS

Three methods were constantly used in the analysis of serum proteins. These will now be described in detail.

Gel filtration chromatography

Theory: Gel filtration is a method by which molecules are fractionated by a sieving process on vertical columns containing a gel matrix. During this chromatographic process Andrews (1964) has shown that globular proteins will pass through the gel matrix at a rate inversely proportional to the volume of the available solvent. This effectively means that the position at which the protein is eluted from the column (Ve) is inversely related to the mean radius of the protein. Since the molecular size of a globular protein is closely related to its molecular weight this means that a mathematical relationship will occur between the elution position and the molecular weight of the protein. This approximates a logarithmic function (Andrews, 1965).

Some proteins deviate from this function. This occurs if:-

1) the protein has a non-globular shape,
2) additional carbohydrate moieties are attached to the protein,
3) aggregation or complex formation by the protein has occurred,
4) interaction occurs between the protein and the gel matrix,
5) the protein is so large that it is totally excluded from all parts of the interstices of the matrix and is eluted in the void volume (V_o) of the column.

The first three factors will tend to cause the exclusion of the protein from the gel matrix and therefore an earlier elution position will be seen, whereas the fourth factor will delay or accelerate elution depending on the nature of the interaction. Anomalous elution due to molecular shape, aggregation or complex formation (not involving peptide or disulphide linkage) can be abolished by performing the elution in denaturing solvents.

Method of gel filtration: Gel filtration chromatography was performed using Pharmacia plexi glass 50 x 2.5 cm columns. These were packed with Sepharose 6B (Pharmacia) or Sephadex G 200 (Pharmacia) according to the instructions of the retailers. The column was filled with phosphate buffered saline (PBS, Oxoid), pH 7.3, and on top of the column was placed a large funnel. A constant downward recirculation of buffer was established. Degassed gel was poured into the funnel, allowed to settle under gravity and the fines and excess gel removed. When packing was completed the top of the column was inserted and the buffer flow reversed. A peristaltic pump (vari-perspex 12000, LKB) provided an upward flow rate of about 10–20 mls/hour and three ml fractions were collected into $3\frac{1}{2}$" x $\frac{1}{2}$" tubes (Sterilin) using an Ultravac (LKB) collector with a photoelectric drip counter. Gel filtration was performed at 4°C and the optical density of the collected eluate fractions was measured in 1 cm quartz glass cuvettes at a wave length of 280 mμ using a Unicam SP 500 spectrophotometer. At this wave-length the aromatic and cyclic peptides have maximal extinction. The PBS buffer contained 0.02% sodium azide (BDH) as a preservative.

The void volume of the column was calibrated with dextran blue 2000

(Pharmacia). During the filtration of this dye the column was checked for irregularities of flow. If satisfactory the column was calibrated with several proteins of known molecular size. These included haemoglobin (M.W. 67,000), human albumin (M.W. 68,000, Koch Light), IgG (M.W. 156,000, Sigma), bovine thyroglobulin (M.W. 670,000, Sigma), and human serum containing large amounts of monomeric or dimeric IgA, and IgM. The position of the "optical" density peak of these marker proteins was taken to represent the elution position, V_e, and the distribution coefficient, K_{av}, of this position was calculated from the formula $K_{av} = V_e - V_t / V_t - V_o$ where V_t is the total volume of the column and V_o the void volume. The total volume, V_t, was determined by measurement.

Characterization of normal and pathological sera using gel filtration chromatography: 1) Normal sera. Sera (0.5 mls) from 26 healthy laboratory subjects were fractionated on a single column of Sepharose 6B (bed dimensions 40 x 2.5 cms). A similar chromatogram was obtained for all these sera and the means $\pm$ 2 standard deviations (M $\pm$ 2SD) of the optical density at five constant elution positions are shown in Figure 2,1.

The concentration of six serum proteins was then measured in each eluate fraction and the elution position of maximal concentration for these proteins is indicated in Figure 2,1. This figure also shows the characteristic elution profile of one of these proteins, viz IgG. Small peptides (and free I^{125}) eluted later and formed a small optical density peak of constant size (Figure 2,1). The inverse relationship between the distribution coefficient, K_{av} of these proteins and the log of their molecular weight is plotted in Figure 2,2.

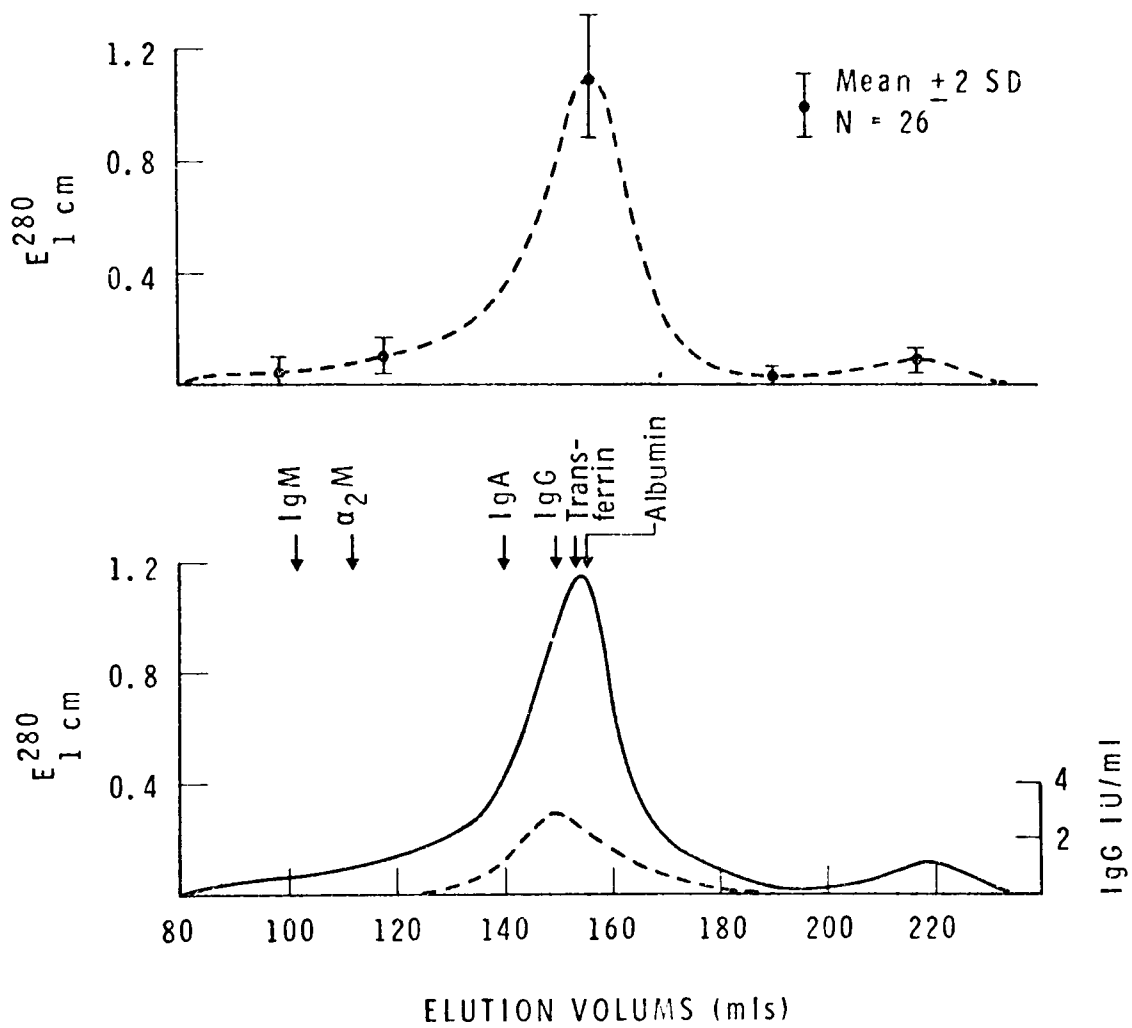


Figure 2,1 Serum specimens from 26 healthy laboratory subjects were fractionated on a single column of Sepharose 6B (bed dimensions 40 x 2.5 cms). The mean and 2 standard deviations of the optical density (read at 280 mμ) measured at five constant elution positions is shown in the upper frame. In the lower frame the optical density elution profile (continuous line) of a representative specimen is illustrated together with the corresponding elution profile of IgG (dashed line). This protein was measured in the eluent by single radial immunodiffusion. The peak elution positions of several other serum proteins are also shown.

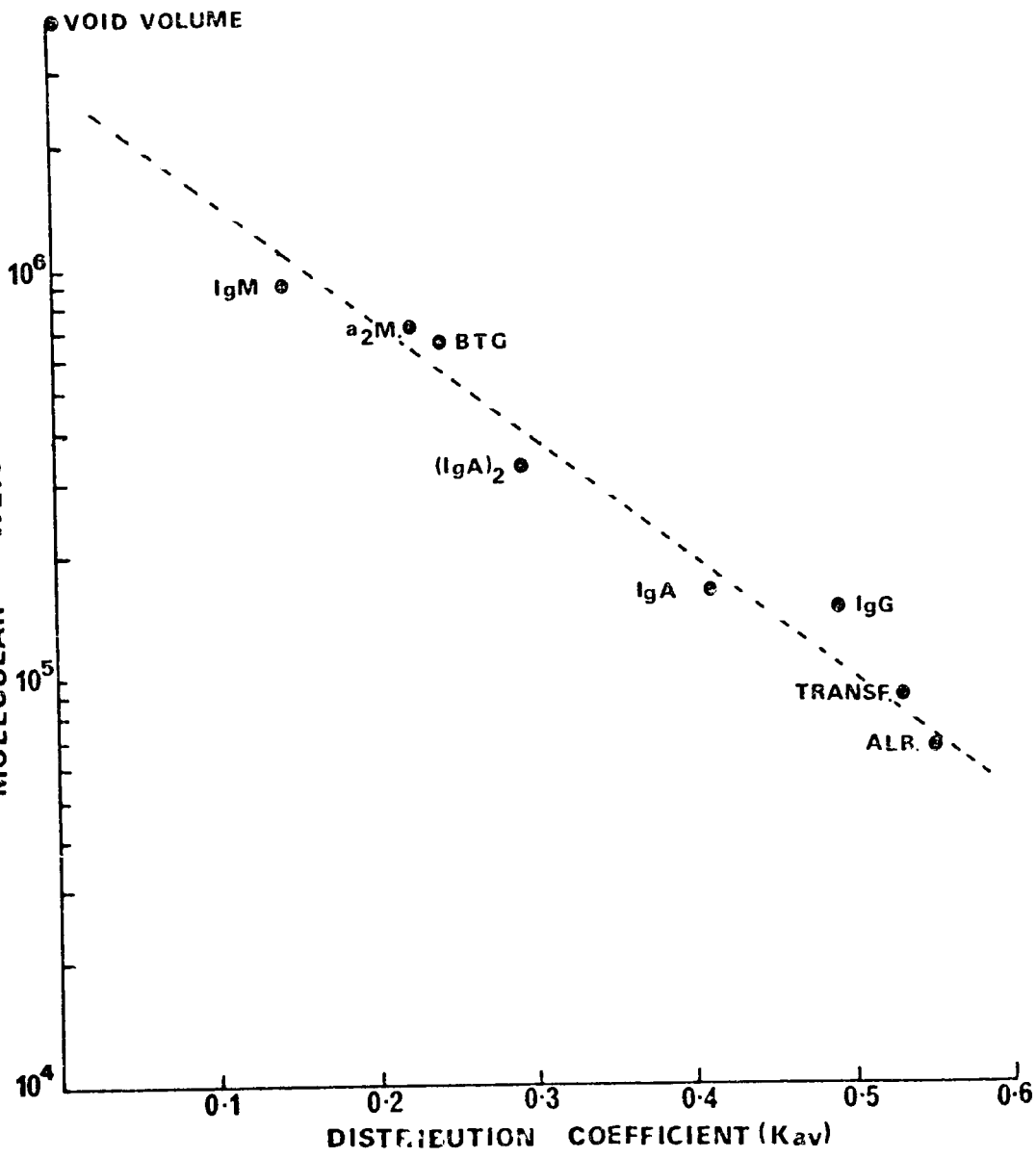


Figure 2,2 The relationship between the distribution coefficient, Kav, of several serum proteins after gel filtration on Sepharose 6 B and their respective molecular weights.

This relationship is not absolutely linear because, as explained above, additional factors besides the molecular weight influence the elution position of a protein.

2) Pathological sera. Sera from patients with a variety of disorders were found to have characteristic changes in the chromatogram. Representative examples of these are shown in Figure 2,3. All these serum abnormalities were confirmed by conventional serum protein analysis. Very low density lipoprotein (VLDL) eluted in the void volume and large peaks in this position were seen if the serum was visibly 'milky'. Smaller void volume peaks occurred in some normal sera, especially if a fatty meal had been recently ingested, and this suggested that VLDL and chylomicra were present in these sera but in minor amounts.

When azotaemia was present the final eluting peak was consistently elevated (Figure 2,3), the elevation being greater in those with a more severe degree of renal failure. Gross haemolysis of the blood specimen could also cause elevation of this peak, but this was easily detected by noting the haemolysis in the specimen.

After gel filtration, the eluate fractions containing a specific protein could be concentrated by low pressure dialysis in Visking 8/32 dialysis membrane to give a semi purified protein preparation. For example it was possible to type the light chain of IgM by concentrating the fractions containing this protein and then determining the light chain type by double immunodiffusion experiments using light chain specific antisera.

Gel filtration chromatography was also performed on Sephadex G 200 columns. Sephadex G 200 has an exclusion limit of 200,000 daltons in contrast

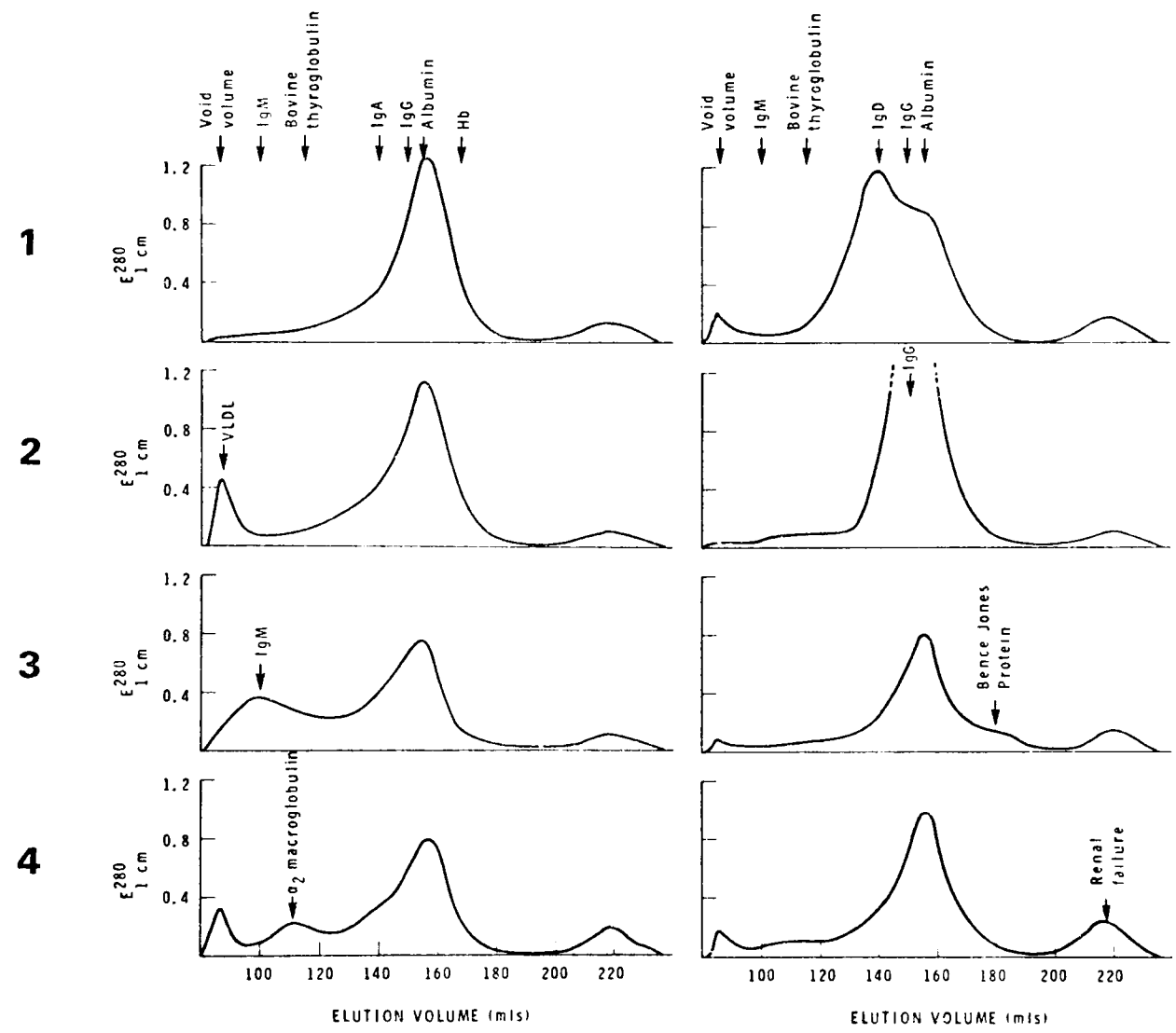


Figure 2,3 Representative elution chromatograms obtained for eight pathological sera showing characteristic abnormalities. The clinical diagnoses were 1) Normal serum, 2) Type IV hyperlipoproteinaemia (WHO classification), 3) Waldenström's macroglobulinaemia, 4) Nephrotic syndrome with elevated levels of α_2 macroglobulin, 5) IgD myeloma, 6) IgG myeloma, 7) Bence Jones proteinaemia, 8) Renal failure with elevated final peak.

to 4 x 10^6 daltons for Sepharose 6B. The chromatograms obtained using G200 will be described in the relevant chapters.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDSPAGE)

Theory: SDSPAGE is a high resolution electrophoretic procedure in polyacrylamide gel, which separates proteins principally according to their molecular size. Polyacrylamide, a synthetic polymer, is used for several reasons. It is transparent, has good chemical stability, is relatively temperature insensitive, and allows a good separation and resolution of proteins. The most important point, however, is that its pore size or lattice structure can be controlled. Polyacrylamide is prepared by the polymerization of its monomer constituent together with a cross linker, bis-acrylamide. Alteration of the concentration or ratio of these two components will result in polyacrylamide having different degrees of lattice structure.

During electrophoresis in polyacrylamide gel proteins migrate according to the charge on the protein and the intrinsic frictional resistance between the protein and the gel. If the lattice structure of the gel is increased, a greater friction occurs and therefore electrophoretic migration is less. However if SDS (an anionic detergent) is included in the procedure the effect of protein charge on electrophoretic mobility will be altered. This occurs because the protein becomes denatured in SDS and binds an amount of detergent which is dependent on the length of the molecule (Reynolds and Tanford, 1970). Thus all protein molecules have the same mass - charge ratio and the same rod like shape due to the mutual repulsion of the negatively charged SDS molecules along their length. Electrophoretic mobility is therefore primarily dependent on

the intrinsic frictional resistance developed and this in turn is dependent on the molecular length of the protein and the degree of lattice of the gel. Thus Shapiro et al. (1967) and Weber and Osborn (1969) were able to confirm that protein electrophoretic mobility in SDSPAGE was inversely proportional to the log of the molecular weight of the protein. However it should be noted that this function holds true only if the protein has no residual secondary structure (i.e. is a rod shaped polypeptide) and has no attached carbohydrate since these accessory side chains are comparatively reluctant in binding SDS (Bretscher, 1971). Under non reducing conditions protein conformation is partially retained (i.e. that due to disulphide bonds) and carbohydrate side chains are not eliminated. Therefore, electrophoretic mobility is not an absolute function of molecular weight, but a close approximation is found (vide infra).

The theoretical considerations of SDSPAGE are discussed by Neville (1971).

Method of SDSPAGE: SDSPAGE was performed as described by Shapiro et al. (1967) and Weber and Osborn (1969) but with some modifications. I am grateful to Mrs H.R. Anderson for teaching me the basics of this method.

Stock solutions were prepared as follows:

A. Acrylamide (Eastman), 28.92 g and N,N'-methylene-bisacrylamide (Eastman), 1.08 g; dissolved in deionized distilled water (H_2O) to make a final volume of 100 mls.

B. 1.0M Tris (hydroxymethyl) methylamine (BDH) - 40 mls

2.0M sodium acetate (AnalaR) - 10 mls

0.2M ethylenediaminetetra-acetic acid (BDH) - 10 mls

Adjust pH to 7.4 with acetic acid and make up to 100 mls with H_2O.

C. 20 g% sodium dodecyl (lauryl) sulphate (BDH)

D. H_2O

E. 1.5 g% ammonium persulphate (May and Baker). Prepare weekly

F. 0.5% v/v N N N' N'-tetramethylethylene diamine (Temed; BDH)

All stock solutions were stored in dark bottles at 4°C. The electrophoretic buffer was prepared from 100 mls of B plus 10 mls of C made up to 1 litre of water. This buffer was replaced after every electrophoretic run. Protein stain was prepared by dissolving 1.25 g of Coomassie brilliant blue (Raymond Lamb) in 454 mls of 50% methanol (BDH) and 46 mls of glacial acetic acid (BDH). This was stirred and filtered. The serum diluent buffer was prepared by mixing normal saline with 2% SDS, 0.125M Tris, 0.004 M iodoacetate (Sigma), pH 7.2 in a ratio of 9 : 11.

Glass tubes, 120 x 10 mm with an internal diameter of 7.5 mm, were soaked in Decon for 24 hours and rinsed several times with water and dried in an oven.

Preparation of gels:- A mixture of the stock solutions was prepared strictly in the following order

A. 1.2 mls

B. 1.0 "

C. 0.1 "

D. 6.2 "

E. 1.0 "

F. 0.5 "

This was then dispensed into the vertical glass tubes until the upper meniscus was 2 cm from the top. Parafilm (Gallenkemp) was used to seal the bottom of the tubes. A small amount of 0.1% v/v Triton X-100 (BDH) was layered onto the upper surface to ensure a flat upper gel surface. The mixture was then allowed to polymerize (10-20 minutes) and after this the Triton was removed by gently flicking the gel cylinder. These gels were then inserted into the electrophoretic apparatus. This consisted of an upper and lower bath containing platinum electrodes and connected to a Shandon power supply (anode at bottom). The gels were placed between the baths by inserting them through rubber grommets in the floor of the upper bath, and each bath was partially filled with electrophoretic buffer.

The serum sample for electrophoresis was prepared as follows:- 10 μl of serum was mixed with 100 μl of serum dilutent buffer and 10 μl of tracking dye (0.05% Bromophenol blue in 0.9% saline, Raymond Lamb). This mixture was incubated for 1 hour at 37^{o}C and then 25 μl was carefully layered onto the upper surface of the gel through the electrophoretic buffer using an Eppendorf pipette. Electrophoresis at 2-6 m amps/gel was terminated when the tracker dye was 1-2 cms from the bottom of the gel. The gel was then removed and the gel length and the migration distance of the tracker dye measured. The gel was then removed from the glass tube by gently infusing water between the side of the glass tube and the gel and the released gel placed in protein stain for 8-12 hours. Destaining of the gel was performed in a Camalco destainer where electrophoresis perpendicular to the long axis of the gel was carried out in 7% acetic acid for 10 minutes. The gel was then quickly rinsed in 50% methanol to remove surface dye and stored in 7% acetic

acid.

Protein samples apart from serum were studied in a similar fashion. In general 0.2-0.5 mgs of protein in diluent fluid was layered onto the upper gel surface and electrophoresis, staining and destaining performed as described above. For protein reduction and alkylation experiments (see Chapter three) protein was reduced with an equal volume of reducing solution containing 8M urea (BDH), 2% SDS, 0.125M Tris and a varying concentration of reducing agent dithiothreitol (complete reduction occurred with 100 mM dithiothreitol). After 1 hour's incubation at 37°C, reduction was terminated by adding 5 μl of 1.0M iodoacetamide and SDSPAGE was performed in the standard fashion.

The protein bands in the stained gels were recorded by drawing, photography, or more commonly by microdensitometry using a Joyce Loebl double beam microdensitometer.

The electrophoretic mobility of the specific protein bands were calculated as follows

$$\text{mobility} = \frac{\text{distance of protein migration}}{\text{gel length after destaining}} \times \frac{\text{gel length before destaining}}{\text{distance of tracker dye migration}}$$

This calculation was performed in order to adjust for the swelling of the gels after staining.

Identification of individual bands in the gels: Identification of proteins in the gels was performed according to the method described by Virella and Coelho (1974). SDSPAGE of a serum sample was carried out on two identically sized gels; one was stained in the usual fashion whereas the other was sliced into 2 mm sections using a Camalco gel slicer and each slice was

sequentially placed into reciprocally sized wells formed in a layer of 1.5% agarose in barbital buffer (see immunoelectrophoresis below). Troughs were cut adjacent to the rows of gel sections and specific antisera were placed in the troughs and allowed to diffuse into the agarose. After several days two forms of precipitation were seen, a non specific linear precipitation formed by the protein of the antiserum and SDS diffusing from the gel sections, and a specific arcuate immunoprecipitation line. Comparison between the region of the sliced gel adjacent to the immunoprecipitation and the corresponding region of the stained gel enabled the definitive identification of a particular band in the stained gel.

It was noted that a visible immunoprecipitation arc would only occur for the minor serum proteins if up to 10 x's the amount of serum was added to the gels as compared with that described above. In addition care was required in differentiating between the non specific and immunoprecipitation. The non specific precipitation was observed adjacent to the trough and was generalized and linear, while the specific immunoprecipitation was localized, between the gel sections and trough, and was arcuate (see also Haupt et al., 1973).

Characterization of normal human serum using SDSPAGE: Gels of 3.6% concentration of acrylamide and 10 cms long were chosen to enable the larger sized serum proteins entry into the gels and to increase the separation distance between protein bands. Acrylamide concentrations less than 3.6% were not found satisfactory due to the lack of rigidity of the gels despite increasing the concentration of the cross linker bisacrylamide. Better resolution of the smaller serum proteins was found if a higher concentration of acrylamide was used but

at the expense of a loss of penetration of the gels by the large proteins.

An electrophoretogram (microdensitometry recording) for normal human serum is shown in Figure 2,4. Eight serum proteins and their migration positions were identified immunochemically by the method described above and the approximate inverse linear relationship between the electrophoretic mobility of these proteins and the log of their known molecular weight (Schultze and Heremans, 1966) is shown in this figure. The electrophoretic mobilities of α_2 macroglobulin (M.W. 820,000) and β lipoprotein (M.W. several million) were atypical. However both these proteins probably dissociate in 0.1% SDS since dissociation occurs in other denaturants (see Turner and Hulme, 1971) and this could be the explanation for their relatively fast migration.

A brief description of the electrophoretogram seen for normal serum will now follow (see Figure 2,4). The bands will be discussed in order of their electrophoretic mobility (i.e. size).

Albumin formed the most prominent band in the electrophoretogram, and due to its small size had the fastest migration. If the acrylamide concentration was increased to 6-9%, two minor bands were seen to immediately precede albumin but these were not identified. Migrating just behind i.e. cathodally to albumin was a band formed by transferrin and the next major band belonged to IgG. Several minor bands were seen between the transferrin and IgG bands but the nature of these were not investigated. IgA migrated just behind the prominent IgG band and in normal concentrations formed a diffuse band which merged with the trailing edge of the IgG band. However if the concentration of IgA was elevated a definite band was observed (as seen in Figure 2,4). Behind this region came a well defined band which was identified at C_3.

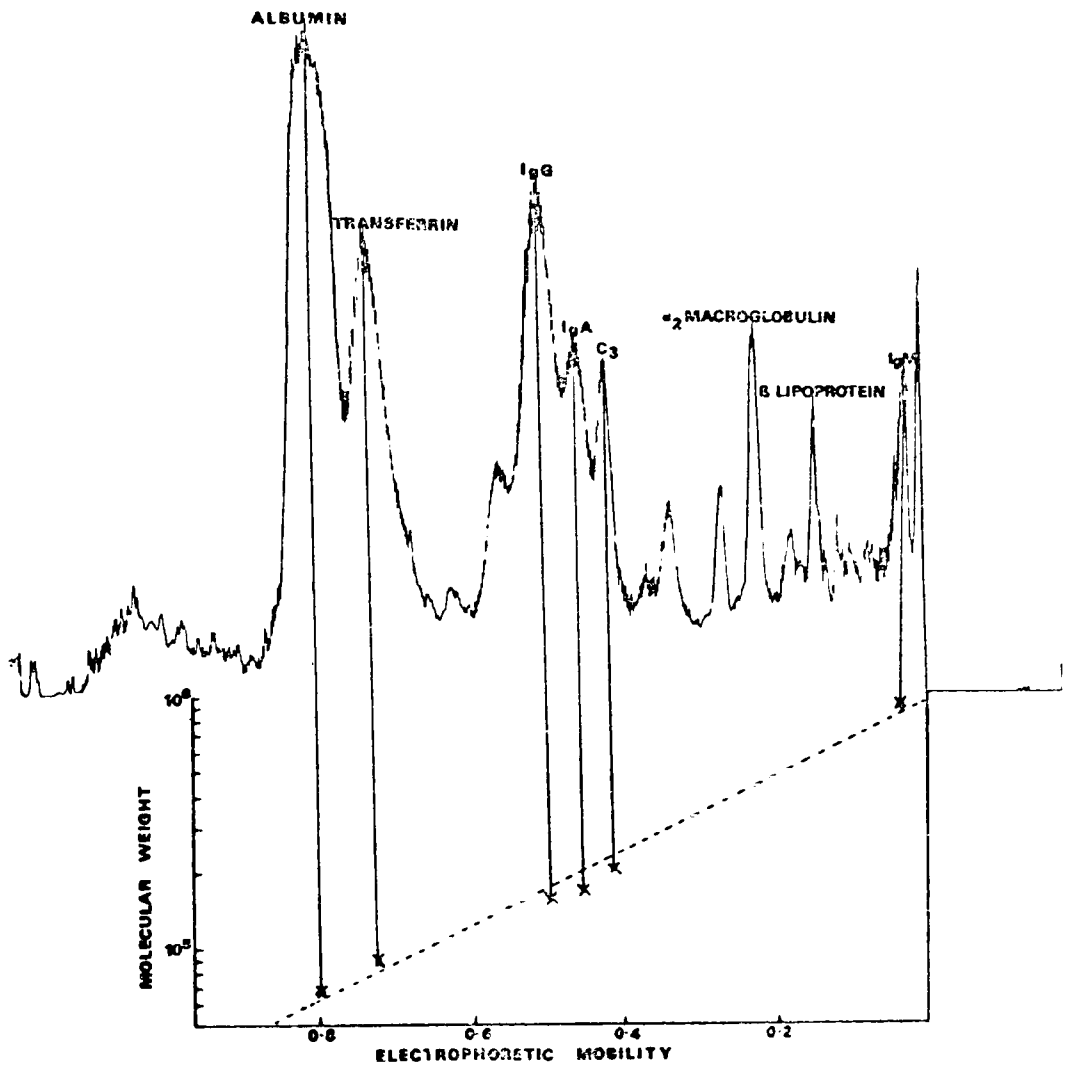


Figure 2,4 Sodium dodecyl sulphate polyacrylamide gel electrophoretogram (microdensitometry recording) and electrophoretic mobilities of specifically identified proteins in normal human serum. The linear relationship between the electrophoretic mobility of these proteins and their corresponding molecular weight is demonstrated.

This band became less obvious or absent in serum which had been stored for 2-4 weeks and this observation is in keeping with the C_3 breakdown that spontaneously occurs in serum after short periods of storage. The next prominent band was α_2 macroglobulin migrating midway between C_3 and the commencement of the gel and this preceded the β-lipoprotein band. This latter band was not seen in old stored sera and its disappearance occurred over several months. The last band seen had just entered the gel and was identified as IgM.

The haptoglobin (Hp) proteins were found to have three different characteristic appearances in SDSPAGE and these were consistent with the three known phenotypes derived from the two genotypes Hp1 and Hp2. These alleles are inherited without dominance. The identification of the Hp position in SDSPAGE was derived from a study of serum Hp proteins in rheumatoid arthritis. This will be enlarged in the appendix but a brief summary of the findings will now be presented. The homozygote Hp 1-1 formed a haptoglobin protein of uniform size and this protein had an electrophoretic mobility immediately preceding that of IgG (Figure 2,5). The homozygote Hp 2-2 formed a series of polymers which migrated between C_3 and the commencement of the gel. The relative position of these bands after SDSPAGE in a serum specimen with elevated levels of Hp is shown in Figure 2,5 while the polymeric nature of these members, and their respective electrophoretic mobility, is shown in Figure 2,6. From this latter figure it is seen that the basic unit for the smallest member of the Hp 2-2 series was about 1.5 x's as large as the Hp 1-1 protein. The heterozygote Hp 2-1 also formed a polymeric series (Figure 2,5 and Figure 2,6). The basic unit for this series was also about 1.5 x's as large as the Hp 1-1 protein and each polymeric member had a slightly greater

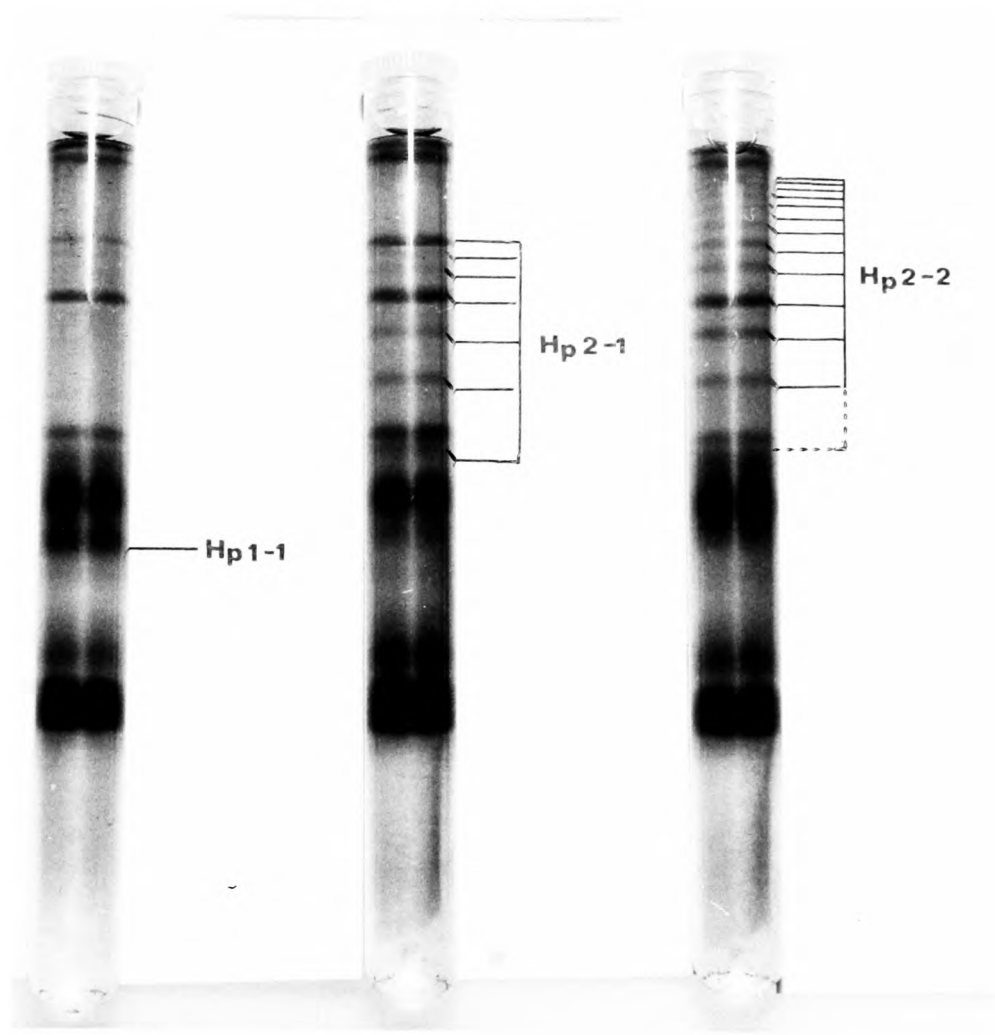


Figure 2,5 The appearance of the three different phenotypes of haptoglobin in 3.6% SDSPAGE. Each serum specimen has been obtained from a patient with elevated levels of this protein.

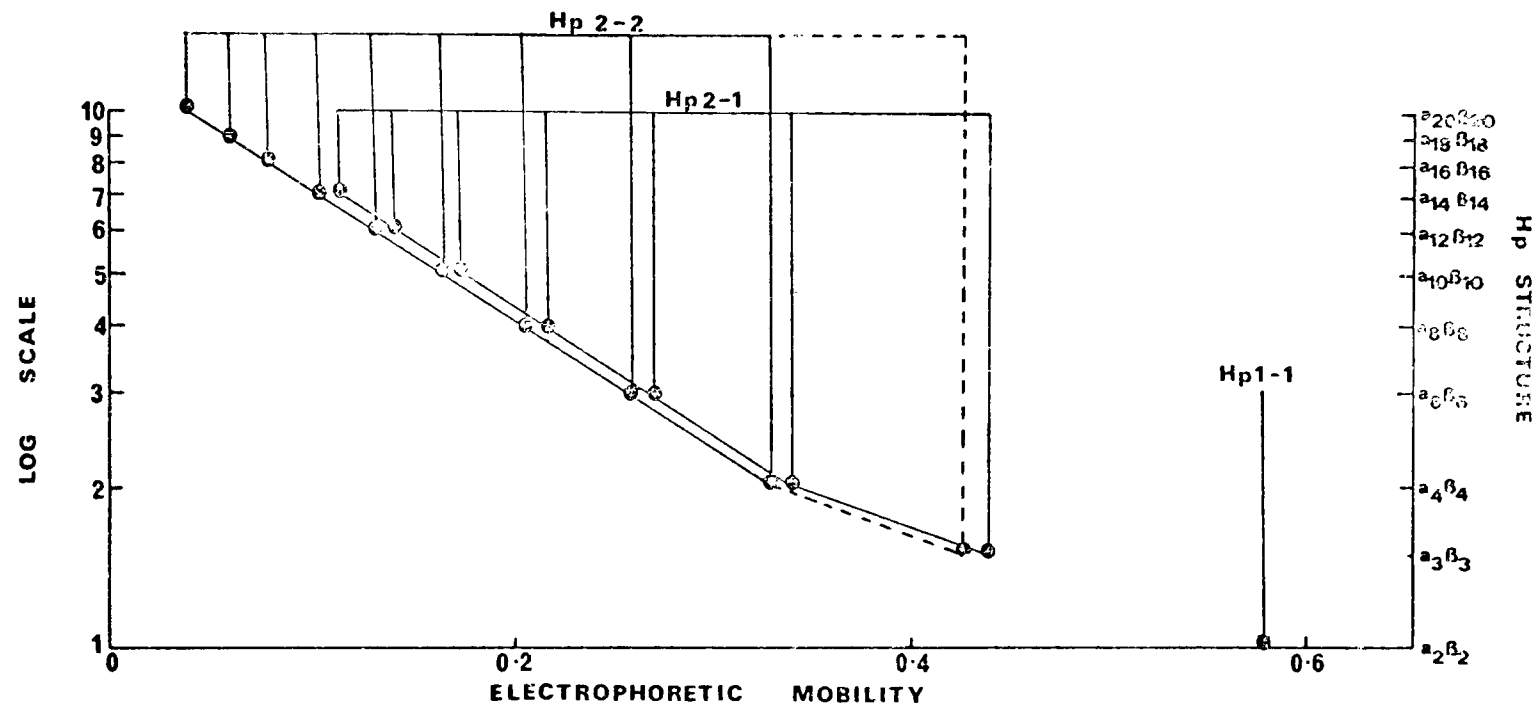


Figure 2,6 Haptoglobin (Hp) proteins can belong to one of three different phenotypes, Hp 1-1, Hp 2-1 and Hp 2-2. The electrophoretic mobility of these phenotypes in 3.6% SDS PA gels is shown together with the postulated number of alpha and beta chains in each Hp member (right ordinate). The linear relationship between the electrophoretic mobility and a log scale (left ordinate) demonstrates the polymeric nature of the Hp members of Hp 2-1 and Hp 2-2. The $a_3\beta_3$ member of these phenotypes did not conform in this relationship (sec text). Each Hp member of the Hp 2-2 polymeric series appears to be a little larger than the corresponding member of the Hp 2-1 series.

electrophoretic mobility than the corresponding polymer of the Hp 2-2 series suggesting a slightly smaller size (Figure 2,6).

The electrophoretic mobilities of haptoglobin proteins in SDSPAGE were consistent with the known molecular size heterogeneity of this protein, (see Gibblett, 1969 for review). The homozygote Hp 1-1 forms a single haptoglobin molecule consisting of 2 alpha and 2 beta chains, the alpha chain having a M.W. of 9000 daltons and the beta chain of 36,000 daltons. The homozygote Hp 2-2 forms a polymeric series of Hp molecules, the basic unit having a calculated molecular weight in SDSPAGE of 203,000 daltons (Barnett et al., 1974). However the M.W. of the alpha chain of Hp 2-2 (alpha 2) is 16,000 daltons while the beta chain is the same (36,000 daltons). Barnett et al. (1974) therefore conclude that the monomeric unit of Hp 2-2 consists of 3 alpha 2 and 3 beta chains. The heterozygote Hp 2-1 is thought to form Hp polymers having both the smaller and larger alpha chain (Ogawa et al., 1970). If so the molecular weight of the polymers would be slightly smaller than the corresponding polymers of the homozygote Hp 2-2. Figure 2,6 shows the postulated number of alpha and beta chains, the electrophoretic mobility of each member is 3.6% SDSPAGE and a log scale on the left ordinate. This latter scale is included to illusirate the polymeric nature of the Hp 2-1 and Hp 2-2 proteins.

Several additional bands, apart from those described above, were seen after SDSPAGE of normal serum. They were present in all the sera at a constant relative position but were difficult to record and were not identified. Other minor bands were also seen which varied in position from serum to serum. Fibrinogen (in plasma) migrated to a position just preceding the α_2

macroglobulin band, and gave a large deflection on microdensitometry.

Pathological sera: Useful information concerning qualitative and quantitative serum protein changes in pathological sera was obtained by an analysis of the electrophoretic pattern after SDSPAGE. Several examples are illustrated in Figure 2,7. All these protein abnormalities were diagnosed by conventional methods using cellulose acetate electrophoresis, immunoelectrophoresis and single radial immunodiffusion.

Paraproteins of different classes or subclasses gave characteristic findings. It must be emphasized, however, that SDSPAGE provides no information about the clonality of any immunoglobulin. Thus both polyclonal and monoclonal immunoglobulin gave the same appearance in SDSPAGE.

IgG paraproteins migrated to the position of normal IgG and if present in high concentrations gave a dense band (Figure 2,7). In three IgG myeloma sera, out of eleven studied, an additional band was seen between albumin and the paraprotein suggesting a molecular deletion of the paraprotein (Hobbs, 1971). The nature of this band was not investigated.

In the single IgD myeloma serum studied a dense band was seen at the position of normal C_3. This suggested a similar size for the IgD paraprotein and was consistent with its published molecular weight of 184,000 daltons (Rowe and Fahey, 1965).

The appearance that IgA paraprotein gives in SDSPAGE will be described in chapter three. The serum from one patient with alpha chain disease was examined. An additional band was seen migrating between transferrin and Hp 1-1. This was considered to be a polymer of the alpha

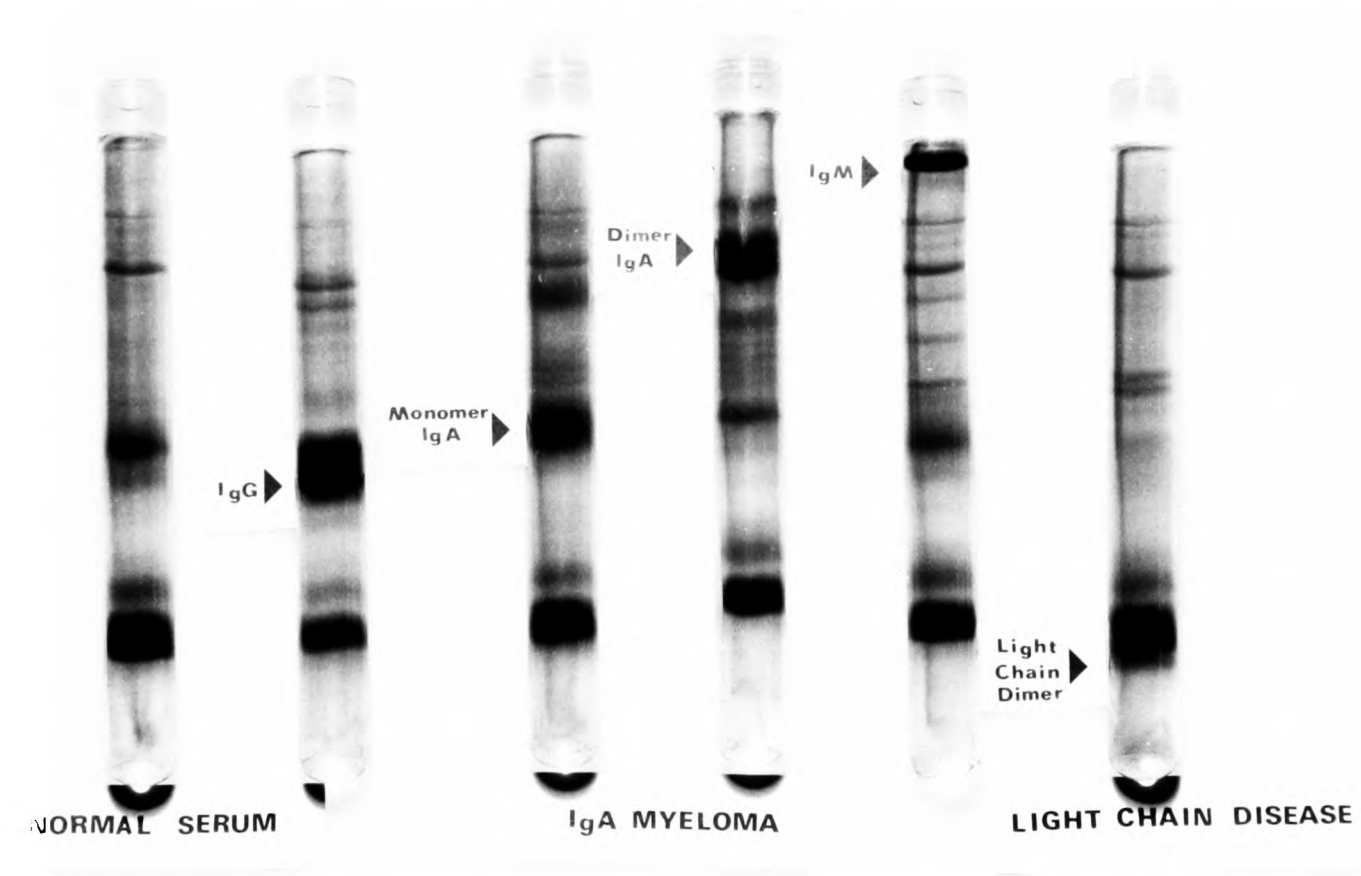


Figure 2,7 Paraproteins of different classes migrate with a characteristic migration in 3.6% SDSPAGE. Examples are shown in this illustration.

chain since polymerization is frequently seen in this condition (Tomasi and Grey, 1972).

Serum from four patients with Waldenström's macroglobulinaemia was studied. In three, the IgM paraprotein migrated to the position of normal IgM. One IgM paraprotein, however, had a slightly greater mobility than normal suggesting a smaller size. It would be of interest to study further sera from patients with this condition to investigate this aspect.

In three out of six sera from patients with light chain disease, and in serum from single patients with IgD and IgG myeloma, a band was noted migrating just before the albumin band. In one of these patients this band was further investigated and definitively identified as a free light chain dimer (by gel filtration chromatography). Polyacrylamide gel concentrations of 6-9% were found more useful in detecting this protein as in 3.6% gels the light chain dimer was poorly separated from the leading edge of the albumin band.

The presence of this light chain dimer and the renal status was not systematically compared. But in at least four patients severe renal impairment was associated with the presence of circulating light chain dimer. However two patients with light chain disease and renal dysfunction had no detectable dimer.

In addition to the immunoglobulin abnormalities described above other protein changes could be assessed by an analysis of the appearance of the gels. Thus gross quantitative changes in the major serum proteins could be detected by noting the density of the appropriate bands. Preliminary experiments showed it was possible to quantitate these changes by a microdensitometric method. In this the area deflected by a particular protein was compared with the area of a

known amount of protein in a reference serum. A basic difficulty in these experiments was adjusting for gel background staining and hence it was considered that single radial immunodiffusion would be more accurate in quantitating these protein changes.

In conclusion it is considered that SDSPAGE is a simple method requiring relatively inexpensive equipment. It can provide another dimension in the analysis of serum proteins by giving information concerning the molecular size of these proteins. Further examples of the usefulness of SDSPAGE in clinical medicine will be given in this thesis.

Single radial immunodiffusion

Theory: Quantitation of individual serum proteins was performed by single radial immunodiffusion according to the method described by Mancini et al. (1965) but with some modifications. Mancini and her co workers noted that when an unknown amount of antigen diffused radially from a well in a uniformly thin layer of antibody containing agar and for a sufficient time to allow all antigen to combine, a precipitate was formed whose final area was directly proportional to the amount of antigen employed and inversely proportional to the concentration of the antibody, and was temperature independent. Thus if known amounts of antigens are employed as standards it is possible to calculate the concentration of an unknown amount of antigen by measuring the area of the unknown precipitate and comparing it with the area of the standards. This method has been extensively used and has a high degree of reproducibility (coefficient of variance 2-10%). Its specificity, however, is dependent on the specificity of the incorporated antisera. The mathematical

consideration of this method is well summarized in the technical brochure issued by Hoechst Pharmaceuticals.

Agar preparation: One gram of purified agar (Difco) and 1.5 g of polyethylene glycol 6000 (Hopkins and Williams) were added to 50 mls of distilled water. This was then autoclaved at 110°C for 10 minutes, mixed, and allowed to cool to 60-70°C. Evaporated water losses were replaced and the mixture placed in a water bath at 56°C. (Polyethylene 6000 was included since it enhances the rate of immunoprecipitation and increases the definition of the precipitation rings (Harrington et al., 1971).)

Antisera preparation: Antisera were obtained from commercial sources (Table 2,1). All antisera showed only one arc in immunoelectrophoresis experiments (vide infra) against normal human serum suggesting specificity The antisera were diluted in water containing 1% sodium azide to form a total volume of 25 mls. The degree of dilution is indicated in the Table. The diluted antisera were then placed in the water bath.

Agar - antisera plates: Twenty five mls of the diluted antiserum was then mixed thoroughly with 50 mls of the agar solution and the mixture then poured into specially prepared moulds. These consisted of two $3\frac{1}{2}$" x $3\frac{1}{2}$" glass slides (Kodak) separated by U shaped plastic frames of similar dimensions and 7 mm wide and 2 mm thick. The bottom glass slide had been previously subbed with 0.5% agar while the top slide had been coated with Repelcote (BDH). The glass slides were firmly applied to the frame by paper clasps. The agar-antibody mixture was introduced into the mould, care being taken to avoid

Table 2,1

Antisera	Batch number	Source	Dilution
anti IgG	K 6554, K 6363	1	1 : 100
anti IgM	K 8800	1	1 : 50
anti IgM	K 8678	1	1 : 10
anti albumin	2448 H,K	2	1 : 14
anti transferrin	2400 M	2	1 : 25
anti haptoglobin	042, T2208T	3,2	1 : 50
anti B_{1C}/B_{1A}	2444 L	2	1 : 25
anti β lipoprotein	2076 AP	2	-
anti α_2 macroglobulin	2520 L	2	1 : 50
anti kappa	023	3	1 : 50
anti lambda	113	3	1 : 50

Standard	Batch number	Source	Dilution
Immunoglobulin	66/97	WHO	neat, 1:2, 1:4, 1:8 1:16, 1:50, 1:100 1:200, 1:400, 1:800
Human serum	1730	2	1:10, 1:25, 1:50, 1:100 1:200, 1:400, 1:1000 1:2000, 1:3000

1 Wellcome

2 Behringwerke

3 Dakopatts Mercia Diagnostics

WHO World Health Organization

air bubbles, and allowed to solidify. The plates were then stored at 4°C in moist airtight containers or prepared for use by sliding the siliconized top glass slide off the agar gel and removing the frame.

Application of antigen samples: Cylindrical wells were punched out of the agar gel using a 2 mm bore needle and a specially prepared plastic frame to space the wells equidistant from each other. The agar was removed from the wells by suction using a Pasteur pipette. In all 49 wells were made in each plate. The antigen sample was deposited in the wells using a Pasteur pipette with a curved capillary tip. The well was filled by gently touching the pipette tip to the side of the well. Filling was stopped when the meniscus of the antigen solution in the well became flat and equal to the height of the surrounding agar. Filling was controlled by mouth suction.

Preparation of reference standards: Four or five dilutions of the reference serum (standards) were included in every plate. The standards for immunoglobulins and other serum proteins are indicated in Table 2,1. The standards were diluted in 0.9% saline to give a range of concentrations that would include all the unknown concentrations; i.e. the area of the unknown would fall between the areas of the standards. A large quantity of the diluted standards was initially prepared and divided into 0.2 ml aliquots and frozen at -20°C. For each protein determination a new batch of standards were thawed; used and then discarded.

Measurement of the area of precipitation and the construction of a reference graph: The agar plates were incubated in a moist chamber at room temperature for 72-120 hours. They were then washed in 3% saline for 6 hours, rinsed with water, and dried on a backing of filter paper. The gel was then stained with Coomassie brilliant blue and destained using a methanol, water, acetic acid mixture in a ratio of 5:5:1. The diameters of the precipitate rings were measured with a Hyland precision viewer to the nearest 0.1 mm. The diameter of the ring was expressed as the mean of two perpendicular diameter measurements. The square of these values for the standards was then plotted against the concentration of these proteins and a standard graph prepared. The concentrations of the unknowns were then calculated from this graph.

Reproducibility of the method: For good reproducibility the thickness of the agar gel layer, the size of the wells and the volume dispensed should be constant. In order to assess the reproducibility under the conditions used in this thesis, the coefficient of variance was calculated for IgG and albumin. The coefficient for 44 replicate determinations of IgG, was 8.15% and for 80 replicate determinations of albumin, 1.0%. No significant differences were observed between values obtained using different plates.

Sensitivity: Single radial immunodiffusion is a sensitive technique. Mancini et al. (1965) noted that as little as 1.25 μg/ml of albumin could be measured by this method. The lowest amount of IgG that could be measured using the method as described above was 1.6 μg/ml. In general most measurements were done in duplicate and the mean of the two results calculated. For the determination of protein elution profiles in gel filtration

chromatography (Figure 2,1) only a single measurement was made, the protein profile being drawn as the best fit through the values found for successive or alternative fractions.

Other methods

Double immunodiffusion, electrophoresis and immunoelectrophoresis were performed in 1.5% agar or agarose and 0.1M barbital buffer (pH 8.6) using the glass slide methods as described in the textbook of Weir (1967).

CHAPTER THREE

RELATIONSHIP BETWEEN PARAPROTEIN POLYMERIZATION AND CLINICAL COURSE IN IgA MULTIPLE MYELOMA

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CHAPTER THREE

RELATIONSHIP BETWEEN PARAPROTEIN POLYMERIZATION AND CLINICAL COURSE IN IgA MULTIPLE MYELOMA

INTRODUCTION

It was demonstrated in chapter two that the methods of gel filtration chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis can be used to separate serum proteins according to their respective molecular size. Examples were given in that chapter of elution profiles or electrophoretic patterns which were of diagnostic value. In the present chapter these methods have been used to study the immunochemical features of twenty five patients with IgA myeloma. In particular a description is given of the molecular size heterogeneity of the IgA paraproteins. This size variation has been related to certain complications seen during the clinical course of the patient's disease and it is concluded that a knowledge of the molecular size of the paraprotein together with its quantity may be of importance in the management of that disease.

Approximately 20% of cases of myeloma are of the IgA class (Hobbs, 1969) and this variety is often considered to be clinically similar to IgG myeloma. There is, however, some evidence that IgA myeloma shows a higher incidence of the hyperviscosity syndrome than does IgG myeloma (Buxbaum, 1972; Bloch and Maki, 1973). Thus one group of observers (Tuddenham et al., 1974) reported the hyperviscosity syndrome to occur in 14% of their IgA myeloma patients whilst Pruzanski and Watt (1972) reported

that only 4.2% of patients with IgG myeloma develop this complication. Elsewhere in the literature there is little recognition of this complication in IgA myeloma (Leading Article, Lancet, 1973). This suggests that the hyperviscosity syndrome may be inadequately diagnosed and treated in these patients.

Another reported difference between these two types of myeloma is the greater tendency of IgA myeloma patients to develop bleeding disorders (Perkins et al., 1970; Vigliano and Horowitz, 1967). This complication may represent interference by the monoclonal protein at several steps in the clotting mechanism (Fiddes et al., 1971).

There is some evidence that IgA myeloma may also differ from IgG myeloma in its association with skin lesions. Both pyoderma gangrenosum and the rare association of hyperlipoproteinaemia with xanthomatosis occur more commonly with IgA myeloma than with IgG (Cream, 1971; Roberts-Thomson et al., 1975). In addition some observers have suggested that the pattern of infection for patients with IgA myeloma differs from that seen in IgG myeloma (Allen, 1970).

IgA myeloma can sometimes be distinguished from IgG myeloma by the cytological appearance of the myelomatous plasma cell in the bone marrow. In IgA myeloma 'flame' cells and thesaurocytes ('storage' cells) have been described (Paraskevas, Heremans and Waldenström, 1961) and the appearance of these cells is believed to be due to the distended endoplasmic cisternae filled with electron dense amorphous material. This material is thought to represent aggregates of the paraprotein (Maldonado, Bayrd and Brown, 1965).

The aim in this present study has been to investigate some biochemical factors in IgA myeloma patients which may be relevant to the clinical manifestations of the disease. One explanation of the clinical difference between IgG and IgA myeloma may accrue from physicochemical properties of these two classes of paraprotein; some IgA monoclonal proteins have a propensity to form polymers whilst polymerization is not normally seen for IgG paraprotein (Laurell, 1961). It is known that the size of a protein is an important factor in determining its contribution to the viscosity of blood (Somer, 1966). The degree of paraprotein polymerization has therefore been measured in the sera of 25 IgA myeloma patients using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDSPAGE) and gel filtration chromatography and it has been possible to divide these patients into two groups depending on the size of the myeloma protein. The first 'polymeric' group contains patients whose IgA paraprotein is more than 50% in a polymerized form whilst in the second 'monomeric' group the myeloma protein is greater than 50% in the monomeric form.

The clinical and biochemical data in these two groups has been compared to determine whether the groups differ appreciably and if so whether these differences can be ascribed to the physicochemical consequences of the paraprotein polymerization.

PATIENTS AND METHODS

Patients

Diagnosis of IgA myeloma: During the period January 1970 to December 1974 31 diagnosed cases of IgA myeloma were seen at the Radcliffe Infirmary. This diagnosis was made by standard haematological, biochemical, radiological and immunochemical criteria. Serum electrophoresis was done on cellulose acetate membrane, radial immunodiffusion assays were performed using commercial plates (Partigen, Boehringwerke) and for immunoelectrophoresis analysis, commercial antisera (Boehringwerke, Dakopatts) were utilized.

Out of the 31 patients seen during this five year period, 25 were studied. These were selected because the respective medical records were complete and dated from the initial presentation and in addition, samples of sera were availoble for examination. Fifteen of these patients were male and 10 female, the mean age at presentation was 63 years with a range from 41 to 82 years.

No patients were included in whom the diagnosis was not fully substantiated by the appropriate investigations. Many patients were entered into the concurrent Medical Research Council myeloma therapy trial where further investigation on urine and serum samples were performed at the Trial References Laboratories. In each case the diagnosis of IgA myeloma was confirmed, and in some cases additional data on light chain typing and immunoglobulin levels were provided.

Complications: Complications seen during the course of the disease were diagnosed and appropriately treated using conventionally accepted principles. A diagnosis of the hyperviscosity syndrome was made if the clinical findings were those as previously described by Fahey et al. (1965). This diagnosis generally included bleeding in two or more sites despite adequate platelet numbers together with the presence of two other features, e.g.: retinopathy, neurological complications, extreme tiredness out of proportion to the degree of anaemia and renal or cardiac failure (with no apparent explanation). Whenever possible this clinical diagnosis was confirmed by the determination of the serum viscosity. Bleeding episodes ranged from widespread bruising or petechae and mucous membrane oozing to more severe haemorrhagic complications.

Immunoparesis was defined as being present if IgG and IgM were both below the 95 percentile of normality, whilst infection was considered to be present if positive clinical findings were found.

Therapy: All patients routinely underwent radiological, biochemical and haematological assessment during their disease. Specific therapeutic measures included steroids, cyclophosphamide, melphalan and irradiation.

Analysis: At the end of the five year period, the clinical and laboratory data were retrospectively analysed from the Medical Records available and assessed with the ongoing special protein studies. The results of these studies were unknown to the clinicians managing the course of the patients.

Patients with benign monoclonal and polyclonal IgA: The degree of IgA polymerization was also studied in thirteen additional patients in whom IgA was in excess of 550 iu /ml (normal less than 188 iu/ml). Two of these had a benign IgA paraproteinaemia while the remaining eleven had polyclonal elevations of IgA. The clinical diagnosis in these patients included carcinoma in five, cirrhosis in two and chronic infections in three. The remaining patients respectively had rheumatoid arthritis, hypercalcaemia and acute myelogenous leukaemia.

Physicochemical analysis of the paraprotein

Serum: Serum specimens for protein studies outlined below were available from all of the patients. In 18 out of 25 of the myeloma patients the serum specimens were obtained both at presentation and during the course of the disease whilst in the remaining 7 patients, serum was available only after presentation. The sera were either stored at -20°C for later examination or during the latter part of the study examined immediately. The sera from 17 of the myeloma patients were studied in the fresh state. In an attempt to determine whether spontaneous paraprotein aggregation occurs over prolonged periods of storage gel filtration chromatography was repeated on three sera which had been stored at -20°C for periods of 10 months, 11 months or 26 months respectively. The optical density elution profiles were identical to those initially seen. However, spontaneous aggregation due to freezing and storage of serum specimens remains a possibility in some cases and two sera were examined in which gross aggregation had occurred. These have been excluded from the results.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis: (SDSPAGE) of serum was performed according to the method described in chapter two. After staining in Coomassie brilliant blue the stained bands seen in the gels were graphically recorded with a Joyce Loebl micro-densitometer. Electrophoretic mobility of individual stained bands were calculated as described in chapter two. Each IgA monomer and polymeric member gave an additional individual band in the densitometry record and the electrophoretic mobility and the corresponding molecular weight of these bands from one representative patient is seen in Figure 3,1. The polymeric nature of the paraprotein is also illustrated in this figure.

The area beneath each paraprotein monomer or polymer densitometry deflection was integrated using a planimetric method; the area subtended by each member was projected onto clear X-ray film and the corresponding area weighed. This allowed calculation of the percentage proportion for each molecular member of the polymeric series. The percent proportion of the polymeric members for an individual paraprotein was found to vary slightly in different SDSPAG electrophoretograms. This was due to the error associated in planimetry of adjusting for different background staining of acrylamide gels seen with different batches of dye and/or acrylamide. To verify that each additional band had IgA antigenic specificity serum electrophoresis for eight selected sera was performed on two gels; one being stained in the routine fashion and the other cut into 2 mm slices and each slice sequentially embedded in agar and allowed to react against anti-IgA in a double diffusion Ouchterlony system. Comparison between the electrophoretic mobility of individual stained bands and the immunoprecipitation reactions allowed antigenic

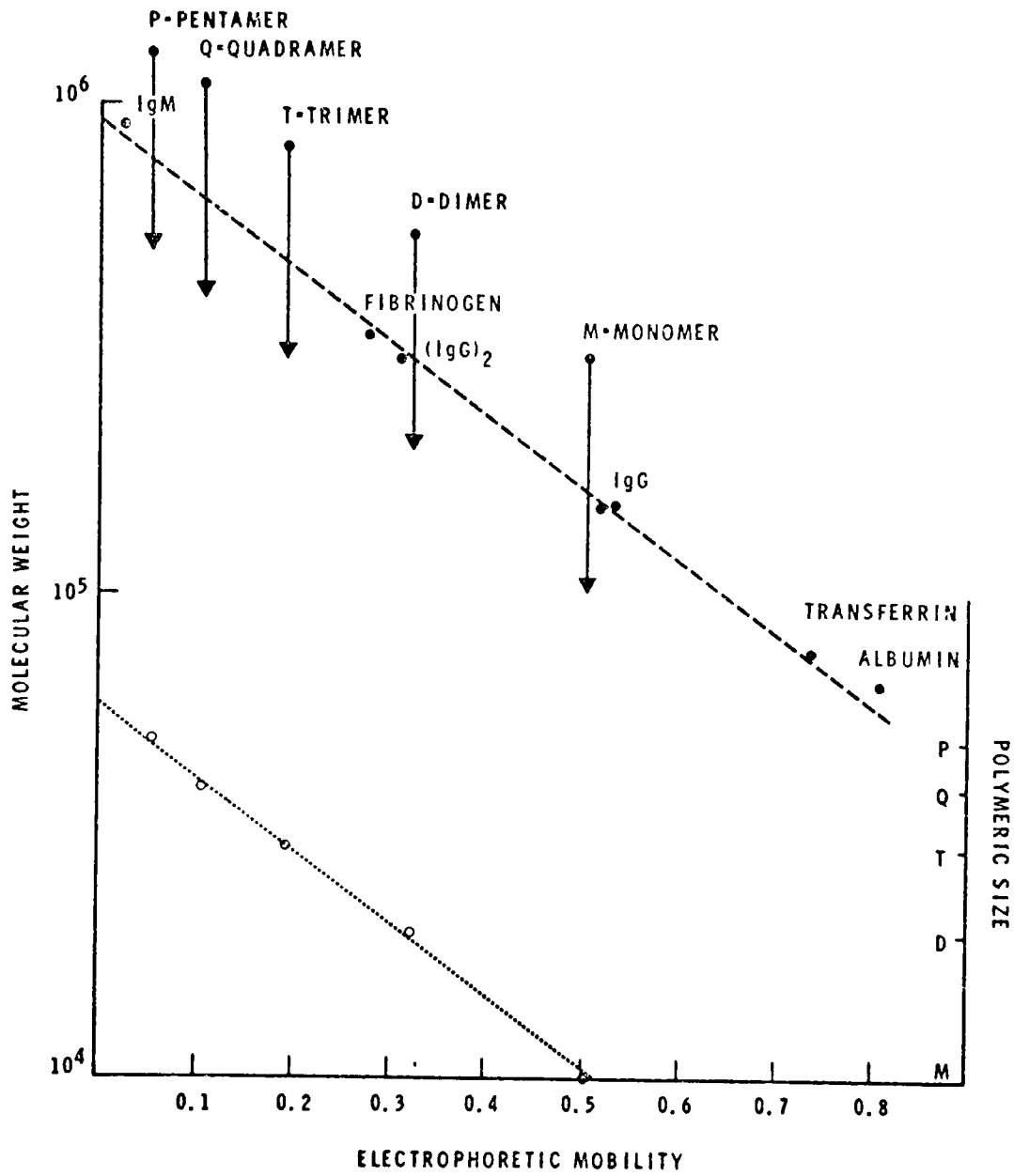


Figure 3,1 A linear relationship is demonstrated in the upper portion of the figure between the electrophoretic mobility of a number of plasma proteins in 3.6% SDS polyacrylamide gels and the log of their respective molecular weight. The vertical arrows indicate the electrophoretic mobility and corresponding molecular weight for the IgA paraprotein members of one representative patient. The lower line demonstrates the linear relationship between the electrophoretic mobilities of these paraprotein members and a logarithmic scale (polymeric size on the right ordinate) indicating that these members form a polymeric series.

identification of the bands. It was observed that an immune precipitate occurred between each additional band and the IgA antisera diffusing from the adjacent trough. This indicated that alpha chain determinants were detectable in both the monomer and the higher polymers.

Gel filtration chromatography: Sera was fractionated at 4°C on Sepharose 6B (Pharmacia) 40 x 2.5 columns in phosphate buffered saline pH 7.3 with 0.02% sodium azide. The conditions were exactly as described in chapter two. The column was calibrated with blue dextran 2000 (Pharmacia) to determine the void volume, bovine thyroglobulin MW 670,000 (Sigma) and monomeric human serum albumin (HSA) MW 68,000. The elution profiles and peak elution positions for albumin, IgG, IgA, IgM were found from the estimation of these proteins in the eluate after chromatography of a number of sera from healthy people. The Kav value for the peak elution position of the paraprotein, (Ve), was calculated from the equation: $Kav = (Ve-Vo) / (Vt-Vo)$ where Vt is the total bed volume occupied by the packed gel and Vo the void volume. The quantity of paraprotein was calculated from the optical density profile, the area subtended by the paraprotein being calculated by planimetry and referred to the area of a known amount of purified IgG since both IgG and IgA have similar extinction coefficients (Tomasi and Grey, 1972). Throughout the study a single batch of Wellcome anti-human IgA lot K7353 was used to estimate the IgA levels in the eluate after gel chromatography. This was done by single radial immunodiffusion as is also described in chapter two. Boehringwerke anti-human albumin batch No. 2448K was used for albumin estimation. The Medical Research Council Working Standard 67/99

was used as a reference serum for IgA estimation whilst Boehringwerke standard human serum batch No. 570AC was used for albumin. Boehringwerke anti-human haptoglobin (batch No. T2208T) was used to determine immuno-electrophoretically if binding could be detected between the paraprotein and haptoglobin (vide infra).

Reduction and alkylation of paraprotein: To determine both the breakdown sequence of the paraprotein under mild reducing conditions and the molecular weight of the final break down products, i.e. the heavy and light chains, reduction and alkylation experiments of several paraproteins were performed as follows. Selected patients' eluate fractions containing predominantly myeloma monomer or polymer IgA were collected, concentrated by low pressure dialysis in Visking tubing 8/32, reduced with an equal volume of dithiothreitol (1, 2, 4 and 100 mM solution - the 100 mM concentration giving complete reduction) in 2% SDS and 8M urea and finally alkylated with excess iodoacetamide. Paraprotein, both partially and completely reduced, was then electrophoresed on SDSPA gels. To determine if there was any variation in the size (i.e. - in electrophoretic mobility) of paraprotein monomers and polymers belonging to different patients, myeloma protein from each patient was mixed with that from a single patient whose paraprotein had been chosen as a standard and the mixture electrophoresed on SDSPA gels.

Serum viscosity: The serum viscosity was measured in as Ostwalt viscometer at 37°C and room temperature. The results were expressed relative to distilled water. Preliminary experiments were also performed using

a single Pasteur pipette as a viscometer. On the barrel were scored three lines, the lower two being 10 and 47 mm distant from the upper line. Serum was drawn up to the upper line, the pipette held vertical and then the serum was allowed to spontaneously drain from the pipette, the time taken for the serum meniscus to pass the two lower lines being measured with a stop watch. The experiments were performed either at room temperature or at 37°C and the pipette was rinsed with temperature equilibrated saline between readings. Good agreement was seen between the relative viscosities of sera measured using the Ostwatt viscometer and the simple pipette viscometer. This latter viscometer is universally available, simple to use and has the added advantage of requiring only 3 mls of serum.

Some sera on which viscosity studies were performed has been stored at -20°C for periods up to 2.5 years. One serum (F.B.) in the fresh state has a relative serum viscosity of 2.8 (at 37°C) and when retested with the same viscometer 28 months later recorded a relative viscosity of 2.5. Similar studies on other serum specimens were not performed due to the limited amount of stored sera.

RESULTS

During the five year period on which this study is based, 81 patients presented to the Radcliffe Infirmary with multiple myeloma. Thirty-eight of these patients had IgG myeloma, 25 had IgA myeloma, 17 had light chain disease and 1 had IgD myeloma. Thus IgA myeloma constitutes about one

third of the cases of myeloma seen in Oxford over this period. The clinical findings and the relevant laboratory findings at initial presentation of the patients with IgA myeloma are illustrated in Figures 3,2, 3,3 and 3,4. Twenty of these patients have to date died from their disease. The clinical details of the death were recorded in thirteen patients. Pneumonia was considered to be contributory to the death in eight of these thirteen patients, renal failure in four, severe cardiac failure in four, hypercalcaemia in three and septicaemia in three.

Paraprotein polymerization

The degree of polymerization of the myeloma protein of these patients was ascertained from the IgA elution profiles after gel filtration chromatography and from an analysis of the microdensitometry record after SDSPAGE of serum. The results are shown in Table 3,1. The Kav value refers to the peak position of the IgA profile and it is seen that Kav values of 0.28 were associated with paraproteins predominantly of the dimeric variety whereas Kav values of 0.4 were seen with monomeric paraproteins. This is further illustrated in Figure 3,5 where the chromatograms of two sera from patients with myeloma are shown. One chromatographic profile (b) shows a predominant monomeric paraprotein (Kav = 0.41) while the other (c) demonstrates the pattern seen with a serum containing predominantly polymerized paraprotein (Kav = 0.29). Some paraproteins were eluted with two IgA peaks in gel filtration chromatography and thus have two Kav values as seen in Table 3,1.

The percentage proportion of monomer or individual polymer was obtained from the electrophoretogram by a planimetric method as described in

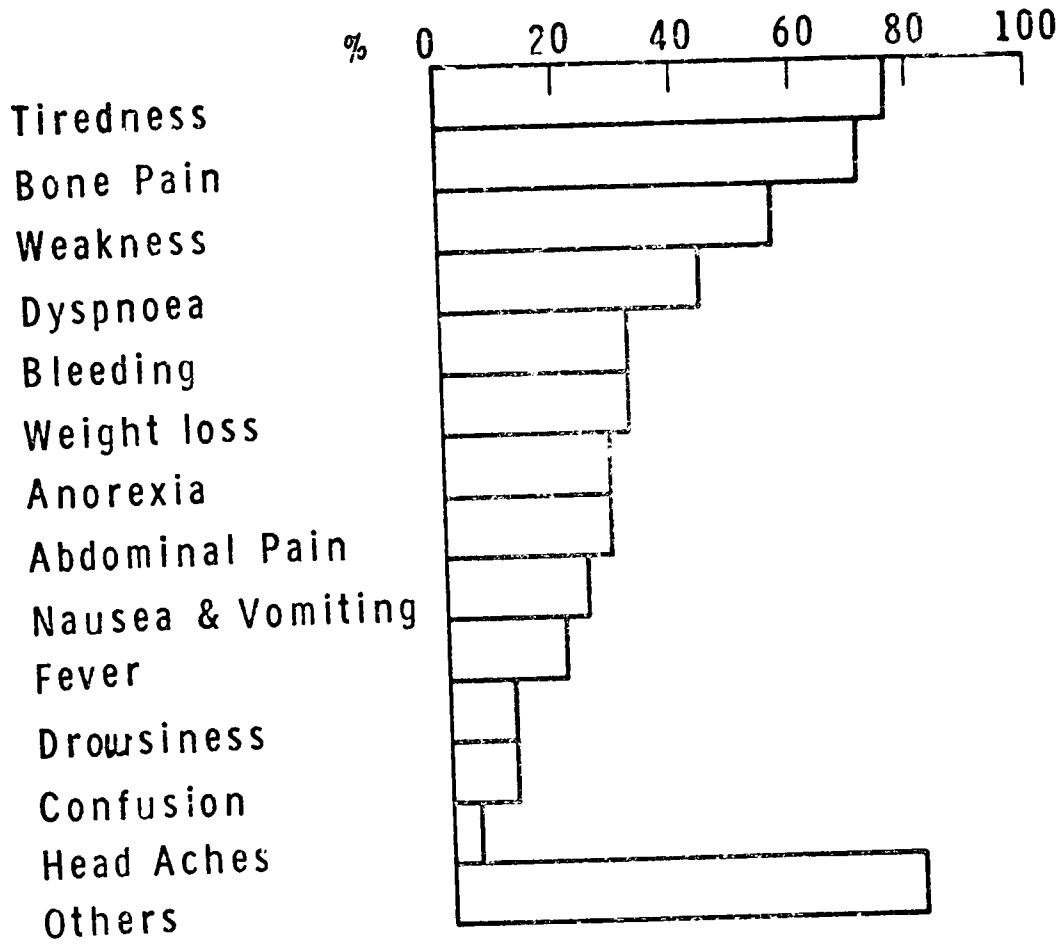


Figure 3,2 Incidence of symptoms seen at presentation in 25 patients with IgA myeloma

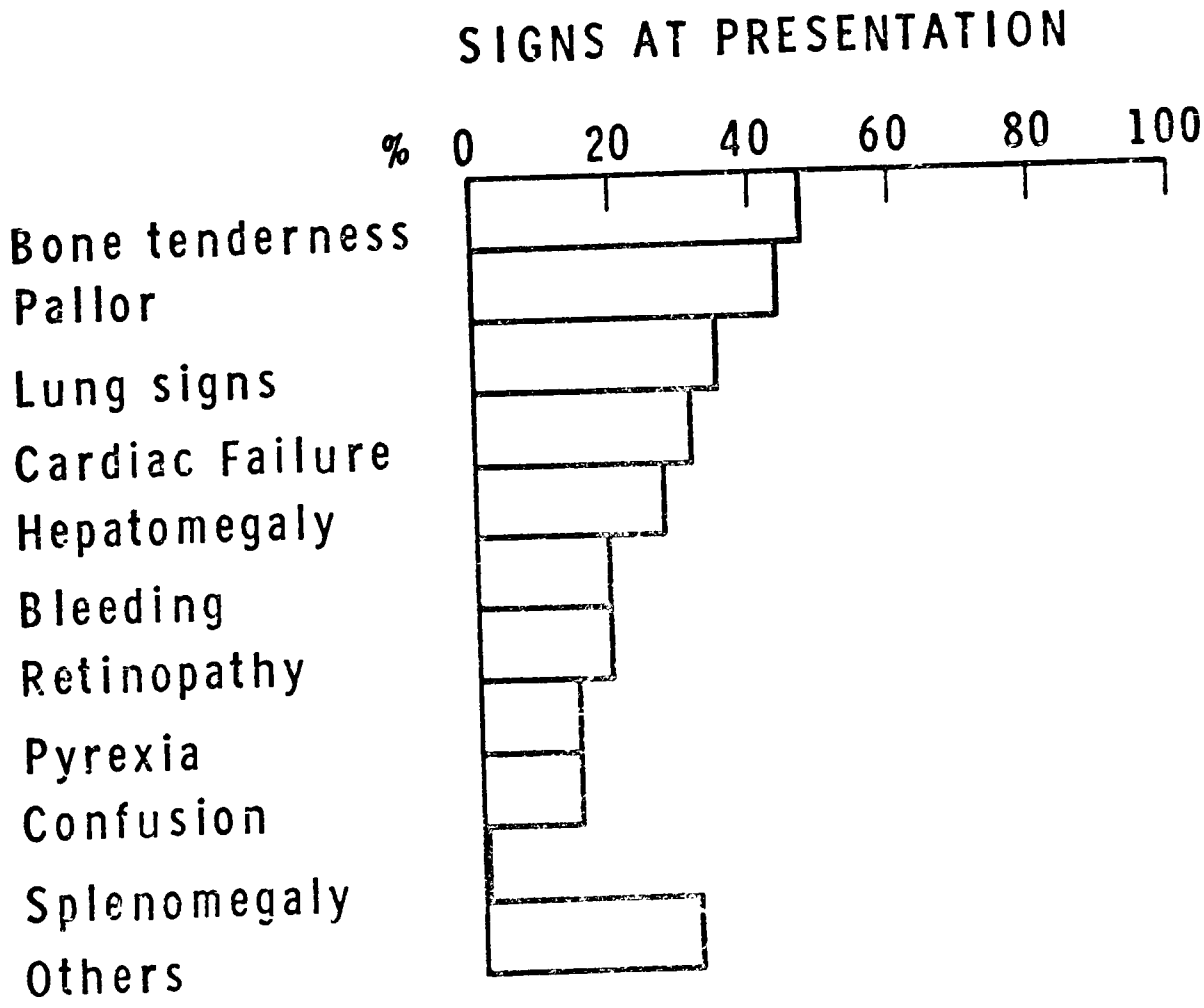


Figure 3,3 Incidence of physical signs seen at presentation in 25 patients with IgA myeloma

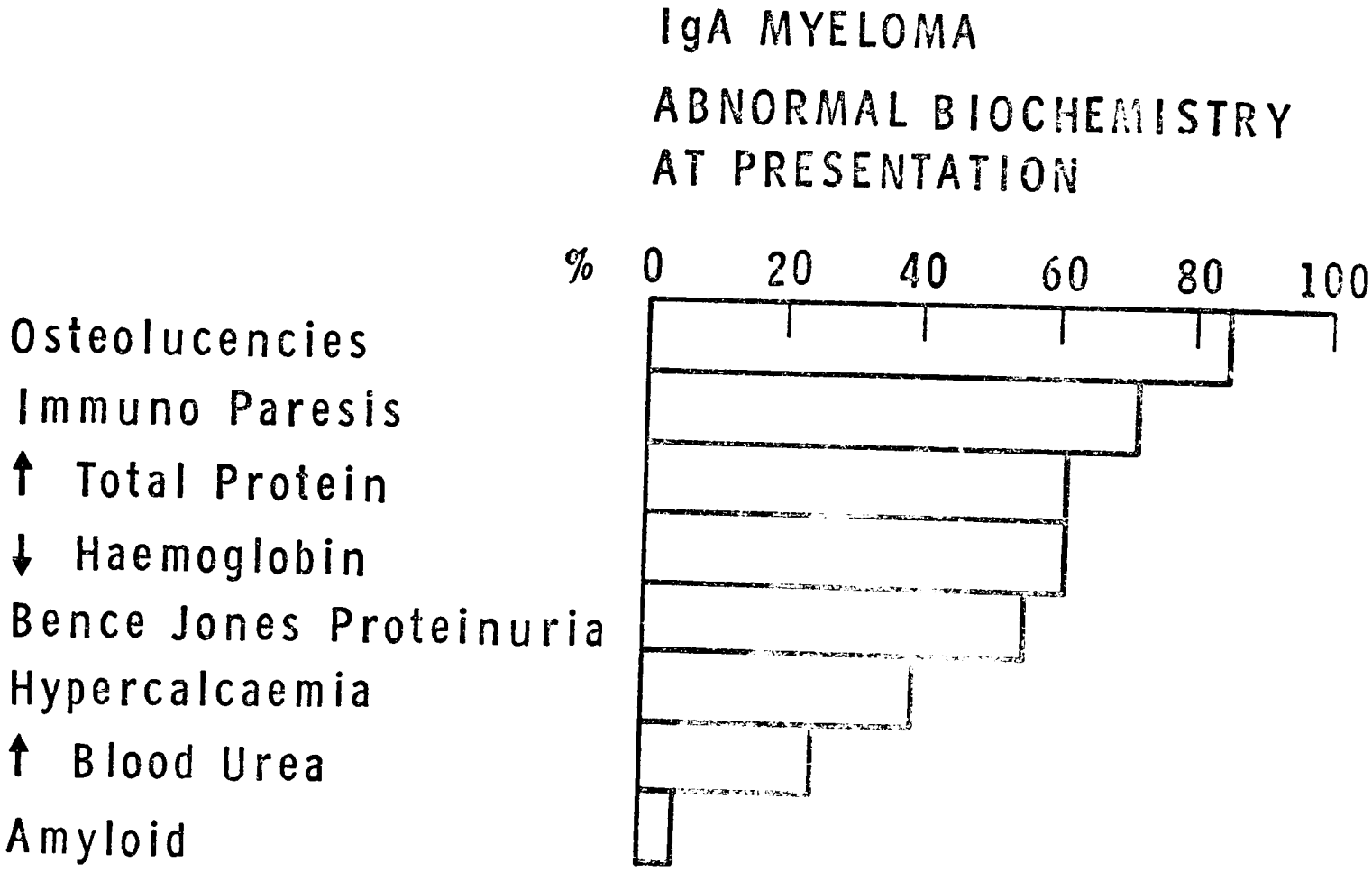


Figure 3,4 Incidence of abnormal radiological and laboratory findings found at presentation in 25 patients with IgA myeloma

Table 3,1 Immunochemical features and the degree of polymerization seen for the paraprotein from 25 patients.

M = monomer D = dimer T = trimer Q = quadramer P = pentamer

	Patient	Age	Sex	M Protein g/l	M Protein i.u./ml	kappa/λ type	Kav	% Polymer M	D	T	Q	P
Polymeric	AH	78	F	76.5	2460	kappa	0.29	7.8	72.2	13.6	5.8	0.6
	FB	60	M	79	4600	kappa	0.28	32.0	49.4	17.9	0.7	- *
	WP	61	M	48.3	2180	kappa	0.40	44.8	44.1	7.2	3.9	-
	GH	62	F	28.5	1500	kappa	0.28	9.7	70.1	76.3	3.9	1.0
	IS	68	F	73.5	6360	λ	($^{0.41}_{0.22}$	< 50	IgA type II			*
	HA	76	F	44	2670	λ	0.29	28.7	62.4	7.1	1.7	- *
	GF	74	F	36.5	1920	λ	0.28	22.8	64.2	11.8	1.2	- *
	CG	61	F	7.6	2780	λ	0.29	23.5	44.5	22.6	7.7	1.7
	AB	55	F	28.5	1650	kappa	0.29	19.7	77	3.3	-	-
	JCI	82	M	7	156	kappa	0.28	19	74	4.5	2.5	- *
	AWa	65	M	N D	1000	kappa	N D	34.1	52.6	13.3	-	-

* = presentation sera N D = not done

	Patient	Age	Sex	M Protein g/L	M Protein i.u./ml	kappa/λ	Kav	% Polymer M	% Polymer D	% Polymer T	% Polymer Q	% Polymer P
	EH	66	M	81	5000	kappa	0.41	60	30.5	6	3	0.5
	BL	44	F	43.5	3640	λ	(0.41 0.31	56.3	35.5	7.1	0.8	0.3
	RB	41	M	37.5	5722	λ	0.42	77.1	21.4	1.5	-	- *
	JCo	75	M	53.5	3480	kappa	0.37	51.4	30.4	13.8	3.3	1.1 *
	PB	78	M	36.5	1600	λ	0.39	66.8	27.7	56	-	- *
	WM	57	M	22.5	550	kappa	0.41	77.5	17.5	5.0	-	-
Monomeric	LT	74	M	36.8	2620	λ	(0.41 0.28	52.5	36.4	10.6	1.5	- *
	IB	78	F	10.5	1000	kappa	0.41	62.5	2.6	11.5	-	- *
	AH	67	M	16.5	2100	λ	0.43	81.4	14.1	2.7	1.8	- *
	AWy	51	M	N D	3500	λ	N D	65.9	26.7	5.3	2.1	-
	CL	42	M	22	2000	kappa	0.40	65	29.7	5.3	-	- *
	RJ	67	M	N D	4700	kappa	N D	60.8	32.8	5.9	1.5	- *
	DN	64	F	N D	2800	kappa	N D	87	9.8	3.2	-	-
	GL	67	M	25.2	3300	λ	0.40	90.6	7.7	1.7	-	-

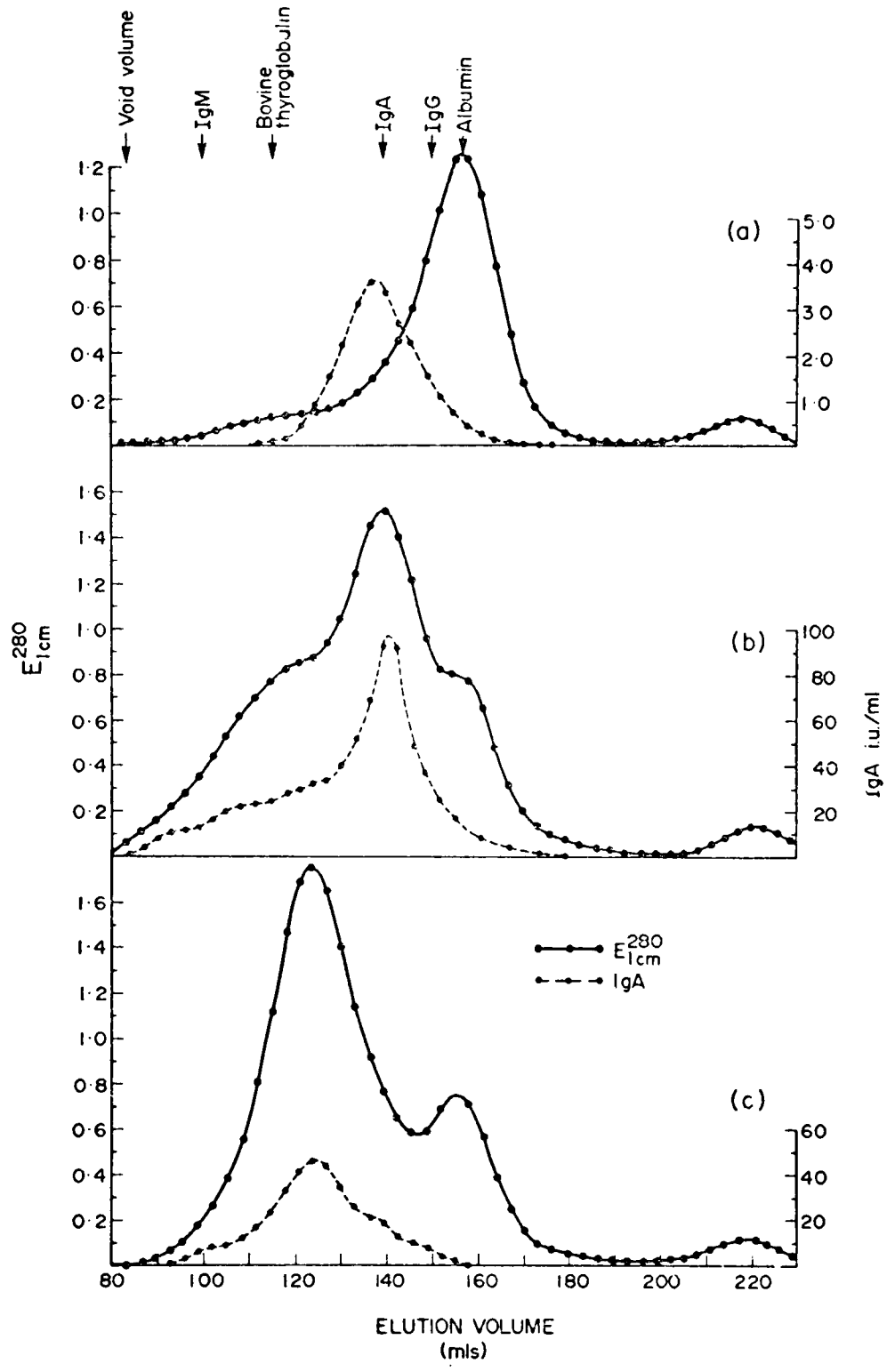


Figure 3,5 Chromatograms (gel filtration elution profiles) for three serum samples showing the relative concentration of protein and of IgA in different fractions.

a) Normal serum. The peak elution positions of several marker proteins are indicated.

b) Demonstration of the profiles seen for a serum with a paraprotein of the monomeric variety.

c) Demonstration of the profiles seen for a serum with a predominantly dimeric paraprotein.

the Methods section. One patient (A.Ho.) presented with a myeloma of the kappa light chain type with a normal serum protein SDSPAG electrophoretogram. Six months later a dense monoclonal IgA band was noted in her serum. The serum SDSPAG electrophoretograms seen in Figure 3,6 demonstrates the evolution of this polymeric paraprotein.

It is noted in Table 3,1 that, in general, a good agreement was observed between the degree of polymerization of the paraprotein as determined from the SDSPAG electrophoretogram and the filtration elution position as expressed by the Kav value. Many of the results tabulated in this table were derived from sera obtained at the time of the initial presentation of the patient.

From Table 3,1 it is seen that the myeloma protein was found predominantly in either the monomeric or the dimeric state whilst the concentration of the remaining polymeric members diminished with increasing polymeric size. No relationship was seen between the percentage proportion of polymers and the concentration of the paraprotein. A wide variation was seen between different individuals in the proportion of each independent polymeric member and its respective molecular size and there was no clear relationship between them. It should be emphasised, however, that no myeloma protein was studied in which the trimer or higher polymer was the predominant species. Figure 3,7 illustrates the proportion of the paraprotein which was in the monomeric state in each patient's serum and this value forms the basis of the division of the patients into two groups according to whether their myeloma protein was greater or less than 50% polymerized.

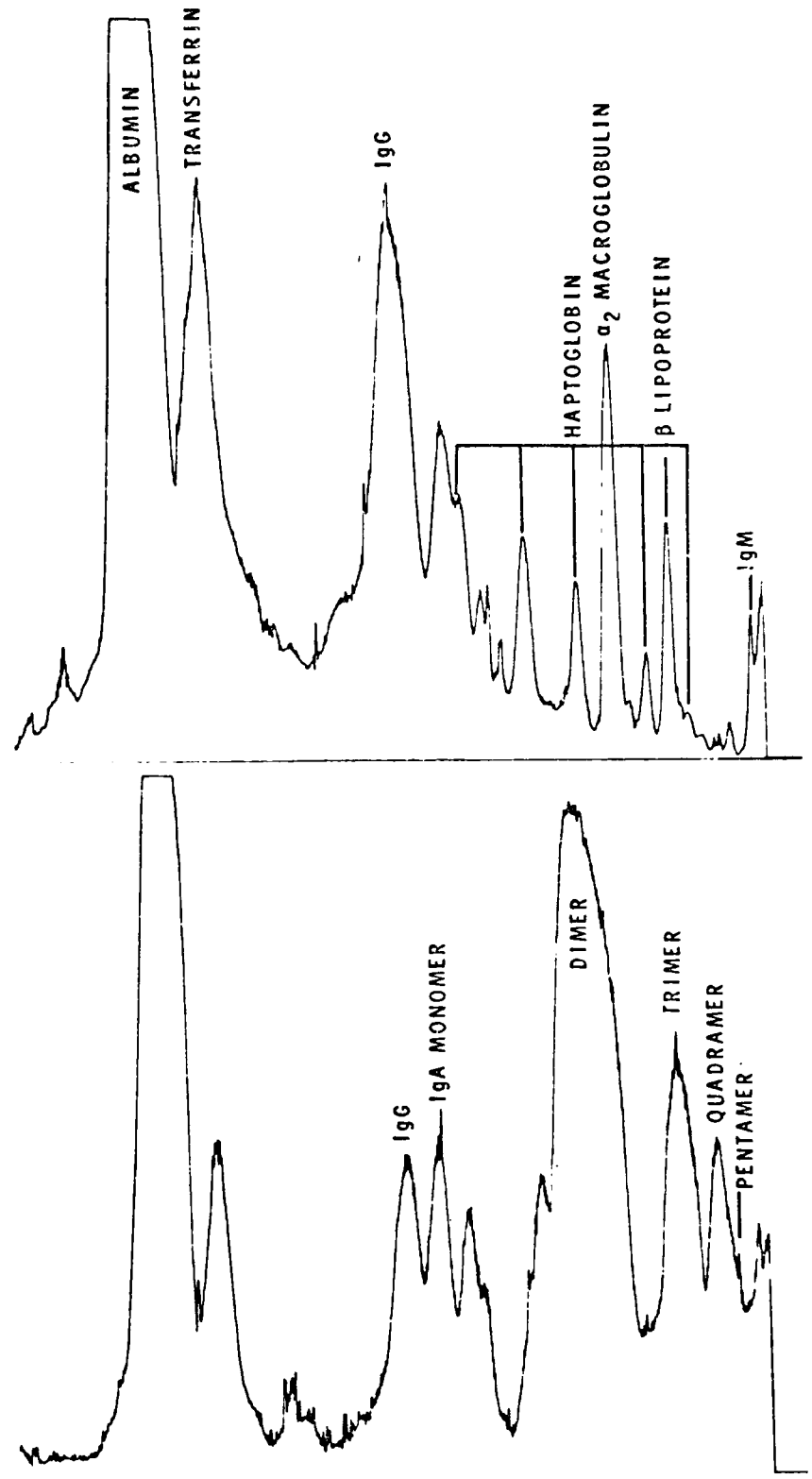


Figure 3,6 Microdensitometry electrophoretogram after SDSPAGE of the serum from 1 patient.

Top: normal pattern - no paraprotein observed

Bottom: paraprotein present and of the polymeric variety

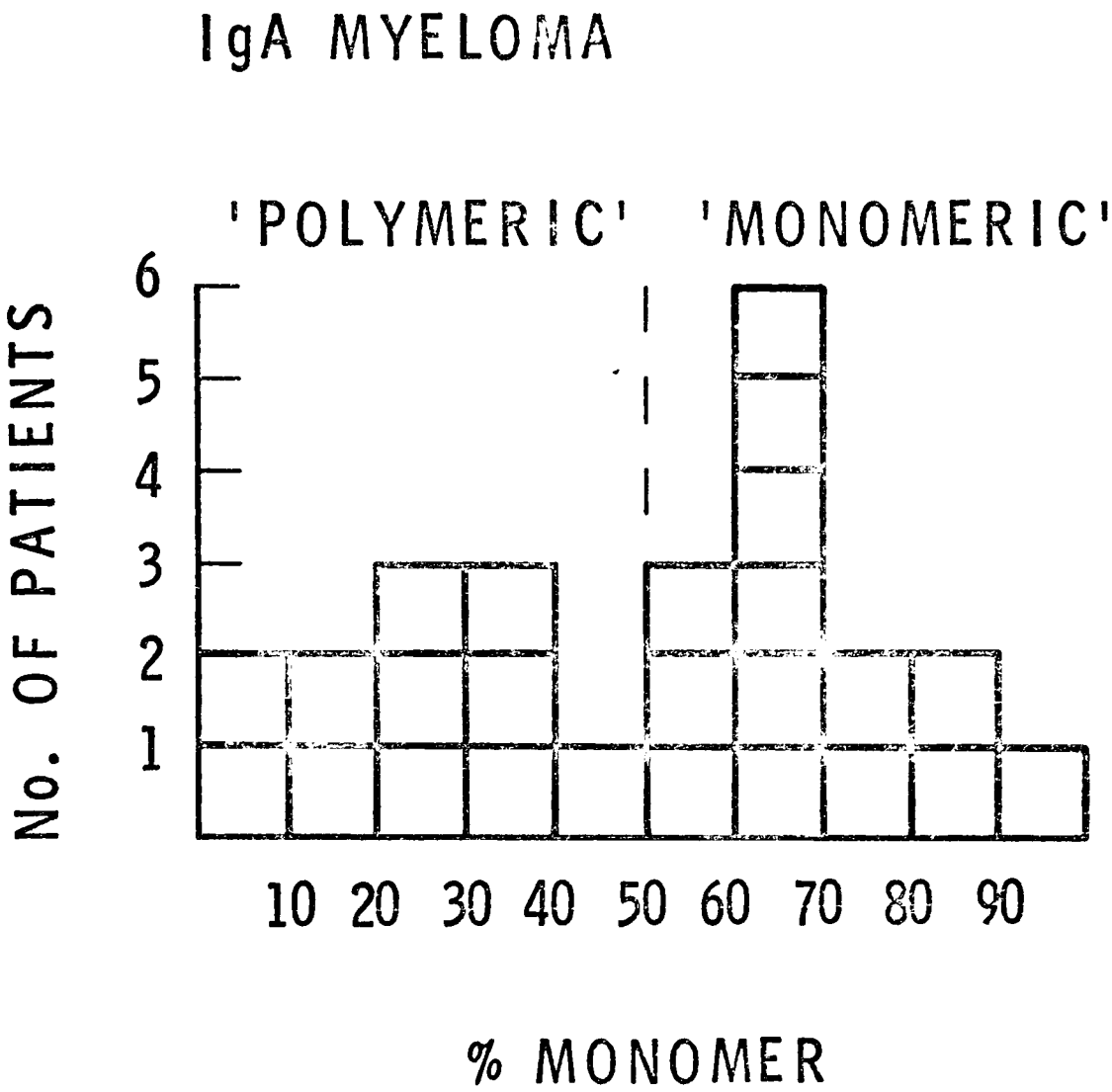


Figure 3,7 The percentage of the paraprotein consisting of the monomeric species is found to vary greatly between paraproteins. The variation seen for 25 patients is demonstrated in this figure. The paraproteins in these patients have been arbitrarily divided into a polymeric or monomeric group according to whether the paraprotein is greater or less than 50% polymerized respectively.

Degree of paraprotein polymerization seen during different stages of the disease

The degree of paraprotein polymerization was determined serially in order to ascertain whether the degree of polymerization remained constant or varied during the course of each individual's disease and with changing paraprotein concentration. The results are depicted in Table 3,II. The mean of the maximum difference between percentage monomer or dimer values obtained at different times for eight patients was 6.6% suggesting that the degree of polymerization of the individual myeloma protein is stable and does not vary during the course of the disease or with the quantity of myeloma protein.

Clinical correlation

A major objective of this study was to assess the clinical and laboratory features during the course of the disease with the polymeric nature of the myeloma protein. The results are summarised in Table 3,III. A striking finding was noted in the polymeric group in that five patients developed the hyperviscosity syndrome during the course of their disease. These patients are the first five listed in Table 3,I and the clinical and laboratory features observed at the time of this complication are seen in Table 3,IV. Serum was available for study during this complication from all five patients and the relationship of the degree of polymerization of the paraprotein to the quantity of the myeloma protein present at the time of this complication is seen in Figure 3.8. Values obtained for other patients without the hyperviscosity syndrome are also included in this figure for comparison. The quantity of paraprotein observed in the patients suffering from the hyperviscosity syndrome

Patient	Date	% Monomer	Kav	Concentration
F.B.	19.1.73	32%	0.28	4600 iu /ml
	23.2.73	31.8%	0.28	3920 iu /ml
	23.3.73	36%	0.28	3600 iu /ml
B.L.	9.1.71	62.6%	0.41	1500 iu /ml
	6.9.73	56.3%	0.41	3640 iu /ml
C.G.	3.3.72	29%	0.29	500 iu /ml
	3.2.74	23.5%	0.29	2780 iu /ml
R.B.	7.10.72	77.1%	0.42	5722 iu /ml
	8.8.73	82.1%	0.43	4800 iu /ml
J.C.	15.8.73	51.4%	0.37	3480 iu /ml
	5.9.73	-	0.38	900 iu /ml
P.B.	24.1.74	66.8%	0.39	1600 iu /ml
	22.9.74	-	0.39	1900 iu /ml

	Date	% Dimer	Kav	Concentration
A.B.	9.8.71	77%	-	-
	20.4.75	84%	0.29	-
A.P.	3.11.69	29.7%	-	2180 iu /ml
	17.10.72	44.1%	-	4500 iu /ml

Table 3,II The percentage proportion of monomer or dimer paraprotein member estimated in 8 patients on several occasions during the disease. The Kav and paraprotein concentration is also shown to demonstrate that the % monomer or dimer and Kav for a particular paraprotein remains generally constant during the course of the disease and with changing paraprotein concentrations.

IgA Myeloma

	Polymeric	Monomeric
Number	11	14
Kappa / lambda	7/4	7/7
Male /female	7/4	11/3
Bence Jones proteinuria	7/10	7/14
Immunoparesis	6/9	10/13
Hypercalcaemia	5/10	6/13
Infection	9/11	9/14
Bleeding	8/11	4/14
BUN > 60 mg %	5/10	6/13
Hyperviscosity syndrome	5/11	0/14

Table 3,III Clinical and laboratory features seen during the course of 25 patients with IgA myeloma. They have been divided into a monomeric or polymeric group according to the degree of polymerization of the paraprotein:- see Figure 3,7. No appreciable differences are seen between these two groups apart from the hyperviscosity syndrome which has occurred in five patients belonging to the polymeric group but in no patient of the monomeric group.

BUN = Blood urea nitrogen

Patient	I.S.	A.Ho.
Lassitude	++ - +++	++
Headaches 'headiness', confusion	terminal confusion	-
Bleeding	skin, retro orbital	epistaxis, skin
Congestive cardiac failure, blood pressure	+++ 130/80	+++ 140/70
Renal failure	+ - ++	terminally
Fundoscopy	bilateral papilloedema	exudates and haemorrhages tortuous dilated veins
Neurological findings	left proptosis and gauze paralysis	fine nystagmus, finger numbness, sudden deafness
Haemoglobin g/l	5.7 - 8.3	9.1 - 6.3
Calcium mg %	9.6	8.3 - 11.0
Total protein g/l	116	91 - 100
Viscosity (relative to water, 37°C)	N D	4.4
Platelet count / mm^3	20,000 - 50,000	20,000 - 50,000
Transfusion in units	11	4
Plasmaphoresis	N D	Yes
Survival after development of H V S	6 weeks	2 months
Cause of death	Cardiac failure, renal failure, pulmonary oedema	Cardiac failure, renal failure, bronchopneumonia
Autopsy findings	widespread bleeding, myeloma	bleeding, pulmonary oedema, myeloma
Diagnosed during life	No	Yes

Table 3, IV Clinical and Laboratory Features of Five Patients with IgA Myeloma and the Hyperviscosity Syndrome (H V S)

F.B.	G.H.	W.P.
+	++	++
'muzzy' headaches	terminal confusion	'muzzy' headaches
epistaxis	haemopytsis, haematuria, skin	epistaxis, malaena, skin
++ 110 / 80	++ N D	- 140/80 - 210/130
-	-	terminally
bilateral haemorrhages and exudates bulbar palsy	not viewed peripheral neuropathy	normal on one occasion progressive deafness left ear
11.2 - 13.0	11.1 - 6.9	6.7 - 10.1
12 - 13	10.7	9.8
100 - 110	92	100 - 118
2.8	N D	3.2
200,000	20,000 - 40,000	60,000 - 80,000
N D	N D	multiple
Yes	N.D	N D
4 months bronchopneumonia	1 month chest infection	5 months cerebral bleed, renal failure, pneumonia
N D	N D	widespread bleeding myeloma
Yes	No	Yes

+ mild
++ moderate
+++ severe

N D not done
- not seen

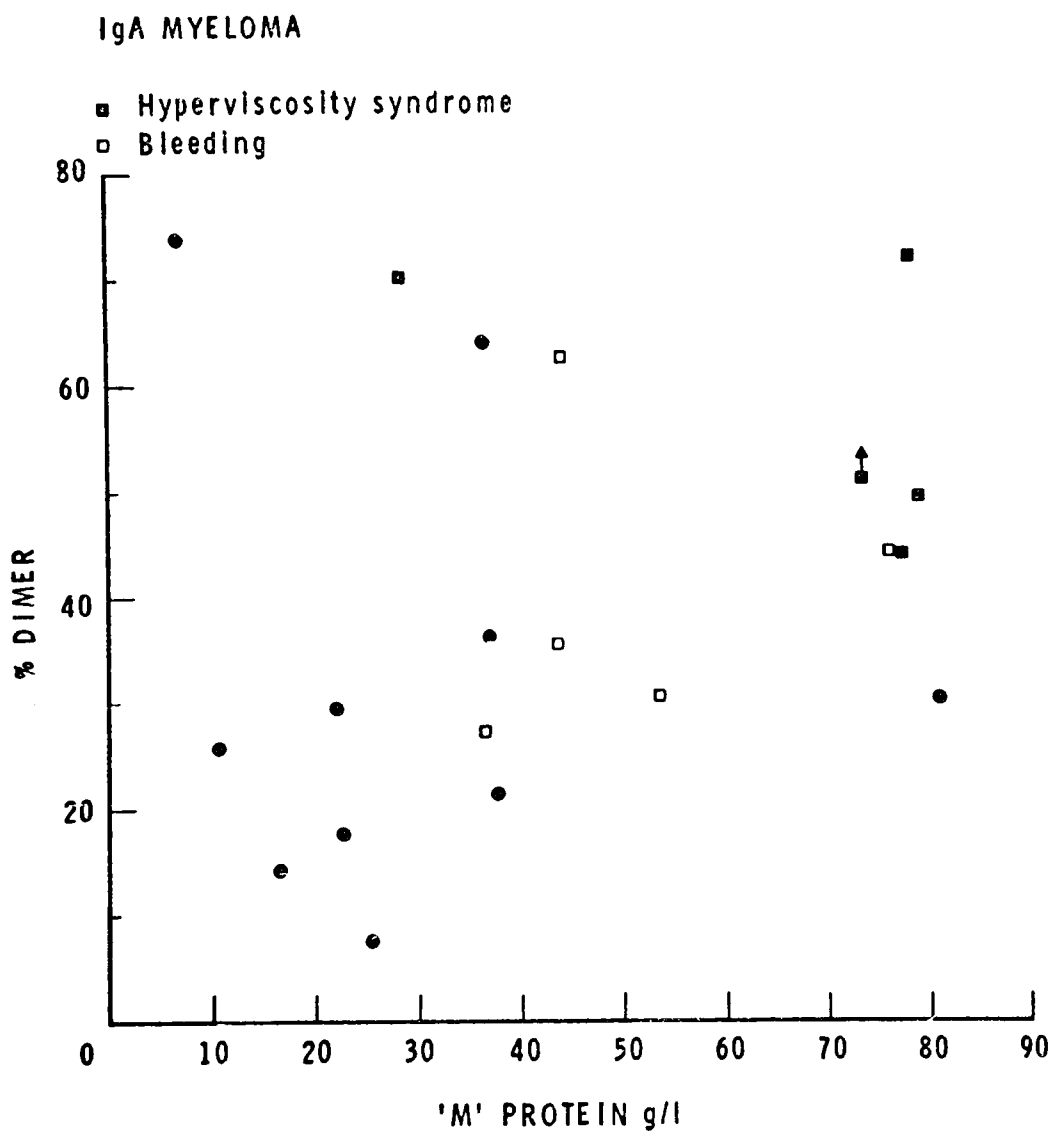


Figure 3,8 Relationship between paraprotein concentration, degree of polymerization and bleeding and hyperviscosity complications. Percentage dimer reflects the degree of polymerization of the paraprotein. Closed circles refer to patients without complications.

was seen to vary from 28.5 - 79 g/l and these values were associated with total plasma proteins between 92 - 118 g/l. During the clinical course of three patients belonging to the monomeric group total protein values between 105 - 120 g/l were observed without the development of the hyperviscosity syndrome.

The serum viscosity was measured in three of the patients at the time of the development of the hyperviscosity syndrome and in each case an elevated value was observed (Table 3,IV). The upper limit of normal for this measurement is 1.8 (η rel 37°C).

Bleeding was observed to be more frequent in the polymeric group than in the monomeric group but this is probably accountable by the constant association of bleeding with the hyperviscosity syndrome. The relationship of bleeding to the polymeric nature of the paraprotein and to the quantity of paraprotein is also indicated in Figure 3,8.

The survival curves for the two groups are seen in Figure 3,9. Although the total numbers are small there is the suggestion that the polymeric group has greater mortality in the first year. The mean survival after the development of the hyperviscosity syndrome was $2\frac{1}{2}$ months despite conventionally accepted methods of treatment including plasmaphoresis in two patients.

Additional findings on paraprotein analysis

The paraproteins from 24 of the patients had electrophoretic mobilities in SDSPAGE suggesting that they were monomers or higher polymers of IgA (Figure 3,1). The paraprotein of the remaining patient (I.S.) dissociated in

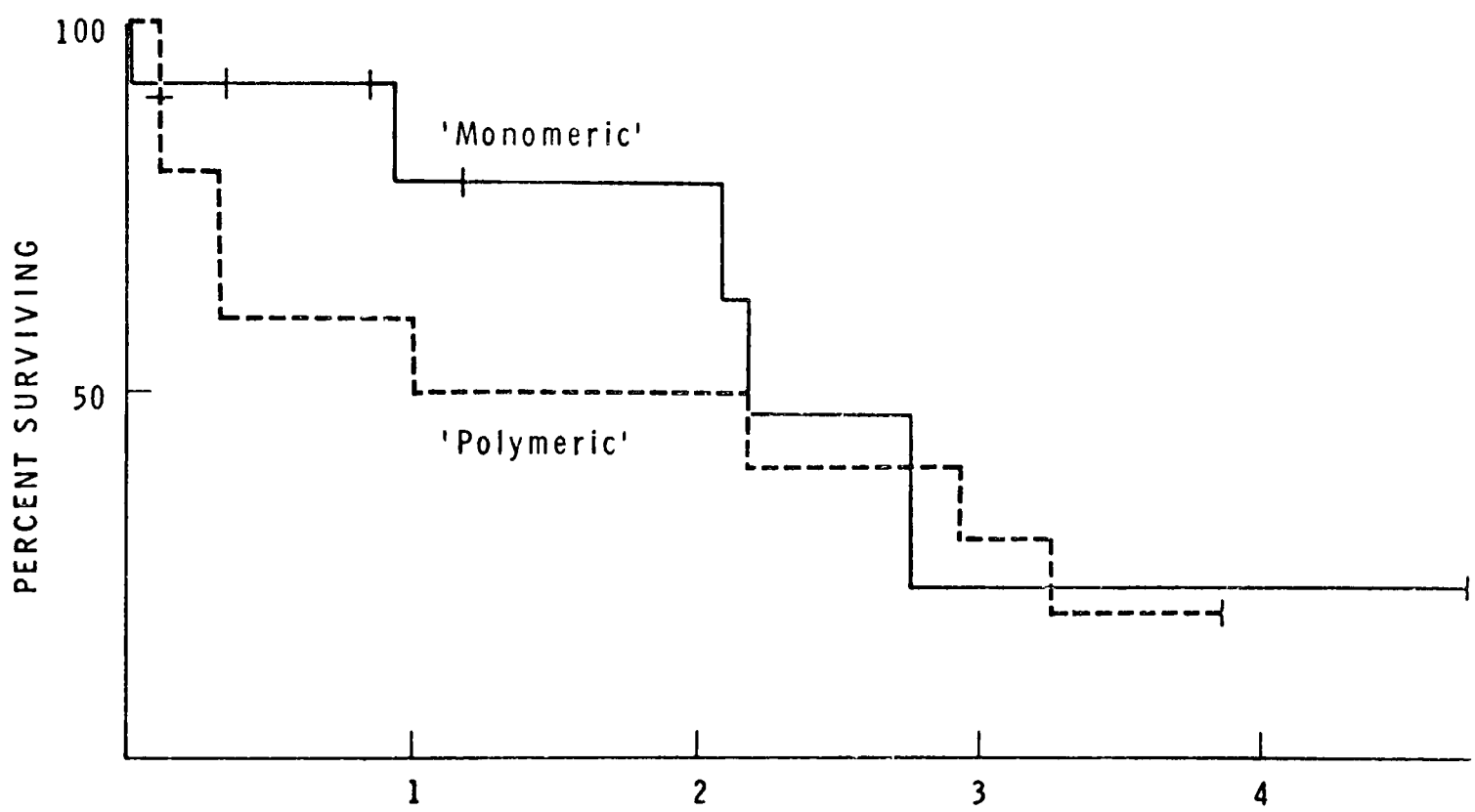


Figure 3,9 Survival for the monomeric and polymeric groups.

0.1% SDS and thus additional bands were observed after SDSPAGE. The appearance and nature of this later paraprotein will be discussed below under the heading 'paraproteins of different sub classes'. Another minor band was seen in three of the sera (F.B., R.B. and J.G.) in addition to the usual polymerizing paraprotein bands. This extra band is illustrated in Figure 3,10 together with the relationship between the corresponding electrophoretic mobilities of the bands and a doubling log scale. This showed that the additional member was only half the size of the monomer but whether it existed as such in the circulation or was artefactual, due to the dissociation of the paraprotein in SDS, was not investigated.

Reduction experiments: Reduction and alklyation experiments were performed on six paraproteins. Two bands were seen in SDSPAGE after complete reduction of the paraprotein with 100 mM dithiothreitol (DTT). The calculated molecular weights of these bands were consistent with them being alpha and light chains. The mean calculated molecular weights for these fragments are listed in Table 3,V.

One IgA λ paraprotein (from patient H.A.) was used on a standard in a series of reduction experiments (see Methods). On SDSPAGE of completely reduced and alkylated paraprotein preparations containing this standard paraprotein plus equal amounts of another paraprotein it was observed that whilst the alpha chain gave a single peak in the electrophoretogram (apart from one patient G.F., vide infra), two peaks were seen for the light chains if they belonged to opposite light chain sub classes. Thus the electrophoretic mobilities for the λ light chain from three patients were found to be slower than the mobilities for the kappa chain from three other patients (Table 3,V).

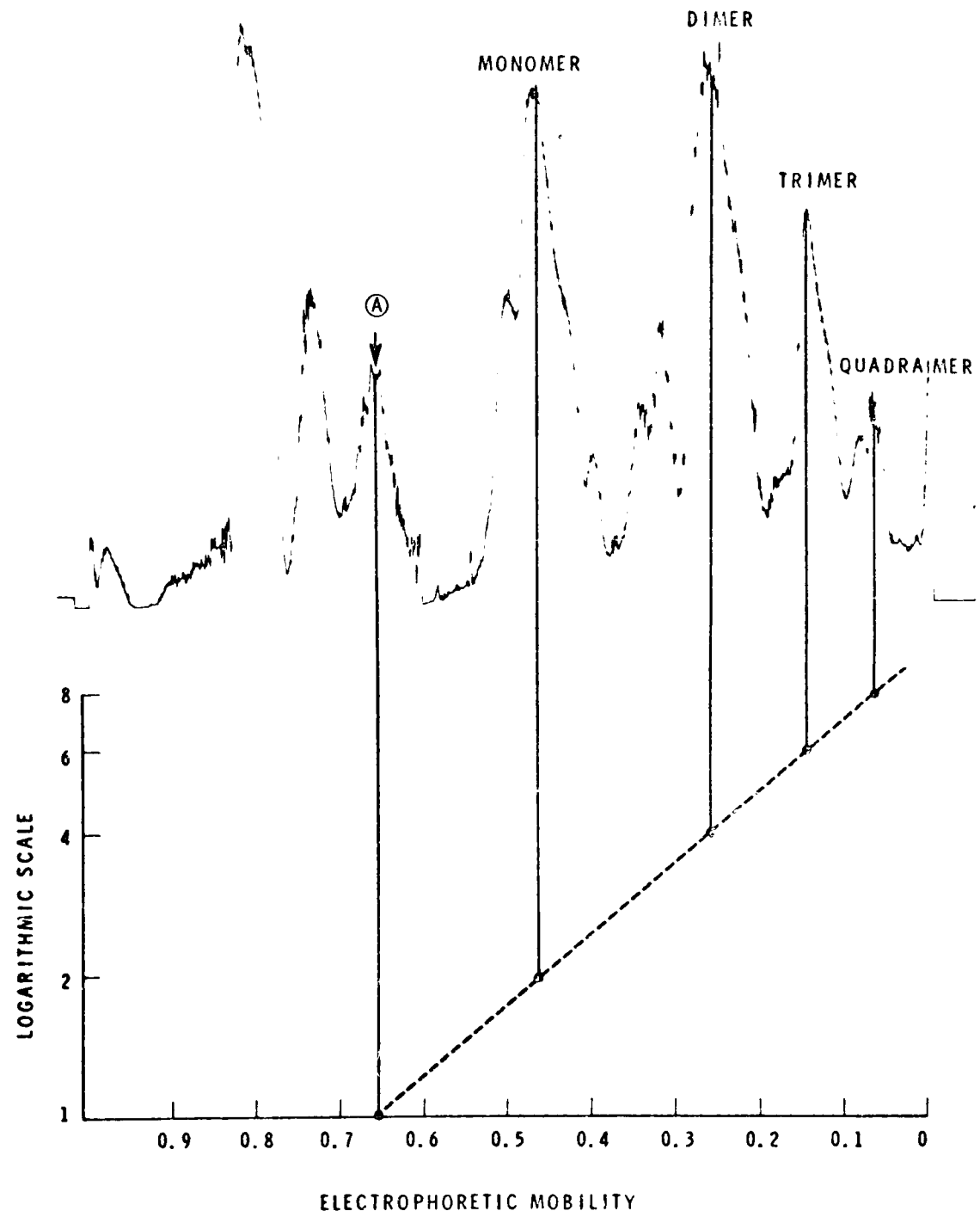


Figure 3,10 Three patients had an additional band (A) in their electrophoretogram. The linear relationship between a log scale and the electrophoretic mobility of this band and the monomers and polymers suggest that this extra band is a half unit of the monomer.

Patient	alpha chain	light chain	
G.H.	70,000	kappa	24,300
J.Cl	61,500	kappa	22,000
C.G.	64,500	λ	16,500
H.A.	61,500	λ	25,100
G.F.	75,000	λ	28,600
F.B.	66,000	kappa	24,000

Table 3,V The molecular weights for the heavy and light chain of the paraprotein from six patients.

In order to elucidate the breakdown sequence in the reduction of IgA polymers, experiments were performed on the polymers of three patients (H.A., G.F. and G.H.). All three showed similar results. Under mild reducing conditions (1.0 mM DTT) four major bands were seen in 3.6% SDSPAGE. A linear relationship was seen between a log doubling scale and the corresponding electrophoretic mobilities for the upper three bands suggesting that the upper band was the dimer, the middle the monomer, and the lower the alpha + light chain component. The calculated molecular weights were consistent with this interpretation. The lowest band in the gel had a molecular weight between 22 - 28,000 suggesting it was the light chain. No free alpha chain was observed under mild reducing conditions. Minor bands were also observed above and below the postulated monomer unit suggesting additional species of molecular weights 20,000 less or 30,000 greater than the monomeric unit. Several minor bands were also observed between the two upper major bands.

Under stronger concentrations of reducing agent (2.0 mM DTT) no higher polymers were seen and the monomeric unit was present in minor amounts. Three prominent bands were present which had molecular weights suggesting that they were light chain, alpha chain and alpha + light chain. Minor bands were also observed with slower electrophoretic mobilities and their calculated molecular weights suggested that they were moieties consisting of two alpha chains and two alpha + one light chain. Stronger reducing conditions (4 mM DTT) gave two principal bands with molecular weights consistent with them being the alpha chain and the light chain.

The monomer and polymers from one patient (G.F.) were observed to migrate more slowly in SDSPAGE than the corresponding members of the

'standard' protein suggesting that the monomer and hence polymers might be abnormally large. This was confirmed by reduction and alkylation of the paraprotein where it was observed that the component alpha chains and λ light chains were found to have slower electrophoretic mobility than the corresponding fragments of the standard paraprotein. Thus the calculated molecular weight of this patient's heavy and light chain was 75,000 daltons and 28,000 daltons as compared to 61,500 daltons and 25,000 daltons obtained for the standard paraprotein (H.A.). These values were the mean of four readings.

IgA paraproteins of different sub classes: The light and heavy chains of IgA proteins of the sub class one are held together by disulphide bonds and thus do not separate in dissociating conditions. However some IgA proteins of the sub class 2 lack these light - heavy bonds and thus dissociation occurs in the presence of denaturants such as 0.1% SDS (Grey et al., 1968).

The paraprotein from one protein (I.S.) partly dissociated in 0.1% SDS to yield 2 additional bands in SDSPA gels; one major band with an electrophoretic mobility of 0.858 and one minor band with a mobility of 0.575. The calculated molecular weight of these two components were 49,000 daltons and 130,000 daltons. Slow moving bands corresponding to high molecular weight species continued to be seen in SDSPA gels. On gel filtration chromatography in non dissociating conditions (PBS, pH 7.3) this paraprotein eluted in both the monomeric position and in an unusually early volume just after the IgM marker.

Paraprotein association: Mannik (1967) has demonstrated disulphide linking of IgA paraproteins to serum albumin. In the sera of eight patients in this study no albumin, as measured by radial immunodiffusion, was detected in the eluates containing the myeloma protein. The albumin profile after gel filtration of one representative serum is seen in Figure 3,11,a.

Association of IgA myeloma protein with other serum proteins including haptoglobin and lipoprotein has been previously reported (Schultze and Heremans, 1966). In four patients prominent haptoglobin bands were observed after serum SDSPAGE. On immunoelectrophoresis examination using a haptoglobin specific antiserum, single uniform haptoglobin arcs were seen migrating in the alpha position suggesting non association of the paraprotein with the haptoglobin protein.

One patient (F.B.) had a 'milky' serum which was due to excess very low density lipoprotein. No association could be found between his serum lipids and his paraprotein even after extensive investigations which have been reported elsewhere (Roberts-Thomson et al., 1975).

Remission: When multiple myeloma patients enter biochemical remission as a result of chemotherapy the serum 'M' band disappears, immunoglobulins tend to revert to normal level and Bence-Jones protein is no longer detectable in the urine (Hobbs, 1971). In Figure 3,11,b the gel filtration elution profile is shown of one such patient. His serum showed no 'M' band on routine cellulose acetate electrophoresis, his IgA was within normal limits (although his IgG and IgM were low) and he had no Bence-Jones proteinuria. However from this figure it is seen that a large proportion of his IgA is of

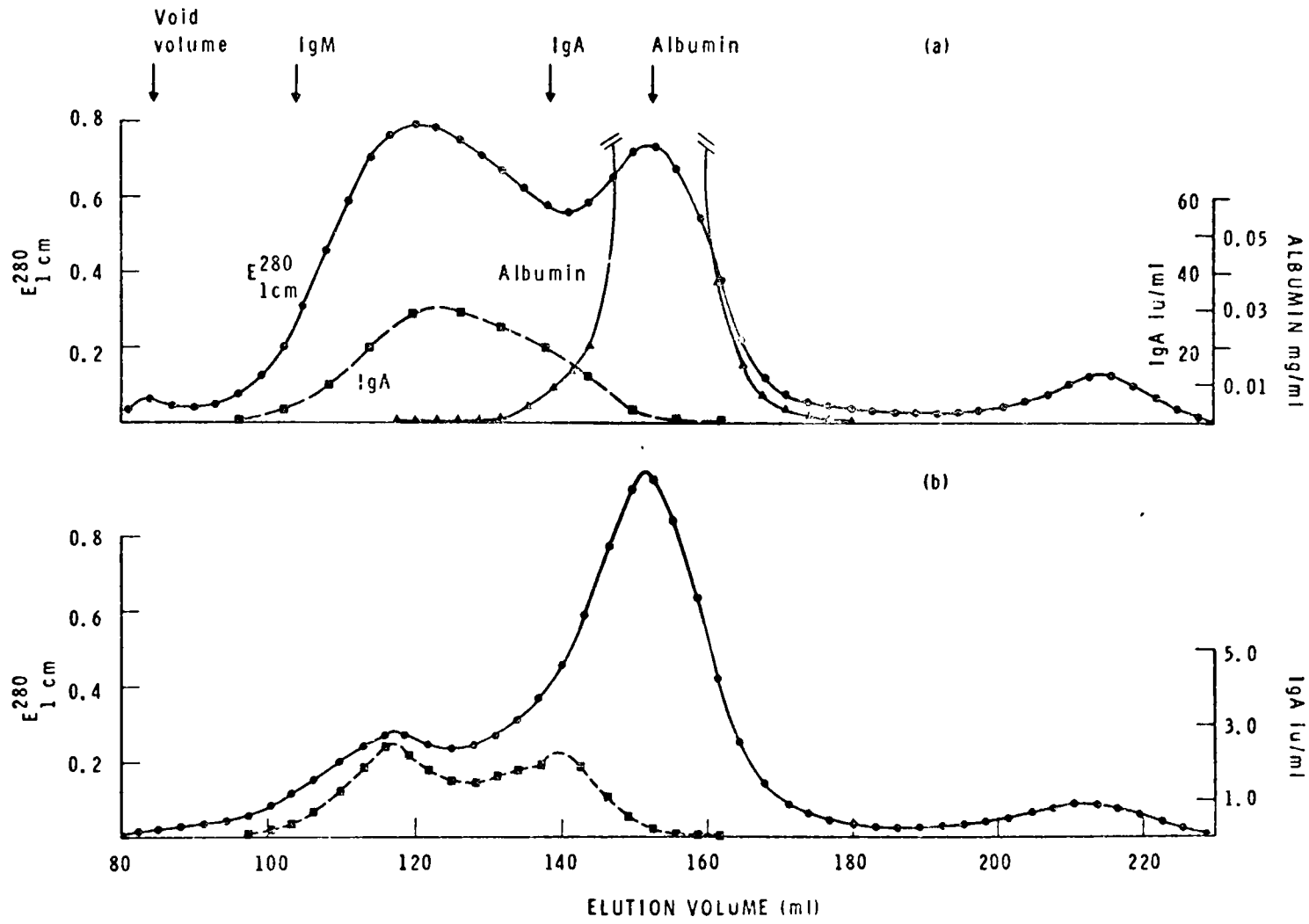


Figure 3,11

a) Gel filtration chromatogram from one representative patient showing lack of association between the paraprotein and albumin

b) Gel filtration chromatogram from one patient in bio-chemical 'remission'. Note the normal amount of IgA but containing a large proportion of polymeric IgA. This patient had a paraprotein of the polymeric variety at presentation.

the dimeric variety. This patient early on in his clinical course secreted moderate amounts of the polymeric form of the IgA paraprotein and the presence of this in low concentrations during 'biochemical remission' suggests that still a small number of the myeloma cells are secreting this paraprotein.

Measurement of polymeric IgA by radial immunodiffusion: From Figure 3,4,b it is seen that the concentration of IgA (as measured by radial immunodiffusion) at the IgA monomer peak per unit of extinction at 280 mμ is greater than the corresponding fraction measured at the peak IgA dimer elution position; the dimer elution position being at the same position as seen in Figure 3,4,c, where a predominantly dimeric paraprotein is seen. If this value (iu /ml /unit O.D.) obtained at the peak monomeric elution position is expressed as a fraction of the value at the dimeric elution position a ratio of 1.6 is obtained.

The mean of the same ratios obtained from similar profiles from 21 patients was 1.4 and suggested that for the same concentration of IgA (as measured by its O.D. extinction) the monomer will give in Mancini radial immunodiffusion a precipitation area which will be on average 1.4 times as large as the area seen for dimeric IgA. This reasoning, however, assumed that the extinction coefficient for both the monomer and dimer was the same.

Serum viscosity studies

Viscosity measurements on the sera of ten patients were related to the concentration of the paraprotein and the results are shown in Figure 3,12. The readings were performed at room temperature using the Pasteur pipette

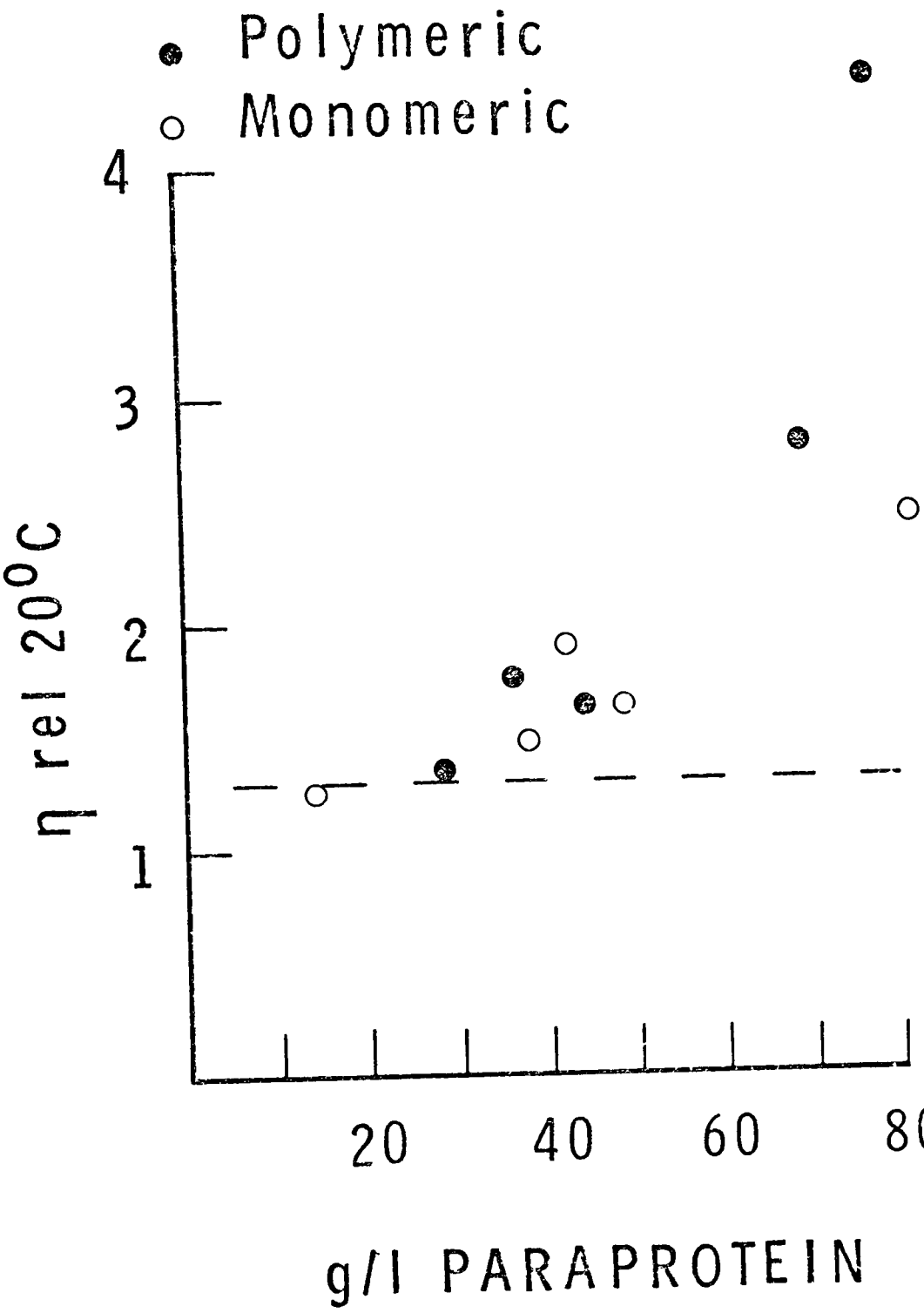


Figure 3,12 Preliminary evidence showing the relationship between relative serum viscosity and the concentration of the paraprotein. These observations are consistent with the suggestion that sera containing paraproteins of the polymeric variety will have relatively higher viscosities than those with monomeric paraproteins at the same concentration. The dashed line depicts the upper limit of serum viscosity obtained using the Pasteur pipette viscometer for 10 normal sera.

method. The limited number of sera were divided into a polymeric or monomeric group as defined above. More recordings are required to confirm the trend noted in this figure that the polymeric group will have higher serum viscosities than the monomeric group for the same concentration of paraprotein.

Elevation of benign monoclonal or polyclonal IgA

The sera from 13 patients with IgA levels in excess of 550 iu /ml were fractionated by gel filtration. In all IgA was found to elute with a maximum concentration at the monomer position. IgA was also seen eluting in earlier volumes in all sera but in small amounts. This indicates the presence of minor amounts of higher polymers. The gel filtration profile for one representative patient is illustrated in Figure 3,13.

DISCUSSION

Multiple myeloma is a neoplastic disease with several characteristic clinical and laboratory features (see Osserman and Isobe, 1972, and Penny, 1974 for a comprehensive review). These include monoclonal plasma cell proliferation with multiple bone involvement; the secretion by these plasma cells of monoclonal immunoglobulin; the presence of monoclonal immunoglobulin light chain in the urine (Bence Jones proteinuria) and a suppression of normal immunoglobulin levels. Atypical forms of myeloma can also occur (Osserman and Isobe, 1972). MacIntyre, in 1850, is thought to be the first to report the clinical features of such a case (MacIntyre, 1850) although the heat characteristics of urinary light chain were described 2 years prior to this

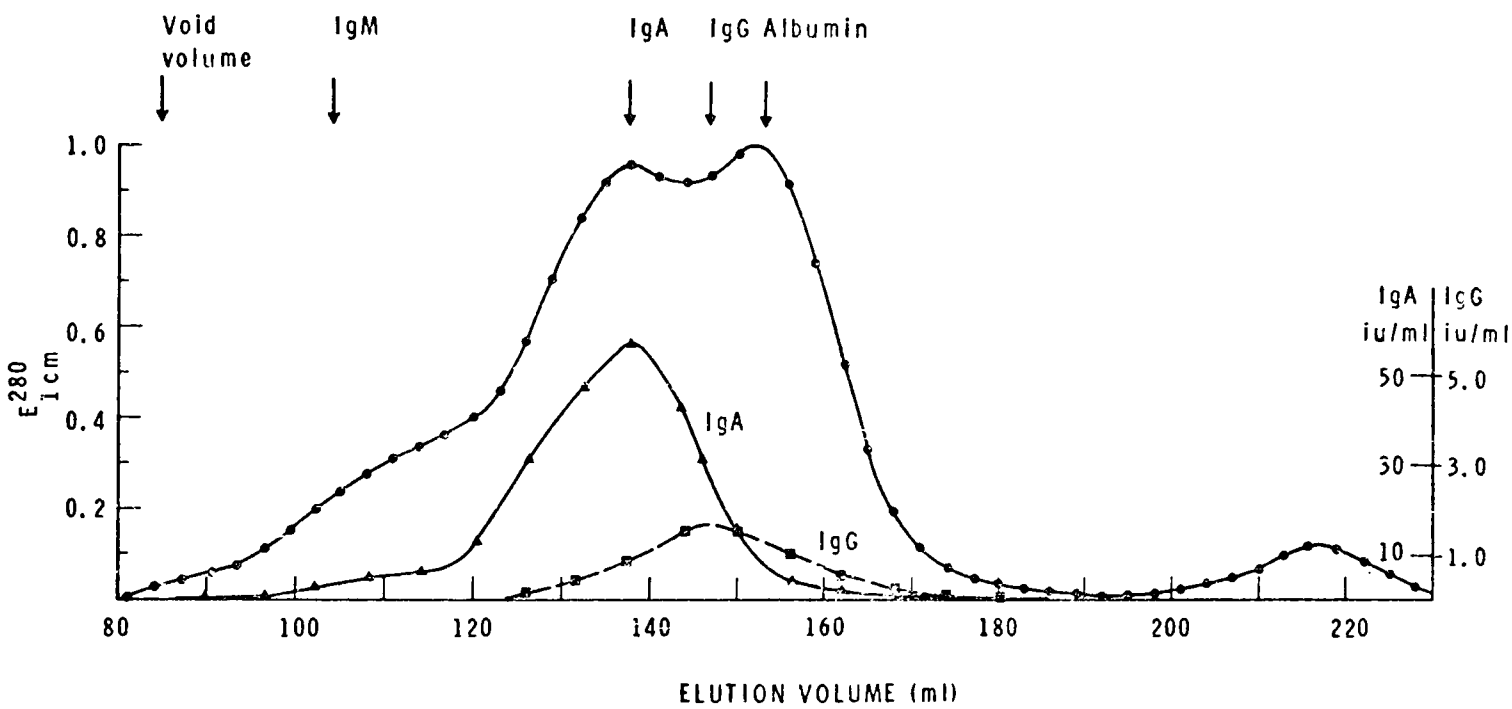


Figure 3,13 Gel filtration chromatogram obtained from one representative patient with benign IgA paraproteinaemia. Note only small amounts of higher IgA polymers are present.

report (Bence Jones, 1848). Rustizky in 1873 commented on the histological features of the disease and coined the term myeloma (Rustizky, 1873). Since then, and especially in the last three decades, numerous publications have stressed many clinical and laboratory features of the disease and much of the present knowledge concerning antibody structure and function has been derived from a study of the monoclonal immunoglobulin obtained from the sera of patients with multiple myeloma. For example the concept of a plasma cell neoplasma producing only a single type of immunoglobulin molecule was one stimulus for Burnet's clonal selection theory of acquired immunity (1959).

Although this disease is now well described much remains to be explained. Its aetiology, as with most neoplasms, is unknown, but genetic and environmental features have been implicated (Osserman and Isobe, 1972). The pathogenesis for many of the manifestations of the disease is understood but for others it remains to be elucidated. Animal models of multiple myeloma are seen in the mouse (Potter, Fahey and Pilgrim, 1957), cat (Farrow and Penny, 1971), dog (Osborne et al., 1968) and hamster (Cotran and Fortner, 1965) and have been of value in explaining some of these pathogenic mechanisms.

Multiple myeloma may present in the third decade but a peak incidence is generally seen in the mid fifties (Osserman and Isobe, 1972). Most of the earlier studies reported a male predominance but recently a change in sex distribution has been detected so that now men and women are equally affected (Martin, 1961). The Registrar General's return in England in 1958 gave an incidence of 2.6 per 100,000 live population (Burnet, 1959) which is similar to that seen for Hodgkin's disease and chronic lymphatic leukaemia.

Patients with multiple myeloma, despite the best medical treatment, often suffer from much pain and debility. Indices of prognosis are known which bear some relationship to the clinical course and duration (Peto, 1971) but there is a relatively early and fatal outcome in most cases. Despite some improvement in survival times induced by cytotoxic drugs the average survival time of 2-3 years (Galton, 1971) is not greatly different to that seen in 1928 without chemotherapy (Geschickter and Copeland, 1928).

Multiple myeloma may be classified into several types according to different clinical and laboratory criteria. One immunochemical classification is based on the class and subclass of the heavy chain and light chain of the paraprotein respectively. Thus acknowledging the pioneering work of Kahler, Ellinger, Waldenström, Heremans, Rowe, Fahey and Ishizaka, multiple myeloma is now generally subdivided into 6 principal types depending on the light and heavy chain of the secreted immunoglobulin molecule i.e. IgG, IgA, IgM, IgD, IgE and light chain. IgM myeloma is rarely seen (Hobbs, 1971), IgM paraprotein being usually associated with Waldenström's macroglobulinaemia.

Some variations between the clinical and laboratory features of these different classes of myeloma are recognized but conventionally they are studied and managed as a single disease. Pruzanski and Ogryzlo (1972) have stressed some of these variations and it has been the intention in the present chapter to investigate the clinical and laboratory features of 25 patients with multiple myeloma entirely of the IgA variety.

IgA myeloma constituted about one third of the cases of myeloma seen in Oxford during the study period. This figure is higher than the 20-25% previously reported (Hobbs, 1969; and Osserman and Isobe, 1972) and could

possibly be biased in favour of IgA due to the selective nature of the study. A male predominance 15 : 10 was found and the average age at presentation was 63 years.

The commonest presenting symptom was tiredness followed by bone pain. Both these symptoms were seen in approximately three quarters of the patients. Bone pain varied from mild to severe. It greatly incapacitated the patient in some cases and usually affected the back and/or the rib cage.

In general the incidence of the signs and symptoms at presentation (Figure 3,3 & 4) with the exception of bleeding was remarkably similar to that reported by Owen (1965) who studied 98 patients with all types of myeloma. In the present study bleeding at presentation was seen in a third of the patients and this incidence is higher than the 18% reported by Owen. Bleeding complications ranged from ecchymoses and purpura to epistaxis, malaena, haematuria, haemoptysis and persistent bleeding per vaginum and were associated with normal or mildly depressed platelet numbers in the majority of cases. Owen noted in his series of patients the association of bleeding with impaired renal function, thrombocytopaenia or infection but these were not consistently seen in the present study. The clinical signs recorded at presentation generally reflected the symptomatology e.g. bone tenderness at sites of pain. However it is instructive to note the occurrence of a retinopathy in two patients at presentation, out of 10 in which fundoscopy was recorded, since this complication was seen in those patients who had symptoms and signs related to elevated serum viscosity.

The abnormal radiological and laboratory findings of the patients presenting with IgA myeloma were also characteristic of those seen in all

types of myeloma (Snapper and Kahn, 1971) and no distinguishable features between the present series and other reports were noted. Similarly the causes of death were also similar to those previously reported (Owen, 1965).

One aspect of IgA myeloma which is of interest is that IgA paraproteins show molecular size heterogeneity. By applying the two methods described in chapter two it was possible to investigate this property of the paraprotein. This size variation was shown to be due to the formation of a series of polymers with the IgA member of molecular weight of approximately 165,000 being the monomeric unit. Polymeric members higher than the pentamer were not seen. The percentage proportion of each individual polymeric member varied greatly between paraproteins with either the monomer or dimer or both together predominating while the higher polymers were seen in decreasing concentration according to their increasing size. No relationship was seen between the degree of polymerization and the total concentration of the paraprotein or with its light chain subclass. It was also observed that the degree of polymerization did not vary during the clinical course of eight patients suggesting that the plasma cell clone in IgA myeloma secretes a paraprotein whose polymeric nature remains constant.

A spectrum was seen between paraproteins which were predominantly monomeric and those predominantly polymeric with perhaps some suggestion of a bimodal distribution (Figure 3,7). These results are in agreement with those reported by Fine et al. (1974) who divided their IgA myeloma proteins into two main groups, defined by the size of their predominant component. These were either 7S or 9S molecules as derived by analytical ultra-centrifugation and were encountered with an incidence of about 55% and 35% respectively.

A third small group characterised by equal 7S and 9S components was also seen.

Polymeric IgA differs from monomeric IgA in that the polymeric variety has the presence of a single J chain (Halpern and Koshland, 1970; Koshland and Wilde, 1974), J chain is a small polypeptide chain of molecular weight 15,000 and is disulphide bound to the penultimate cysteine residue of the heavy chain of the dimer (Koshland and Wilde, 1974). In a mouse model of IgA myeloma it is found that both J chain and the presence of a disulphide exchanging enzyme are the important prerequisites for polymerization (Parkhouse and Della Corte, 1974). The major factor which determines whether the monomer is secreted as such or is polymerized intracellularly to a higher polymeric form is thought to be the amount of intracellular J chain. When this is the limiting factor the monomeric variety of the paraprotein is preferentially secreted. It is possible that a similar restriction on the amount of intracellular J chain determines the characteristic degree of polymerization in human IgA myeloma. Once secreted the degree of polymerization does not vary and is characteristic for that particular myeloma clone (Fine et al., 1974).

In normal human plasma 5-15% of the circulating IgA is in a polymeric state whilst in secretions the dimer secretory IgA and higher polymers predominate (Tomasi et al., 1965). It is of interest that IgA is seen circulating predominantly in the polymeric state in many other mammals but often at relatively low total concentration (Heremans and Vaerman, 1972). Whether the smaller size of circulating IgA in man reflects a different site of production is not known. Heremans (1974) suggests that the origin of monomeric IgA is from the bone marrow, spleen and lymph nodes, whilst

polymeric IgA is derived from the mucosal linings, especially the gut wall. However, Radl et al. (1974) have studied bone marrow cells using antisera directed against J chain and polymeric determinants. They conclude that human bone marrow can be regarded as a significant source of both the polymeric and monomeric varieties of IgA.

From our studies of IgA elevations without evidence of myeloma it could appear that predominantly polymerized IgA is only seen in multiple myeloma. Tomasi and Grey (1972) have observed polymeric IgA in various pathological sera but they make no mention of the proportion that the higher polymers contribute to the total IgA.

A major objective of this study was to compare the clinical and laboratory feature in IgA myeloma with the degree of polymerization of the paraprotein. In general no clinical or pathological differences could be related to the degree of paraprotein polymerization apart from that directly attributable to the physicochemical effect of the larger size of the polymers. Thus five patients whose paraprotein was of the polymeric variety deveioped symptoms and signs similar to those described by Fahey, Barth and Solomon (1965) and called the hyperviscosity syndrome.

The hyperviscosity syndrome is a disease entity which is due to the blood having a grossly elevated viscosity (see Wells, 1970 for review). It is most commonly observed in Waldenström's macroglobulinaemia, infrequently in multiple myeloma and rarely in the collagen disorders. In all these conditions associated with this complication elevated serum viscosities have been recorded. (Other causes associated with this complaint include leukaemia, polycythaemia and states in which the erythrocyte becomes less deformable e.g. hereditary

spherocytosis. In these conditions the serum viscosity is normal but the whole blood viscosity is elevated.) In the present study five patients had symptoms and signs consistent with this diagnosis. In three of these patients serum viscosities were measured and in each case an elevated value was found thus confirming the diagnosis. In the remaining two patients this measurement was not done but these patients have been considered to have this complication due to the characteristic clinical features (see Table 3,IV).

The clinical features of the hyperviscosity syndrome, first described by Waldenström (1944), includes bleeding, often intractable mucous membrane oozing despite normal platelet counts; retinopathy with findings ranging from mild distension and 'irregularity' of retinal veins to frank papilloedema; varied neurological disorders such as 'muzzy' headaches, vertigo, nystagmus, impaired hearing, peripheral neuropathy and coma etc alone or in combination; intractable cardiac failure; mild to severe lassitude and sometimes renal failure. A large variation is seen between individuals with this complaint and no constant relationship has been found between the development and severity of symptoms and the serum viscosity level. Fahey and others (1965) have suggested that for patients with Waldenström's macroglobulinaemia hyperviscous symptoms are rare if the relative serum viscosity is below 4 (normal is 1.4 to 1.8). Two of the patients in this study with clinical features of the hyperviscosity syndrome had serum viscosity values below 4.0 (rel.). In one (F.B.) the value of 2.8 (rel.) was determined after his total plasma protein had fallen with chemotherapy from 110 to 100 g/l. In addition this patient was not anaemic (Table 3,IV) and thus the blood viscosity would be relatively greater (see Mannick, 1974). In the second patient (W.P.) the serum viscosity

of 3.2 (rel.) was determined only when the major clinical manifestation was bleeding. This patient later developed a total plasma protein of 118 g/l and this high level was associated with multiple clinical features of the hyperviscosity syndrome. However despite these caveats Tuddenham et al. (1974) have reported two IgA myeloma patients with the hyperviscosity syndrome in whom relatively low serum viscosities of 3.2 (rel.) and 3.9 centipoise were seen.

Thus the observations in this present study and of others suggest that the hyperviscosity syndrome in IgA myeloma may occur with relatively low serum viscosities (when compared to Waldenström's macroglobulinaemia) and care is required in assessing viscosity values between 3 and 4 (relative to water) for patients with this condition.

A review of the literature has revealed the hyperviscosity syndrome to be described in 16 patients with IgA myeloma (Virella et al., 1975; Tuddenham et al., 1974; Pruzanski et al., 1973; Freel et al., 1972; Dine et al., 1972; Sugai, 1972; Slavin et al., 1971; Wright and Jenkins, 1970; Azzena et al., 1968; Vigliano and Horowitz, 1967, and Beaumont et al., 1967), together with elevated serum viscosities in several more (Hammock and Mestecky, 1972; Somer, 1966; Vaerman et al., 1965). The polymerizing nature of the paraprotein was studied in 9 patients with the hyperviscosity syndrome. In 8 of these marked polymerization of the paraprotein was detected. One paraprotein (Pruzanski et al., 1973) was principally monomeric but it was present in high quantities (total protein 127 g/l) and gelled at low temperatures. A notable feature was the absence of Bence Jones protein in patients with polymerizing IgA myeloma and the hyperviscosity syndrome. All patients described with this complication had

total proteins in excess of 98 g/l .

In the five patients of the present study who developed this complication the paraprotein was of the polymeric variety and was associated with total plasma protein levels between 91 - 118 g/l . In three of these patients Bence Jones proteinuria was present.

The incidence of the hyperviscosity syndrome in IgA myeloma in this study was 20% and is comparable to the 14% recorded by Tuddenham and others (1974). These values are greater than the 4.2% recorded for IgG myeloma (Pruzanski and Watt, 1972). However it is known that the size of a protein is an important factor in the contribution of that protein to the viscosity of blood (Somer, 1966) and it has been shown in this study that the patients who developed this complication of hyperviscosity had the polymeric form of the paraprotein. Preliminary experiments also suggested that sera with the polymeric form of IgA paraprotein had a higher relative viscosity than did those with the monomeric form for the same concentration of paraprotein. These results could well then supplement the experimental findings of Somers (1966) who noted that the slope of the regression line between IgA paraprotein concentration and serum viscosity was intermediate between the steep slope seen for larger IgM paraproteins and the shallow slope seen for smaller IgG paraproteins. Thus the present study suggests that the slope for patients with IgA myeloma may also depend on the degree of polymerization of the paraprotein, those with polymeric form of paraprotein having steeper slopes than those seen for paraprotein of the monomeric variety. However Virella and others (1975) have noted that an occasional patient with a large amount of polymeric paraprotein has a relatively low serum viscosity and have

therefore suggested that the molecular configuration of the paraprotein is also of importance. It is possible that a combination of the size of the protein and its configuration will both be critical factors contributing to serum viscosity.

The mean survival time after the development of the hyperviscosity syndrome was $2\frac{1}{2}$ months. Two patients received packed cell transfusions whilst manifesting some of the clinical features of the hyperviscosity syndrome and this was rapidly followed in both by clinical deterioration. As it is known that there is a direct relationship between the whole blood viscosity and the volume of packed red cells (Wells, 1970) this suggests that blood transfusion in patients with the hyperviscosity syndrome may be dangerous. Penny (1974) suggests that for bleeding anaemic patients with elevated blood viscosities, exchange transfusions or transfusions after plasmaphoresis is the correct form of treatment.

Bleeding disorders are a common feature of IgA myeloma (Perkins et al., 1970). The common causes include defects in platelet adhesiveness and deficiencies in some clotting factors. Perkins and others (1970) noted that patients with high levels of paraprotein tend to bleed and this has also been seen in the present study. Other described defects include inhibition of thrombin activity (Sugai, 1972) and fibrin polymerization, and collagen coating leading to prolonged bleeding times (Vigliano and Horowitz, 1967).

Bleeding complications were seen frequently in the present study and were uniformly present in those patients with the hyperviscosity syndrome. The cause of this disorder was not studied systematically in any of these patients.

Although the total numbers were small the survival curves for the two groups appeared to show a greater mortality for the polymeric group in the first year as compared to the monomeric group. It would be of interest to study a larger group of patients with IgA myeloma prospectively to see if this trend was confirmed.

In conclusion it has been shown in this study that IgA paraproteins vary considerably in their degree of polymerization but this degree is a characteristic feature of the particular clone of myeloma cells and does not change with time. No appreciable differences have been shown between those clones secreting principally polymeric or monomeric paraprotein apart from those manifestations arising from the physicochemical properties of the paraprotein, in particular the polymeric myeloma patients being prone to develop the hyperviscosity syndrome. A knowledge of the molecular size of the paraprotein together with its quantity is of importance in the management of the disease.

In addition to the main objective of the study further experiments were performed to gain information concerning the structure and properties of the paraproteins. The results of these experiments will now be discussed.

On mild reduction and alkylation of the paraprotein from three patients it was observed that the polymers reduced initially through several intermediates to the monomer unit with the release of some light chain. With stronger concentrations of reducing agent, reduction of the monomer and intermediates proceeded further to yield free alpha and light chain through an alpha + light chain moiety.

Parkhouse, Virella and Dourmashkin(1971) have subjected a dimeric IgA paraprotein to mild reduction using similar experimental conditions. These investigations also noted the preferential release of light chains relative to single heavy chains during mild reduction but did not observe the preferential formation of the monomeric unit. The nature of this discrepancy between the two studies is unknown but may relate to the individual paraprotein since Schultze and Heremans (1966) carrying out similar reduction experiments noted that IgA polymers reduced through the monomer species.

When strong concentrations of reducing agent were used the paraprotein yielded free alpha chain and light chain only. It was observed that the electrophoretic mobility in SDSPAGE of 3 kappa light chains studied were greater than the mobility for 3 λ light chains. This phenomenon has also been recently reported by Virella and Coelho (1974). Using similar reducing conditions to those described in the present study and 5% polyacrylamide gels they calculated the molecular weight of 12 λ and 12 kappa light chains prepared from isolated IgG, IgM and IgA paraproteins. The mean and 1 standard deviation for the λ light chain was 28,395 $\pm$ 1873, and for the kappa chain, 22,845 $\pm$ 673. These values are similar to those obtained in the present study. The significance of the difference between the electrophoretic mobilities of the two light chains has been discussed by Virella and Coelho (1974). They suggested that differences in molecular configuration between the light chains may be an explanation for this differential mobility.

The paraprotein from one patient (I.S.) was observed to partially dissociate in 0.1% SDS to yield light and heavy chain dimers. Thus this

paraprotein falls into the $Am_2(+)$ subclass of IgA which is characterized both serologically and by the absence of disulphide linkage between the light and heavy chains (Grey et al., 1968). Dissociation is thus seen in the presence of denaturants without the requirement of a reducing agent. The IgA_2 subclass constitutes some 7-14% of normal serum IgA (see Jerry and Kunkel, 1974, for review) whilst in external secretions it is found in higher relative concentrations compared with serum. It was of interest that the polymers of this paraprotein in 0.1% SDS did not dissociate to heavy chain dimers although it was probable that light chain dimers were released from these polymers. This suggested that light dimers were not disulphide bound to either the monomer or the polymers of this protein.

An unexpected finding resulting from this study was that half molecules of IgA, consisting of alpha + light chain, were seen in the electrophoretogram of the sera from three patients. It is interesting to speculate that this alpha + light chain moiety does indeed exist in the circulation of some patients with IgA myeloma and that its presence may reflect a degree of biochemical dedifferentiation of the plasma cell clone as Hobbs (1971) has postulated to occur in myeloma. No previous reports can be found in the literature concerning the existence of circulating half molecule of IgA paraprotein in man although Potter and Kuff (1964) have reported a mouse plasma cytoma, 47A, which secretes a 3.9S paraprotein which appears to be a half molecule of IgA consisting of one light chain linked to one heavy chain. However half molecules of IgG have been seen in the serum of five patients with IgG myeloma. These molecules were also associated with small amounts of whole IgG paraprotein in at least two of

the patients (Spiegelberg et al., 1975; Hobbs and Jacobs, 1969). If the presence of circulating half molecules of IgA paraprotein can be confirmed, the relationship between this moiety and the nature of the assembly of the paraprotein will be of interest as current thought suggests that dimerization of the heavy chains precedes light chain attachment (Askonas and Williamson, 1968). Thus if a half molecule occurs it may suggest that a synthetic defect has occurred in some of the plasma cell clone to eliminate or block the normal site necessary on the heavy chain for dimerization. Alternatively half molecules of IgA paraprotein may occur normally during synthesis but at low levels of production or they may be a post secretory phenomena.

No evidence was obtained in the present study to suggest any association between the IgA paraprotein and albumin, haptoglobin or lipoprotein as has been described by Mannik (1967) and Beaumont et al. (1967). Mannik studied several IgA myeloma sera and noted that portion of the paraprotein was disulphide bound to albumin when examined immunoelectrophoretically. But he also observed the paradoxical dissociation of this complex during gel filtration in neutral buffer. This dissociation could be blocked by the addition of iodoacetamide to the buffer indicating that the dissociation was dependent on a disulphide interchange reaction. However the nature of this reaction and why it should occur during gel filtration and not in neat sera is not known. Thus the absence of paraprotein association from gel chromatographic studies is consistent with the results of Mannik.

CONCLUSION

The clinical manifestations of IgA multiple myeloma are usually not considered distinguishable from those of IgG myeloma despite the fact that IgA differs from IgG in several characteristics and in particular with respect to molecular size heterogeneity.

The clinical and laboratory features of 25 patients with IgA myeloma seen during a five year period are presented. The degree of paraprotein polymerization was observed to vary greatly in these patients but remained chronologically constant in 8 individuals studied on several occasions over this period. The patients were divided into two groups on the basis of the degree of paraprotein polymerization. The first group comprised those patients in whom the IgA paraprotein was greater than 50% polymerized, whilst in the second group the paraprotein was predominantly monomeric. No clinical or pathological differences were seen between the 'polymeric' and 'monomeric' groups of myeloma apart from that directly attributable to the physicochemical effect of the paraprotein polymerization. Thus, five patients out of 11 of the 'polymeric' group developed the hyperviscosity syndrome, whilst no patients in the 'monomeric' group developed this complication. The concentration of the paraprotein during the course of the disease was comparable in both groups.

This syndrome is considered to be relatively common in IgA myeloma and adds to the morbidity and mortality of the disease. Its anticipation and treatment may improve the quality and survival of patients likely to develop this complication.

CHAPTER FOUR

THE IMPLICATIONS OF IMMUNE COMPLEXES IN THE CIRCULATION OF MAN

Historical introduction

Do circulating Ag/Ab complexes cause tissue injury?

Fate of circulating immune complexes

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CHAPTER FOUR

THE IMPLICATIONS OF IMMUNE COMPLEXES IN THE CIRCULATION OF MAN

A large body of experimental and clinical evidence has implicated circulating immune complexes in the pathogenesis of many human diseases. In this chapter I will critically review some of this evidence. The current methods used for their detection will also be discussed and finally the clinical relevance of these complexes will be considered. This chapter will serve as an introduction to the subsequent two chapters in which experimental data will be presented relating to the detection of immune complexes in several human diseases.

Historical introduction

In 1905 von Pirquet and Schick published their classic monograph on the clinical and laboratory features of serum sickness. This iatrogenic condition was then common and was caused by the therapeutic intravenous or intramuscular injection of immune horse serum into patients suffering from diphtheria (or rabies, tetanus or scarlet fever). As much as 200 mls of horse serum was injected and was followed, 8-12 days later, by a syndrome consisting of urticaria, fever, arthralgia, lymphadenopathy, neutropaenia and sometimes, a peripheral neuritis. This syndrome was generally non-fatal and was seen in a variable number of treated patients, the number depending on the amount of injected horse serum (see Freeman, 1971, pg 71). The

pathological features in the few patients who died during the acute phase included a necrotizing vasculitis, a non-specific lymphadenopathy and hyperplasia, and occasionally, an acute nephritis. Von Pirquet suggested that the condition of serum sickness was an allergic phenomenon and that the mode of the disease was mediated through the toxic effect of a circulating neutralizing agent reacting against the foreign serum constituents (cited by Notkins and Koprowski, 1973). However the time was not then technologically ripe for this hypothesis to be confirmed or refuted.

During the next fifty years further advances in immunological methodology and theory together with the availability of suitable animal models of serum sickness enabled this hypothesis to be substantially confirmed. Thus in 1958, Weigle and Dixon, and Dixon and others, showed in a rabbit model the presence of circulating complexes (i.e. the neutralizing agent reacting against the foreign protein) during the development of serum sickness, while Dixon et al. (1958) investigated the pathogenic role of these complexes in the production of the morphological lesions of serum sickness. Nine years later Vaughan and his colleagues (1967) described a case of human serum sickness and presented evidence for the existence of immune complexes in the circulation during the acute phase of the disease.

In addition to serum sickness, evidence was also accumulating suggesting that nephritis was an immune complex mediated disorder. Schick (1907) had originally noted a similarity between serum sickness and scarletinal nephritis. Both diseases had a 6-8 day delay between the initial immunological insult and the subsequent clinical manifestations and both diseases had known renal involvement (Rackeman et al., 1916). These observations had suggested to

some that nephritis might be due to circulating 'toxins' generated in an allergic response (see Rich, 1956). This concept was further substantiated by Germuth in 1953 when he showed, in a study of serum sickness in rabbits, that glomerular lesions developed during the immune phase of antigen elimination, and regressed when excess antibody was present in the circulation. He proposed that soluble antigen-antibody (Ag/Ab) complexes were formed in the circulation during the active antigen elimination and that these complexes were transported to the kidney where they caused the renal lesion of the type seen in serum sickness.

Thus these early studies had suggested that circulating immune complexes might have a primary role in the causation of tissue injury both in serum sickness and nephritis.

Do circulating Ag/Ab complexes cause tissue injury?

Soluble immune complexes, once localized to the tissue spaces, are capable of producing injury. Ishizaka and Campbell (1958) injected complexes, formed in antigen excess directly into the skin of experimental animals and saw variable tissue reactions. These ranged from altered capillary permeability to frank haemorrhagic necrosis and their severity depended on the amount of injected complexes. Spector and Heesom (1969) also injected complexes, but formed at antibody-antigen equivalence, into the skin of mice and noted the formation of granulomata.

But do immune complexes in the circulation have a primary role in the causation of tissue injury? Does tissue injury always occur in animals with circulating soluble complexes? Does tissue injury follow the injection or

infusion of Ag/Ab complexes into the circulation of unsensitized animals? What factors are concerned in the localization of circulating complexes? Much controversy surrounds these questions. Germuth and McKinnon (1957) suggested a pathological role for circulating complexes when they produced anaphylactic shock in unsensitized guinea pigs after injecting them with large amounts of soluble bovine serum albumin (BSA) - anti BSA complexes prepared in moderate antigen excess. The shock was associated with a necrotizing pulmonary arteritis and a slight glomerulonephritis, but these features differed from those characteristically seen in serum sickness. Germuth and Pollack (1958) also observed mild vasculitic changes after infusing specific antibody into the circulation of rabbits which had been primed with antigen. McClusky and Benacerraf (1959) infused soluble complexes into mice and obtained a more intense vascular reaction characterised by arteritis and nephritis. These reactions occurred in some of the mice within 36 hours after the first injection. But it should be noted in these experiments that many of the animals had an accompanying septicaemia most likely due to the presence of indwelling catheters, confined soiled quarters,and non sterile techniques.

These findings have not been easily reproduced. Cochrane and Weigle (1958) and later Fish and others (1966) infused preformed immune complexes into 80 unsensitized rabbits and 200 mice, and were not able to show reproducible injury. In particular, no vascular or renal lesions were produced. Indeed Weigle (1961) in a comprehensive early review stated "it is evident from an accumulation of data that something besides the mere presence of soluble Ag/Ab complexes in the circulation or tissue baths plays an

important role in the pathological changes produced by Ag/Ab complexes" and it has been the experimental work of the last 15 years that has thrown a glimmer of light on the mechanisms by which circulating immune complexes interact with other biological mechanisms to cause tissue injury.

Fate of circulating immune complexes

Circulating immune complexes are either removed by the reticulo-endothelial system (mononuclear phagocyte system, vide infra) or are deposited in the vessel walls or tissue spaces. Their fate is dependent on both the nature of the antibody and antigen in the complex, and the capacity of the reticulo-endothelial system to remove them (see Mannik et al., 1974; Cochrane and Koffler, 1973, for review).

Deposition: The mechanisms by which these immune complexes are deposited in tissues are now known in some detail. The majority of this knowledge has been derived from the study of experimental serum sickness in rabbits and much of this pioneering work has been done by Germuth, Dixon, Cochrane and their colleagues. In the acute serum sickness disease of rabbits, a bolus of intravenously injected Ag e.g. BSA disappears from the circulation in three phases (Cochrane and Koffler, 1973). The first phase is one of intra and extra-vascular equilibration. In the second phase the protein antigen is slowly lost from the circulation due to catabolism, while the third phase, beginning at about the eighth day, is one of immune elimination. During the latter phase circulating immune complexes of Ag and newly formed Ab are seen (Weigle and Dixon, 1958), total haemolytic complement may fall

and lesions of endocarditis, arteritis and glomerulitis are seen. These lesions are closely associated with the deposition of complex and complement (C_3) in the vessel walls and glomerular basement membranes (see Unane and Dixon, 1967 for review). The arteritis is prominent at the coronary ostia, points of aortic branching and bifurcation, and in the pulmonary arteries, and is, to a large extent, neutrophil and complement dependent in contrast to the glomerulitis which is not (Knicker and Cochrane, 1965; Henson and Cochrane, 1971). With the development of these lesions Ag/Ab complexes are lost from the circulation and free Ab is seen. After the disappearance of the complexes the vasculitic lesions commence to heal. Deposited complex and complement rapidly vanish from the affected vessel wall and are often only detectable for 1-2 days after the onset of the arteritis.

In the chronic serum sickness disease of rabbits a variable amount of Ag is injected daily by the intravenous route (Unane and Dixon, 1967). The amount of Ag that is required is that which guarantees the formation of circulating Ag/Ab complexes after each injection of Ag. Some rabbits will produce different amounts, and avidities, of Ab and hence differing amounts of dosing Ag are required. Using this regime rabbits which produce strongly avid Ab will develop a chronic membranous glomerulonephritis in about five weeks. Those with a weaker Ab response will take longer periods to develop this complication and in the rabbits with little or no Ab response to the injected antigen no renal pathology is seen. In the responders granular deposits of complex and complement are observed frequently in the glomerulus lying externally to the glomerular basement membrane. With continued injection of Ag in the high antibody responders, epithelial crescents and

neutrophil infiltration of the glomerulus are seen and these animals usually die in renal failure. However arteritis and endocarditis are not found in the chronic serum sickness disease of rabbits. It has been stated that only those rabbits which produce an antibody immune response to the injected protein will develop nephritis. Christian and Lightfoot (1970) suggested that the avidity of this Ab is an important factor in predisposing rabbits to this complication since they found that rabbits producing poorly precipitating Ab were particularly prone to develop nephritis. However Dixon and others (1961) and Wilson and Dixon (1971) found that all immunologically responsive rabbits will develop nephritis provided circulating immune complexes are formed in the circulation. Therefore, perhaps quantity and quality of the antibody are both closely involved in the development of this disease. In the chronic serum sickness model it is also known that the presence of a large excess of Ag in the circulation will lead to improvement of the renal lesion (Valdes et al., 1969).

In both these acute and chronic experimental models of serum sickness the mechanisms by which the circulating immune complexes are deposited in the tissues have been extensively studied and a number of factors have been defined.

Cochrane and Koffler (1973) have stressed the importance of vascular permeability factors. Thus in the acute serum sickness model in the rabbit the mechanism can be succinctly summarized as follows (Henson and Cochrane, 1971; and Cochrane, 1971). Immune complexes and free antigen are present in the circulation during the early immune elimination of the antigen. This free antigen is able to initiate the release of soluble factors from the granules of circulating basophils. This process is mediated through a reaginic immune

response, the reaginic antibody binding to and sensitizing the basophil to that particular antigen. The soluble factors released from the basophils include platelet activating factor which causes local platelets to clump and release vasoactive amines (histamine etc) which in turn cause extravasation from local vessels. During this process large complexes (vide infra) enter and become entrapped in the vessel wall and initiate further injury. Therefore agents antagonistic to the vasoactive amines (e.g. anti-histamines) can protect against the deposition of these large complexes. However, the direct significance of these findings to human disease is hard to infer, since human platelets do not contain histamine.

The lattice formation / size of circulating complexes are also important factors in the deposition of immune complexes. The lattice formation generally implies a three dimensional structure and the degree of lattice is dependent on the ratio of Ag to Ab in the complex, i.e. the valency of the Ab and the number of determinants on the Ag. Depending on the size of the Ag, complexes with small lattice are usually of small size. In a correlative study of both acute and chronic serum sickness disease of guinea pigs and rabbits, only those animals whose sera contained immune complexes greater than 19 S in size (since small antigens were used this implies complexes of moderate lattice formation) were shown to have glomerular and / or arterial lesions (Cochrane and Hawkins, 1968; Wilson and Dixon, 1971). Complexes whose size was less than 19 S were observed in those animals who were not affected. Cochrane and Hawkins (1968) suggested that Ag/Ab complexes had to exceed a certain critical size and that vascular permeability had to be increased before complexes were entrapped by vascular or glomerular basement membranes.

However in similar studies of acute serum sickness in rabbits performed by Dreesman and Germuth (1972), small circulating complexes of lattice $AbAg_2$ and Ab_2 Ag_3 and containing low avid IgG Ab were found in association with an acute diffuse proliferative glomerulonephritis in half of the rabbits. The quantity of deposited immune complexes correlated poorly with the severity of the lesions. These experiments therefore suggested that complexes with small lattice may also be involved in the production of glomerular lesions.

Germuth et al. (1972) in another series of experiments injected a constant amount of B S A into rabbits daily. In those few animals that responded with low levels of antibody, a diffuse proliferative glomerulonephritis was found and this was consistently associated with circulating complexes of molecular weight 5-7 x 10^5 daltons containing highly avid IgG antibody. Rabbits producing intermediate levels of Ab had circulating immune complexes of larger size (greater than 10^6 daltons) and immune complexes were noted to be deposited exclusively in the glomerular mesangium and this deposition was associated with a focal mesangial cellular proliferation without inflammatory changes. It was suggested that glomerular injury occurs when the amount of small to intermediate sized complex delivered to the glomerulus exceeds the phagocytic and catabolic capacities of the mesangium so that the complex accumulates within the subendothelial space of the glomerular loop. This juxta-mesangial deposition of immune complex is thought to produce localized proliferative and/or inflammatory changes characteristic of focal glomerulonephritis.

It is of interest that in both these studies of Germuth and others the circulating complexes did not appear to fix complement although complement

was seen in the glomerular lesions.

Hydrodynamic factors are again important in the deposition. Arteritis in experimental serum sickness is most commonly seen at sites of vessel bifurcation and branching and adjacent to turbulent flow (Knicker and Cochrane, 1968; Germuth, 1953).

Hydrostatic pressure differences are other factors concerned in the localization of circulating immune complexes. Germuth and others (1967) have shown that experimentally produced renal artery stenosis protects the rabbit kidney from immune complex nephritis. This has also been seen in man (Leading article, Lancet, 1975).

Germuth has also noted in this same study that low concentration of soluble complexes will, if present over prolonged periods, cause a glomerulonephritis in contrast to those states characterized by large amounts of circulating immune complexes in which arterial localization occurs.

Many other factors are probably involved in complex deposition but these are unknown or poorly understood. The microcirculation and its effects on local soluble complex concentration and lattice formation will perhaps explain the predilection for complex deposition in such sites as the vascular basement membrane of the glomerulus, skin, and joints and nerves.

Following localization of the immune complex in the tissue, a complex sequence of biological mechanisms may be initiated leading to tissue injury (see Goldstein and Weissman, 1974, for review). This includes activation of the complement system and the subsequent attraction of polymorphonuclear leucocytes. These cells can release intracellular proteolytic lysosomal enzymes (Ward et al., 1965) causing tissue injury. The kinin, clotting and fibrinolytic

system may also be activated directly or indirectly. The subsequent histological features are varied, ranging from Arthus-like reactions or granuloma formation to florid vasculitis, depending on the interaction of the many biological mechanisms mentioned above (Humphrey and White, chapter 11, 1970). It can be appreciated that many of these effects vary according to the nature of the complex and in particular on the class and subclass of the antibody. Thus the clinical and histological features, related to the deposition of circulating immune complexes, might be expected to show a large variation.

Reticuloendothelial system removal: The great majority of the circulating immune complex load is taken up by the phagocytic cells of the reticuloendothelial system (RES). Mannik and colleagues in their extensive review of the subject (1974) have stressed several features in this removal. These include the degree of lattice formation of the immune complex, the state of the RES and the nature of the Ab and the Ag of the complex. These conclusions have resulted from experiments in which preformed soluble Ag/Ab complexes were infused into the circulation of unsensitized rabbits and monkeys. In these experiments less than 1% of the complexes were deposited in the tissues outside of the RES.

The lattice of an Ag/Ab complex is dependent on the nature of the antibody, the nature of the antigen and the relative concentrations of each. In slight Ag excess soluble complexes were analysed by sucrose gradient ultracentrifugation and were found to consist of small amounts of Ag_1 Ab_1 complexes, a larger proportion of Ag_2 Ab_2 complexes and a spectrum of complexes with larger lattice than Ag_2 Ab_2 (Arend et al., 1972). In greater Ag excess lattice formation decreases with loss of the larger size

complex and a predominance of the Ag_2 Ab_2 and Ag Ab components.

Soluble complexes with lattice that exceeded two IgG Ab molecules were selectively and rapidly removed from the circulation whereas those which contained two Ab's or less, persisted in the circulation (Mannik et al., 1971). The Kupffer cells of the liver appeared to be a major site of uptake of these complexes. It was also found that Ag with 1-2 antigenic determinants formed complexes with only small degrees of lattice and that these complexes persisted in the circulation. It was also noted that although the immune complexes whose lattice exceeded Ag_2 Ab_2 were capable of fixing the first component of complement, their uptake by the RES was complement independent. It was however dependent on an intact and functional Fc portion of the IgG molecule of the complex. Haakenstad and Mannik (1974) observed that the RES could be saturated with immune complexes in mice thus prolonging the survival of complexes with high degrees of lattice formation. It is not presently known if RES saturation predisposes to deposition of immune complexes or whether RES uptake varies between individuals or changes in disease.

Little information is available concerning the fate of immune complexes of differing classes or subclasses of antibody and for complexes composed of differing forms of Ag.

Thus at this stage it is possible to summarize present knowledge concerning soluble circulating immune complexes and it can be stated that the vast majority of Ag/Ab complexes with lattice greater than 2-3 IgG molecules are rapidly and selectively taken up by the RES. A minor amount may be deposited in the tissues and initiate injury but this appears to be closely associated in the experimental animal with additional allergic biological mechanisms. Immune complexes with smaller degrees of lattice

tend to persist in the circulation with half lives in the rabbit of 1-2 days (Arend et al., 1972). Little is known concerning the relevance of these smaller IgG containing complexes or the relevance of immune complexes containing antibody of differing class (or subclass) to that of IgG. The role of the RES in immune complex states has not yet been studied in any detail.

Methods of detecting circulating immune complexes

Many types of circulating immune complexes may exist differing in Ab class, subclass, lattice formation and survival. Therefore it is unlikely that all these varieties of complexes will be detected by any single method. To date a large number of methods have been developed for this task but none has found widespread or routine clinical use. This deficiency may also reflect the fact that the clinical relevance of detecting circulating immune complexes is not yet appreciated nor has the mode of treatment for such a condition been established.

The presence of circulating Ag/Ab complexes may be definitely demonstrated or indirectly suggested by several techniques. These include physicochemical and biological methods all of which have difficulties in application and interpretation. These methods are listed in Table 4,1. together with the reference in which they were described, their sensitivity and their limitations. The majority of methods detect immune complexes containing IgG antibody.

Immune complexes inhibit or activate many biological mechanisms. A number of methods are based upon the principle the same complexes fix Clq the first component of complement. However it should be noted that not all

Method	Author	Limitations	Sensitivity
Analytical ultracentrifugation	Kunkel et al., 1961	Insensitive, requires ultracentrifuge	+
Gel filtration chromatography	Jewell & MacLennan, 1973 Soothill & Hendrickse, 1967	Dilution dissociates complexes	++
Sucrose gradient density ultracentrifugation	Bombardieri et al., 1973		++
Electron microscopy of centrifuged pellet	Almeida & Waterson, 1969	Requires large or particulate antigen	++
Poly ethylene glycol precipitation	Creighton et al., 1973	Requires strict standardization	+
Differential solubility	Vaughan et al., 1969	Dependent on nature of antigen	+

Table 4,1 Physicochemical methods for detecting immune complexes

Method	Author	Limitations	Sensitivity
Platelet aggregation	Penttinen et al., 1969	Other serum factors cause platelet aggregation. Standardization difficult	+++
Histamine release	Baumal & Broder, 1968	Other serum factors cause histamine release. Requires perfused guinea pig lung preparation and gel chromatography of serum specimen	+++
K cell cytotoxicity inhibition	MacLennan, 1972	Other serum factors are inhibitory	+++
Macrophage uptake inhibition	Onyewot et al., 1974	Serum rheumatoid factors are inhibitory	+++
Clq fixation	Agnello et al., 1971 Nydegger et al., 1974	Other serum factors fix Clq Small complexes may not fix Clq	+ +++
Monoclonal IgM rheumatoid factor	Winchester et al., 1971	Dependent on the specificity of the rheumatoid factor. The rheumatoid factor may not react with small complexes	+
Polyclonal IgM rheumatoid factor	Cowdery et al., 1975		+++
Anti complementary activity	Johnson et al., 1975	Requires strict standardization Small complexes may not be anti complementary	+++

Table 4,1 Biological methods for detecting immune complexes

+++ very sensitive
++ sensitive
+ insensitive

complexes fix complement as Hyslop et al. (1970) found that complexes made up with divalent haptens and containing less than three IgG molecules did not fix complement. A simple test for immune complex fixation of Clq is described by Agnello et al. (1970) using purified Clq and agar immunodiffusion while Nydegger et al. (1974) has developed a more complicated radio-immunoassay. But endotoxin, DNA and other anionic substances are known to fix Clq (Agnello et al., 1970, 1971a) and it is also possible that most larger complexes have spontaneously fixed Clq·in vivo and thus being fully saturated will not slow Clq fixation. DNA has recently been detected in the free state in Systemic Lupus Erythematosus (SLE) (Koffler et al., 1973) and the presence of this in the serum may partly explain why Clq precipitation is commonly found in this disease (Agnello et al., 1971b).

If serum containing immune complexes is heated to 56°C, the complex bound complement is destroyed and the serum becomes anti-complementary (Johnson et al., 1975). In the anticomplementary method a known amount of guinea pig complement is added to the heated serum, some is fixed by the complex, and then the remaining complement is measured by a total haemolytic assay, the loss of available complement reflecting the presence of complexes (Johnson et al., 1975). Careful standardization of this technique is required as it appears that serum IgG may undergo spontaneous aggregation during heating and become anticomplementary (Thompson, 1974).

Both monoclonal and polyclonal IgM rheumatoid factors have been used to detect circulating complexes (Winchester et al., 1971; and Cowdery et al., 1975). In these tests the rheumatoid factor is allowed to bind with the immune complex and this phenomenon is measured either directly as in agar precipitation

(Winchester et al., 1971) or indirectly as in an inhibition assay where the binding of the rheumatoid factor to radio-iodinated heat aggregated IgG is inhibited by preincubation of the rheumatoid factor with the immune complex (Cowdery et al., 1975). However natural rheumatoid factors may be present in sera containing immune complexes and this will interfere in the assay, and the specificities of different purified rheumatoid factors vary. The size of the complex is also important in this method since Cowdery and others (1975) and Norberg (1974) suggest that rheumatoid factors may not react with complexes of small size.

Immune complexes may initiate platelet aggregation (Penttinen et al., 1969) or cause the release of histamine from the perfused guinea pig lung preparation (Baumal and Broder, 1968). These complicated techniques are very sensitive but other serum factors are known which initiate these reactions and both methods require a large amount of preparation and standardization. The nature of the complexes and the size and degree of lattice formation required to initiate these reactions are unclear but it appears that both histamine release and platelet aggregation are activated only by complexes of a moderate to large size (Baumal and Broder, 1968; Pettinen et al., 1971).

Immune complexes can inhibit both the uptake by guinea pig macrophages of radio labelled aggregated IgG (Onyewotu et al., 1974) and the lymphocyte dependent antibody mediated cytolysis of target cells (MacLennan, 1972). In the former assay naturally occurring free rheumatoid factor appears also to inhibit the uptake (or enhance it if soluble complexes are present, (Onyewotu et al., 1975) whilst in the latter assay other serum inhibitory factors are known (Hersey et al., 1973). Immune complexes of

small size are able to inhibit the lymphocyte dependent antibody mediated cytolysis assay (MacLennan, 1972; Hallberg, 1974) whilst the minimal size required for inhibition of macrophage uptake is not known.

Several physicochemical methods for the detection of circulating immune complexes are known and depend on the separation of complexes from normal immunoglobulin due to differences in size, charge and solubility.

Immune complexes containing IgG are larger than uncomplexed monomeric IgG. This size difference is applied in several methods where complexed IgG separates in higher sized fractions than does normal IgG. Examples of these methods are analytical ultracentrifugation (Kunkel et al., 1961) gel filtration chromatography (Jewell and MacLennan, 1973) and sucrose density gradient ultracentrifugation (Bombardieri et al., 1973). The immune complex can be viewed directly as in analytical ultracentrifugation where an additional sedimenting member is seen, or may be suggested by the measurement of high molecular sized IgG_1 or by the assay of a property of that complex e.g. the anti-complementary activity. The higher molecular size of a complex has also been utilized in the method of ultracentrifugation followed by electron microscopy of the sedimenting pellet (Almeida and Waterson, 1969). This is suitable for conditions in which heavy or particulate antigens are postulated. The larger size of complexes is also a decisive factor in the selective precipitation of immune complexes by standardized concentrations of polyethylene glycol (Creighton et al., 1973).

Immune complexes may also differ from non-complexed IgG in their charge and this phenomenon is thought to underlie the solubility differences of some complexes. This method was used to detect circulating complexes for the

first time in human serum sickness (Vaughan et al., 1969). A limitation of this technique is that solubility is dependent on many factors including the nature of the antigen (Farr, 1958).

If the nature of the antigen in the circulating immune complex is known or postulated it is possible to degrade or dissociate the complex and measure the increase in free antibody and/or antigen (Harbeck et al., 1973; Phillips and Draper, 1975). The nature of the antigen in most immune complex states is not known.

The size and/or degree of lattice formation are important factors in detecting immune complexes since different assays vary in their sensitivity to complexes of different size/lattice formation. It has previously been stated that one might expect immune complexes with small degrees of lattice to predominate in the circulation due to their prolonged survival. This fact must be recognized in attempts to detect circulating immune complexes.

In all biological assays there is little knowledge concerning the properties of immune complexes other than IgG containing complexes. IgG can vary in its subclass and different subclasses of IgG vary in their biological properties (MacLennan, 1972). The sensitivity of the biological assays varies with these subclasses, thus immune complexes differing in their subclass may or may not be detected using different assays.

In general it appears that the biological methods are more sensitive than the physicochemical methods in detecting complexes but are more susceptible to false positives. The latter methods are more cumbersome and time consuming than the former and may require expensive equipment but a positive finding is generally thought to definitely indicate the presence of

complexes (providing care is exercised in the handling and storage of sera). Many investigators are now using more than one method or even combinations of methods (Soothill and Hendrickse, 1967; Mowbray et al., 1973) in an attempt to increase the specificity of the search for immune complexes. These usually consist of both a physicochemical and a biological assay.

Several indirect methods suggesting the presence of an immune complex state are known. These include the detection of cryoglobulin (see Chapter six) and the assessment of the complement pathway. Immune complex diseases may be associated with depressed levels of complement components and/or with the presence of inactive complement components in the circulation (Gotoff et al., 1969; Soothill and Hendrickse, 1967).

Human diseases in which circulating immune complexes have been identified

A variety of human diseases have clinical and histological features similar to those seen in human and experimental serum sickness and much research has been directed towards the identification and implication of circulating Ag/Ab complexes in the pathogenesis of these diseases. Many diseases are now known in which circulating complexes have been clearly detected whilst an even greater number have indirect or suggestive evidence for the presence of these complexes. Deposits of immunoglobulin and/or complement is often taken as indirect evidence for the existence of circulating complexes, but in this chapter the discussion will be primarily concerned with diseases in which complexes have been definitely detected in the blood.

Rheumatoid arthritis and hypergammaglobulinaemic purpura were the first disorders in which circulating complexes were directly visualized (Kunkel

et al., 1961). In rheumatoid arthritis two types of complexes are known, a '22S' composed of IgM rheumatoid factor and IgG (Franklin et al., 1957) and 'intermediate' complexes composed of IgG rheumatoid factor and IgG. These intermediate complexes have also been described in SLE and Sjögren's syndrome (Blaylock et al., 1974; Alarcon-Segovia et al., 1974).

IgG containing complexes of small size have been detected in the sera from patients with inflammatory bowel disease (Jewell and MacLennan, 1973), coeliac disease and dermatitis herpetiformis (Mowbray et al., 1973; Doe et al., 1973) and thyroid disorders (Calder et al., 1974). The nature of the antigen in these disorders is unknown.

The pathogenesis of SLE may be mediated by soluble complexes and this disease has a great similarity to the spontaneous autoimmune disease of NZW / NZB F_1 hybrid mice in which soluble complexes are found (see Christian, 1969; Howie and Helyer, 1968). In the human disease DNA / anti DNA complexes have been detected in the circulation and are also seen deposited in the basement membranes of the glomeruli and skin (Koffler et al., 1967; Harbeck et al., 1973). Natali and Tan (1972) have shown in the animal that DNA - anti DNA complexes are nephrotoxic but Gilliam and others (1975) consider that they are unlikely to have a causative role in the production of the inflammatory skin lesions of human SLE.

Immune complexes have also been detected in the serum of patients with illnesses associated with the Australia antigen. These illnesses include serum hepatitis, polyarteritis nodosa, active chronic hepatitis, and glomerulonephritis in children (Almeida and Waterson, 1969; Gocke et al., 1970; Prince and Trepo, 1971; Brzosko et al., 1974). Circulating immune

complexes have been detected in other viral illnesses including infectious mononucleosis (Behan et al., 1973) and measles (Myllyla et al., 1971).

A number of bacterial and parasitic diseases are known in which there is evidence for circulating immune complexes. Examples include leprosy (Moran et al., 1972), schistosomiasis (Phillips and Draper, 1975), and infectious endocarditis (Boulton-Jones et al., 1974). Diseases of unknown aetiology may also have circulating soluble complexes as illustrated in sarcoid (Hedfors and Norberg, 1974) and cystic fibrosis of the pancreas (McFarlane et al., 1975). It is surprising that there is little direct evidence for circulating complexes in many of the nephridities, although there is strong indirect evidence for their presence (see McCluskey and Klassen, 1973 for review). Finally circulating complexes have been detected in the sera of patients with cutaneous vasculitis (see Cream and Turk, 1971 for review) and in some forms of cancer such as malignant melanoma (Cowdery et al., 1975).

Significance of circulating immune complexes in clinical medicine

Three questions may be immediately asked, 1) what is the significance of detecting immune complexes in the circulation? 2) what should be done if these complexes are found? 3) what is the significance of not detecting circulating immune complexes? Several facts relevant to these questions are known. Immune complexes are not seen in the sera of normal healthy people (Johnson et al., 1975; Cowdery et al., 1975), although it is probable that they occur transiently during immune elimination of any foreign antigens. Circulating immune complexes are known to occur in various diseases of the 'immune complex' type as outlined above. Finally immune complexes can

either be safely removed by the RES or they may be deposited in the tissues where injury may result. The mechanisms involving deposition of complexes in the tissues are poorly understood and probably multifactorial. Furthermore circulating complexes are not necessarily 'toxic' to the tissues in themselves. This is illustrated in rheumatoid arthritis where circulating immune complexes are seen for prolonged periods and in high concentrations but their presence is unassociated with any of the systemic manifestations of the 'immune complex' disorders e.g. vasculitis and nephritis (see Chapter five).

Therefore the detection of immune complexes in the circulation implies a pathological state and signifies that additional investigations should be instigated into the nature of the complex, especially the nature of the antigen as this may provide an aetiological agent for that disease.

If immune complexes are suspected of being involved in the pathogenesis of any disease their elimination is of therapeutic importance. This fundamentally implies the elimination of the antigen contributing to the formation of the complex but as this antigen is generally unknown or is an auto antigen other steps must be taken. Potential therapeutic measures include the removal of the complex from the circulation by plasmaphoresis, and measures that inhibit the deposition of the complex in the tissues or potentiates the RES removal. The practicalities of these latter measures, in our present state of knowledge, are not defined nor has the benefit of such therapy been shown but this is an exciting area for future development.

The absence of circulating immune complexes may suggest that the assays used were insensitive or it may truly reflect the absence of the complex. It is likely that some immune complexes may be present only transiently in the

circulation, being either rapidly eliminated by the RES or deposited. Hence the inability to detect complexes in the blood should never be taken to imply the exclusion of an 'immune complex' state.

Conclusion

Immune complexes have been implicated in the pathogenesis of a large number of human diseases. From experimental work it is known that circulating complexes containing IgG are either removed by the reticulo-endothelial system or deposited in the tissues. Deposition may cause tissue injury. Both the deposition of the complexes and the resultant injury generally involve additional biological mechanisms. Immune complexes with large degrees of lattice formation are present only transiently in the circulation (unless RES saturation has occurred). Small complexes, with less than three IgG molecules, persist in the circulation and can be detected by a variety of methods.

While there is no simple method currently available for the detection of all types of circulating complexes several techniques are known which can show the presence of complexes and may give additional information concerning the biological properties of these complexes.

In the clinical situation the detection of circulating complexes may be of primary pathogenic importance. However these complexes may also occur as a secondary phenomenon and thus the implication of identifying complexes may or may not be of direct relevance to the disease state. Circulating immune complexes have not yet been found in all the 'immune complex' disorders.

CHAPTER FIVE

FACTORS RELATING TO CIRCULATING IMMUNE COMPLEXES IN RHEUMATOID ARTHRITIS

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CHAPTER FIVE

FACTORS RELATING TO CIRCULATING IMMUNE COMPLEXES IN RHEUMATOID ARTHRITIS

INTRODUCTION

In the preceding chapter a critical review was undertaken of the methods of detection and clinical implications of circulating immune complexes. One physicochemical method discussed for the detection of these complexes was gel filtration chromatography. It was indicated that the rationale for using this technique was based upon the fact that complexed immunoglobulin has a larger molecular size than normal immunoglobulin and thus separates in earlier fractions of the chromatogram. This bound immunoglobulin can be directly measured or indirectly assessed using different biological assays. This method has been previously used to detect circulating complexes in animal experiments (Gotch, 1973) and in human disease including inflammatory bowel disease (Jewell and MacLennan, 1973).

In this and the subsequent chapter this method of gel filtration chromatography has been used in an attempt to detect IgG containing complexes in the sera of patients with rheumatoid arthritis, other joint diseases, acute myeloid leukaemia, multiple sclerosis and cutaneous vasculitis. The experimental data concerning the detection of these complexes in the sera of patients with joint disease will be presented in this chapter.

Kunkel and his colleagues in 1961 were the first to observe the presence of 'intermediate' sized IgG containing complexes in the sera of

patients with rheumatoid arthritis. These complexes were in contrast to the larger IgM containing '22S' complexes previously reported (Franklin et al., 1957). Kunkel et al. (1961) noted that these smaller complexes sedimented between the 7 and 19S markers in the analytical centrifuge and this finding suggested that less than five IgG molecules were involved in the formation of these complexes.

Other workers have subsequently demonstrated that these intermediate complexes will dissociate at low pH to yield IgG rheumatoid factor and IgG (Schroenloher, 1966; Capra et al., 1971).

Recenily Jasin et al. (1970) have shown these complexes to be a cause of the hyperviscosity syndrome but this complication is only seen if the complexes are present in very high concentration (Editorial, Annals of Internal Medicine, 1974). Other pathophysiological consequences may also result from the presence of these circulating intermediate complexes and they have been suggested as a cause of the vasculitis commonly seen in rheumatoid arthritis but there is little available data to substantiate this view (see Zvaifler, 1973, for a review).

Currently, no study of rheumatoid arthritis has been reported in which the presence of these complexes has been systematically measured and compared with other clinical and laboratory features of the disease. The aim of the present study has been to do this, using gel filtration chromatography as a means to detect and assess these complexes.

The results of this physicochemical method have also been compared with the results obtained using another method for the detection of soluble immune complexes. This second technique is based upon the principle that lymphocyte dependent antibody mediated cytotoxicity or K cell mediated

cytotoxicity can be competitively inhibited by immune complexes occupying the receptors for antibody on K cells and so preventing K cell access to target cell sensitized with antibody.

Finally the presence of complexes as assessed by these assays has been compared with the impairment of polymorphonuclear leucocyte chemotaxis which is seen in rheumatoid arthritis (Mowat and Baum, 1971). This phenomenon has been suggested to be due to the prior ingestion of immune complexes by these phagocytic cells (Mowat and Baum, 1971).

MATERIALS AND METHODS

Patients, clinical and biochemical assessment and controls

I am much indebted to Dr. B. Hazleman for obtaining the blood samples and compiling the clinical details and to Dr. A. Mowat for allowing me to study patients under his care.

Forty four in-patients were studied. Of these thirty five had rheumatoid arthritis, and all had definite or classical disease (American Rheumatism Association criteria). Twenty four had a positive differential agglutination titre (DAT) of 1:32 or greater. The remainder had been sero-negative for at least six months. In the final analysis three patients were excluded because of multiple pathology. The remaining group of patients was small, consisting of two men with active ankylosing spondylitis, one boy with mild chronic juvenile polyarthritis and six patients with degenerative joint disease. The mean age of the patients and the mean duration of disease were

similar between groups. The frequency distribution plots for the age, age at onset, and duration of the disease, for the rheumatoid patients are shown in Figures 5,1; 5,2 and 5,3.

All patients were receiving anti-inflammatory drugs and, in addition, eleven patients were taking prednisone, mean dose 6.5 mg (range 5 - 10 mg); the dose had been stable for at least the preceding three months.

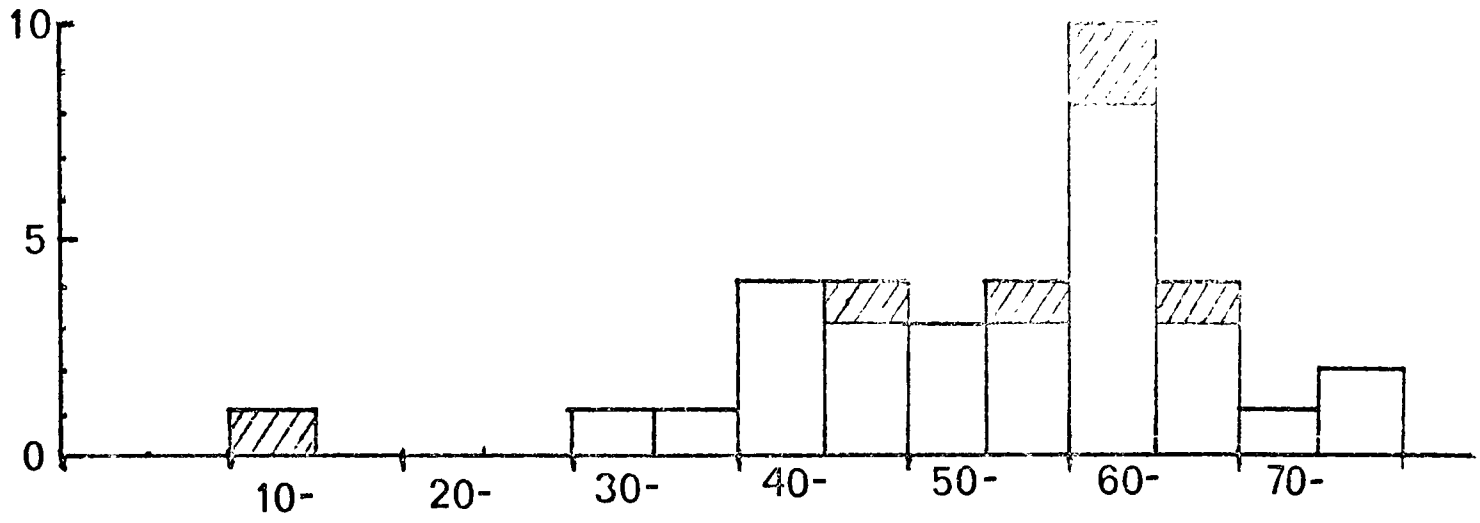
Assessments of clinical activity used included the duration of morning stiffness and a measure of articular index based on that of Ritchie et al. (1968); and extended by the addition of a quantitative assessment of joint swelling as described by Camp (1971).

Laboratory investigations included measurements of sedimentation rate (Westergren method), haemoglobin and total and differential white ceil count, blood urea and serum creatinine, total protein and albumin globulin ratio, DAT using latex particles and antinuclear antibody. Urine was tested for protein and the sediment examined.

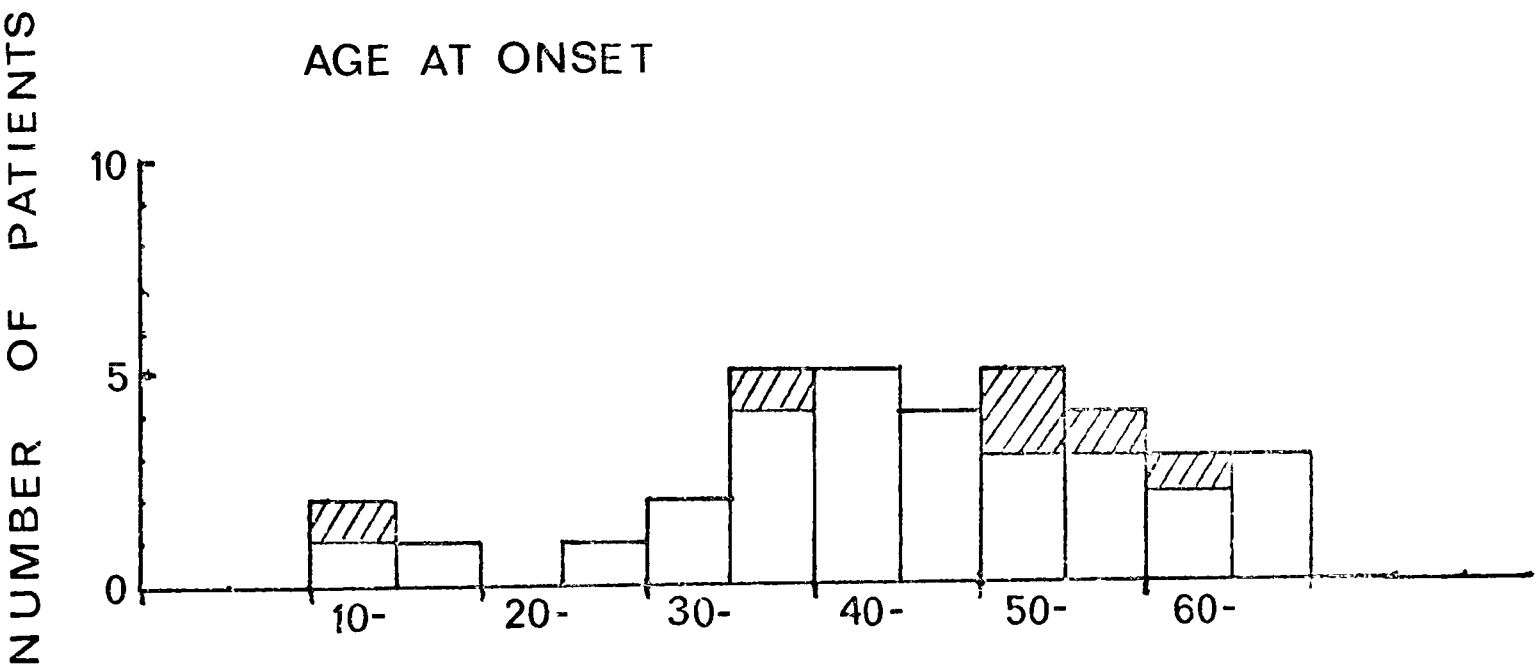
Eighteen healthy laboratory personnel were used as controls. There were ten females and eight males and their ages varied from 18 to 57 years with a mean of 35 years.

Venous blood was aspirated from the ante cubital vein of patients and controls, and allowed to clot at room temperature. The clot was centrifuged within four hours and the serum collected. In general, serum was immediately studied without freezing. In four patients, however, it was frozen at -20°C and examined 2-4 weeks later.

AGE



AGE AT ONSET



DURATION OF DISEASE

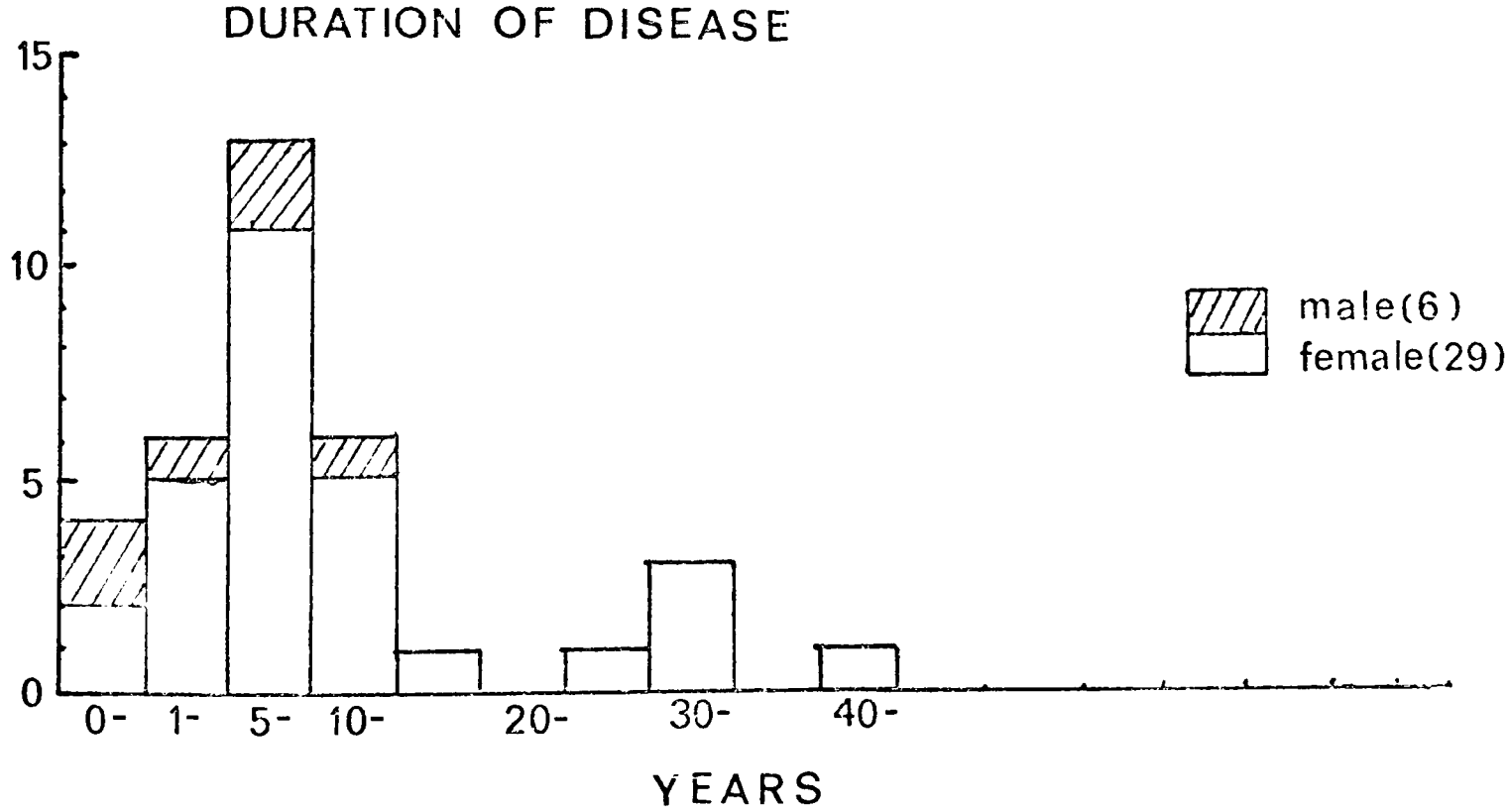


Figure 5, 1-3 Age, Age at Onset and duration of the disease for 35 patients with rheumatoid arthritis.

Gel filtration chromatography

Gel filtration was performed on Sepharose 6B (Pharmacia) columns using the same technique and conditions as described previously. IgG was measured in the eluate by single radial immunodiffusion (see chapter two).

The rationale for using gel filtration chromatography as a method for detecting small IgG containing immune complexes is based on the experimental findings of Gotch (1973). This investigator noted that if preformed immune complexes consisting of radio iodinated human serum albumin (HSA) and anti HSA, and prepared in slight antigenic excess, were injected intraperitoneally into the rat, about half the quantity of complexes entered the circulation and disappeared with a half life of 12 hours. If the serum from one of these rats was fractionated by Sepharose 6B chromatography a radioactive profile was obtained similar to that illustrated in Figure 5,4. This consisted of a peak and shelf, the elution position and profile of the peak being similar to that of monomeric HSA. The shelf eluted in earlier fractions than the peak and it was shown to consist of complexed HSA (as demonstrated by the Farr technique in which bound HSA is precipitated with half saturated ammonium sulphate in contrast to unbound HSA which is soluble).

A similar peak and shelf profile has also been obtained for complexed IgG as shown by Jewell and MacLennan (1973) when they showed small IgG containing complexes in the sera of patients with inflammatory bowel disease.

With these preliminary remarks it is now possible to define some chromatographic measurements which were used in the assessment of these IgG containing complexes. The explanation of these is given below and should be read in conjunction with Figure 5,5.

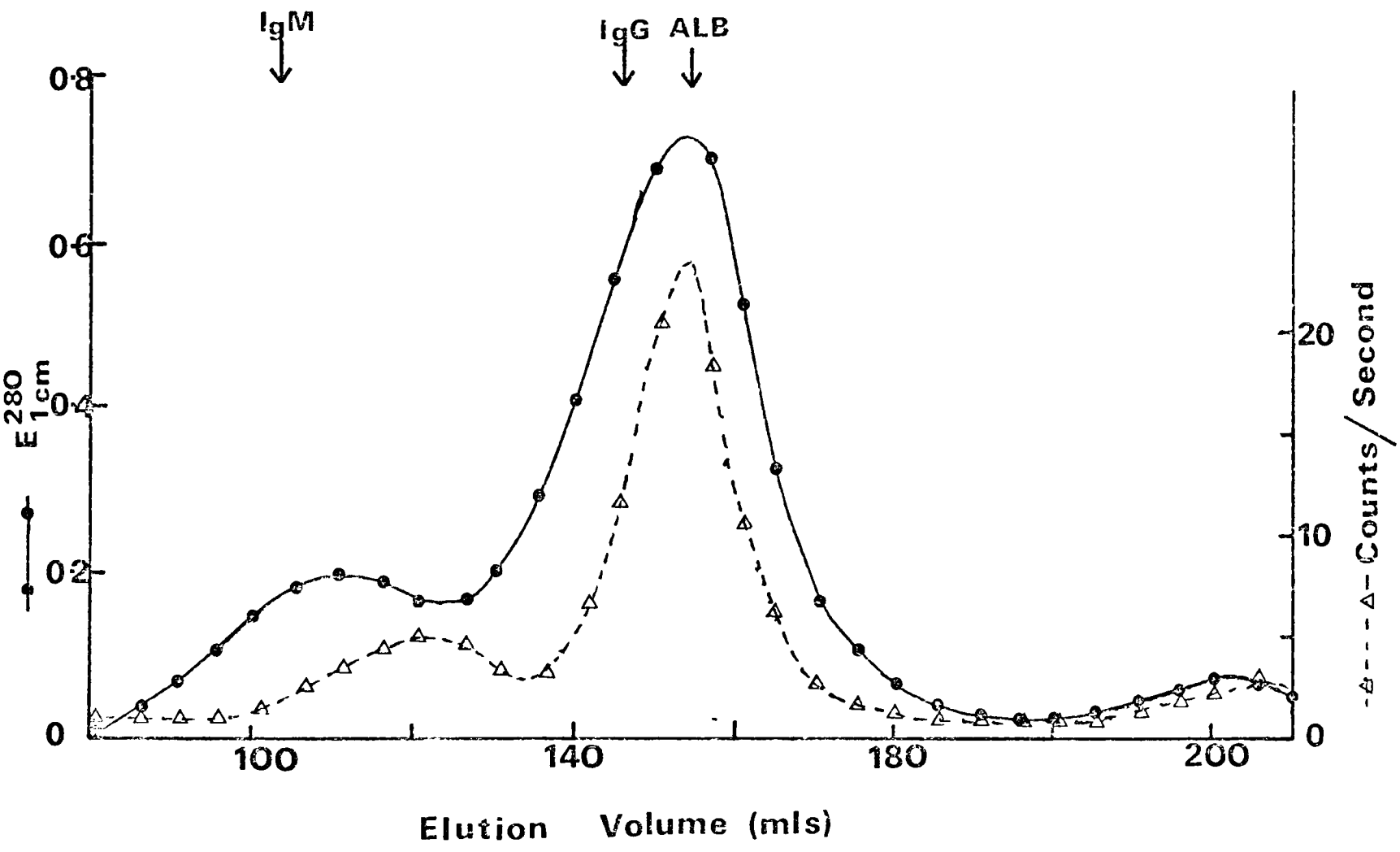


Figure 5,4 The optical density and radioactive profile obtained after fractionation of rat serum on Sepharose 6B. The rat had been previously injected intraperitoneally with I^{125} HSA/anti HSA immune complexes. Note the shelf of the radioactive profile preceding the main peak, indicating the presence of complexed HSA in the serum.

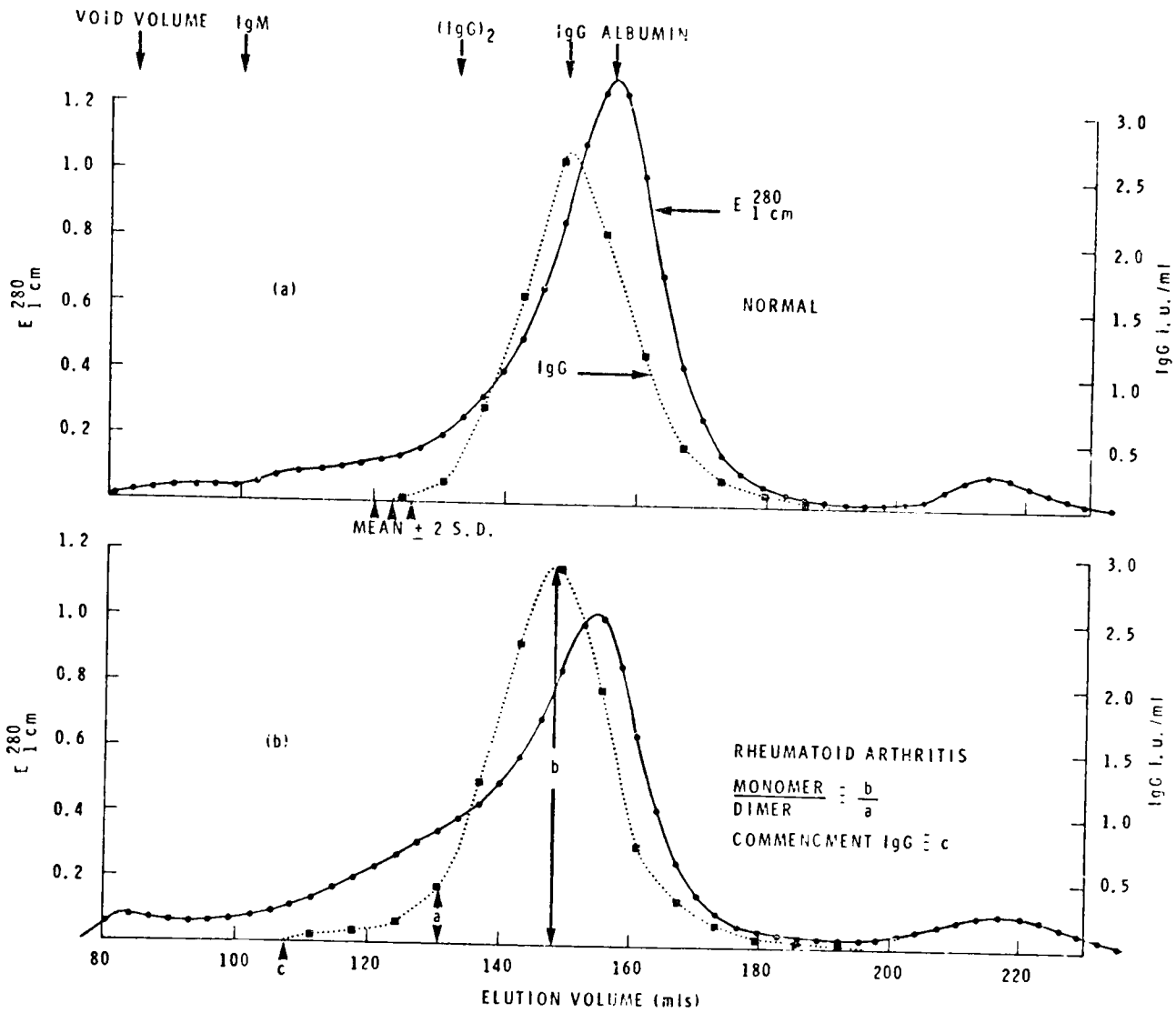


Figure 5,5 Elution profiles obtained from gel chromatography of serum on Sepharose 6B. In the upper part of the figure the continuous line signifies the optical density of the eluate read at 280 mμ, while the lower intermittent line is the profile of IgG in international unit per ml (i.u./ml) estimated in the eluate fractions. Peak elution positions of different marker proteins used in the calibration of the column are shown. $(IgG)_2$ = elution position where dimeric IgG is expected to peak. Commencement IgG (=C) is the elution position where IgG is first detected. Mean ± 2 standard deviation (M ± 2 SD) for commencement IgG obtained from 18 sera from normal healthy subjects is shown. In the lower part of the figure the monomer/dimer ratio = $(^{b}/_{a})$ is the concentration of IgG at peak monomeric IgG elution position (b) to concentration of IgG at peak dimeric IgG elution position (a). The results obtained from a normal healthy subject (upper profiles) and that from a patient with rheumatoid arthritis (lower profiles) are shown for comparison.

IgG commencement (IgG(c)): IgG(c) was defined as that elution position where IgG was first detected in the eluate and this position for one representative serum is arrowed ——> c in Figure 5,5(b). The method for its calculation involved two steps

1) The elution volume difference (IgG_x) between the elution volume at the peak of the optical density profile and at IgG(c) was measured. This was done since slight variations in the elution position of the optical density peak occurred in different chromatographic runs and this was not explicable on quantitative differences in albumin or IgG. It was assumed that similar slight differences in IgG(c) also occurred in different runs. Thus the optical density peak position was taken as an internal marker eluting at a position common to all sera.

2) The IgG(c) was then calculated by subtracting this elution volume difference (IgG_x) from the optical density peak elution volume of a reference elution profile, which was taken as a standard, and the resultant volume was taken to indicate the elution position of IgG(c).

Monomer : dimer ratio: Before defining this ratio it is necessary to discuss some relevant experimental work. Mannik and Arend (1971) have shown that IgG immune complexes that persist in the circulation of the rabbit and monkey have small degrees of lattice with perhaps one or two IgG antibodies per complex. Thus complexes, if present in human sera, would probably also have small degrees of lattice and, if a small antigen is postulated, this complex would only be a little larger than normal monomeric IgG.

Gotch (1973) has also observed that during gel filtration chromatography, HSA-anti HSA immune complexes dissociate to a varying extent as they become diluted in their chromatographic passage through a column. The degree of dissociation depends on the affinity of the antibody, low affinity antibody dissociating to a far greater extent. During the dissociation this previously complexed HSA is still partially separated from non complexed HSA and is found in the ascending limb of the monomeric HSA profile. As the concentration of HSA in the ascending limb is also dependent on the concentration of non complexed HSA it is necessary to take this into account when the concentration of previously complexed HSA at the position of the ascending limb is measured.

Identical reasoning can be applied to the gel filtration of complexed IgG in human serum. Thus in an attempt to assess small IgG complexes and IgG which has been in a complexed form, a ratio of the amount of IgG found at the monomeric IgG marker compared to that at the dimeric IgG marker was established. This monomeric to dimeric IgG ratio (M:D) is signified as $^{b}/_{a}$ in Figure 5,5(b) and has been taken as an index of the amount of IgG complexes present.

The expected peak elution position for dimeric IgG was extrapolated from the graph plotting the $\log_{10}$ of the molecular weight of several marker proteins against their respective elution position (see chapter two). However as long as standard position is taken the exact elution position of 'a' is not critical.

Comparison of results from different columns: Three similarly sized columns of Sepharose 6B were used. One column had dimensions of 42 x 2.5 cms and was designated the 'master' column while the other two 'minor' columns had dimensions of 40 x 2.5 cms and 39 x 2.5 cms (actual dimensions of Sepharose gel after packing). Due to these slight size differences the elution positions of the marker proteins predictively varied between columns. Thus correction factors were necessary when comparing IgG(c) and monomer:dimer ratios for sera filtered on different columns.

The serum for four laboratory members was filtered during the period of these studies on all three columns and the mean $IgG_{(x)}$ and M:D ratio for the sera calculated for each column. The correction factors for each of the two minor columns were then calculated from the ratio

$$\frac{\text{Mean } IgG_x \text{ master column}}{\text{Mean } IgG_x \text{ minor column}} \quad \text{and} \quad \frac{\text{M:D master column}}{\text{M:D minor column}}$$

These correction factors were then applied to the IgG_x and M:D values obtained from the filtration of other sera on the two minor columns. Thus it was possible to compare IgG_x and M:D ratio for each sera filtered on the minor columns to those obtained on the master column. Over half of all sera were filtered on the master column.

Reproducibility: After deriving these correction factors an attempt was made to assess the reproducibility between different columns. The sera from five healthy controls were additionally separated on two or all three columns. The results are seen in Table 5,1. In addition the serum from one patient and one control was refiltered on the master column to assess the

Name	Measurement	Columns		
		Master	1	2
F.R.*	IgG(c)	107 111	- -	- -
	M:D	3.74 4.94	- -	- -
P.R-T.	IgG(c)	121 123.5	123.2	123.9
	M:D	7.86 7.75	6.8	9.0
B.W.	IgG(c) M:D	122.5 8.83	- -	121.6 5.9
D.G.	IgG(c) M:D	123.2 5.9	124.5 9.4	120.6 7.8
I.C.M.M.	IgG(c) M:D	123.5 10	- -	124.4 8.2
P.H.	IgG(c) M:D	123 7.84	124.4 10	- -

* rheumatoid arthritis

Table 5,1 Reproducibility between commencement IgG(c) and monomer:dimer ratio obtained from the IgG profile after gel filtration of serum on 2 or 3 different agarose columns, or on separate runs on the same column. The three columns are designated master, and minor, 1 and 2. The sera were from five healthy laboratory subjects and one patient with rheumatoid arthritis.

reproducibility for the method using a single column. The results are also seen in this Table. A good agreement was found for repetitive IgG(c) values between columns whereas a larger variation was seen for the M:D ratio. More separations are required to assess the reproducibility of the method using a single column but close repeated values were found for IgG(c) and monomer : dimer ratio for two sera studied.

K cell cytotoxicity inhibition assay (lymphocyte dependent antibody mediated cytotoxicity inhibition assay)

K cell cytotoxicity is a mechanism by which target cells are killed by K cells in the presence of small amounts of specific IgG anti-target cell antibody (see MacLennan, 1972, and Perlmann, Perlmann and Wigzell, 1972, for review). The K cell involved in the killing, when the Chang cell is used as a target cell, has been characterized to be a non-adherent non-phagocytic mononuclear cell with the morphological appearance of a small to intermediate sized lymphocyte (MacLennan, 1972). It does not share the properties of T or B lymphocytes as assessed by spontaneous sheep red cell rosetting and surface immunoglobulin respectively. The K cell does, however, have surface receptors for the Fc part of the IgG molecule. When specific antibody combines with the Chang target cell membrane, determinants on the antibody molecule are thought to be exposed which react with the Fc receptor on the K cell and initiate cytolysis.

^{51}Cr release is a convenient method to assess target cell damage since chromium is released on cell lysis of labelled target cells but is not taken up by surviving cells. The K cell cytotoxicity assay, using chromium release

to detect cytolysis, is accurate and reproducible (MacLennan, 1970) and can be easily adapted for the detection of serum inhibitory factors such as soluble immune complexes (Jewell and MacLennan, 1973).

Soluble immune complexes and heat aggregated human IgG myeloma proteins of subclass 1,2,3 and to a lesser extent 4, inhibit K cell mediated cytolysis by competing with the antibody, bound to the target cell, for the Fc receptors on the K cell (MacLennan, 1972; MacLennan et al., 1973). As little as 30 ng of antigen, present in the form of a soluble complex, will give a measurable amount of inhibition of K cell killing (MacLennan, 1972). However since many serum inhibitory factors apart from immune complexes can effect K cell cytotoxicity (vide infra) this assay can not be considered as giving absolute evidence for the presence of immune complexes. However it does act as a screening test and in addition provides information concerning the biological properties of complexes that are definitively detectable by physicochemical means.

K cells: K cells used in this assay were isolated from peripheral blood using the method of Coulson and Chalmers (1964). Venous blood from one individual (I.C.M.M.) was defibrinated in a universal container using sterile 4 mm glass beads. Defibrinated blood was mixed with equal volumes of 3% gelatin (British Glue and Gelatin Research) in 0.9% saline and the mixture allowed to stand for 15 - 30 minutes at 37°C. Sedimentation of the blood occurred, and the supernatant was removed. The blood was resuspended in equal volumes of gelatin and the process repeated. The supernatants were pooled and the mononuclear cells so obtained collected by gentle centrifugation.

These cells were washed in Eagle's minimum essential medium containing 10% foetal bovine serum (M.E.M.10), resuspended in Hepes buffered Eagle's Basal medium enriched with 10% foetal bovine serum (B.M.E. 10) and counted in an Improved Neubauer counting chamber using phase contrast microscopy.

^{51}Cr labelled Chang cells: Chang cells (Flow laboratories), a continuous human liver cell line, were used as target cells. They were grown in suspension cultures in 8 oz medical flat bottles at 37°C. The culture medium used was M.E.M.10, 1% glutamine, 1% non-essential amino acids, penicillin 200 units/ml and streptomycin 10 mg/%. Single cell suspensions were obtained every 24 - 48 hours by shaking the bottle, pouring off the medium, and replenishing with fresh medium for a further growth. The eluate containing suspended Chang cells was then centrifuged at 200 g for ten minutes. The resultant pellet of cells was resuspended in about 1 ml of M.E.M. and incubated with 100 μc [^{51}Cr] sodium chromate (Radiochemical Centre, Amersham, specific activity 2 - 10 μg Cr/mC) for 1 hour at 37°C. The cells were then suspended in M.E.M. and washed through a 3 - 4 ml layer of foetal bovine serum, resuspended in B.M.E. and counted using phase contrast microscopy. Only viable Chang cells were counted as assessed by their regular size and smooth surface.

Rabbit anti-Chang antiserum: This was prepared using the method of MacLennan et al. (1969). One ml of 10^7 Chang cells in Freund's complete adjuvent was injected intradermally into multiple sites of a rabbit. Three weeks later 10^7 Chang cells in saline were injected intravenously. Two weeks after

this the animal was bled, the serum collected and heated to 56°C for 30 minutes to destroy the complement, diluted 1 : 10 in M.E.M., aliquoted into 0.05 ml volumes and stored at -20°C until required.

Cytotoxicity assay: Cultures of 0.3 mls were set up in sterile 3" x $\frac{1}{2}$" tubes (M. and H. Plastics (Romford) Ltd.). The basic medium was B.M.E. enriched with 1% foetal bovine serum (Biocult Batch 00239). Each culture contained 2×10^4 Chang target cells sensitized with 1 : 10,000 anti Chang antibody, and 3×10^5 peripheral blood lymphocytes. The cell suspensions were constantly mixed while the cells were being dispensed in order to ensure even distribution of cells. In addition each culture contained dilutions of patients' serum or control serum. All dispensing and dilutions were done using Eppendorf pipettes. Cultures were set up in triplicate.

Each experiment also included cultures containing the following two standards: 1) 500 μg of aggregated human IgG obtained from aliquots of a single batch. This was prepared as follows: IgG was purified from normal human serum by half saturated ammonium sulphate precipitation followed by DEAE 32 ion exchange chromatography (Whatmans) and Sephadex G 200 gel chromatography. The purified IgG in P.B.S. was then measured spectrophotometrically, heated at 63°C until a slight turbidity of the solution was apparent, centrifuged, and the supernatant diluted to give a total IgG concentration of 5 mg/ml. This was aliquoted.

2) Medium only, i.e. no inhibitor

Test sera were diluted to final concentrations of 1:3, 1:9 and 1:27. Cultures were incubated at 37°C for $3\frac{1}{2}$ hours. After this time, 1 ml of cold saline

was added to each tube and the cultures were centrifuged at 200 g for 7 minutes. Exactly 1 ml of supernatant was then removed from each tube, the residue and supernatant tubes being finally counted using a Wallac 500 sample automatic gamma counter which contained a 3" x 3" NaI (TL) crystal. Each tube was counted to 5000 - 10,000 counts at 100 - 200 seconds.

The percent ^{51}Cr release was calculated as follows:

$$\% \ ^{51}Cr = \frac{1.3\ (A - K)}{(A - K) + (B - K)} \times 100$$

where A = activity of supernatant in counts per second (C.P.S.)

B = activity of residuum in C.P.S.

K = background counts in C.P.S.

The percent cytotoxicity inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\% \ ^{51}Cr \text{ release in medium} - \% \ ^{51}Cr \text{ release in test serum}}{\% \ ^{51}Cr \text{ release in medium} - \% \ ^{51}Cr \text{ release with aggregated IgG}} \times 100$$

Polymorphonuclear leucocyte (PMN) chemotactic index

This test was kindly performed by Dr. A. Mowat in his laboratory. The method will be only briefly described (Mowat and Baum, 1971). A cytocentrifuge was used to sediment a small number of PMN onto a 3μ Millipore filter. This was then placed in a Sykes-Moore tissue culture chamber with the surface of filter containing the PMN facing up. Both compartments of chamber were filled, the upper with Hanks's solution, and the lower with

a solution that attracts PMN (Hanks's solution containing casein and human complement). The chamber was incubated for 3 hours at 37°C and the PMN on each side of the millipore filter were then counted using microscopy. The chemotactic index C.I. was calculated as follows -

$$C.I. = \frac{\text{Number PMN (attracting side)}}{\text{Number PMN (starting side)}} \times 1000$$

Thus a low index implies impairment of chemotaxis.

Serum protein estimations

The immunoglobulins G, A and M, haptoglobin (Hp), alpha-2-macroglobulin (α_2M), and albumin (alb) were measured by single radial immunodiffusion as described in chapter two.

Analysis of results

The assessment of the clinical features and the measurement of PNN chemotaxis and other laboratory tests were made independently of the results of the assays for immune complexes. The values for percentage inhibition of K cell cytotoxicity were calculated without knowledge of the results obtained from gel filtration chromatography.

Many of the estimations could not be considered to be normally distributed and hence non parametric statistical tests were used. The Wilcoxon sum of ranks test was used when comparing two groups. Correlation between variables within a group were performed on the Oxford University computer

using Spearman rank correlation coefficients. I am indebted to Miss S. Howard for help with the statistical analysis.

RESULTS

Gel filtration chromatography

The elution position where IgG was first detected (IgG(c)) was determined for 18 healthy control sera. For this group it was found that IgG(c) was confined to a narrow range (Fig. 5,5,a) and each individual value is indicated in Figure 5,6. A significant correlation was observed in this group between IgG(c) and the total concentration of IgG as assessed by the height of the IgG profile, $r = 0.998$. This finding suggests that in health IgG(c) will vary over a small range according to the concentration of IgG.

Eighty percent of the sera of 35 patients with rheumatoid arthritis (RA) tested in this way had IgG separating in higher molecular size fractions than any of the healthy controls (Figure 5,6). Four patients with other joint diseases showed early emerging IgG but one had active ankylosing spondylitis and two patients with clinical osteoarthritis were atypical in respect of elevated sedimentation rates (71 and 20 mm / first hour).

In the rheumatoid group there was no significant correlation between the concentration of IgG and IgG(c), $r = 0.393$, $p = 0.5$, and the commencement IgG differed from that of the small group of patients with other joint diseases, $0.1 > p > 0.05$.

The monomer : dimer ratios ($^{b}/_{a}$, Figure 5,5,b) obtained for the sera of

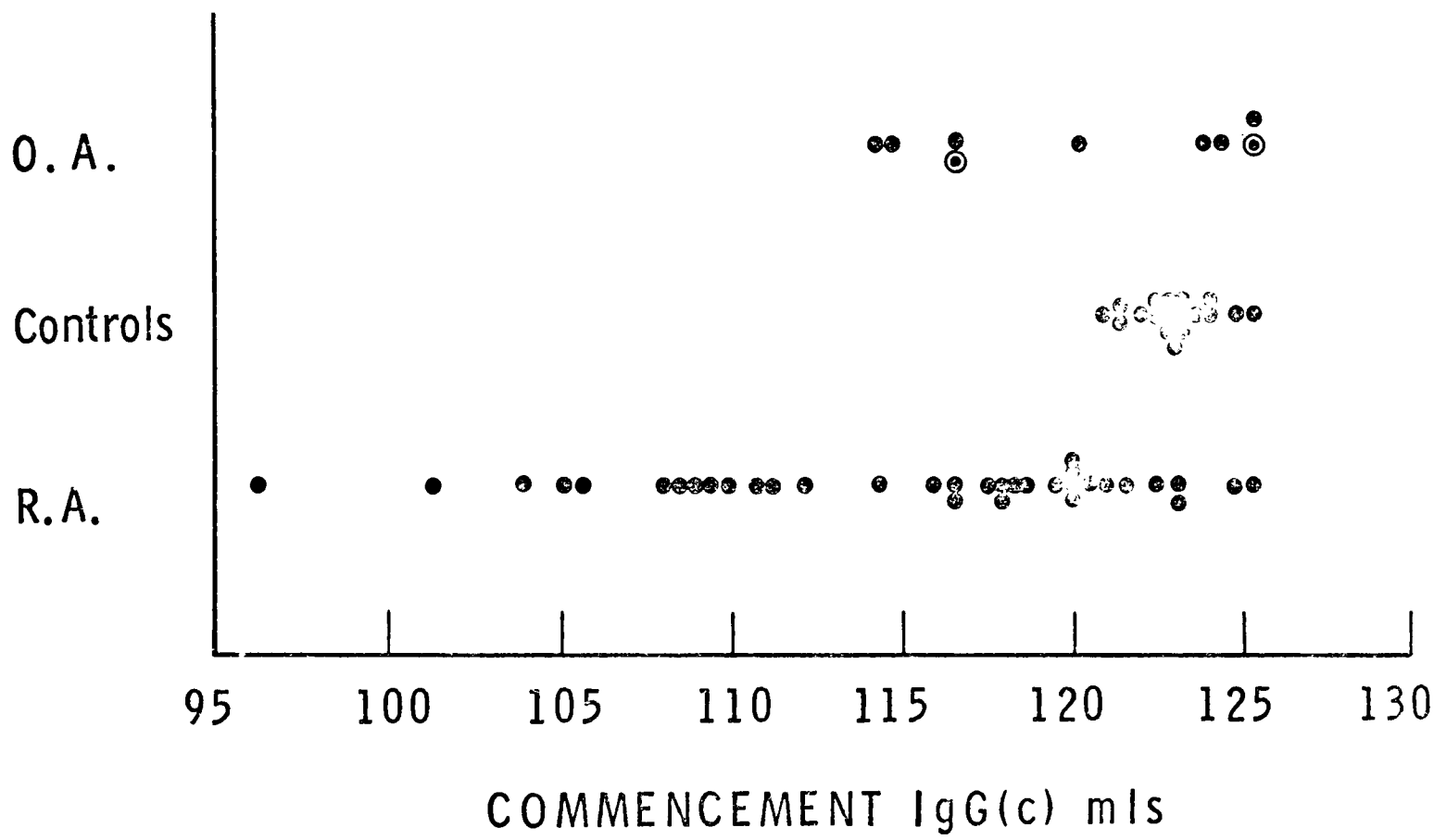


Figure 5,6 Commencement IgG(c) is the elution position at which IgG is initially detected. This position (in mls of eluate) is plotted for patients and controls.

Controls = healthy laboratory subjects

O.A. = Patients with osteoarthrosis. The patients with ankylosing spondylitis have been encircled

R.A. = rheumatoid arthritis patients

patients and controls are plotted in Figure 5,7. The ratios obtained for the rheumatoid group were significantly lower than those found for the other two groups; RA < healthy controls $p < 0.005$; RA < other joint disease $p < 0.05$.

In the 18 sera studied from the healthy controls, the concentration 'a' measured at the dimer IgG marker varied directly with the concentration 'b' measured at the monomer IgG marker ($r = 0.909$). This showed that the concentration of IgG in the ascending limb of the IgG profile varied directly with the total concentration of IgG for this control group. In addition it was noted that the monomer concentration 'b' (Figure 5,5,b) was similar in all three groups (in fact the mean for the RA group was slightly higher than that for the other two groups). Since the ratio b/a was significantly low for the RA group this requires that 'a' is abnormally large and strongly suggests the presence of small complexed IgG in this group.

Three patients in the other joint disease group had low monomer : dimer ratios. These consisted of the two patients with active ankylosing spondylitis and one of the patients with osteoarthrosis whose serum also gave a high degree of inhibition of K cell cytotoxicity (vide infra).

Comparison of monomer : dimer ratios with IgG commencements in the rheumatoid group showed no significant correlation $p = 0.779$. This would suggest that these are measurements of two different variables, perhaps indicating two types of complexes with distinct and independent origins.

K cell cytotoxicity inhibition

The percentage inhibition of cytotoxicity obtained with sera diluted 1:3 for the three different groups are shown in Figure 5,8. The 1:9 and 1:27

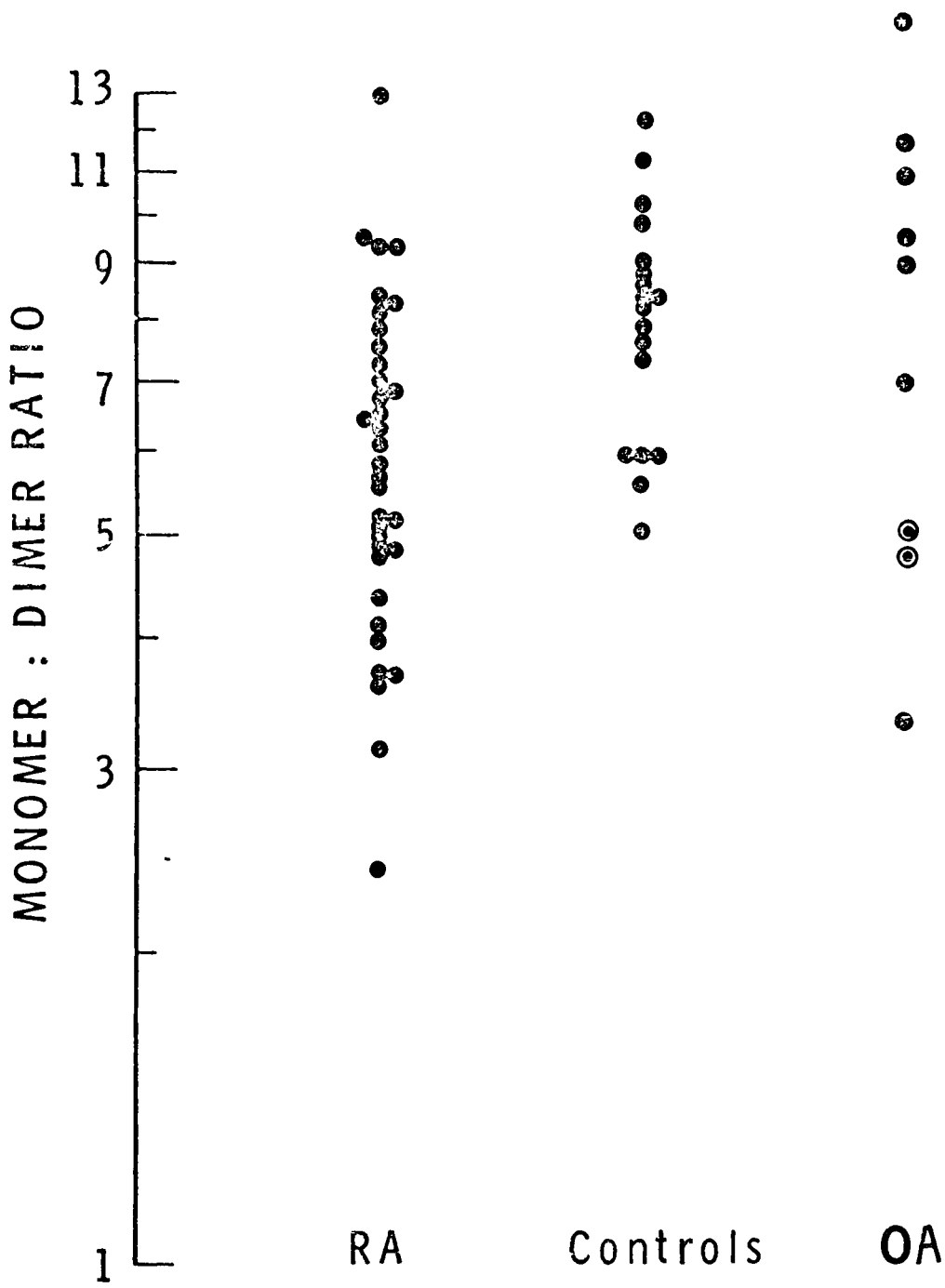


Figure 5,7 The monomer: dimer ratios for patients and controls

R.A. = rheumatoid patients

Controls = healthy subjects

O.A. = patients with other joint disease, the patients with ankylosing spondylitis being encircled

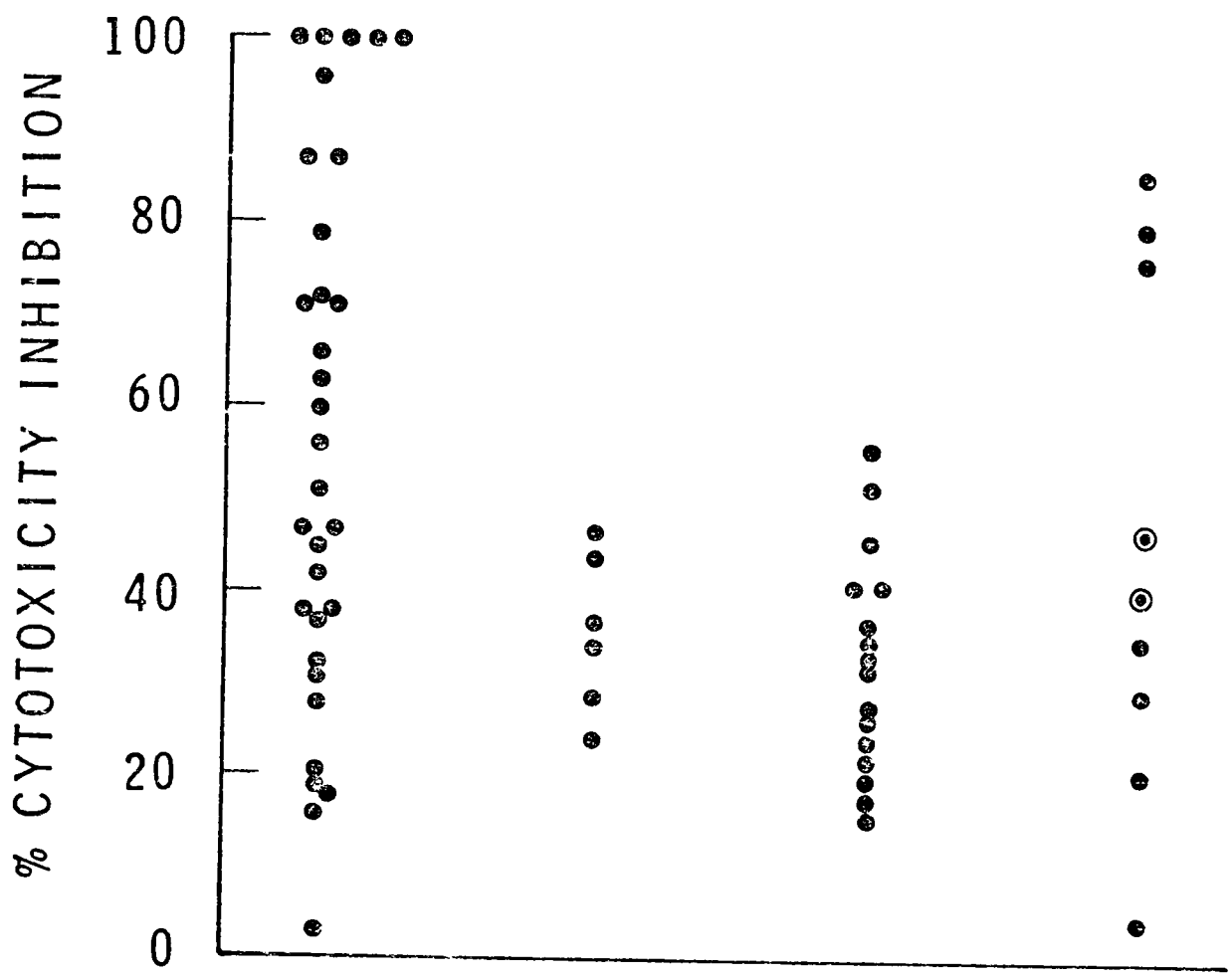


Figure 5,8 The percentage inhibition of K cell cytotoxicity obtained with sera from patients and controls

R.A. = patients with rheumatoid arthritis

Controls = healthy laboratory subjects

O.A. = patients with other arthropathies, the 2 encircled points representing patients with ankylosing spondylitis

I.C.M.M. = the six specimens of serum obtained from one individual (I.C.M.M.) and tested on six separate occasions

dilutions of sera gave correspondingly less inhibition than was seen with the 1:3 dilution.

The rheumatoid group showed a significantly greater inhibition than the healthy controls, $p < 0.005$. This group, however, did not differ significantly from the other joint diseases group.

The sera from three patients of the other joint diseases group inhibited K cell cytotoxicity more than any of the control group. These patients all had osteoarthrosis of recent onset.

The sera from the control group showed some inhibition of cytotoxicity but none gave values greater than 56%. This upper limit was a little greater than that seen by Jewell and MacLennan (1972) although those workers used slightly different assay conditions. The variation in the degree of inhibition seen for the serum of one individual (I.C.M.M.) studied on six occasions is shown in Figure 5,8. No correlation was observed between percentage inhibition and the age or sex for the control sera.

Small aggregates of IgG can inhibit K cell cytotoxicity (Hallberg, 1974). However no direct correlation was seen between percentage inhibition of cytotoxicity and serum IgG concentration as might be expected if spontaneous aggregation of serum IgG was the cause of the inhibitory material. In fact an inverse correlation was seen for the rheumatoid group (Table 5,II).

Two rheumatoid sera which strongly inhibited K cell cytotoxicity were analysed by Sephadex G200 and Sepharose 6B chromatography. The eluates after filtration were divided into several fractions and each fraction was concentrated by low pressure dialysis back to the original serum volume. Each concentrated fraction was then tested for inhibitory activity at a 1:3 dilution

in the standard manner. The results for these experiments are seen in Figure 5, 9 and 10. Both these experiments showed that inhibitory material predominantly occurred in a molecular size range that varied between 200,000 - 600,000 daltons, but higher sized fractions also showed some inhibition. Further attempts to characterize this inhibitory material by precipitated IgG absorption were technically unsatisfactory.

Correlation between assays for immune complexes

As is shown in Table 5, II, K cell cytotoxicity inhibition for the rheumatoid arthritis group correlated significantly with the monomer : dimer ratio, $p = 0.013$, but not with IgG commencement, $p = 0.963$. No significant correlations were seen between these measurements in the small number of patients constituting the other joint disease group.

Polymorphonuclear leucocyte chemotaxis

It has previously been suggested that impairment in neutrophil chemotaxis in patients with RA may be related to the prior investigation of immune complexes (Mowat and Baum, 1971). The mean value for the chemotactic index for the 21 patients in the rheumatoid group studied was 417 with a range of 166 to 601. (The mean $\pm$ one standard deviation for healthy adults is 553 ± 69 (Mowat and Baum, 1971)). A significant correlation was observed between the neutrophil chemotactic index and the serum monomer: dimer ratio for these rheumatoid patients $0.02 < p < 0.05$ (Figure 5,11). The chemotactic index was inversely correlated $0.001 < p < 0.01$ with the

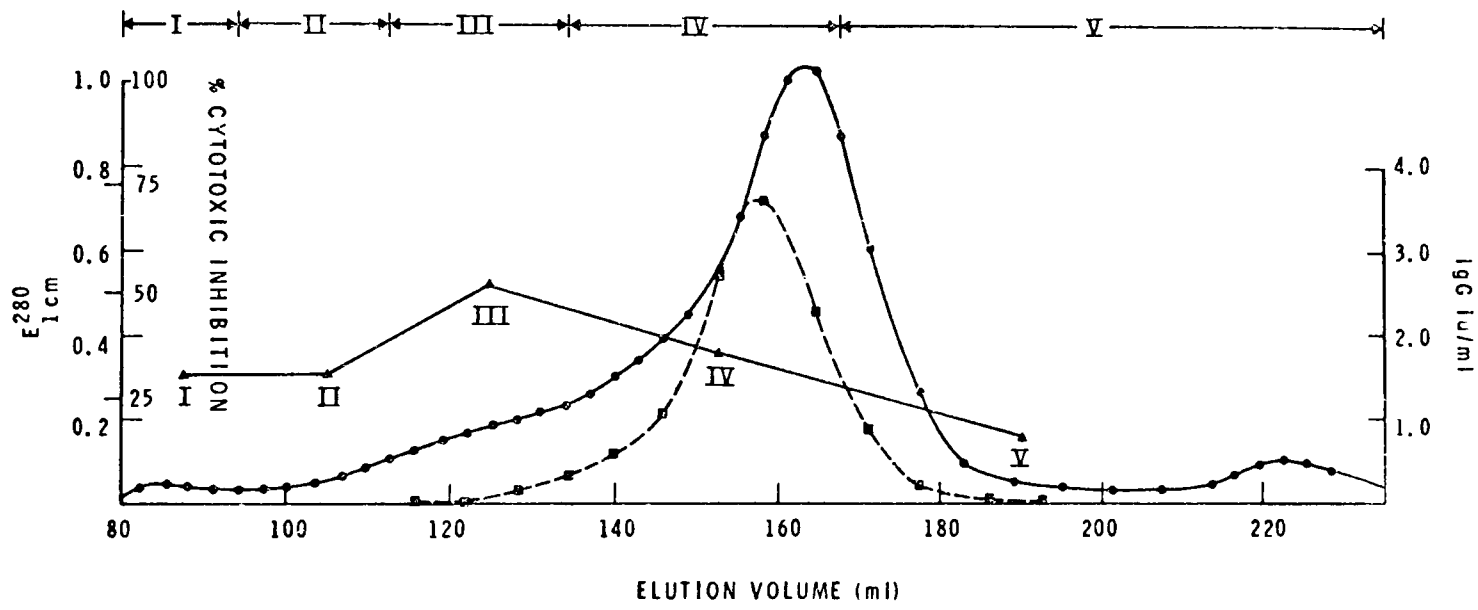


Figure 5.9 Serum from a patient with rheumatoid arthritis has been fractionated on Sepharose and the eluate divided into five fractions I - V. The percentage inhibition of K cell cytotoxicity obtained with each fraction is shown. Note the maximal inhibition and corresponding elution position of fraction III

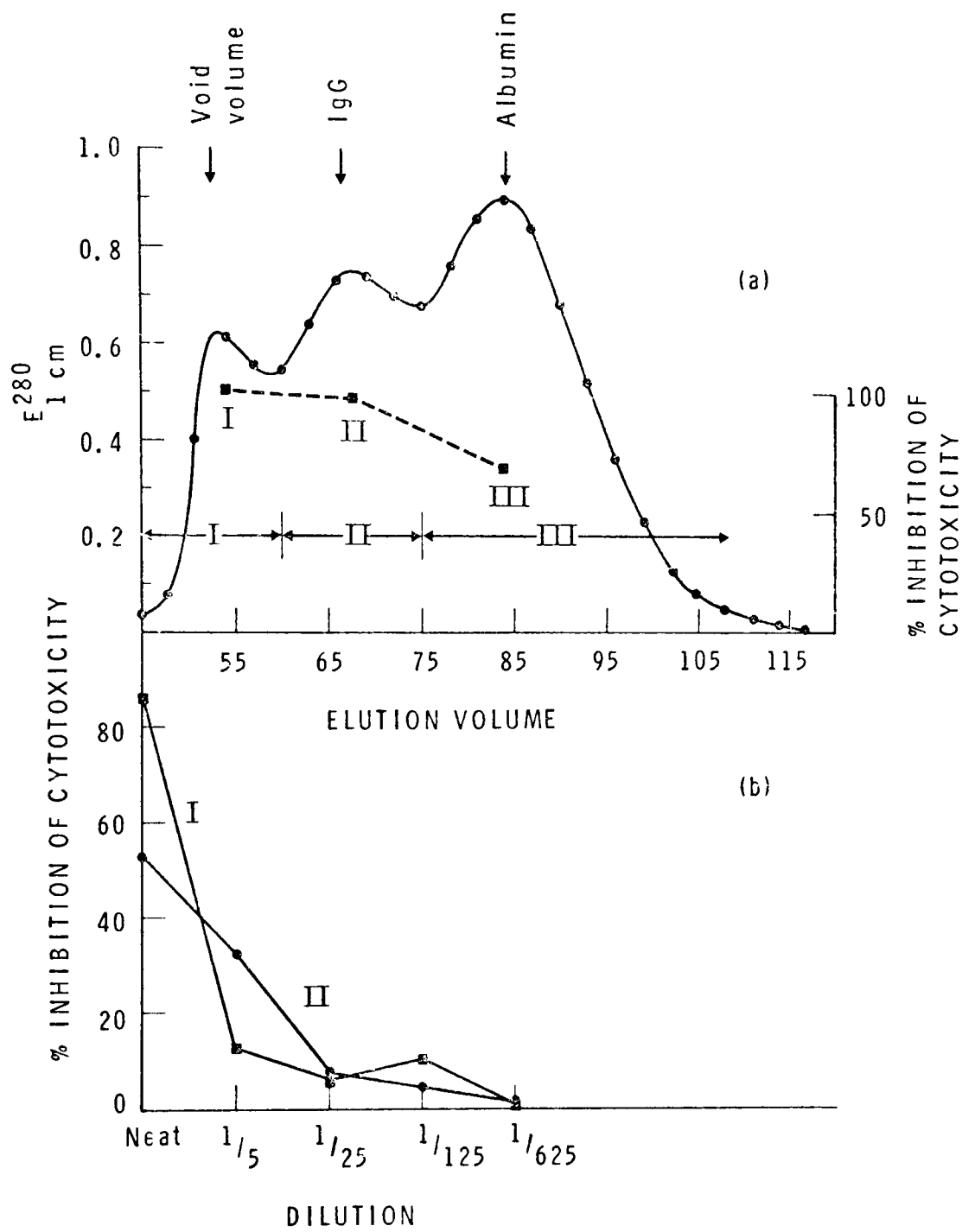


Figure 5,10 Serum from a patient with rheumatoid arthritis has been fractionated on Sephadex G200 and the eluate separated into three fractions I - III. The degree of inhibition of cytotoxicity obtained with each fraction is shown in the upper frame while the titre for fraction I and II is plotted in the lower frame

	Age	Duration	Clinical activity	Morning stiffness	ESR	Log latex titre	Hb	W.C.C.	IgG	IgA	IgM	Albumin	α_2 Macroglobulin	Haptoglobin	Steroids	% Inhibition	IgG Commencement	Monomer . Dimer
Age		+0.224	+0.314	+0.967	+0.138	+0.111	-0.848	-0.473	+0.967			-0.469	-0.891	+0.388	+0.612	+0.111	+0.696	-0.717
Duration			-0.640		-0.897	+0.735	+0.491	-172	-0.966	+0.438	-0.878	+0.694	+0.919	-0.429	+0.652	+0.099	+0.791	+0.678
Clinical activity				+0.133	+0.063		-0.876	+0.271	-0.453		-0.322	-0.298	-0.435	+0.615	+0.955	-0.729	+0.162	-0.827
Morning stiffness		-0.009			+0.172	+0.984	-0.471	+0.598	-0.809	+0.578	+0.180	+0.620	-0.973	+0.111	+0.513	-0.100	+0.405	+0.620
ESR						+0.559		-0.136	+0.270	+0.963	-0.813	-0.151	+0.796		-0.599	-0.745	+0.146	+0.291
Log latex titre			+0.047				+0.168	+0.363	-0.325	+0.074	+0.114	-0.150	-0.921	-0.181	+0.193	+0.587	-0.552	-0.800
Hb					-0.001					+0.730	+0.721	+0.279	-0.520			+0.089	-0.302	-0.450
W.C.C.							+0.03		-0.153	-0.778	-0.942	+0.368	+0.855	-0.592	+0.153	+0.323	-0.893	-0.414
IgG							-0.001			+0.706	+0.185	+0.870	+0.138	+0.304			+0.508	+0.111
IgA	+0.003		+0.036							+0.001		-0.167	-0.358	-0.366	-0.165	+0.818	-0.476	-0.775
IgM	+0.005											-0.265	+0.539	-0.523	-0.777	+0.845	-0.608	-0.817
Albumin												+0.04		-0.589	+0.778	+0.918	-0.474	-0.640
α_2 Macro-globulin														-0.366	-0.743	-0.636	+0.084	-0.173
Haptoglobin					+0.005		-0.008								-0.586	-0.088	+0.249	
Steroids							+0.006		-0.001							+0.112	+0.273	-0.256
% Inhibition									-0.009								-0.963	
IgG Commencement																		-0.779
Monomer Dimer														+0.031		-0.013		

Table 5,11 Probability 'p' values for correlation coefficients of clinical, haematological and laboratory data in assessment of rheumatoid arthritis.

All p values are given above the diagonal - the values below being significant $p < 0.05$

\+ = direct correlation

\- = inverse correlation

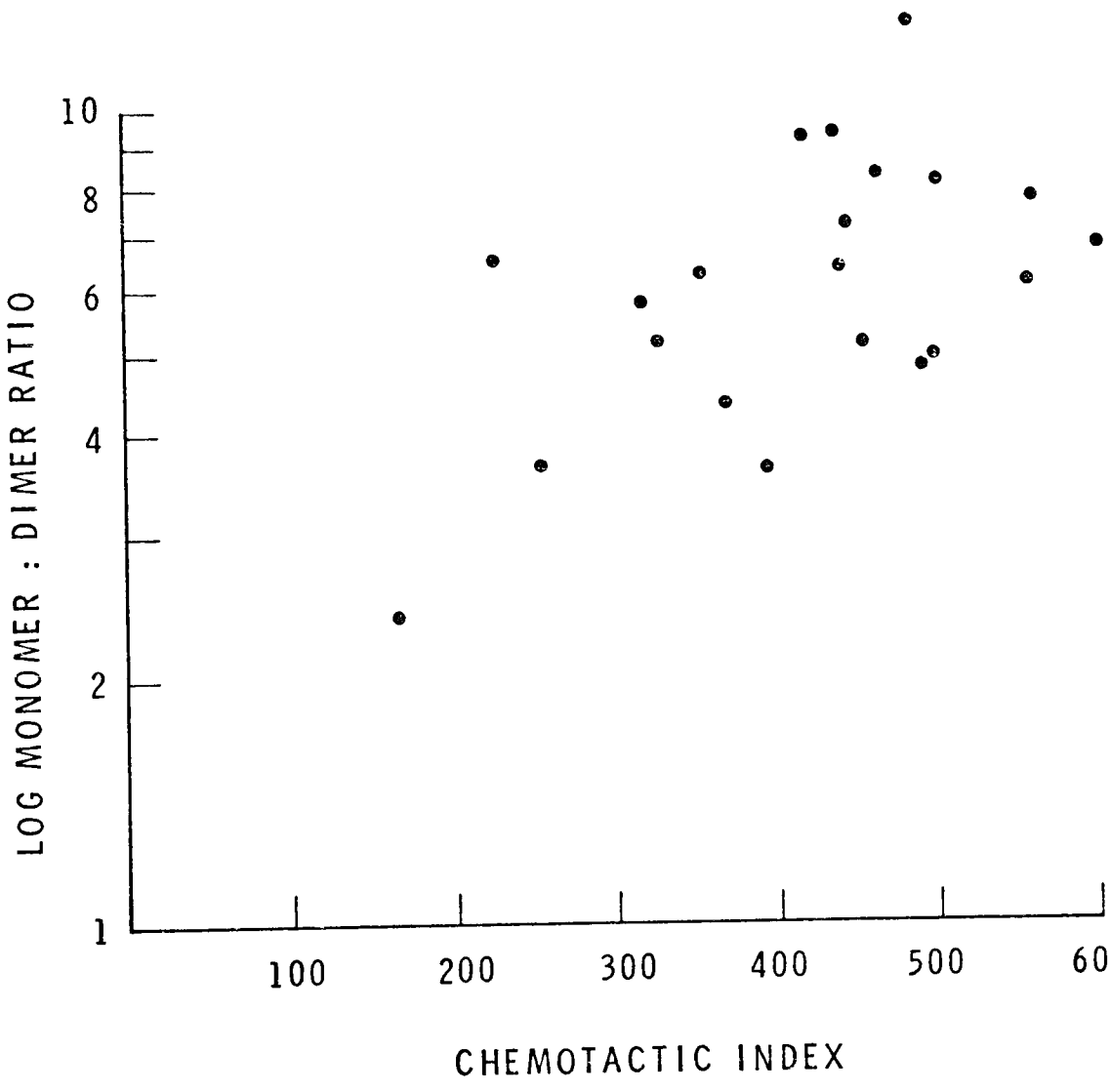


Figure 5,11 Correlation between neutrophil chemotaxis index and monomer : dimer ratio. Correlation coefficient 0.462, $p < 0.05$

percentage inhibition of K cell mediated cytotoxicity after the exclusion of the rheumatoid sera which maximally inhibited to 100% (Figure 5,12). If these sera were included an inverse correlation was still apparent but not significant at conventional levels $0.1 < p < 0.2$.

Serum proteins

The result of the serum protein estimations for the three groups will be described in detail in Appendix I. They are included in this chapter only for comparison with other clinical and laboratory features obtained for the rheumatoid patients and specifically with the results of gel filtration chromatography and K cell cytotoxicity inhibition.

Clinical correlations

The clinical and laboratory data obtained for each of the 35 patients with rheumatoid arthritis were compared with the results obtained from gel filtration chromatography and K cell cytotoxicity inhibition. Probability values for correlation coefficients in this comparison are listed in Table 5, II. Three patients were excluded from this comparison as they had multiple pathology. It might be expected that some of the correlations would be significant due to chance, as a large number of bivariate data was compared.

It is of interest that no significant correlation was seen between the assays for complexes and with the IgM rheumatoid factor titre. Furthermore only two of the eight patients with sero-negative rheumatoid arthritis had normal results for both assays for complexes. Four of these patients had values outside

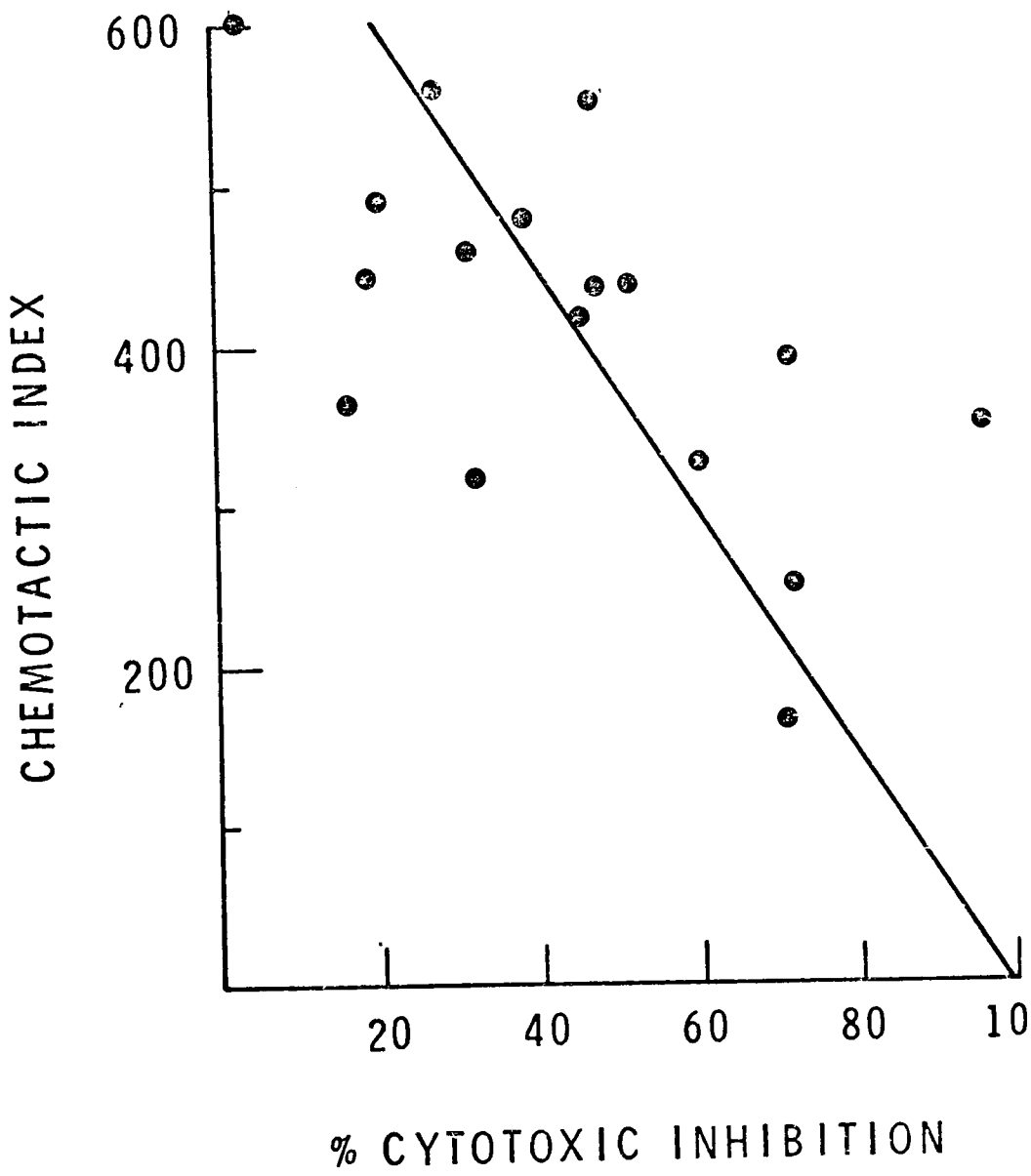


Figure 5,12 Correlation between percentage inhibition of cytotoxicity and chemotactic index. Correlation coefficient 0.608, $p < 0.01$. Three sera which inhibited cytotoxicity to 100% have been excluded.

the normal range in one assay, and two patients had abnormal values in both assays.

No indices for the severity of rheumatoid arthritis correlated with the results obtained from the assays for immune complexes. A significant correlation was observed between the haptoglobin concentration and the monomer : dimer ratio, $p = 0.031$, but the implication of this is obscure.

Several significant correlations were seen between the clinical and laboratory findings. In particular clinical activity correlated with log latex titre, $p = 0.047$; and the sedimentation rate correlated with the haptoglobin level, $p = 0.005$, and inversely with the haemoglobin concentration, $p = 0.001$. IgG levels were significantly lower in patients on steroids, $p = 0.001$.

Data

The basic data obtained for the patient group has been tabulated in Appendix II. The means and standard deviations for IgG(c), monomer : dimer ratio and percentage inhibition for the three groups are listed in Table 5,III.

DISCUSSION

Rheumatoid arthritis may be considered as the 'model' for an immune complex disorder due to several characteristic clinical and laboratory features (Zvaifler, 1973). These include the widespread systemic nature of the disease and the pathological tissue reactions of an erosive arthritis, granulomatous like skin nodules and variably, a vasculitis. Laboratory findings are characterized

	Normal Subjects			Rheumatoid Arthritis			Other Joint Diseases		
	IgG Commencement	Monomer:Dimer	% Inhibition	IgG Commencement	Monomer:Dimer	% Inhibition	IgG Commencement	Monomer-Dimer	% Inhibition
Mean	123.1	8.22	31.0	115.1	6.2	56.7	120.4	9.7	46.5
No.	18	17	18	35	35	32	9	9	9
S.D.	1.4	2.1	13.8	7.0	2.1	29.4	4.7	5.9	28.9

Table 5,III The means and standard deviations (S.D.) for IgG(c) (in mls of eluate), monomer:dimer ratio, percentage inhibition of cytotoxicity for 18 healthy controls, 35 patients with rheumatoid arthritis and 9 patients with other joint diseases.

by the presence of rheumatoid factors (Stage and Mannik, 1973), immunoglobulin abnormalities (Pruzanski et al., 1973), cryoglobulins (Marcus and Townes, 1971), extracellular depositions of immunoglobulin and complement (Munthe and Natvig, 1971), and complement abnormalities (Schur and Austen, 1971). Immune complexes have been detected in both the serum (Kunkel et al., 1961) and the synovial fluid (Winchester et al., 1970) and they may be involved in the pathogenesis of the disease (Zvaifler, 1974). Two types of circulating soluble immune complexes have been detected to date. Franklin et al. (1957) have described a large complex composed of IgM rheumatoid factor and IgG and sedimenting at the 22 S position while Kunkel and others (1961) have described smaller 'intermediate' complexes. These latter complexes are composed of IgG rheumatoid factor and IgG and form the object of the present study.

This study was initiated with two major aims in mind:

1) Is it possible to assess the presence of these intermediate complexes in rheumatoid sera using the method of gel filtration chromatography?

2) Are there any relationships between the presence of these complexes and other clinical and laboratory features of the disease? Before replying to these questions it is necessary to critically examine the methods and the results.

Gel filtration chromatography has been previously used to detect circulating immune complexes in inflammatory bowel disease (Jewell and MacLennan, 1973). In that study the sera from six patients and four healthy subjects were analysed by gel chromatography and it was observed that some

of the pathological sera had as much as 20% of their IgG eluting in abnormally early fractions when compared to the healthy control. Apart from this report there was little information in the literature, prior to 1974, concerning the value of gel filtration chromatography as a means of detecting soluble immune complexes.

In this present study it was demonstrated that the sera from the majority of patients with rheumatoid arthritis contained IgG which emerged after gel filtration, in higher molecular size fractions than seen for the healthy controls. In the control sera it was observed that IgG emerged in earlier fractions when greater IgG concentrations were present. However this phenomenon is not the explanation for the early emerging IgG in the rheumatoid group since IgG concentrations were similar in both groups (see Appendix I) and no correlation was seen in the rheumatoid group between commencement IgG and the total IgG concentration.

Aggregates of IgG would also elute in this early region. However, aggregates of IgG are unlikely to be the explanation for the early emerging IgG in view of the lack of correlation between commencement IgG and the total IgG (which might be expected if concentration dependent aggregation occurred). In addition aggregation of IgG is usually seen after heating or freezing of serum specimens (Hansson, 1968; Yokohari et al., 1973). In this study serum was, in general, studied in its fresh state.

What then is the nature of this high molecular sized IgG? Norberg (1974) has recently examined the sera of 33 sero positive patients with rheumatoid arthritis by sedimentation analysis and gel filtration chromatography on Sephadex G200 and Sepharose 4B. High molecular weight IgG was

detected in 23 of these sera (70%) and in some formed a continuum from the 19S to the 7S marker. These findings are remarkably similar to those described in this present chapter.

This investigator was also able to show that this complexed or high molecular weight IgG, as detected by gel filtration using neutral buffers, was not present when filtration was performed in buffers of low pH, suggesting that disocciation had occurred. This observation is strong evidence in favour of this early eluting IgG being in the form of an immune complex since disulphide bound polymers or aggregates would not dissociate in the presence of denaturants (see chapter four).

Similar experiments were performed in the present study but were technically unsuccessful and thus no definite evidence was obtained to show that this high molecular weight IgG was present in the form of an immune complex. However the significant correlation seen between the index used to calculate the quantity of this complexed IgG (i.e. monomer : dimer ratio) and the other assay used to detect immune complexes (viz, K cell cytotoxicity inhibition) is strongly suggestive that this high molecular weight IgG is present in the form of an immune complex. The definitive evidence for this will require the isolation of this complexed IgG and the demonstration that the $F(ab)_2$ portion of the IgG molecule has activity against the isolated antigen.

Early eluting IgG was seen in the sera of both sero-negative and sero-positive rheumatoid patients; in particular 4 sero-negative patients, out of eight studied, had high molecular weight IgG. There was no correlation between commencement IgG and the latex titre. In this respect these results differ from those reported by Norberg. This investigator studied seven

sero-negative patients and in all cases found a normal IgG elution profile. In addition he observed a higher frequency of complexed IgG when the rheumatoid factor titre was equal to or greater than 1 : 1280 compared to those less sero-positive (80% compared with 54% respectively).

In some patients in the present study IgG was observed to elute in the region of the IgM marker (103 mls, Figure 5,5). A 22S immune complex containing IgM rheumatoid factor and IgG has been previously described in the sera of patients with rheumatoid arthritis (Franklin et al., 1957). It is known that IgM rheumatoid factor has a weak association constant (Normansell, 1972) and such a complex might dissociate with dilution as occurs in gel filtration. If so this may be the explanation for this early eluting IgG (in the region of the IgM marker) and also explain the lack of correlation between IgG commencement and the monomer : dimer ratio. However IgG commencement did not correlate with the latex titre, but whether this titre correlates with the presence of a 22S complex is not known.

The monomer : dimer ratio obtained from the IgG profile was measured in an attempt to quantitate the complexed IgG. The reasoning for using this ratio as an index of the amount of complexed IgG has been discussed in the methods section. The monomer : dimer ratio for the rheumatoid group was significantly lower than that seen for the healthy controls and this strongly suggests the presence of small IgG containing complexes. In addition this ratio correlated significantly with the results of the second assay used to assess circulating complexes, viz, inhibition of K cell cytotoxicity and this result further supports the view that the monomer : dimer ratio was a measure of these small immune complexes.

The K cell cytotoxicity assay can be inhibited by a number of serum factors. MacLennan (1972) has shown that preformed soluble complexes in antigen antibody equilibrium or slight antigenic excess will optimally inhibit K cell mediated cytolysis of sensitized Chang target cells. Even after centrifugation of the complexes at 140,000 g for 90 minutes, more than half of the inhibition was retained by the supernatant suggesting that complexes of small size are inhibitory.

But other factors apart from immune complexes can inhibit K cell cytotoxicity. Hallberg (1974) has noted that small aggregates (9 to 10S) of IgG obtained by repeated freezing of IgG are potent inhibitors of K cell killing while MacLennan and others (1973) have shown that heat aggregated myeloma proteins also inhibit this system. Small aggregates of IgG occur during heating or freezing serum specimens (Hansson, 1968, and Yokohari et al., 1973) and thus care is required if such specimens are used in this assay.

In the present study the majority of sera examined were in the fresh state. In addition an inverse correlation was seen between the percentage inhibition of cytotoxicity and the serum IgG concentration. This result was again evidence against the suggestion that aggregates of IgG were the source of the inhibitory material for if so a positive correlation would be expected (if a IgG concentration dependent aggregation process had occurred).

Inhibition of K cell induced cytolysis also occurs due to anti-HLA antibody directed against tissue antigens on the effector cell (Hersey et al., 1973). When this form of inhibition occurs very little cytotoxicity may result. Five rheumatoid sera at 1:3 dilution maximally inhibited to 100%. It would

be of interest to know what degree of inhibition would occur with these sera using effector cells of different HLA type since if little inhibition was seen this would suggest that immune complexes were not the source of this form of inhibition.

Inhibition in this assay may also be due to non immunoglobulin factors. This is illustrated by the finding that different batches of foetal bovine serum which contains little or no immunoglobulin will permit somewhat different degrees of cytolysis.

Jewell and MacLennan (1973) have used this assay to demonstrate the presence of inhibitory material in the sera of patients with inflammatory bowel disease. The degree of inhibition correlated significantly with the severity of the disease, the height of the ESR and with the severity of the inflammation seen on sigmoidoscopy. The inhibitory material eluted between IgG and IgM on gel filtration and these investigators concluded that it was most probably in the form of IgG containing small immune complexes.

A preliminary study using this technique also indicated the presence of inhibitory material in rheumatoid sera (Barnett and MacLennan, 1972) and Hallberg (1972) using chicken erythrocytes as his target cells showed enhanced inhibition in 21 sero positive rheumatoid sera compared to 17 blood donor controls. In both studies no significant correlation occurred between inhibitory activity and the rheumatoid factor titre.

In the present study it was demonstrated that the sera from the patients with rheumatoid arthritis were significantly more inhibitory to K cell mediated cytotoxicity than the sera from the healthy controls. In two rheumatoid sera, which showed strong inhibition further experiments suggested that the inhibitory

activity was maximal in fractions a little larger than monomeric IgG. This result was consistent with the view that the inhibition was caused by small IgG containing immune complexes. This view was further strengthened by the significant correlation seen between percentage inhibition of K cell cytotoxicity and the monomer : dimer ratio obtained from the IgG filtration profile. This correlation also suggested that there was a direct relationship between the amount of the complexes and the degree of inhibition of cytotoxicity.

The sera from three patients with osteoarthrosis strongly inhibited K cell cytotoxicity. This was an unexpected finding and can not be explained. One of these patients had complexed IgG as detected by filtration chromatography and this therefore, suggested that the source of the inhibitory activity in this particular patient was complexed IgG. The other two patients had normal IgG elution profiles.

The third assay performed was that of polymorphonuclear leucocyte (PMN) chemotaxis. This was done in an attempt to see if there was any relationship between the known impairment of chemotaxis in RA and the presence of immune complexes in the serum.

A significant inverse correlation was observed between the chemotactic index of the circulating neutrophils and the amount of complexes as assessed by the monomer : dimer ratio and the degree of inhibition of K cell cytotoxicity. These results support the earlier suggestion of Mowat and Baum (1971) that the impairment in neutrophil chemotaxis in rheumatoid arthritis patients could be related to the prior ingestion of immune complexes.

Mowat and Baum (1971) showed that a significant correlation occurred between the chemotactic index and the serum C_3 level suggesting a relationship

between complement consumption and the impairment of chemotaxis. The results of the present study would therefore tend to suggest that the complement consumption may be related to the presence of these complexes. A future study into this aspect would be of interest. Mowat and Baum (1971) have been unable to show any other correlation between the clinical and laboratory features of rheumatoid arthritis and the chemotactic index.

In an attempt to assess further the clinical significance of these intermediate complexes the results of the assays for immune complexes were compared with several clinical and laboratory measurements. No significant correlation was observed between the amount of the complexes and with the clinical activity and duration of the disease. No laboratory measurement, including the sedimentation rate and the latex titre, correlated significantly with the assays for complexes. In many cases however, see Table 5, II, the direction of the correlation indicated that a meaningful relationship was apparent but not significant at the conventional level of $p < 0.05$.

It is now possible to briefly summarize the main findings of this study and answer the original aims. It was demonstrated that small IgG containing complexes could be detected in rheumatoid sera using the method of gel filtration chromatography and that these complexes were frequently present. The quantity of these complexes as estimated from the IgG monomer : dimer ratio in 35 rheumatoid sera correlated significantly with the degree of inhibition in vitro of K cell cytotoxicity and with the degree of inhibition in vitro of PMN chemotaxis. No other clinical or laboratory measurement correlated significantly with the quantity of these complexes.

I shall now review the relevant literature relating to these results.

Other investigators using different methods have detected immune complexes in the serum of patients with rheumatoid arthritis. Kunkel and others (1961) demonstrated intermediate IgG containing complexes in the sera of a small number of severely affected rheumatoid patients but made no mention of the incidence of such complexes in unselected patients.

Baumal and Broder (1968) detected a factor (rheumatoid biologically active factor) in 56% of their rheumatoid sera. This factor resembled a soluble immune complex and was detected by the capacity to release histamine from the perfused guinea pig lung. The presence of this serum factor was correlated with some variable of the disease process but it did not correlate with the intermediate complexes as observed by analytical ultracentrifugation or with IgM rheumatoid factor (Gorden et al., 1969). The molecular weight of this factor was 10^6 or greater and its presence was associated with anti complementary activity. This factor thus appears to be separate and additional to the intermediate complexes detected by analytical ultracentrifugation and gel filtration chromatography.

Winchester and Kunkel (1968) used monoclonal IgM rheumatoid factor to precipitate immune complexes in the sera of patients with rheumatoid arthritis and 57% of such sera gave a positive precipitation. However it has been recently suggested that IgM rheumatoid factor may not precipitate with very small complexes (Norberg - unpublished observations, Norberg (1974)).

Luthra and others (1975) have also used monoclonal rheumatoid factor in a radio immunoassay to detect circulating immune complexes in rheumatoid arthritis. They studied 51 patients and found complexes in 12 (26%). However their rheumatoid factor also appeared to be reacting with large molecular sized

material sedimenting between 20-30S. They were able to show a correlation between the presence of this material and the severity of the disease.

Thus it appears from the studies cited that the incidence of circulating complexes in rheumatoid arthritis is very much dependent on the nature of the assay used for their detection. It is most probable that different types of immune complexes will explain these different results. However with regard to the small intermediate complexes it is apparent from the present study and from the work of others (Norberg, 1974; Kunkel et al., 1961) that these complexes are seen in the sera of over half of the patients with rheumatoid arthritis.

The nature of these intermediate complexes, their aetiology and their relationship to the pathogenesis of rheumatoid arthritis is obscure. Kunkel and others (1961) demonstrated that these complexes would dissociate in conditions of low pH to yield monomeric IgG. Other investigators showed that IgG rheumatoid factor was a constituent of these complexes (Schroenloher, 1966; Capra et al., 1971) and it was postulated that the intermediate complexes might be composed of IgG rheumatoid factor, acting as an antibody, and conformationally altered autologous IgG, acting as the antigen. However little evidence has been produced to suggest that IgG obtained from the dissociation of these complexes differs in its conformation from normal IgG and in particular no other protein has ever been shown to be a part of this complex (Hay and Nineham, 1974). Pope and his coworkers have recently suggested that these intermediate complexes might represent complexes formed by the self association of IgG rheumatoid factor (Pope et al., 1975). Their conclusions were reached after studying immune complexes isolated from three patients with high levels of these complexes. They found that IgG rheumatoid factor

underwent a self association process to form a cyclic dimer; this complex being thermodynamically stable because each IgG molecule served as both antibody and antigen. They postulated that further concentration dependent polymerization of these dimers could occur to form complexes of increasing size. However it is not known if this self association of IgG rheumatoid factor is a general phenomenon and occurs in the formation of the immune complexes found in the rheumatoid synovia (Munthe and Natvig, 1971), synovial fluid and serum (Winchester et al., 1971).

From the evidence cited it appears that IgG rheumatoid factor is a constant feature of the intermediate complex. This factor occurs very commonly in the sera of adults and children with rheumatoid arthritis (Hay and Nineham, 1974). It also occurs in low concentrations in degenerative joint disease and in healthy adults (Abruzzo and Heimer, 1974). The presence of IgG rheumatoid factor is only poorly correlated with the presence of IgM rheumatoid factor (Heimer and Abruzzo, 1972) and it is consistently present in patients with sero-negative rheumatoid arthritis (Torrigiani et al., 1970).

IgG rheumatoid factor is produced by the plasma cells found in the hypertrophied rheumatoid synovial membrane that lines the joint space (Munthe and Natvig, 1970). Two theories are in vogue for its production (see Ziff, 1974). The first theory postulates that chronic immunization occurs to autologous IgG that is conformationally altered, as may occur in the formation of an immune complex (Givol et al., 1974). The second theory suggests that T lymphocytes are stimulated by exogenous antigens and this leads to the release of enhancing factors which participate in the stimulation of non-tolerant B cells to synthesize IgG anti-IgG.

Apart from being present in the circulation IgG/IgG rheumatoid factor complexes also occur in the synovial fluid (Winchester et al., 1970), and there is now strong evidence that these complexes play an important role in the pathogenesis of the synovial inflammatory response (see Zvaifler, 1973; Ziff, 1974 for review). In the synovial fluid these complexes are complement fixing, have high degrees of lattice formation (Winchester et al., 1970) and are phagocytosed by the synovial PMN and macrophages (Hurd et al., 1970; Kinsella et al., 1970). During this phagocytosis, it is postulated that lysosomal proteolytic enzymes are released which cause local inflammation and activate effector mechanisms such as the complement system. The subsequent release of chemotactic and inflammatory mediators attracts further circulating PMN into the synovial space.

It is possible that the origin of the intermediate complexes, seen in the circulation of patients with rheumatoid arthritis, is from the affected joints. The mechanism of production could be as follows - IgG rheumatoid factor is locally synthesized and secreted into the joint fluid with perhaps a little being released systemically. In the synovial fluid this factor complexes with autologous IgG as mentioned above, initiates and activates several biological effector mechanisms, and free complex flows into the circulation via the draining lymphatic channels which are known to be non selective in terms of molecular size (Brown et al., 1969). Complexes with small degrees of lattice persist whereas the larger complexes are rapidly taken up by the reticulo endothelial system. Thus from this hypothesis one might expect that these complexes would be present in relative high concentrations in the draining lymph and with greater degrees of lattice formation than is seen in the circulation. Such a

suggestion could be easily confirmed or refuted.

Once in the circulation the question arises as to whether these small complexes have any clinical significance. It was demonstrated in this study that these complexes can inhibit K cell cytotoxicity. This inhibition is thought to proceed through the complex attaching to the Fc receptor on the K cell and preventing access of this cell to the sensitized target cell. Do the complexes attach to other Fc receptors and block their function in vivo? The answer to this question at present can only be speculative. Lymphocytes of the B and K variety (Basten et al., 1972; and MacLennan, 1972), macrophages (Huber et al., 1968) and neutrophils (Henson, 1969) all have Fc receptors on their surface. K. cell function is blocked by small third party immune complexes in vitro (MacLennan, 1972) but as yet the K cell has no defined function in vivo. Fixed macrophage function in the mouse can be blocked in vivo by high concentrations of circulating immune complexes (Haakenstad and Mannik, 1974). Do the intermediate complexes seen in rheumatoid arthritis do likewise?

In this study it was shown that impairment of PMN chemotaxis was associated with the presence of complexes. This result suggested that these complexes might play a causative role in this phenomenon. Is it possible that the immune complexes exert their effect through the PMN Fc receptor? Do these complexes impair PMN phagocytosis and bactericidal activity as well? Are these mechanisms the explanation for the susceptibility of rheumatoid patients to bacterial infections (Uddin et al., 1970). Preliminary experiments by Gotch (personal communication) suggest that these intermediate immune complexes do not impair PMN killing of bacteria in vitro and this result is consistent with the conclusions of Brandt and Hedberg (1969) who reported that

phagocytosis by PMN is normal in rheumatoid arthritis. However other types of immune complexes can interfere with PMN phagocytosis and bactericidal activity (Gotch, personal communication). This aspect needs to be further explored and may provide information concerning the varying biological effects that different immune complexes have.

The intermediate complexes have also been implicated in other rheumatoid manifestations. Thus Jasin et al. (1970) and other investigators have observed the hyperviscosity syndrome in rheumatoid patients who had large amounts of these complexes. The symptoms and signs of this syndrome were markedly improved when these complexes were removed by plasmaphoresis, suggesting an aetiological role. In general, the patients described with this complication usually had a severe arthritis with multiple joint involvement and high IgG rheumatoid factor titre. In addition, Pope et al. (1975) noted parenchymal liver and lung damage in his rheumatoid patients with the hyperviscosity syndrome. Pope and his colleagues suggested that the constant presence of large amounts of circulating complexes may have a role in the causation of these tissue lesions.

Theofilopoulos and others (1974) have presented experimental evidence suggesting that intermediate complexes (and possibly incorporating low molecular weight IgM rheumatoid factor) may be involved in the pathogenesis of rheumatoid vasculitis. These workers also noted the close agreement between the presence of IgG rheumatoid factor and an accompanying vasculitis. Deposited complement, IgG and IgG rheumatoid factor have also been detected in the wall of the vasa nervosum of the sural nerve in rheumatoid patients with

a vasculitis. Both these studies, therefore, suggest that immune complexes may be involved in the rheumatoid vasculitis.

Thus direct and indirect evidence is now beginning to accumulate suggesting that the circulating complexes found in rheumatoid arthritis are not innocent byproducts but are active participants in the pathogenesis of the disease. The delineation of the effects of these small complexes and their relationship to the pathogenesis of the rheumatoid state will be an exciting avenue for future research and may provide additional information concerning the nature and mechanisms of other immune complex disorders.

CONCLUSION

Evidence has been presented suggesting that circulating immune complexes occur in over half of the sera of patients with rheumatoid arthritis. These IgG containing complexes were small, eluting between IgG and IgM on gel filtration on Sepharose 6B and were not seen in the sera of healthy control subjects. These complexes were detected in the sera of both sero-negative and sero-positive patients and their quantity did not correlate with IgM rheumatoid factor titre. A monomer to complexed IgG ratio was used for quantitation of the complexes. This ratio was derived from the IgG profile obtained by gel filtration of the serum. The quantity of complexes correlated significantly with inhibition obtained by the rheumatoid sera of cytolysis *in vitro* of IgG sensitised target cells by K cells from human peripheral blood. A significant inverse correlation was observed between the quantity of serum

complexes and the chemotactic index of the circulating polymorphonuclear leucocytes obtained from the same rheumatoid patient. This suggests that these complexes may be implicated in the suppression of polymorphonuclear leucocyte chemotaxis observed in patients with rheumatoid arthritis.

There was no correlation between the quantity of the complexes in the sera and other clinical, haematological and biochemical measurements. The presence of complexes in the sera of patients with other joint diseases has also been assessed.

CHAPTER SIX

DETECTION OF CIRCULATING IMMUNE COMPLEXES IN NON RHEUMATOID DISEASES

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CHAPTER SIX

DETECTION OF CIRCULATING IMMUNE COMPLEXES IN NON RHEUMATOID DISEASES

INTRODUCTION

The aim of the present chapter has been to look for circulating immune complexes in the serum of patients with acute myeloid leukaemia, cutaneous vasculitis, multiple sclerosis, and a small number of miscellaneous diseases. The two principal methods used for this task were gel filtration chromatography and inhibition of K cell mediated cytotoxicity.

Acute myeloid leukaemia is a neoplastic disease of the bone marrow and is characterized by an excessive production of cells of the myeloid element. Typically a gross increase in the number of circulating leucocytes occurs and many of these cells show primitive morphological features. Less common forms of this condition are recognized where only a few or no abnormal cells are seen in the blood and the marrow shows a hypoplastic state associated with bizarre anomalies of the myeloid cells. Currently a large amount of research is being performed to increase our knowledge concerning the immunological features of this disease. There is some evidence that the primitive myeloid cells contain leukaemia associated, or even specific, antigen(s) on their cell surface (Harris, 1973). If this is so the question arises as to whether the body is intrinsically tolerant to these neoantigens or whether the immunological system, which would normally recognize, and perhaps reject, these foreign antigens, has been blocked. If the concept of an immunological

'reflex arc' is used for explanatory purposes such blockage could occur at an efferent, central, or afferent level. Immune complexes are known to be able to block immunological mechanisms especially at the efferent level (Currie, 1974). The identification of circulating immune complexes in acute myeloid leukaemia has therefore been considered an important and worthy objective.

Cutaneous vasculitis is a relatively common condition of poorly understood aetiology. The prominent clinical feature is an episodic attack of purpura, commonly occurring on the lower extremities, and in some patients associated with an urticarial-like reaction. Histologically the appearance is a vasculitis and polymorphonuclear leucocytes are seen infiltrating the walls of the affected vessels. In some cases, though, cells of the lymphoid series may dominate. Both the clinical and histological features of this condition vary considerably and this is reflected in the large number of alternative names available for this disease (see Ryan, 1974 for review). Many patients with vasculitis will however, have characteristic clinical and laboratory findings which are similar to other disorders associated with circulating immune complexes and in some of these patients soluble complexes have been detected (Cream and Turk, 1971). The nature and significance of these complexes is but poorly understood and additional information is urgently required.

Multiple sclerosis is the commonest chronic neurological disorder. Clinically the outstanding features are its multiple sites of central nervous system involvement and its relapsing and remitting course (see MacAlpine et al., 1972 for review). Many immunological abnormalities have been described in this disease including the production of IgG in the central nervous system (Tourtellotte, 1970) and the suppression of some aspects of

lymphocyte function (Arnason and Chelmicka-Szore, 1975). To date there are no reports in the literature concerning attempts to detect immune complexes in the circulation of these patients. Thus the serum from a number of patients with this condition, and from a neurological control group, has been examined for immune complexes and, in addition, the cerebrospinal fluid has also been examined both for central nervous system IgG production (see Chapter 8) and for biologically active immune complexes.

During these studies the serum from other patients has also been examined. These patients generally had disorders of a suspected immune complex nature of the type discussed in Chapter four.

PATIENTS AND METHODS

Patients and Controls

Four groups of patients were studied. The first group comprised 19 patients with newly diagnosed acute myeloid leukaemia. This diagnosis was made if abnormal myeloblasts were present in the bone marrow. Some patients had large numbers of primitive myeloid cells in the circulation whereas others showed a depressed white cell count with a hypoplastic marrow. All patients were entered into the Medical Research Council's 6th acute myeloid leukaemia trial. Further clinical and laboratory features of these patients will not be described as they are not of direct relevance to this present study. Defibrinated blood was obtained from these patients at presentation and sent by urgent postal delivery to the laboratory of Dr. I.C.M. MacLennan for immunological studies. If the blood was older than 24 hours it was discarded.

Once the blood was received, the serum was separated and immediately studied by gel filtration chromatography.

The second group consisted of 11 patients with cutaneous vasculitis. I am grateful to Dr. T. Ryan for introducing me to the subject of cutaneous vasculitis and allowing me to study these patients. The age, sex and clinical features of these patients are listed in Table 6,1. Activity in this table refers to the state of the purpuric rash on the day when blood was aspirated. Blood was collected into a warmed syringe and divided into two aliquots, one in EDTA for complement studies and another for column chromatography and cryoglobulin studies.

A third group consisted of 49 patients with neurological disorders. Twenty seven of these patients were suffering from multiple sclerosis (MS) while the remaining patients, who served as a neurological control group, had a variety of neurological diseases. These patients formed part of a larger series of patients which will be described in more detail in chapter eight. The neurological diagnosis was made in each case by the attending neurologist and no patient was included in whom the diagnosis was uncertain. There were 15 females and 12 males with MS. Their average age was 36 years (range 21 - 55 years), and the average duration of their disease was 3.4 years. This group was further divided into those patients with definite MS, or those with probable or possible MS according to the criteria of MacAlpine et al. 1972. Ten females and 12 males formed the neurological control group. Their average age was 37.5 years and their diagnosis included four patients with cerebro-vascular disease, three patients with psychiatric disorders, three patients with structural disorders of the spine, and two patients with dementia. The

remaining patients suffered from a variety of unrelated disorders.

Cerebrospinal fluid was also obtained from all these patients at the same time as the blood specimen. After cell counting and separation the cerebrospinal fluid supernatant was stored at -20°C until further examination.

A fourth small group consisted of patients having a variety of disorders. Their diagnoses were made according to conventional criteria and are listed in Table 6,II.

Healthy laboratory subjects (see chapter five) served as controls and their serum was studied in the same fashion as for the other groups.

Gel filtration chromatography

Serum from patients with acute myeloid leukaemia was fractionated on Sepharose 6B columns using exactly the same techniques and conditions as described in chapter five. Commencement IgG(c) and monomer : dimer ratios were calculated from the IgG profiles as previously described.

A preliminary experiment had shown large molecular sized IgG in the serum of a single patient with florid vasculitis. Thus in an attempt to simplify the column chromatographic procedure for the detection of complexed IgG, different measurements were used for the vasculitic group. Serum was fractionated on a single Sepharose 6B column (bed dimensions, 43 x 2.5 cms) as before and the IgG concentration at the peak position of the IgG profile (i.e. 'b' Fig. 5,5) was measured by radial immunodiffusion. In addition the 6 mls of eluate between the elution volumes 118 mls to 124 mls (see Fig. 5,5) was pooled and 5 mls of this was concentrated 25 x s using an Amicon Minicon 3 concentration unit. The IgG of this concentrated specimen was

then measured. Thus the aim using this different procedure was to both simplify, and increase the sensitivity of the method in detecting early eluting IgG. The IgG levels were measured in duplicate for all specimens and the results expressed as the mean of the two readings.

Inhibition of K cell mediated cytotoxicity

The K cell cytotoxicity assay described in the previous chapter requires a considerable amount of peripheral blood in order to prepare mononuclear effector cells. However Gale and MacLennan (1975) have shown that whole blood can be used in place of purified mononuclear cells without altering significantly the kinetics of the cytotoxic reaction and with little change in the specificity or sensitivity of killing. Moreover, Gale, Roberts-Thomson and MacLennan (unpublished) have also shown that preformed immune complexes of tetanus toxoid/antitoxoid or aggregated IgG inhibits this whole blood cytotoxicity reaction in a similar fashion and to a similar degree as found using purified effector cells. Thus the K cell cytotoxicity assay was modified in the present chapter by using whole blood as the source of the effector cells. This was done in an attempt to make the assay simpler and less time consuming when screening a large number of serum specimens for circulating immune complexes.

The procedure was as follows. The effector cells were prepared by diluting defibrinated whole blood from a healthy subject 1 : 3 with M.E.M.10 (see chapter five). Chromium labelled sensitized Chang cells were prepared as previously described and made up to a concentration of 2×10^4/ml in M.E.M.10. Serum was used neat or diluted 1 : 5 with 0.9% saline. All

cultures were done in triplicate. The following were added to 3" x $\frac{1}{2}$" sterile tubes. 1) 500 μl of sensitized Chang cell suspension. 2) 300 μl of whole blood dilution. 3) 200 μl of test serum or serum dilution. The tubes were then capped and incubated for 20 hours at 37°C. The cytotoxic reaction was stopped by centrifugation for 10 mins at 200 g and at 4°C, and 500 μl of supernatant removed from the culture and placed in a supernatant tube. The radioactivity of the residue and supernatant was counted and the mean percentage chromium release calculated in the standard fashion as described in chapter five.

In every experiment the following standards were included. a) 500 μl Chang cell suspension and 500 μl M.E.M.10. b) 500 μl Chang cell suspension, 300 μl of whole blood dilution and 200 μl FBS. c) 500 μl Chang cell suspension, 300 μl of whole blood dilution and 200 μl of aggregated IgG (5 mg/ml); this culture being used as a positive control. Percentage inhibition of cytotoxicity was calculated using the formula X - Y / X - Z where X is the mean percentage chromium release for the standard culture 'b'; Y is the release for the test serum and Z is the background release for the Chang cell suspension alone (standard 'a').

Detection of cryoglobulins

The detection of cryoglobulins was limited to the vasculitic group of patients. Venous blood was aspirated into a warmed syringe and then delivered into a warmed universal container. This was then placed in a 37°C water bath (or incubator if a water bath was not available) and the blood allowed to clot. Serum was collected by centrifugation and divided into two aliquots; 0.5 mls

being fractionated at 4°C by gel filtration chromatography and the remainder allowed to stand at 4°C in a narrow bore test tube. This serum was then inspected daily for four days for the appearance of cloudiness or a precipitate. If either of these appeared the serum was then heated to 37°C to see if this would disappear and thus confirm the presence of a cryoglobulin. The serum was then returned to 4°C and three days later it was centrifuged at 1000 g for 30 minutes and the precipitate collected.

This was washed 4 x's in cold PBS, redissolved in 1 ml of 0.9% saline, warmed to 37°C, and the optical density at 215 and 225 mμ measured. The concentration of the cryoglobulin was then calculated as described in chapter seven and the results expressed as the amount of cryoglobulin precipitate formed from a unit volume of serum. The cryoglobulin was further examined for the presence of immunoglobulin and C_3 by immunodiffusion in agar. Immunoelectrophoresis was used to type the light chain subclass of any immunoglobulin and SDSPAGE was used in an attempt to detect any other proteins in the cryoglobulin. The methods used for these three tests were as described previously. Rheumatoid factor activity was detected by allowing the serum of cryoglobulin to diffuse in agar at 37°C against a sample of heat aggregated IgG prepared as described in chapter five.

Complement studies

These were kindly performed by Mr. J. Clarke. The concentration of the third component of complement (C_3) was measured by radial immunodiffusion and the results compared to the normal limits ascertained in the laboratory for a number of healthy individuals. Aliquots of a serum specimen obtained from

a healthy individual and stored in liquid nitrogen were used as standards in these experiments. The method of crossed electrophoresis in EDTA buffered agarose using a monospecific antiserum to C_3 was used to detect any C_3 conversion products. During conversion of C_3, due to in vivo activation a faster migrating β_{1A} component occurs and this can be detected by crossed electrophoresis as a forward shelf to the main β_{1C} peak. However as a similar appearance is seen in old or poorly stored serum, care in the interpretation of this test is required.

RESULTS

Patients with acute myeloid leukaemia

Gel filtration chromatography: The optical density elution profiles obtained for the sera of these patients were of normal appearance. The IgG concentration was measured in each alternate 3 ml collected fraction and the commencement IgG position (IgG (c)) and the monomer : dimer ratio determined. The results are plotted in Fig. 6,1, and Fig. 6,2. The same measurements obtained from the rheumatoid patients (chapter five) are included for comparative purposes. Two leukaemic patients had IgG eluting in earlier fractions than seen for any of the healthy controls. However there was no significant difference between the control group and the patient group ($p = 0.6$, using the Wilcoxon sum of ranks statistical test). The monomer : dimer ratio for the acute myeloid leukaemia group showed a wider scatter than the normal controls but again no significant difference was apparent between these groups.

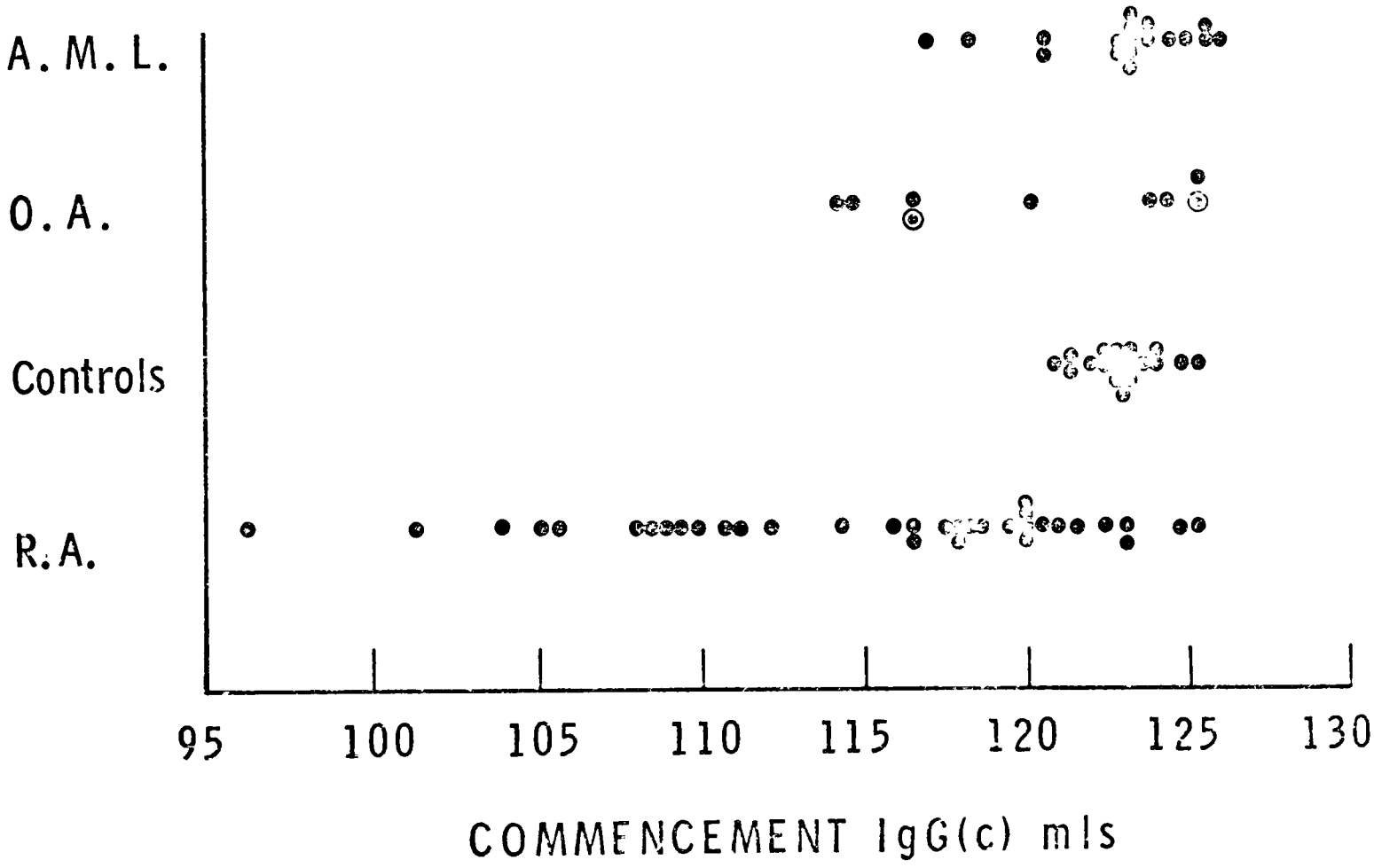


Figure 6,1 IgG(c) is the position at which IgG commences to elute. The values obtained for the patients with acute myeloid leukaemia (AML) are shown. In addiion the values obtained for patients with rheumatoid arthritis (RA), other joint diseases (OA) and healthy controls are shown for comparison.

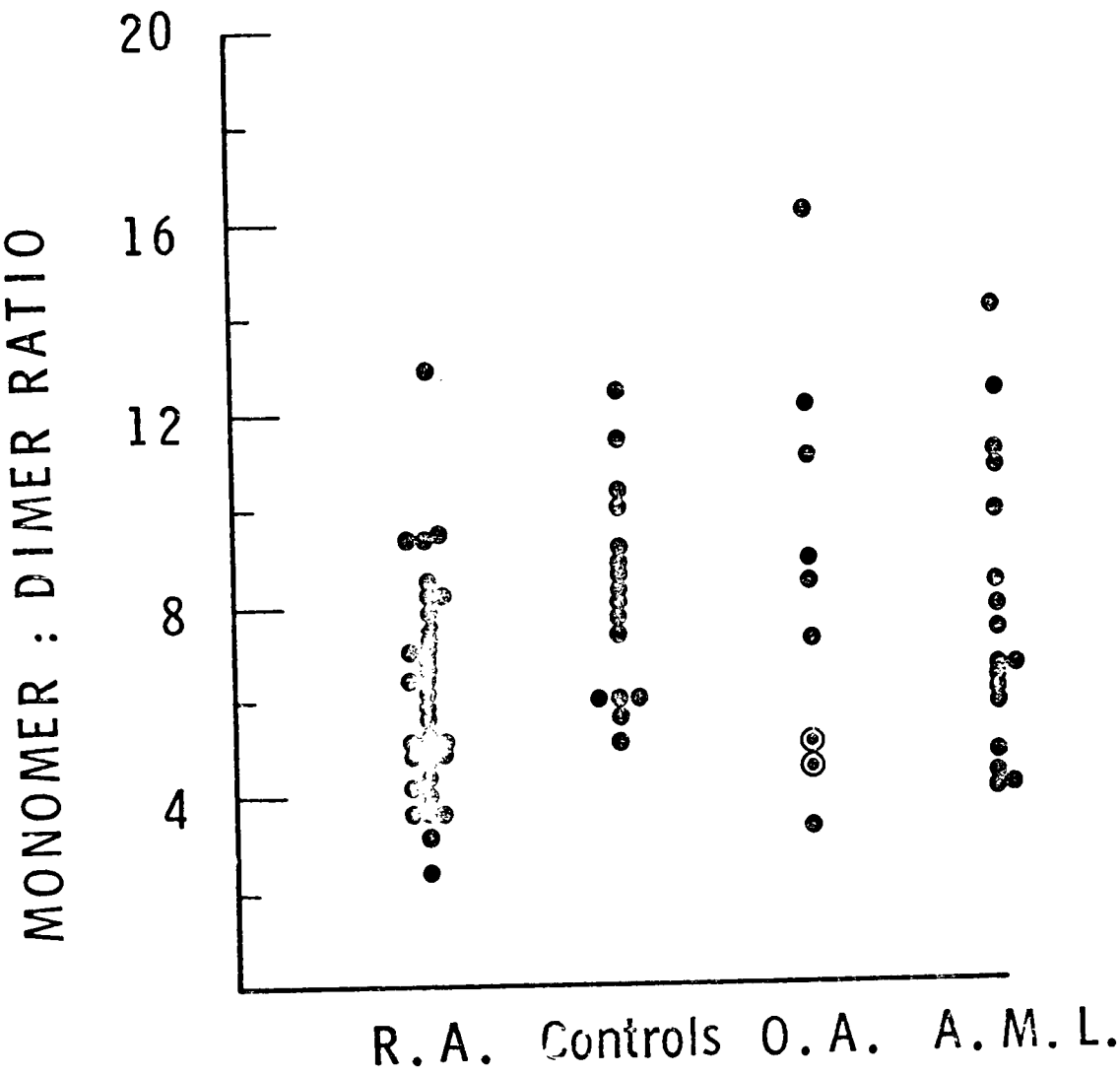


Figure 6.2 Monomer : dimer ratios obtained for the patients with myeloid leukaemia (AML). The values obtained for patients with rheumatoid arthritis (RA), other arthropathies (OA), and healthy controls, have been included for comparison.

Patients with cutaneous vasculitis

Gel filtration chromatography: The optical density elution profile was normal for nine of the patients whereas the remaining two had elevations of the main peak. Both these patients (Ra. and Ni., Table 6,1) had increased levels of IgG in their serum. The concentration of IgG measured at the peak position of the IgG profile ('b', Fig. 5,5) and in the early concentrated eluate (see methods) was measured and the result for each patient is listed in Table 6,1.

In chapter five it was shown that the position at which IgG commences to elute, i.e. IgG(c), was dependent on the serum IgG concentration. Thus it would be expected that a greater amount of IgG would be found in the early concentrated eluate fraction if a high total concentration of IgG was present, i.e. if 'b' was large. In an attempt to correct for this phenomenon a ratio was derived where the IgG concentration at the peak position of the IgG profile was divided by the IgG concentration measured in the 25 x's concentrated fraction. The ratios so obtained for the vasculitic patients and for six healthy controls are plotted in Fig. 6,3 and listed in Table 6,1. It is seen that six out of eleven vasculitic patients had low ratios when compared to the controls, but there was no significant difference between these two groups $0.3 > p > 0.2$. Since this ratio was low in patients who had high, low, or normal levels of IgG it is suggested that this ratio can be considered as an index of the presence of early emerging complexed IgG.

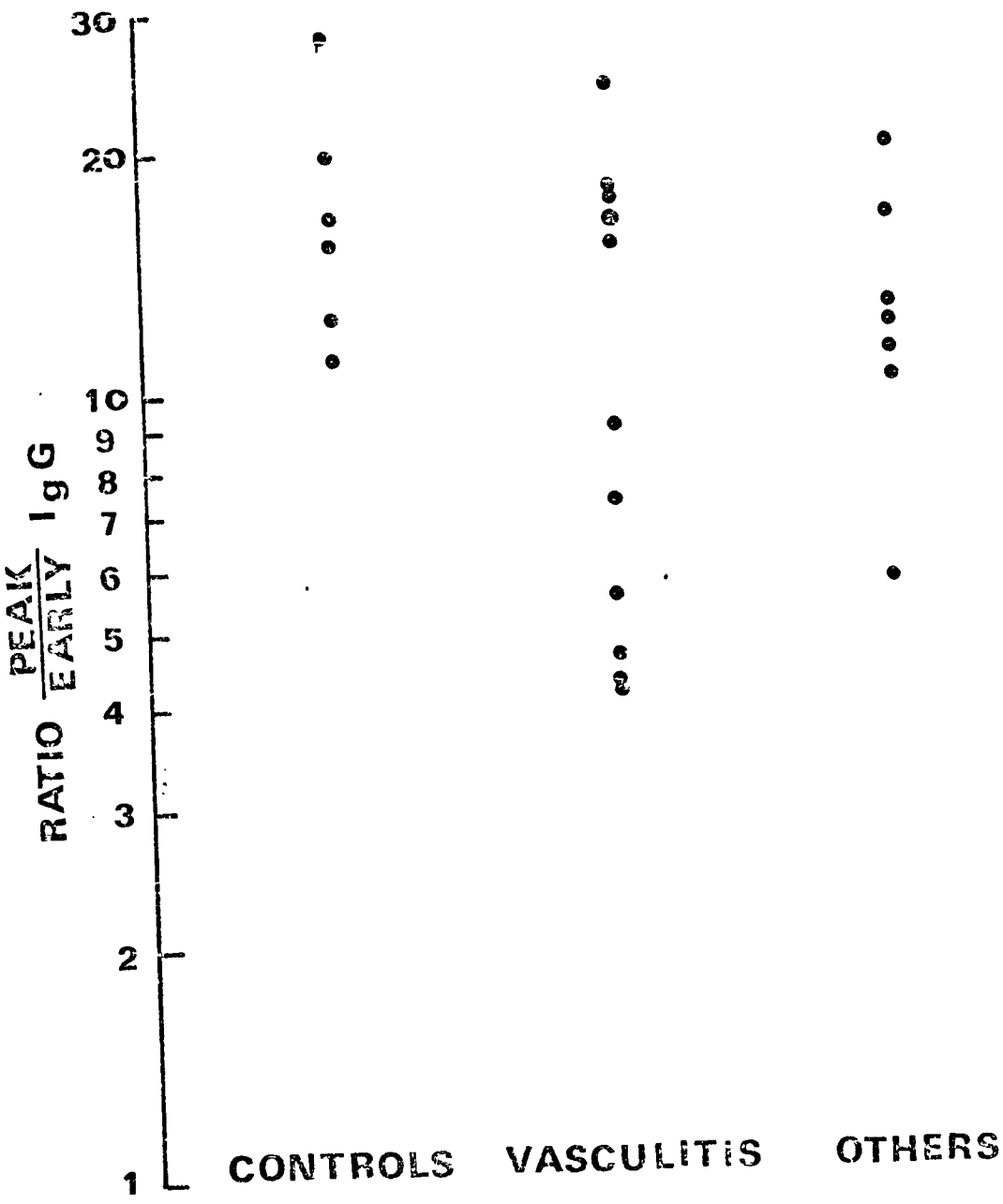


Figure 6.3 The peak : early IgG ratio obtained after chromatography of serum on Sepharose 6B columns (see methods). The values obtained for 6 healthy controls, 11 patients with cutaneous vasculitis and 7 patients with other diseases are shown.

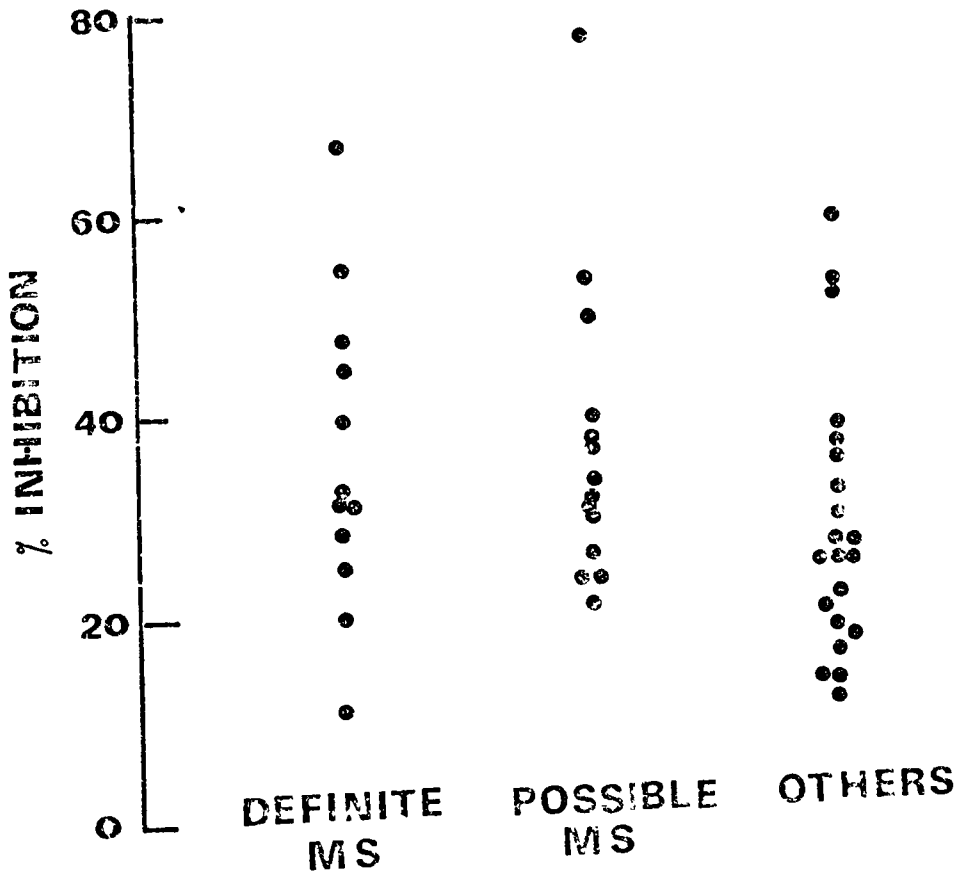


Figure 6.4 The % inhibition of cytotoxicity obtained by the serum from patients with multiple sclerosis or other neurological disorders. There was no significant difference between the groups.

Patient	Sex	Age	Clinical features	Activity	Rheumatoid factor
Pa.	F	65	Bronchiectasis 4 years lower limb purpura 1 yr arthralgia and polyneuropathy	Active	+
Se.	F	58	Purpura legs 1 year	Active	+
Ga.	F	27	Purpura lower legs 4 yrs	Active	-
Ra.	F	58	Purpura legs 1 year	Inactive	+
Ni.	M	14	Purpura ankles 1 month arthralgia, weight loss liver granuloma	Active	-
Ki.	M	55	Rheumatoid arthritis 17 years purpura legs 1 year	Active	+
Wi.	F	60	Widespread urticarial leucocytoclastic vasculitis for 15 years	Active	-
As.	F	25	Purpura legs	Active	-
Ho.	F	22	Henoch-Schonlein purpura 2 months	Inactive	-
Cu.	M	50	Purpura legs for 10 years	Active	-
Br.	M	69	Rheumatoid arthritis vasculitis/ulceration legs benign IgA gammapathy	Active	-

Table 6.1 Clinical and laboratory features of 11 patients with cutaneous vasculitis

Cryoglobulins (mg%)	C_3 and C_3 Conversion	% inhibition of cytotoxicity	Column IgG profile (i.u./ml) peak	early	peak:early ratio
40. IgA & IgM (K & λ) + rheumatoid factor	NAD	77.7	1.28	0.14	9.1
150. IgG (K)	NAD	40.6	1.09	0.23	4.7
IgA (K & λ) 2.8 IgA, IgG & IgM (K & λ)	NAD	17.9	1.58	0.28	5.6
-	NAD	6.6	4.2	0.27	15.5
-	C_3 elevated no conversion	67.0	3.08	0.7	4.4
20. IgG & IgM (K & λ)	ND	71.3	1.78	0.24	* 7.4
8.5 IgG & IgA (K & λ)	NAD	55.7	2.02	0.12	16.8
-	NAD	49.8	2.02	0.11	17.7
-	ND	39.0	2.2	0.13	17.5
-	ND	32.0	1.46	0.06	24.3
-	ND	27.9	0.9	0.21	4.3

NAD no abnormalities detected

ND not done

\+ detected

\- negative

* serum supernatant

Inhibition of K cell cytotoxicity: All sera in this group were tested for inhibition in a single experiment together with the sera from seven healthy controls. The percentage inhibition obtained for the patients' neat serum are listed in Table 6,1. No control serum inhibited to more than 40% (M $\pm$ 1 SD was 25.1% $\pm$ 15.5%) and little or no inhibition was seen for test sera diluted 1 : 5. The comparison between the values obtained from gel filtration and the percentage inhibition of cytotoxicity is shown in Table 6,1. Some patients (e.g. Pa., Ni. and Ki.) both strongly inhibited and had complexed IgG as assessed by the peak / early IgG ratio; others (e.g. As., Ra., Ho., Cu. and Wi.) had normal values for both, while two patients (Br. and Ga.) had normal levels of inhibition but depressed ratios.

Cryoglobulins: In two patients cryoprecipitates were seen after 24 hours standing at 4°C, in a third within two days whilst in a fourth (Se.) a cloudly appearance was apparent within a few hours. All disappeared on warming and returned after further standing in the cold. The cloudiness settled after centrifugation to form a precipitate with a clear serum supernatant. The concentration and contents of the cryoprecipitates are indicated in Table 6,1 together with the light chain subclass of the immunoglobulin. Rheumatoid activity was detected in one cryoprecipitate. SDSPAGE revealed traces of other serum proteins in all cryoprecipitates and moderate amounts of albumin in two. This was despite four washings. In the cryoprecipitate of Ki. a dense band was seen migrating midway between IgM and IgG. This band had the same electrophoretic mobility as fibrinogen but further identification was not attempted. In the cryoprecipitate of Ga. two minor bands were seen migrating between IgG and albumin and a further additional band was seen migrating in

front of albumin. The identity of these bands was not determined. In the cryoprecipitate of patient Se. an unidentified band, migrating a little slower than IgA, was observed.

Complement studies: The results of the complement studies are listed in Table 6,I. C_3 was elevated in only one patient (Ni.) and no serum had demonstrable C_3 conversion.

Patients with multiple sclerosis and other neurological disorders

Inhibition of K cell cytotoxicity: In Fig. 6,4 is listed the percentage inhibition found for the neat serum of patients belonging to this group. The serum from two patients with MS inhibited K cell cytotoxicity more than any of the neurological control sera. The more inhibitory serum specimen was further investigated. Inhibition to a similar degree was found using K effector cells from three donors having different HLA phenotypes. This indicated that the inhibition was not due to anti-HLA activity. However, the IgG profile obtained after gel filtration of this specimen was of normal appearance and in particular a normal peak / early IgG ratio was obtained (Table 6,II). This suggests that the inhibition was not due to small complexes of IgG.

In addition to the main studies the CSF from each patient was tested for inhibition. In a simple large experiment, 200 μl of CSF was added to each culture instead of 200 μl of serum. For all CSF specimens the degree of cytotoxicity was similar to that obtained in the control cultures which contained 200 μl of 10% F.B.S. This indicated that no CSF specimen inhibited K cell cytotoxicity to any significant degree.

PATIENTS

Name	Sex	Age	Diagnosis	Column IgG profile (i.u./ml) peak	early	peak:early ratio
Yo.	F	33	Multiple sclerosis	2.2	0.17	13.0
Hi.	F	?	Untreated coeliac	2.22	0.13	16.8
Cl.	M	34	Nephritis	2.06	0.10	20.6
We.	F	?	Pre-eclampsia	0.93	0.16	5.8
Wa.	F	35	Nephritis	1.58	0.13	12.1
Ho.	M	38	Untreated coeliac	1.92	0.17	11.3
Ba.	M	54	Untreated coeliac	1.56	0.15	10.4

CONTROLS

Ba.	F	26	-	1.25	0.1	12.5
Ro.	M	28	-	1.34	0.12	11.1
Wa.	F	25	-	1.68	0.06	28.0
Hg.	F	36	-	1.66	0.1	16.8
Ho.	F	62	-	1.52	0.1	15.2
No.	M	24	-	1.5	0.075	20.0

Table 6.11 IgG concentrations seen at the peak IgG position and at an early elution position (see methods) for patients with other diseases and for healthy controls.

Patients with other diseases

Gel filtration chromotography: The peak / early IgG ratio obtained for these seven patients is shown in Table 6,II together with the diagnosis of the respective patient. The values obtained for six healthy controls are also included in this table and the figures for both groups have been plotted in Fig. 6,3. One patient was observed to have a low ratio when compared to the controls. This patient was suffering from severe pre eclampsia of pregnancy. Further clinical details were not obtained.

Complement studies: No patient had any detectable abnormalities of C_3.

DISCUSSION

Serum specimens from groups of patients having acute myeloid leukaemia, MS or cutaneous vasculitis have been examined for circulating immune complexes using methods similar to those described in chapter five. Apart from a small number of patients with cutaneous vasculitis, little evidence was found in favour of complexes being present in these disorders. These findings can be contrasted to those described in chapter five where it was shown that the majority of patients with rheumatoid arthritis had small immune complexes present in the circulation.

There is little available data in the literature concerning the detection of circulating immune complexes in acute myeloid leukaemia. indirect evidence for soluble complexes in this disease has been provided by Sutherland and others (1974) who noted IgG and C_3 deposited in 14.3% of 83 kidneys

from patients with acute leukaemia studied at post mortem. No light microscopy abnormalities were seen in any of these kidneys. However 11% of 33 kidneys from patients without leukaemia also gave positive immunofluorescence. Thus the significance of these findings is obscure. Immune complex nephritis is a common finding in AKR mice with spontaneous leukaemia (Oldstone et al., 1972) but others consider that immune complex nephritis is ubiquitous in normal laboratory mice (Markham et al., 1973).

Anderson, Gale and MacLennan (personal communication) have also tried to detect circulating immune complexes in acute myeloid leukaemia using the K cell cytotoxicity assay. However no inhibition of cytotoxicity was seen which could be attributed to serum immune complexes. Thus there is little evidence contrary to the findings presented in this chapter suggesting the absence of circulating IgG containing immune complexes in newly diagnosed cases of acute myeloid leukaemia.

The relationship between cryoglobulins, immune complexes and cutaneous vasculitis is controversial (Cream, 1974). Cryoglobulins are serum proteins or protein complexes which precipitate out on cooling below 37°C and redissolve on warming. They were first described by Wintrobe and Buell (1933) and the term cryoglobulin was coined by Lerner and Watson (1947). Single component cryoglobulins (containing a monoclonal immunoglobulin) are generally seen in multiple myeloma or Waldenström's macroglobulinaemia while mixed cryoglobulins, containing two or more types of immunoglobulin, have been detected in a large number of diseases. Most of these have clinical and laboratory stigmata suggestive of an immune complex pathogenesis. The nature of cryoglobulins and the diseases in which they occur is well reviewed

by Barnett, 1970. Mixed cryoglobulins at 4°C generally consist of an antiglobulin directed against an autologous immunoglobulin (usually IgG) and they are often complement fixing. But it is not presently known if other antigens are a feature of the immune complex or whether cryoglobulins are still complexed in vivo. The relationship between cryoglobulins and the pathogenesis of the disease in which they occur is poorly understood.

Some mixed cryoglobulins can cause inflammatory reactions when injected into the skin of animals (McIntosh et al., 1971) and man (Whitsed and Penny, 1971). But cryoglobulinaemia may be found without any associated vasculitis (Cream, 1974). Thus it is likely that additional factors are involved in the pathogenesis of vasculitis associated with the presence of a cryoglobulin. Cryoglobulinaemia in vasculitis could well be a secondary phenomena (Cream, 1974). This is suggested by the following facts. 1) The concentration of cryoglobulins tends to correlate with the duration of the vasculitis (Cream, 1972). 2) Antiglobulins are usually a component of cryoglobulins and no other antigens apart from C_3, fibrinogen and IgG have been consistently detected. Therefore cryoglobulins might be regarded as a marker of an immunological disorder perhaps involving circulating immune complexes (Kunkel, 1971).

In a similar vein small 'intermediate' complexes consisting of IgG and IgG rheumatoid factor, but without showing cryoprecipitation, can be associated with a vasculitis (Kyle et al., 1971; Capra et al., 1971). However similar complexes, as found in rheumatoid arthritis (see chapter five), are not necessarily associated with a vasculitis. But as discussed in chapter four, small complexes may reflect the transient presence of larger more harmful

complexes and thus in this study the aim was to find if small complexes could be detected in the serum of patients with vasculitis and if so whether their presence was related to the presence of cryoglobulins or to the inhibition of K cell cytotoxicity in vitro.

A low chromatographic peak / early IgG ratio was found in six out of eleven sera from patients with vasculitis. This low ratio was considered to reflect the presence of complexed IgG since there was no relation between early IgG and the total concentration of IgG and furthermore it was considered that spontaneous aggregation or polymerization of IgG was unlikely to have occurred in fresh sera. But could such aggregation of IgG occur in vivo in the circulation of patients with vasculitis? No evidence in this present study can be presented to refute this suggestion. However, Capra et al. (1971) have shown that these small IgG containing complexes seen in some cases of vasculitis can be dissociated at low pH to give monomeric IgG, some with rheumatoid factor activity. This strongly suggests that the nature of this complexed igG is an immune complex which can be dissociated to form its constituent antigen and antibody.

The serum of two vasculitic patients had demonstrable complexed IgG but no detectable cryoglobulin while four patients showed both phenomena One serum had a small amount of cryoglobulin without any complexed IgG. These findings suggest that small soluble IgG containing complexes, as detected by gel filtration at $4^{o}C$ differ from cold precipitable complexes which would probably precipitate out during gel filtration. In one patient, Ki., the serum was fractioned after the isolation of the cryoglobulin and complexed IgG was still detected. The patient's neat serum and supernatant serum also strongly

inhibited K cell cytotoxicity whereas the cryoglobulin showed much less inhibition. These observations again suggest the duality of the cryoglobulin and the small complexes of IgG. Moreover the findings of cryoglobulins in the serum of patient Wi. without the presence of complexed IgG would also tend to support this view. However even if the suggestion of the occurrence of two forms of complex is accepted the relevance of these findings to the state in vivo remains somewhat speculative. Are the small complexes of IgG as detected by gel filtration at 4°C also seen in vivo and if so what is their relationship to the pathogenesis of the vasculitis? Since three sera from patients with active vasculitis had no detectable complexed IgG this would imply that additional factors are important in the production of the vasculitis.

Thus these preliminary findings suggest that small complexes of IgG may or may not be associated with vasculitis and are distinct from cold precipitable cryoglobulin. However, since complexes were detected in four out of five sera which also contained cryoglobulin, this would suggest a close relationship between the two.

In one patient, Ni., no cryoglobulins were found but a considerable amount of complexed IgG was detected by gel filtration. This patient's serum strongly inhibited K cell cytotoxicity and also polymorphonuclear leucocyte (PMN)* killing of E.Coli after eight hours incubation of the PMNs in the serum. Both these assays are known to be inhibited by certain types of preformed immune complexes (MacLennan and Gotch, personal communication). Since it was noted that this patient's own K cell cytotoxicity and PMN killing were grossly depressed, this raises the interesting question as to whether this depression was secondary to the presence of circulating complexes. At this

* PMN were from a healthy donor

stage no answers can be given but from experiments to be described elsewhere it would appear that K cell cytotoxicity may be depressed by autologous serum complexes in vitro. The significance of these findings in vivo is an exciting arena for future research.

The nature of cryoglobulins has aroused much interest. Using SDSPAGE to analyse the constituents of the cryoglobulins it was found in this study that several additional bands were apparent in three cryoglobulins besides immunoglobulin. Further investigations into the nature of these bands were not attempted but this method might provide an interesting approach for future analysis. In one cryoglobulin a dense band was seen with an electrophoretic mobility identical to fibrinogen. In renal glomerular diseases McIntosh et al. (1974) have shown that cryoglobulins which contain fibrinogen are often associated with a rapidly progressive renal disease. Apart from this little is known of the significance of other protein constituents of cryoglobulins.

I will now turn from the topic of cryoglobulins and vasculitis to discuss the results of other diseases. Paired serum and cerebrospinal fluid specimens from patients with MS were examined for circulating complexes and the results compared to those obtained for a group of patients with other neurological diseases. No significant differences were found between the groups. In addition these sera also gave a degree of inhibition of K cell mediated cytotoxicity similar to that which is normally obtained with sera from healthy subjects. This suggests that complexes are not likely to be present in the majority of the neurological disorders.

There are minimal reasons to suspect the presence of circulating complexes in the CSF or serum of patients with MS. However immune alterations are

evident in MS (see MacAlpine et al., 1972) and some investigators classify this disease under the autoimmune heading. Immune complexes have been detected in other autoimmune disorders including rheumatoid arthritis, systemic lupus erythematosis and thyroid disorder (see chapter four) and thus patients with MS were investigated with this similarity in mind. No complexes were found.

Other patients with nephritis or coeliac disease were also investigated for circulating complexes but with negative results. Immune complexes have been detected in a proportion of patients with both these conditions (see chapter four) but due to the small numbers in the present study, comparisons with other results would be unwarranted. It was of interest, however, that one patient with pre-eclampsia had complexed IgG in her serum. Further studies to detect complexes in this condition appear worthwhile especially as IgG and complement have been detected by immunofluorescent means, in the placentae of these patients (see Edwards and Coombs, 1975).

CONCLUSION

Four groups of patients were studied. The first group comprised 19 patients having newly diagnosed acute myeloid leukaemia. The serum of these patients was examined by gel filtration chromatography. The IgG in the majority of these sera eluted in a normal position and complexed IgG was not detected. It was concluded that IgG containing immune complexes of small size were not present in the circulation of these patients in any detectable quantity.

The second group was comprised of 11 patients with cutaneous vasculitis. Serum from these patients was examined for cryoglobulin and for complexed IgG. It was observed that complexed IgG was present in some of these sera and was generally associated with the presence of cryoglobulin and with a significant inhibition of K cell mediated cytotoxicity but not always so. In addition active vasculitis was seen without the presence of either a cryoglobulin or complexed IgG. In the analysis of the cryoglobulins it was concluded that SDSPAGE might be a rewarding method.

The third group consisted of 26 patients with multiple sclerosis. The percentage inhibition of K cell mediated cytotoxicity by the serum of these patients was similar to that obtained by serum from patients with other neurological disorders. In addition no significant inhibition of cytotoxicity was obtained with any of the cerebrospinal fluid of these patients. Immune complexes are unlikely to be present in the serum or cerebrospinal fluid of these patients.

The fourth group consisted of 7 patients with a variety of diseases. One serum from a woman suffering from severe pre-eclampsia was found to have complexed IgG.

CHAPTER SEVEN

PROTEIN MOLECULAR SIZE AND THE BLOOD / CEREBROSPINAL FLUID BARRIER

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CHAPTER SEVEN

PROTEIN MOLECULAR SIZE AND THE BLOOD / CEREBROSPINAL FLUID BARRIER

Most proteins in the cerebrospinal fluid (CSF) are derived from plasma by simple diffusion across the blood / CSF "barrier" (Frick, 1960; Laterre, 1965; Tourtellotte, 1970; and Schultze and Heremans, 1966 for a review). The concentration of these proteins is much less than that seen in plasma and in health the larger proteins, such as β lipoprotein and IgM, are totally excluded (Burton, 1964). Rosenthal and Soothill (1962) were the first to clearly demonstrate the 'sieving' effect of the blood / CSF barrier to proteins when they showed that the concentration of a particular protein in the CSF was directly related to its serum concentration and inversely related to its molecular size. Furthermore, these workers also noted that when the permeability of the blood / CSF barrier was impaired, as occurs in various neurological disorders, the molecular size selectivity of the barrier was partially lost and larger sized proteins entered the CSF.

In this chapter I will review the literature relating to the protein sieving effect of blood / CSF barrier and will present experimental data which, in essence, has confirmed the previous findings of Rosenthal and Soothill. This present study will serve as an introduction to the next chapter in which a more extensive examination of CSF proteins in various neurological diseases will be described. In particular, a CSF/serum protein quotient is defined in the present chapter which has been found useful in assessing the molecular size

selectivity of the blood/CSF barrier. This quotient will be compared with other more commonly used CSF protein quotients.

The cerebrospinal fluid

The CSF is the crystal clear fluid which surrounds and permeates the interstitium of the central nervous system (CNS) and is enclosed by the arachnoid layer of the leptomeninges. Davson (1967) gives a lucid description of the anatomy and physiology of the CSF. The total volume of the CSF in the healthy adult is approximately 120 mls and this volume forms the third largest collection of extracellular fluid (being exceeded by the interstitial fluid and the plasma). The CSF is composed of water, electrolyte and a low concentration of protein and it acts as a mechanical, thermal and biochemical buffer for the sensitive cells of the CNS. It is in continuity with the extracellular fluid of these cells via large fenestrations in the ependymal layer (Brightman, 1965; Tschirgi, 1960; and Fishman, 1974). But it is separated from the vascular compartment either by the tightly knit endothelial cells of the neurocapillaries (Brightman et al., 1970; Pappas, 1970) or in some CNS regions by epithelial and glial cells (Brightman et al., 1970). Davson (1967) and Oldendorf (1967) have suggested that, due to the close proximity of the CSF to the CNS cells, this fluid can be considered to have a "sink action" for the metabolic activities of these cells and thus be a sensitive indicator of the biochemical events occurring within the CNS.

Most of the CSF is produced in the choroid plexis of the ventricular system and about 500 mls is secreted daily (Cutler et al., 1968). A slow circulation of CSF occurs from the ventricles into the subarachnoid space,

including the lumbar sac, and over the convexities of the cerebral hemispheres to its site of absorption. The absorption of the CSF has been the subject of much controversy but it appears that a transcellular flow of CSF occurs into the venous blood of the draining sinuses at the sites of the arachnoid granulations. This flow is non-selective with respect to protein size (Cutler et al., 1968; Editorial, Lancet, 1973).

Blood/cerebrospinal fluid barrier

The CSF differs from other body fluids (apart from the aqueous of the eye) in that it contains a very low concentration of protein (15 - 45 mg %). The explanation of this low protein concentration requires a consideration of the blood/CSF barrier and of its twin the blood/brain barrier.

The concept of the blood/brain barrier arose from studies which showed that certain dyes, injected into the systemic circulation, stained most organs but not the brain (Ehrlich, 1885; Goldman, 1913). It was later shown that these dyes were bound to plasma proteins so that the barrier to dyes was in fact a barrier to protein. With the introduction of radiolabelled proteins, electron microscopy, and fluorescent and enzymic technology it became possible to define this barrier morphologically and it is currently considered to be formed by the endothelial cells of the vessels of the CNS. These cells are bound tightly to each other at the pentalaminar junctions (Pappas, 1970; Gardner, 1972).

The concept of the blood/CSF barrier arose from the anatomical localization of the CSF, from its low total protein and its slight electrolyte differences when compared with plasma. The nature of this latter barrier

requires a comment on the sites of origin of the CSF protein for it is at these sites that the blood/CSF barrier is operative.

It is generally accepted that most CSF proteins in health originate from the plasma (Schultze and Heremans, 1966). These proteins enter the CSF at the choroid plexii and elsewhere. These latter sites have been implicated for the following reasons 1) Surgical excision of the choroid plexii does not stop CSF production (Milhorat, 1969). 2) The CSF protein concentration increases along the neuroaxis. This phenomenon can not be explained by fluid absorption occurring along this axis (Hochwald, 1970). 3) Hochwald and Wallenstein (1967) and Hochwald (1970) have shown in animal experiments that a percentage of the CSF albumin and IgG originates from the circulation in the regional nervous tissue and that only about 40% of the CSF albumin is derived from the choroid plexii.

It is thus apparent that CSF (and its proteins) is derived from both the choroid plexii and from the large surface area in contact with the CSF. The blood/CSF barrier must then be considered to occur at all levels at which CSF formation occurs, and in addition, at the boundaries of the CSF compartment. It is not surprising, then, that Gardner (1972) has described the blood / CSF and blood / brain barrier as being one and the same.

The detailed mechanism by which plasma proteins (and fluid) enter the CSF is obscure. This is vitally relevant to the present discussion because the explanation must allow for the phenomenon of protein sieving which, as will be described, operates at the blood/CSF barrier. One hypothesis for the 'secretion' of CSF is via pinocytotic transport across the endothelial cell, the process being the result of filtration pressure (Gardner, 1972). Alternative

mechanisms include the concept of fenestration cr windows in the neurocapillary endothelium similar to that which are found in the glomeruli, the pressure gradient across the endothelium determining the direction of flow. Gardner (1972) has also proposed a process incorporating both concepts, the fenestra developing when the distance between the two limiting unit membranes of the endothelial cell becomes less than 300 Å, a 'critical' value. However the actual mechanism is unknown.

'Permeability' of the blood / cerebrospinal fluid barrier

The permeability of the blood/CSF barrier relates to the ease at which plasma proteins enter the CSF. Normally this barrier is relatively impermeable to plasma proteins since the ratio of CSF/serum albumin is about 1:200. In certain disease states though, this ratio may increase and if this occurs the permeability of the blood/CSF barrier is said to be increased since there is no evidence for the cerebrogenic origin of CSF albumin (Tourtellotte, 1970). Because of the close correlation between CSF albumin and CSF total protein (Kabat et al., 1948) most investigators consider that the measurement of CSF total protein (or albumin) is an accurate index of the permeability of the blood/CSF barrier.

The permeability of this barrier varies physiologically. Thus neonates show a high normal CSF total protein (up to 150 mg%, Widell, 1958) while in later life a gradual increase in the mean levels is found (Mandonick and Weissman, 1955).

The CSF total protein in the lumbar sac is higher than in the ventricular fluid. Hochwald (1970) suggests from ventriculo-cisternal perfusion experiments

in the cat, that the explanation for this is due to the sluggish CSF flow in this region associated with a continuous protein influx. There is no evidence that the permeability of the barrier differs in the lumbar sac from elsewhere or that CSF concentration occurs in this region (Hochwald, 1970).

Protein sieving effect of the blood/cerebrospinal fluid barrier

As plasma proteins pass from the circulation into the CSF they are selectively sieved by the blood/CSF barrier. The evidence for this is both circumstantial and definitive. It was originally noted during immunoelectrophoretic analysis of CSF proteins that large sized molecules were conspicuously absent both in health and most diseases (Burtin, 1964; Dencker et al., 1961). Other workers also noted that the functional exchange rates of several radio-labelled proteins measured between CSF and plasma (and vice versa) were totally consistent with the known ratios (Fishman, 1953; Frick and Scheid-Seydal, 1958). This suggested that these CSF proteins were entirely derived from the plasma and in proportion to their molecular weight.

In the first comprehensive study of the sieving effect of the blood/CSF barrier Rosenthal and Soothill (1962) were able to show that the CSF/serum concentration ratio of five CSF proteins (and expressed as a percentage of the CSF/serum albumin ratio) was inversely proportional to the respective molecular weight of these proteins. They also observed that the large proteins IgM and α_2 macroglobulin were either totally excluded from the CSF under normal conditions or only present in very low amounts. However when the CSF total protein was elevated these proteins were detected and in addition a rise in the CSF/serum ratio of all the CSF proteins had occurred. Furthermore these

investigators noted in both health and disease, lines of identity in immuno-diffusion experiments between the CSF and corresponding serum proteins. The combination of all these findings suggested to Rosenthal and Soothill that CSF proteins were derived from the plasma and that the concentration of these proteins was proportional to the permeability of the blood/CSF barrier, the plasma concentration and the reciprocal of the molecular weight of the protein.

One protein, transferrin consistenily deviated from these conclusions. As a relatively high CSF/serum ratio was found, Rosenthal and Soothill suggested that this protein was being locally produced in the CNS. The alternative suggestions of a preferential secretion of this protein into the CSF or an impaired absorption from the CSF were considered very unlikely.

Aim of present study

The aim of the present study has been to re-examine the sieving effect of the blood/CSF barrier in health and disease. This was done using two methods. The first method was similar to that used by Rosenthal and Soothill. Individual CSF and serum proteins of differing size were quantified by single radial immunodiffusion (a more precise method than used by Rosenthal and Soothill) and the CSF/serum ratio calculated and compared to the respective molecular weight of these proteins. The second method used was SDSPAGE of CSF. The CSF from patients with normal or elevated CSF total proteins were examined.

PATIENTS AND METHODS

Patients

CSF was obtained from 27 patients by lumbar puncture. On the same day venous blood was aspirated from the antecubital vein and the serum collected. The patients consisted of 13 females and 14 males with an average age of 44 years (range 12 - 74 years). They suffered from a variety of neurological diseases but none were considered to have a demylinating or infectious neurological condition in which CNS production of immunoglobulin may occur (see Chapter eight). Eight patients had non haemorrhagic cerebrovascular disorders, six patients had structural disorders of the spine with neurological complications, three patients had a neurosis or psychosis, one had dementia and the remaining nine patients had miscellaneous disorders.

Cerebrospinal fluid and serum

The CSF cell count was performed in an Improved Neubauer counting chamber using phase contrast microscopy. The CSF specimen was centrifuged to remove the cells (200 g for 10 minutes) and the total protein measured. The CSF was then stored at -20^{o}C for future protein estimation. This was done in large batches within 6 months of storage. The serum was stored in a similar fashion and the protein measurements performed at the same time and in the same radial immunodiffusion plates as were used for the corresponding CSF specimen.

Protein estimations

The proteins, albumin, transferrin, IgG, IgA and IgM were measured in the CSF and serum by single radial immunodiffusion exactly as described in Chapter two. Each protein estimation was performed in duplicate and the mean of the two values calculated. To convert i.u. /ml to mg/ml the formulae of Humphrey and Batty (1974) were used.

Cerebrospinal fluid total protein

The CSF total protein was measured using a modification of the ultra violet spectrophotometric method of Waddell and Hill (1956). In this method the CSF was diluted 1:50 with normal saline and the extinction at 215 mμ and 225 mμ measured. (Normal saline was used as a blank in this measurement.) At this wavelength the peptide bond absorbs intensely and therefore small amounts of protein can be measured. The protein concentration of the CSF at the two wavelengths was then read from a calibration graph and the mean of the two values calculated. The calibration graph was made using known concentrations of standard protein solutions which were prepared as follows. Equal amounts of desiccated human serum albumin (Koch Light) and gamma globulin (Sigma) were dissolved in saline to achieve a total concentration of 15, 30, 60 and 120 mg% and the optical density at 215 and 220 mμ measured for a 1:50 dilution of each solution. A calibration graph was then prepared for each wave length. In every batch of CSF measured the 60 mg% solution was included as a reference solution. The coefficient of variation for 20 different measurements of this solution was 1.2%.

SDSPAGE

SDSPAGE of sera and CSF was performed using 3.6% polyacrylamide gels and the method described in Chapter two. To 50 μl of CSF was added 5 μl of 20% SDS and after incubation for 30 minutes 50 μl of this preparation was layered onto the upper surface of the gels. SDSPAGE was then performed and the gel stained and destained in the standard fashion. CSF was also concentrated 25 x's using a Amicon Minicon B concentration unit. To 100 μl of this specimen was added 5 μl of 20% SDS and 50 μl of this mixture was then applied to the upper gel surface.

Gel filtration chromatography

Two mls of CSF were fractionated using 50 x 2.5 cm Sephadex G200 columns. The eluent was PBS and the flow rate was 10 mls/hour. Three ml eluate fractions were collected. The optical density of the eluate was measured in 1 cm cuvettes at a wavelength of 220 mμ.

RESULTS

Cerebrospinal fluid total protein

The total protein for the 27 CSF specimens varied between 39 and 586 mg%. A highly significant linear regression was found between the CSF total protein and the corresponding albumin level ($p < 0.001$) and this is plotted in Figure 7,1. Regression coefficients of a similar significance were also obtained between the total protein and the IgG, IgA and transferrin levels (Table 7,1). From the equation of regression indicated in Figure 7,1 it

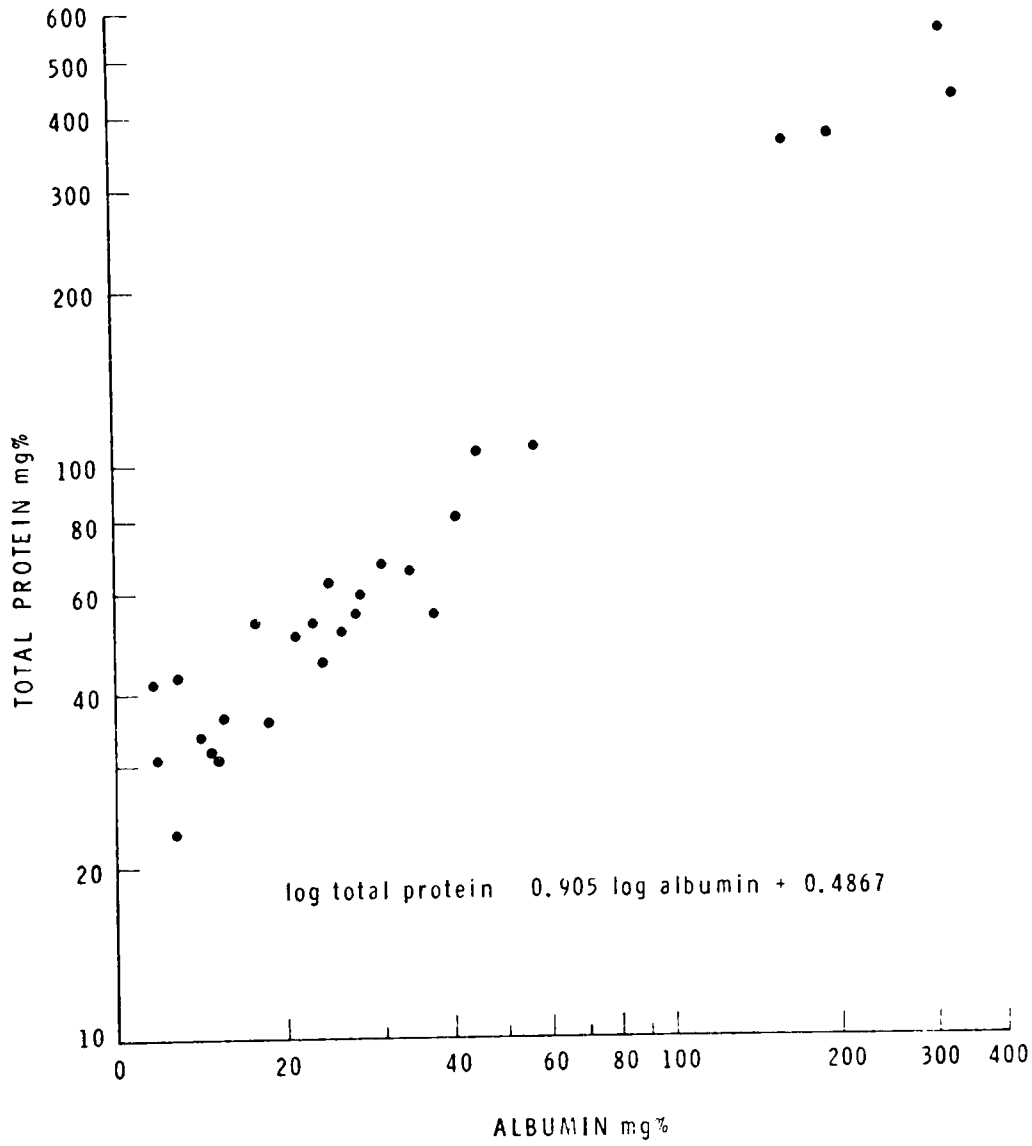


Figure 7,1 The relationship between the CSF total protein and the albumin level in 27 specimens of CSF. The equation of regression is indicated.

Specific CSF protein	Correlation coefficient	Probability 'p' value
Albumin	0.975	< 0.001
IgG	0.956	< 0.001
IgA	0.845	< 0.001
transferrin	0.940	< 0.001

Table 7,1 Correlation coefficients and probability values between CSF total protein and specific CSF proteins (by regression analysis using the method of least squares).

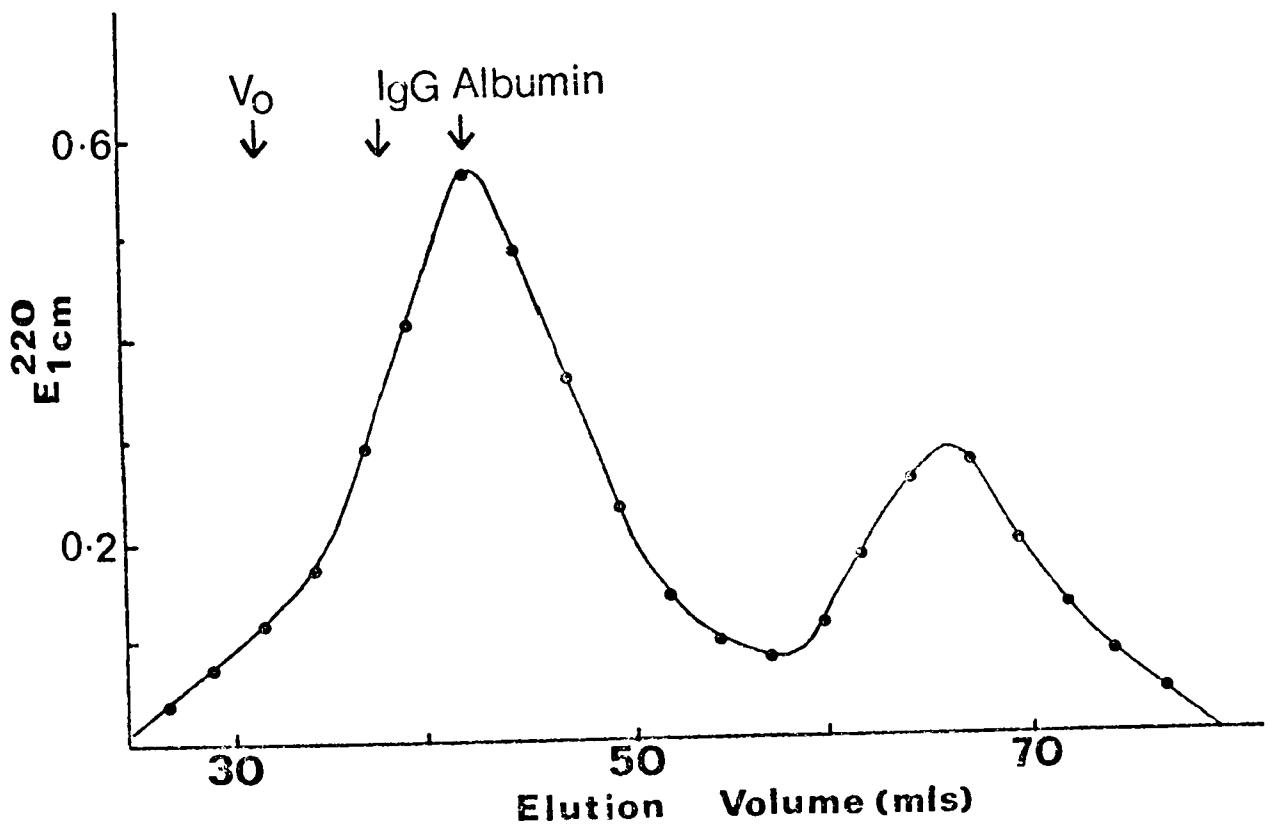


Figure 7,2 Characteristic elution profile of CSF after gel filtration on Sephadex G 200 (read at 220 mμ).

is seen that when the CSF albumin is zero the CSF total protein (as measured by the spectrophotometric method) will give a value of about 30 mg%. In examining this finding further, 15 CSF specimens of varying total protein concentration were fractionated on Sephadex G200. An illustrative profile for one of these specimens is shown in Figure 7,2. The first elution peak corresponded to the peak position of the radio labelled monomeric albumin marker, whereas the second peak corresponded to the position where free radioactive iodine was eluted. It was also noted that the height of the first eluting peak varied according to the concentration of the CSF protein while the later emerging smaller peak had a constant height. If the area of this later peak was expressed as a percentage of the area subtended by the optical density profile and this percentage then multiplied by the total protein obtained for that CSF specimen, a value of 15.7 + 1.2 mg% was obtained (mean $\pm$ one standard deviation). This indicates that if the spectrophotometric method is used to measure CSF total protein, a constant 16 mg% is made up of low molecular weight material and this amount is independent of the total CSF protein.

Molecular size selectivity of the blood/cerebrospinal fluid barrier

CSF proteins: In an endeavour to assess the sieving effect of the blood/CSF barrier the proteins albumin, transferrin, IgG, IgA and IgM were measured in serum and CSF. The resultant concentrations were then used in deriving quotients in which the CSF/serum ratio was calculated for each protein and expressed as a percentage of the corresponding CSF/serum albumin ratio. The quotients so obtained for each protein were then divided into two

groups depending on whether the CSF total protein was greater or less than 75 mg%. In Figure 7,3 the mean $\pm$ 2 standard deviates (M $\pm$ 2 SD) of these quotients for each protein has been plotted against the respective molecular weight of these proteins. (For albumin the CSF/serum ratio has been taken as 100% in every specimen.) It is seen that as the molecular weight increases the quotient falls, but less so for the group with elevated CSF total proteins. The quotients obtained for transferrin, however, were noted to all lie above 100%, and the mean for the group in which the CSF total protein was in excess of 75 mg% was lower than the mean of the other group.

IgM was measurable in three specimens only, and all had CSF total proteins in excess of 350 mg%. Thus the M $\pm$ 2 SD of the quotient for this protein was not calculated.

SDSPAGE: Paired serum and CSF from eight patients were studied using SDSPAGE. In three of these patients the CSF total protein was greater than 100 mg%. A representative example of the electrophoretic appearance seen for CSF with total protein less than 75 mg% is seen in Figure 7,4 while Figure 7,5 shows an example for a specimen with an elevated total protein of 456 mg%.

It is seen from the first illustration that even after the CSF was concentrated to approximately the same relative concentration as the corresponding serum specimen, an absolute deficiency of the larger sized proteins was apparent, while the medium sized proteins (of the order 100,000 - 200,000 daltons) were present in the CSF in relatively low concentration when compared with the serum specimen. When the CSF total protein was elevated (Figure 7,5) the larger proteins were more conspicuous but still in relatively

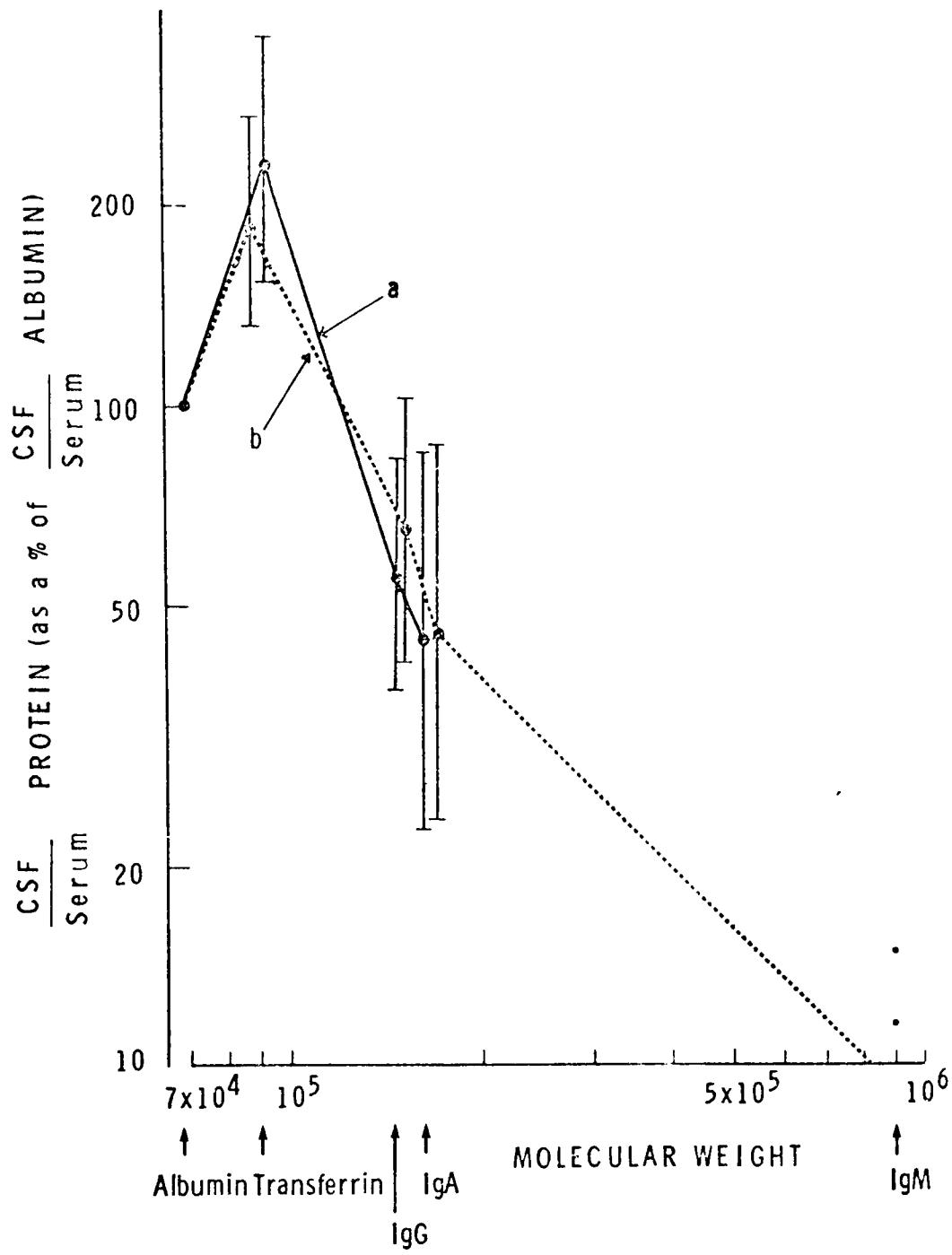


Figure 7,3 This figure demonstrates the sieving effect of the blood/CSF barrier. The concentration of albumin, transferrin, IgG, IgA and IgM was measured in 27 paired specimens of CSF and serum. The vertical lines represent the mean $\pm$ 2 standard deviations (M $\pm$ 2 S D) of the CSF/serum ratios of these proteins (expressed as a percentage of the corresponding CSF/serum albumin) and these values have been plotted against the molecular weight of the corresponding protein. The M $\pm$ S D obtained for each protein has been subdivided according to whether the respective total protein was less than 75 mg% (line a) or greater than 75 mg% (line b). IgM was measurable in only three specimens (out of seven) of this latter subdivision and the M $\pm$ S D was not calculated.

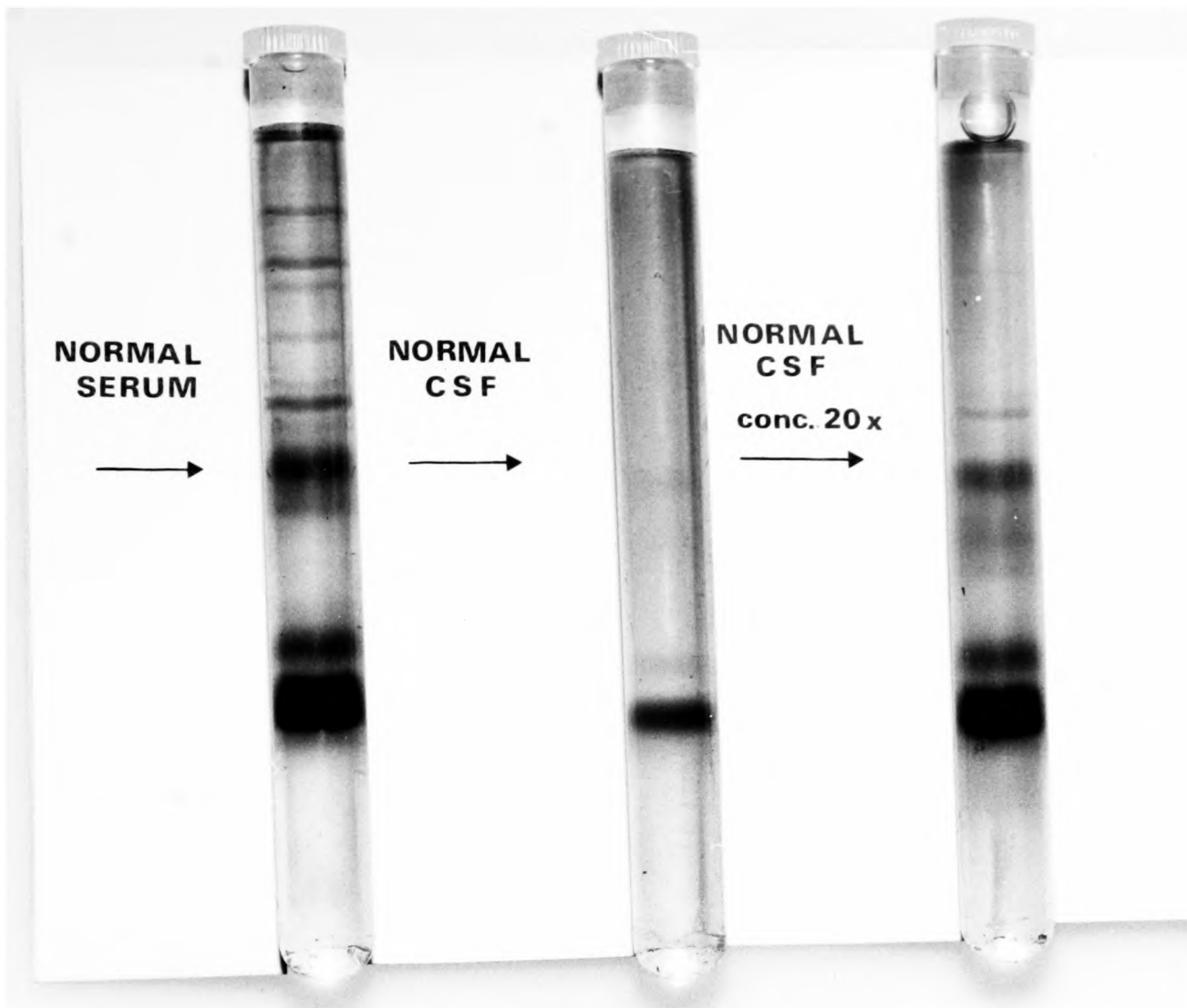


Figure 7,4 Appearance of paired serum and CSF using 3.6% SDSPAGE. The total protein of the CSF specimen was normal.

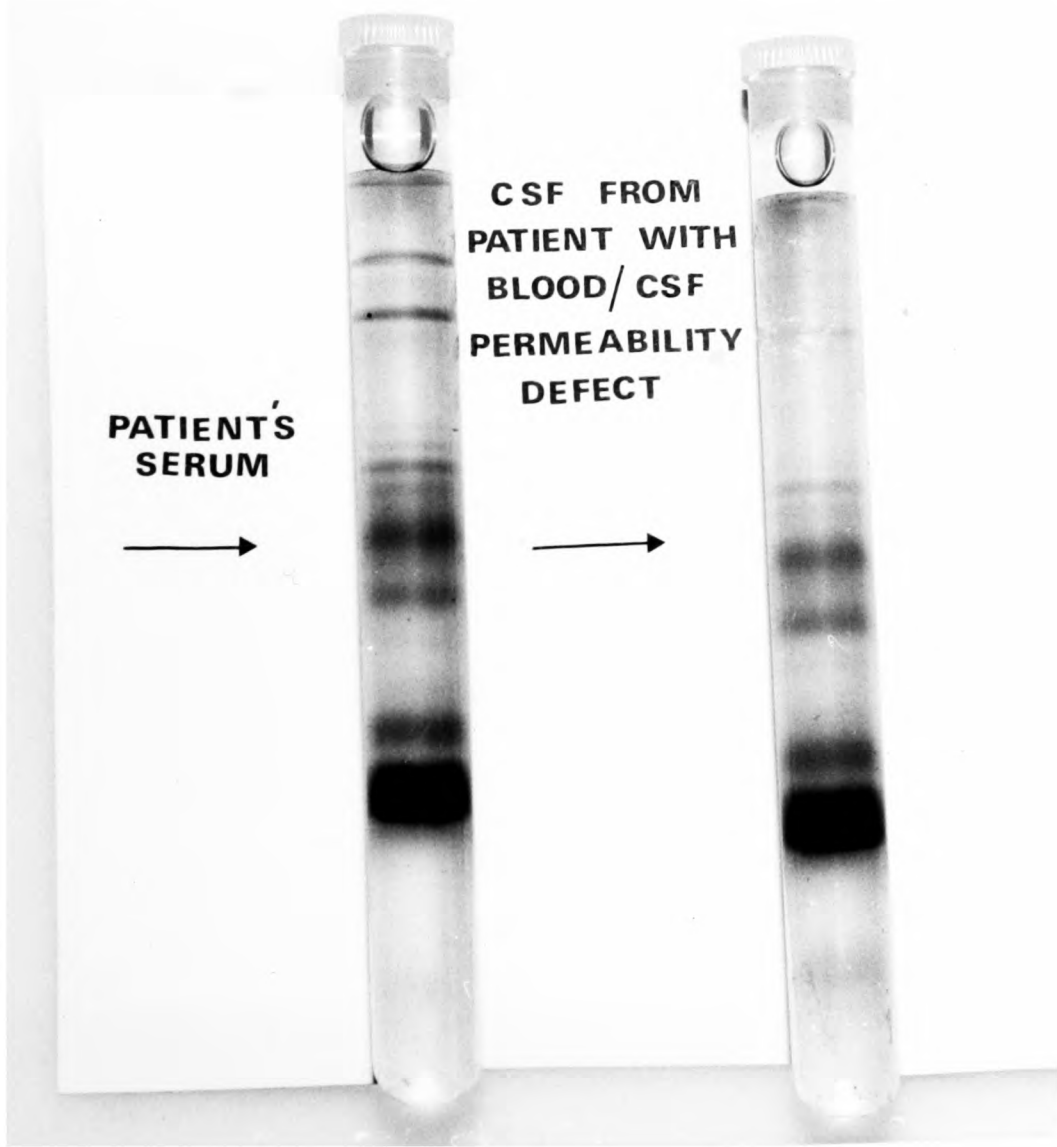


Figure 7,5 Appearance of paired CSF and serum using 3.6% SDSPAGE. The total protein of the CSF specimen was 456 mg%.

low amounts when compared with the corresponding serum sample. When this CSF specimen was concentrated 2 fold so as to give a relative concentration of the same order as its corresponding serum sample, the larger proteins were again relatively inconspicuous whilst the largest (e.g. IgM) were only just evident.

In Figure 7,6 the relationship between protein molecular size and the relative CSF/ serum quotient is plotted for two representative patients, one with a CSF total protein of 456 mg% and the other with a normal total protein. This figure is similar to Figure 7,3 but in this case the CSF/serum protein quotient (expressed as a percentage of the CSF/serum albumin ratio as before) was derived from the corresponding area of deflection for that protein as seen in the CSF and serum electrophoretogram. A decrease in the quotient, with increasing molecular weight, is seen for all proteins (except for transferrin in one specimen); the decline for the specimen with normal total protein being steeper than that of the specimen with elevated total protein.

DISCUSSION

An examination of several proteins in 27 specimens of CSF revealed that 1) the concentration of these proteins was directly proportional to the permeability of the blood/CSF barrier

2) the CSF/serum ratio (and expressed as a percentage of the CSF/serum albumin) was inversely proportional to molecular weight of these proteins. These findings, therefore, confirm the observations of Rosenthal and Soothill (1962) and strongly suggest that the blood/CSF barrier acts as a protein sieve.

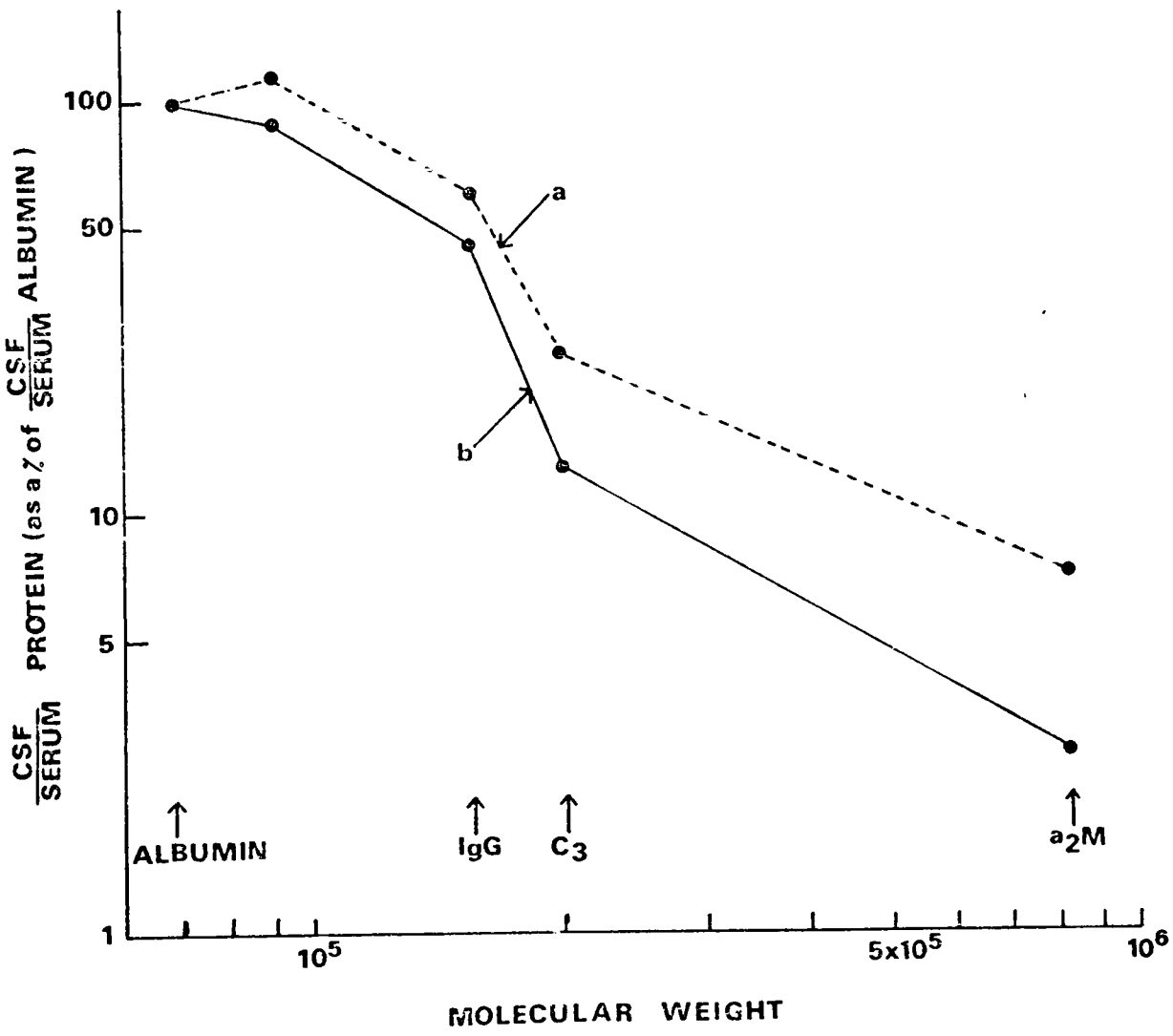


Figure 7,6 The CSF/serum quotient (see text) was derived for four proteins by integration of the area beneath the respective SDSPAGE microdensitometric deflection. These quotients have been plotted against the molecular weight of the proteins. Line 'b' demonstrates the relationship for a CSF specimen with normal total protein while line 'a' demonstrates the relationship for a specimen with a total protein of 456 mg%.

It was also shown that during neurological diseases associated with elevated CSF total proteins (and this was taken to indicate an increased permeability of the blood/CSF barrier) this sieving effect was still apparent but to a less degree; i.e. the slope relating relative CSF/serum concentration to molecular size was more horizontal (Figure 7,3). Finally the simplicity of SDSPAGE in assessing this sieving effect was demonstrated in contrast to the more laborious immunoquantitative method.

Several points for discussion arise from these results. Why does the sieve occur and what is its nature? These interrelated questions can not be immediately answered. However it is known that the sieving effect is not unique to the blood/CSF barrier as a similar phenomenon is also seen at the blood/synovial fluid barrier (Kushner and Somerville, 1971; Brown et al., 1969; Pruzanski et al., 1973, and Svartman et al., 1975) and probably the barriers of the peritoneal, pleural and pericardial spaces (Schultze and Heremans, 1966). The evidence for these other barriers is based on experimental findings using similar analytical techniques as described for the blood/CSF barrier and in particular the relative deficiency of high molecular weight serum proteins. These barriers differ, however, from the blood/CSF barrier in that they are relatively permeable to serum proteins. Thus the synovial fluid/serum albumin ratio is about 0.4 in contrast to the CSF/serum ratio of 0.005. It is tempting to equate the relatively impermeability of the blood / CSF barrier to the blood/brain barrier which is characterized morphologically by the tight endothelial pentalaminar junctions.

Since this sieving effect is seen at several biological barriers this implies similar or possibly the same basic mechanism. Whether this mechanism

operates via fenestra of limiting size in the barrier lining cells or through cellular pinocytosis is not yet known. However it is difficult to visualize a sieving process during pinocytosis.

Another finding seen in the present study was that in conditions associated with elevated CSF total protein, most probably a result of an increased permeability in the blood/CSF barrier (since no CSF haemorrhage had occurred, there was no block to CSF flow, the serum proteins were normal and there was no reason to suspect a lesion of the choroid plexii), the sieving effect was still present but was less marked. This was shown by two means. The first was relating relative CSF/serum concentrations to molecular size. In conditions with elevated CSF total proteins the slope between these two variables became more horizontal (Figure 7,3). The second was the detection of large molecules in the CSF as the total protein increased. This was simply accomplished using SDSPAGE.

Raimondi (1966) and Clawson et al. (1966) have suggested that the mechanism for the increased CSF total protein in pathological conditions is due to an intensified pinocytotic activity ferrying appreciable amounts of plasma proteins across the endothelium. However other investigators (see Brightman et al., 1970 for review) suggest from electronmicrographic studies that the most likely routes of protein leakage are via penetration between endothelial cells or across severely damaged endothelial cells. Either mechanism would appear compatible with the loss in protein size selectivity that occurs with defects in the blood/CSF barrier.

The existence of the blood/CSF sieve appears to be an integral part of the blood/brain barrier. Thus to explain the necessity of the sieve it is

necessary to explain the requirement of the blood/brain barrier; and this is not fully known at the present time. Apart from maintaining a constant 'milieu' for the nervous elements it may also protect the CNS cells from the protein of the plasma. Rubinstein et al. (1962) have shown that the acute extravascular spread of serum through the white matter induces a prompt astrocytic reaction expressed by their enlargement formation of fibrils and by several enzymic changes. It is not strictly clear whether this reaction is secondary to the presence of serum or whether it is the initiating primary event with a secondary leak. Chronic extravasation of serum tends to be associated with myelin destruction (Rubenstein et al., 1962).

Another finding of interest arising from the present study was that the CSF/serum ratio of transferrin was abnormally high when related to the molecular weight. This suggests either a preferential secretion of this protein into the CSF, an impaired absorption, or a local CNS production. Frick and Scheid-Seydal (1960) injected labelled serum β globulin (transferrin) intravenously into 20 patients with different diseases. When the dynamic equilibrium between CSF and serum was established the relationship between blood and CSF specific activity (see Cutler et al., 1970 for definition) indicated that only some of the CSF β globulin was derived from the serum, the rest probably being formed locally in the CNS. No evidence was obtained in favour of the preferential secretion or impaired absorption of CSF transferrin.

A cerebral origin for portion of CSF transferrin would also be compatible with the lower mean seen in the present study, of the relative CSF/serum transferrin ratio for the group of patients with elevated CSF total protein levels when compared to those patients with normal levels. This finding

was in contrast to all other proteins studied where the mean of the ratio increased as the CSF total protein rose. As the CSF total protein increases it would be expected that more plasma derived transferrin would appear in the CSF and dilute the locally formed transferrin. Thus the relative CSF/serum transferrin ratio would fall towards its expected position (less than that of albumin, see Figure 7,3).

Laterre (1965) has also studied CSF transferrin and his results are in accord with the view that portion of CSF transferrin may be locally produced. Laterre found two electrophoretic forms of transferrin. One had a β_2 migration, as seen in serum whereas the other migrated in the post β or tau position. This latter transferrin was deficient in neuramic acid. It is still not known whether this form of transferrin is locally synthesized in the CNS.

In some neurological disorders abnormally high relative CSF/serum ratios of IgG may occur. This suggests a local production of this immunoglobulin in the CNS and has important diagnostic relevance. This aspect will be considered in detail in the next chapter. In passing it might also be mentioned that an elevation in the relative synovial fluid/serum IgG ratio is felt by many to reflect the known production of IgG in the rheumatoid joint (Pruzanski et al., 1973).

Finally the simplicity of SDSPAGE in assessing the sieving effect of the blood/CSF barrier was demonstrated. Application of this method to assess other protein sieves, such as the renal glomerulus, might be rewarding.

CONCLUSION

SDSPAGE and single radial immunodiffusion were used in the examination of several proteins in 27 specimens of CSF obtained from patients with non demylinating and non infectious neurological disorders. The results revealed that the CSF concentration of these proteins was directly proportional to permeability of the blood/CSF barrier and that the relative CSF/serum concentration was inversely proportional to the molecular weight of these proteins. These findings strongly suggest that the blood/CSF barrier acts as a molecular sieve to plasma proteins. Furthermore it was shown that in neurological diseases associated with elevated CSF total proteins, this sieving effect was still evident but to a diminished degree. Finally SDSPAGE was shown to be a simple but useful method in demonstrating this molecular sieve and assessing its integrity.

CHAPTER EIGHT

CEREBROSPINAL FLUID IMMUNOGLOBULINS IN NEUROLOGICAL DISEASE

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CHAPTER EIGHT

CEREBROSPINAL FLUID IMMUNOGLOBULINS IN NEUROLOGICAL DISEASE

INTRODUCTION

In the preceding chapter the evidence relating to the blood/CSF barrier was reviewed. It was considered that most of the proteins in the CSF, including the immunoglobulins, were derived from the plasma by diffusion across the barrier and that this barrier acted as an efficient molecular sieve allowing the entry of smaller proteins in preference to the larger proteins.

During the last twenty years a considerable amount of clinical and experimental evidence has accumulated which strongly suggests that immunoglobulin may be additionally synthesized in the central nervous system (CNS) in certain demyelinating and infectious neurological diseases (reviewed by Tourtellotte, 1970).

The CNS produced immunoglobulin may be indirectly assessed by measuring the CSF immunoglobulin. However in doing this it is necessary to correct for the permeability of the blood/CSF barrier in order to account for the immunoglobulin derived from the plasma. Tourtellotte (1970) and others - (also see Chapter seven) - have shown that the permeability of the blood/CSF barrier is accurately reflected by the CSF total protein or albumin level and thus most investigators have related the absolute CSF immunoglobulin level to the CSF total protein or albumin level in order to derive a relative immunoglobulin concentration. If this relative concentration (or immunoglobulin quotient) is elevated this has been taken to indicate the local synthesis of

immunoglobulin within the CNS.

In many of the neurological diseases in which this quotient is increased, the immunoglobulin belongs to the G class and gives a banded appearance in the electrophoretic γ region suggesting that it is derived from a small number of plasma cell clones (Laterre et al., 1970). Further support for this oligoclonal concept has been given by Zettervall and Link (1970) who observed the preferential synthesis of IgG of the kappa light chain subclass in many cases of multiple sclerosis (MS).

The function of this CNS derived immunoglobulin is obscure. In subacute sclerosing panencephalitis (SSPE), a disease characterized by a marked selective increase in CSF IgG, Link and his colleagues (1973) have shown that portion of the oligoclonal IgG has specific antibody activity against the measles virus, a probable aetiological agent. However the function of the immunoglobulin in MS and other diseases is unknown although in some MS patients specific anti-viral activity has been detected against several viruses including measles (Haire et al., 1973).

The aim of the present study has been to measure the CSF immunoglobulin / albumin quotient in patients suffering from a variety of neurological disorders and to compare groups of patients with demyelinating, infectious and polyneuritic diseases with the remaining patients. An endeavour has also been made to find which CSF quotient, out of several which have been described, is best at distinguishing the different groups. In addition, in certain patients the IgG /albumin quotient has been compared with the CSF kappa / lambda ratio and the CSF measles, mumps and herpes simplex antibody titre to see the relationship between these measurements and to determine whether a further

refinement in the separation between these diagnostic groups can be obtained.

PATIENTS AND METHODS

Patients

The CSF was obtained from 355 patients suffering from a variety of neurological disorders. At the end of the period of study the clinical and laboratory details were reviewed from the case notes and a comparison made with the CSF findings.

There were 236 patients who formed the neurological control group. They consisted of 92 females and 141 males (3 were unknown) and had an average age of 46.4 years. Thirty-eight of these patients had cerebrovascular disease, 14 degenerative disease, 17 epileptic disorders, 17 senile dementias, 41 cervical and lumbar osteopathies, 18 CNS tumours, 26 neuroses or different forms of headache, 53 had other disorders and 12 were undiagnosed.

There were 83 patients who had or were suspected of having MS. These patients were subdivided into two groups; the first consisting of patients with definite MS and the second of patients with probable or possible MS. This subdivision was, in general, based on the diagnostic criteria of McAlpine (1972). Thus patients with definite MS had evidence of multiple sites of CNS involvement associated with a history of a previous acute neurological episode followed by improvement and then one or more relapses. There were 23 females and 13 males in this subdivision; their mean age was 36.7 years (range 17 - 60), the mean duration of the disease was 5.8 years (range 1 week - 28 years) and the mean number of neurological episodes was

3.1 (range 2 - 6). There were 25 females and 22 males with probable or possible MS. The mean age was 40.7 (12 - 64) years, the mean duration 3.7 years (1 week - 23 years) and the mean number of attacks 1.8 (1 - 8).

Twenty-four patients suffered from a CNS infection. Patients were not included in this category unless an infectious agent was positively identified or strongly implicated. Nine patients had HSVE, the diagnosis being proven by viral isolation from brain biopsy or CSF, positive immunofluorescence, or a rising titre to herpes simplex virus (HSV) in both the serum and CSF. Five patients had bacterial meningitis, four patients had neurosyphylis, two patients had tuberculous meningitis, two patients had herpes zoster meningoencephalitis and one patient each had SSPE and a cerebral abscess.

The remaining 12 patients had a polyneuropathy including four with characteristic features of the Guillain-Barré syndrome.

Cerebrospinal fluid

CSF was obtained by the lumbar route in all patients. These specimens consisted of the majority received by the laboratory over a one year period. Seven specimens from patients with HSVE had been collected and stored at -20°C over a 8 year period (these were kindly donated by Dr. F.O. MacCallum). Specimens of CSF were eliminated if heavily blood stained (> 2000 red blood cells/mm^3). The CSF cell count was routinely performed and the cells separated by gentle centrifugation. The total protein was then measured and the specimens stored at -20°C for future protein estimation. This was performed in large batches within 6 months of storage.

Serum was available for study from 62 of the patients. It was collected

at the time of the lumbar puncture and studied simultaneously with its corresponding CSF.

Cerebrospinal fluid total protein and specific protein estimations

The CSF total protein and albumin, IgG, IgA and IgM were measured as described in Chapters two and seven. The CSF kappa/lambda ratio was measured by single radial immunodiffusion using Dakopatts antihuman kappa and lambda (lot 113 and 023 respectively). The World Health Organization reference serum 66/97 was used as standard for this ratio and was arbitrarily assigned a ratio of 1.35.

Viral antibody titres

Complement fixing antibodies against measles, mumps and HSV were measured in serum and CSF using the method of Smith, Peutherer and MacCallum (1967). I am indebted to Dr. F.O. MacCallum and his staff for performing these measurements.

In this method neat or dilutions of CSF and serum (heat inactivated at 56°C for 30 minutes after dilution) were tested in agglutination trays (WHO) using an optimum dose of viral antigen, complement (Burroughs Wellcome) and sensitized sheep red blood cells and incubated overnight at 4°C. The viral antigens were obtained commercially or prepared from amnion cell cultures. Controls of each dilution of serum, CSF and antigen were included in each plate together with the appropriate complement control. The plates were read visually.

In the CSF of healthy subjects with intact blood/CSF barriers, no

complement fixing antibody is normally detected against these viruses. However if the serum titre is high or the blood/CSF barrier impaired, antibody may diffuse into the CSF and be detected. To correct for this anomaly the CSF/serum antibody titre ratio was used in assessing any antibody titre in the CSF and the CSF total protein noted. In some cases, however, it was not possible to measure the serum titre.

Analysis of results

Protein concentrations in the healthy population approximate to a normal distribution when plotted on a logarithmic scale (Weeke and Krasilnikoff, 1970). In addition a close approximation to a normal distribution was obtained for the IgG/albumin quotient and kappa/lambda ratio of the neurological control group when plotted on a log scale. Accordingly, all quotients have been plotted using this scale and the means and standard deviations calculated from the log of the quotients. Correlation coefficients between two variables have been calculated on the log measurements using the method of least squares.

RESULTS

Assessment of cerebrospinal fluid immunoglubulins

The absolute measurement of CSF immunoglobulins was of little use in distinguishing between the various diagnostic groups. This was because a variable impairment in the permeability of the blood/CSF barrier occurred between different patients. Thus a high level of CSF immunoglobulin was seen

when the CSF total protein was high (also see Chapter seven). In order to detect CNS produced immunoglobulin it was necessary to correct for the immunoglobulin derived from plasma. This was done by deriving a quotient i.e. where the CSF immunoglobulin was expressed as a percentage of the CSF albumin. The results for these quotients will now be presented.

IgG/albumin quotient (see Figure 8,1)

Neurological control group: The mean + 2 standard deviations (M + 2 SD) of the CSF IgG/albumin quotients obtained for 236 patients in this group was 13.9 + 14% (Fig. 8,1).

Six patients had a quotient greater than the M + 2 SD. Two of these had long standing dementia and in one this elevated quotient was associated with an elevated K/λ ratio (vide infra) and 6 lymphocytes/mm^3 in the CSF. The second patient had a mildly elevated quotient and at autopsy the only pathological finding was a cerebral infarct in an atrophied brain. A further two patients had suffered from sub-arachnoid haemorrhages and in both elevated IgA/albumin quotients were also seen. Another patient had post streptococcal nephritis, cerebral oedema, epilepsy and cortical blindness while the final patient had a low CSF albumin (and total protein) but with a normal CSF IgG concentration.

Of the 10 patients with quotients less than the M - 2 SD (6.9%, Fig. 8,1), two were children with hydrocephalus. A third infant of two months of age was also noted with a low quotient and, predictably, had low levels of serum and CSF IgG. The remaining seven patients had a variety of unrelated diseases.

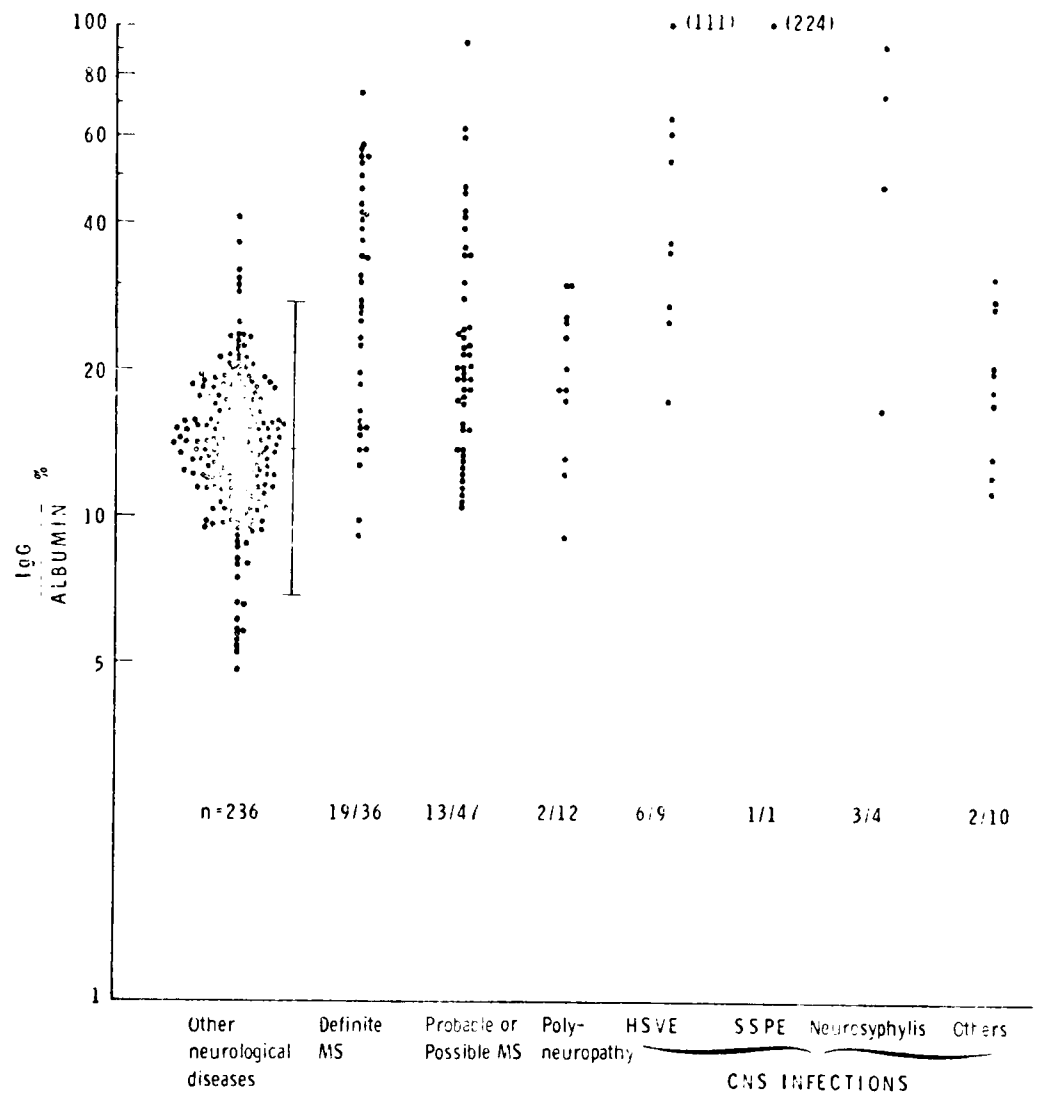


Figure 8,1 CSF IgG/albumin quotients (expressed as a percentage) obtained for 355 patients with various neurological diseases. The vertical line represents the mean $\pm$ 2 standard deviations (M $\pm$ 2 SD) of the quotients for the neurological control group. The M + 2 SD (27.9%) has been used as an upper limit when calculating the number of patients with elevated quotients in the various diagnostic groups (e.g. 19/36 patients with definite multiple sclerosis have elevated quotients).

The M + 2 SD of the IgG/albumin quotients of a group of 36 patients suffering from myelographically defined cervical spondylosis with myelopathy or lumbar disc herniation was 14.5 + 10.2%. If 24.7% is taken as an upper limit for this later group this is similar to the value (27.9%) obtained for all the neurological control patients combined.

Patients with multiple sclerosis: The IgG/albumin quotient was elevated in 19/36 (53%) of definite and 13/47 (28%) of probable or possible cases of MS when compared to the M + 2 SD of the neurological control group, Fig. 8,1. This is interpreted as indicating synthesis of IgG in the CNS in the patients with raised values.

The M $\pm$ SD for the mononuclear leucocyte cell counts in the CSF for patients with definite MS was 12.5 $\pm$ 16/mm^3, and for patients with suspected MS, 4.6 $\pm$ 4.3/mm^3. Twenty-one out of 34 (62%) of the definite MS patients and 15/39 (39%) of the suspected MS patients had CSF mononuclear leucocyte counts in excess of 5 cells/mm^3. A significant correlation was seen between this cell count and the IgG/albumin quotient when both MS groups were combined ($p = 0.01$) but not for the definite MS group taken alone. Significant correlations were also seen for both groups combined between the IgG/albumin quotient and the duration of the disease ($p = 0.02$), and the reciprocal of the albumin level ($p = 0.05$). These correlations were not significant, however, in the definite MS group alone. No correlation was observed with the age of the patients in either group or in both groups combined.

Patients with CNS infections: Several CSF specimens from patients comprising the CNS infection group had elevated IgG/albumin quotients (Fig. 8,1). Of interest was the finding that 6/9 patients with proven HSVE had elevated quotients. Of the three CSF specimens with normal quotients one was from a patient who had survived the encephalitic episode for over a year while another was from a patient whose encephalitis had been present for only two days. The clinical details of the third patient were unobtainable. The CSF of the remaining six patients were all obtained between the 12th - 200th day of the illness.

Three out of four patients with neurosyphylis had elevated quotients. The single patient with a normal quotient had been twice treated for tabies dorsalis over a 20 year period. Two other patients were untreated while the remaining patient had been diagnosed and treated seven years previously.

Three other patients, one with a cerebral abscess, one with SSPE and one with bacterial meningitis had elevated quotients.

Patients with polyneuropathies: Two patients out of 11 in this group showed an elevated IgG/albumin quotient (Fig. 8,1). In one of these a small monoclonal IgG band was noted in the serum.

Quotient used to adjust for variations in the blood/CSF barrier and in the serum levels of IgG albumin

A high CSF IgG/albumin ratio may merely reflect a high serum level of IgG or, alternatively, a low serum albumin (since both these proteins are in dynamic equilibrium with the CSF). To correct for variations in the plasma levels of these proteins a refinement in the CSF IgG/albumin quotient was used. This was the quotient CSF IgG/serum IgG (expressed as a percentage of the CSF/serum albumin ratio), The aim in using this quotient was to see if a better division could be obtained between a

neurological control group and a group of patients with MS. The relationship between this quotient and the quotients IgG/albumin and IgG/total protein is shown in Fig. 8,2 together with the proportion of values in the MS group which were above the M + 2 SD of the corresponding quotient in the other neurological disease group. All three groups have been subdivided depending on whether the CSF total protein was above or below 75 mg%. Thus it is seen in this Figure that the upper limit for the neurological control group was higher if a breach in the blood/CSF barrier had occurred. Comparison of these three quotients shows that the proportion of MS patients with an elevated IgG/albumin or IgG/total protein quotient is less than that seen using the third quotient.

Kappa/lambda ratio (Figure 8,3)

Recent evidence has shown a preferential increase in the kappa light chain subclass of CSF IgG in some patients with MS (Zettervall and Link, 1970). Consequently the CSF kappa/lambda ratio was measured for many of the patients in the present study.

The mean of the ratio found for 63 patients of the neurological control group was 1.28; the upper second standard deviation 1.63; and the lower 2 SD 1.01 (Figure 8,3). The one grossly elevated value (2.8) was from the patient with dementia who also had an elevated IgG/albumin quotient (vide supra).

An elevated K/λ ratio was observed in 10/30 (33%) of patients with definite MS and 10/38 (26%) of patients with possible or probable MS when compared with the neurological control group.

A significant correlation was seen for these MS patients between this ratio and the IgG/albumin quotient $p < 0.001$. However in six of these

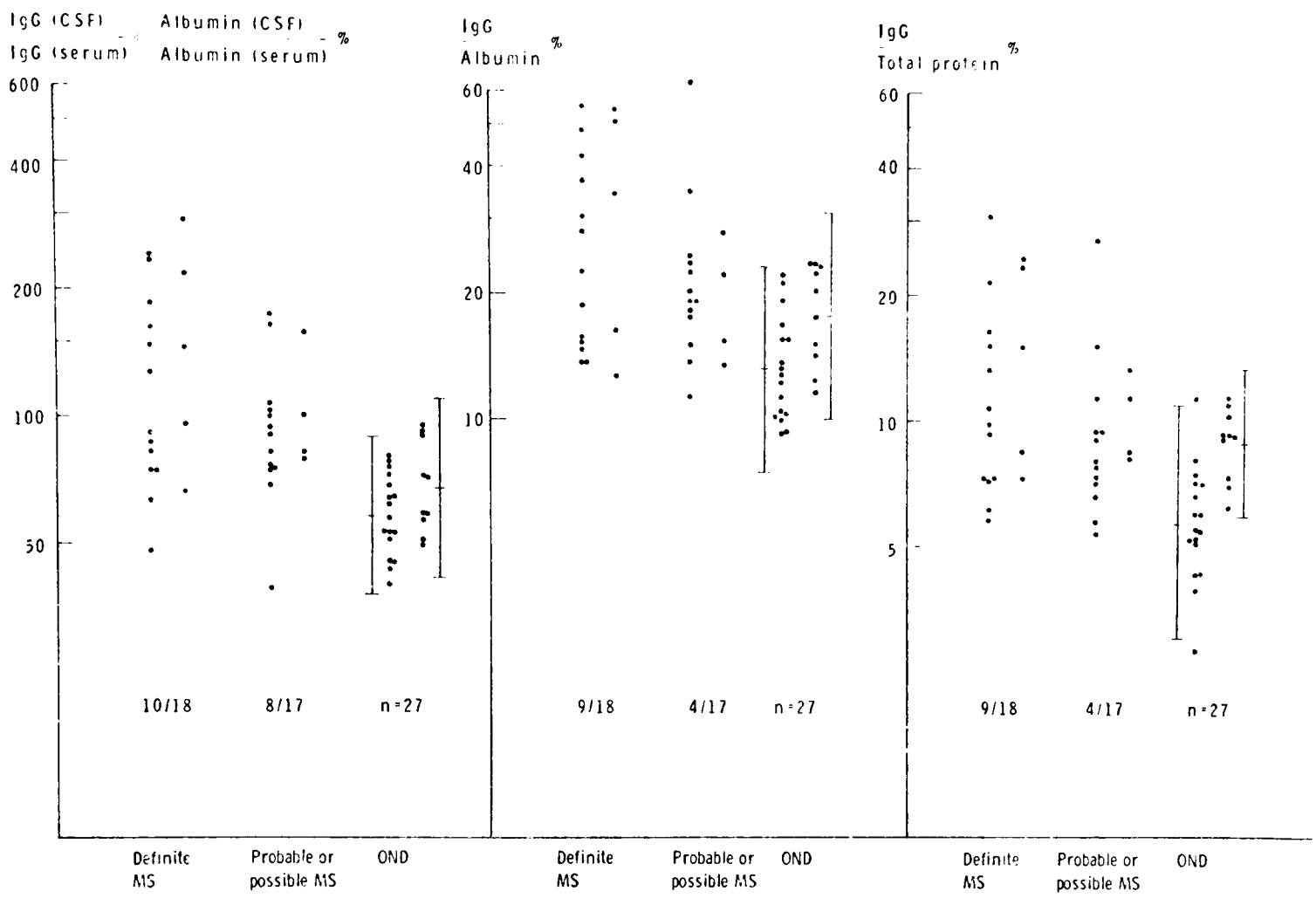


Figure 8,2 The proportion of elevated quotients seen for the same group of 35 patients with multiple sclerosis when compared with 27 patients with other neurological diseases. All groups have been subdivided into two depending on whether the CSF total protein was normal (left bracket of each group) or greater than 75 mg% (right bracket of each group). In comparing the three different quotients note the higher proportion of multiple sclerosis patients with an elevated CSF/serum IgG ÷ CSF/serum albumin quotient. This suggests that this quotient is more discriminating in distinguishing between patients with multiple sclerosis and other neurological disorders.

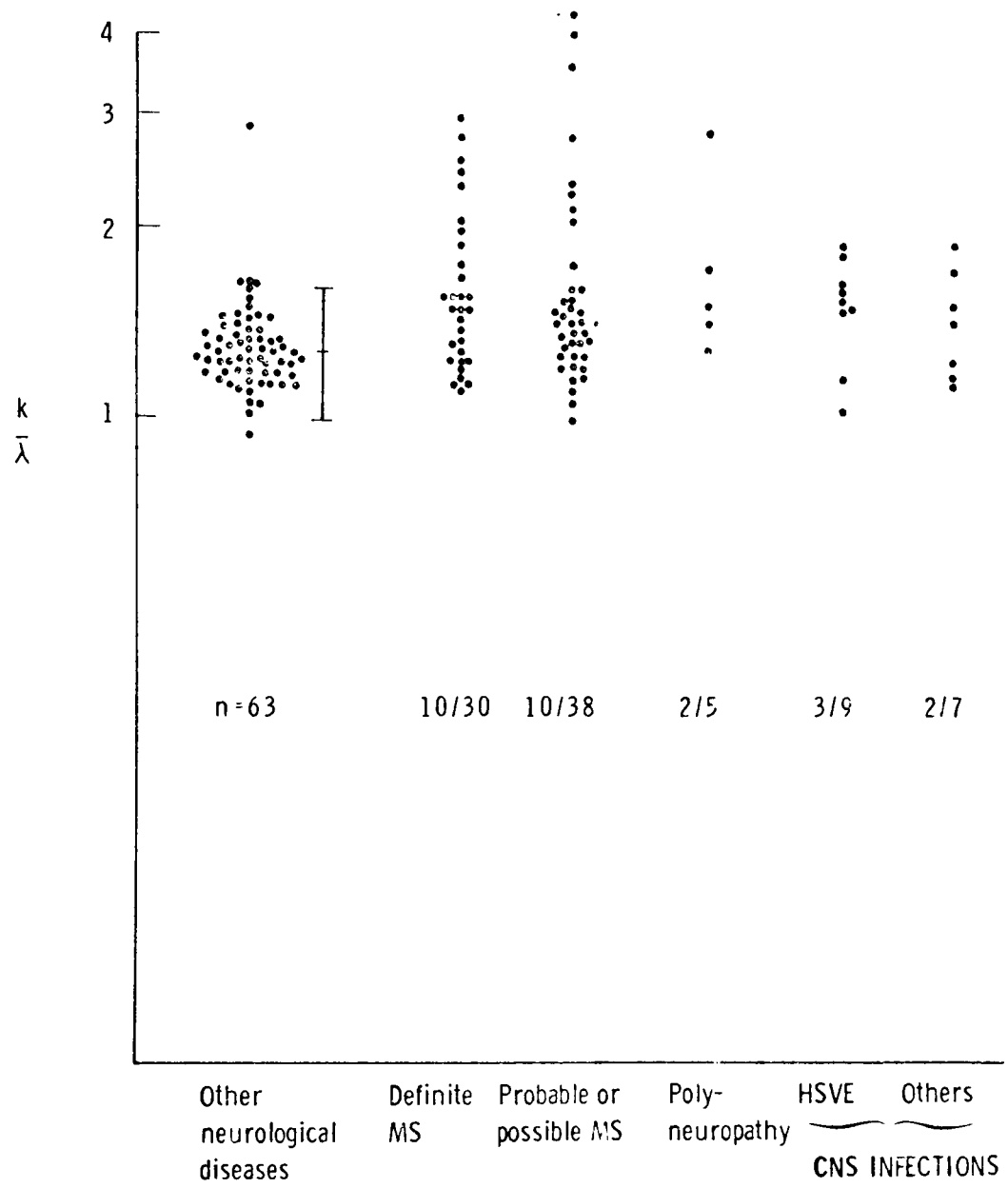


Figure 8,3 CSF kappa/lambda ratios obtained for the various diagnostic groups. The vertical line represents the mean ± 2 standard deviations obtained for the neurological control group while the number of patients in each group with an elevated ratio is indicated.

patients, normal IgG/albumin quotients were found associated with elevated K/λ ratios (Fig. 8,4).

Elevated K/λ ratios were also seen in one patient with SSPE, two patients with HSVE and one patient with tuberculous meningitis (Fig. 8,3).

IgA/albumin quotient and CSF IgM (Figure 8,5 and Table 8,1)

The IgA/albumin quotient was of little value in distinguishing between the various diagnostic groups (Figure 8,6). However it was interesting to note that two patients with neurosyphylis, one with HSVE and one with a cerebral abscess, had elevated IgA/albumin quotients when compared to the neurological control group. (An upper limit for the IgA/albumin quotient was arbitrarily set at 5% in order to exclude the upper 2.5% of specimens of the neurological control group.) It was additionally noted that three other patients with HSVE had high normal values between 4% and 5%.

CSF IgM was measured in some of the patients, including all those with MS or neurological infections. IgM was not measurable in the CSF of any patients with MS. Six patients with CNS infection had measurable CSF IgM. These consisted of four patients with HSVE, one with neurosyphylis and one with tuberculous meningitis. Two patients with polyneuritis also had IgM in the CSF. The relationship between the CSF immunoglobulins and the total proteins for all these patients is shown in Table 8,1.

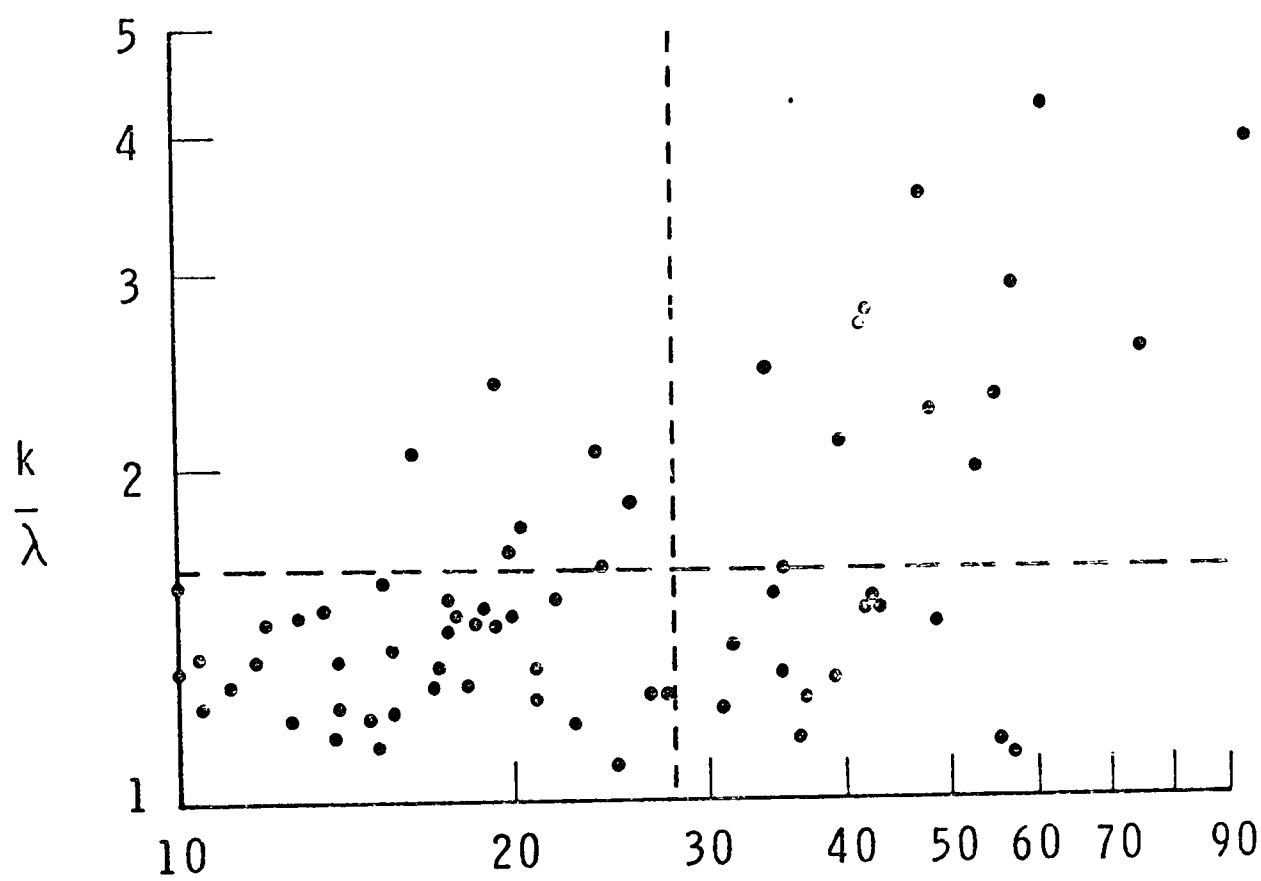


Figure 8,4 Scattergram of the CSF kappa/lambda ratio and the IgG/albumin quotient obtained for 68 patients with definite or suspected multiple sclerosis. A significant correlation exists between these values $r = 0.604$, $p < 0.001$. Note that six specimens with normal IgG/albumin quotients have elevated kappa/lambda ratios. The dashed lines indicate the upper limits for the neurological control group.

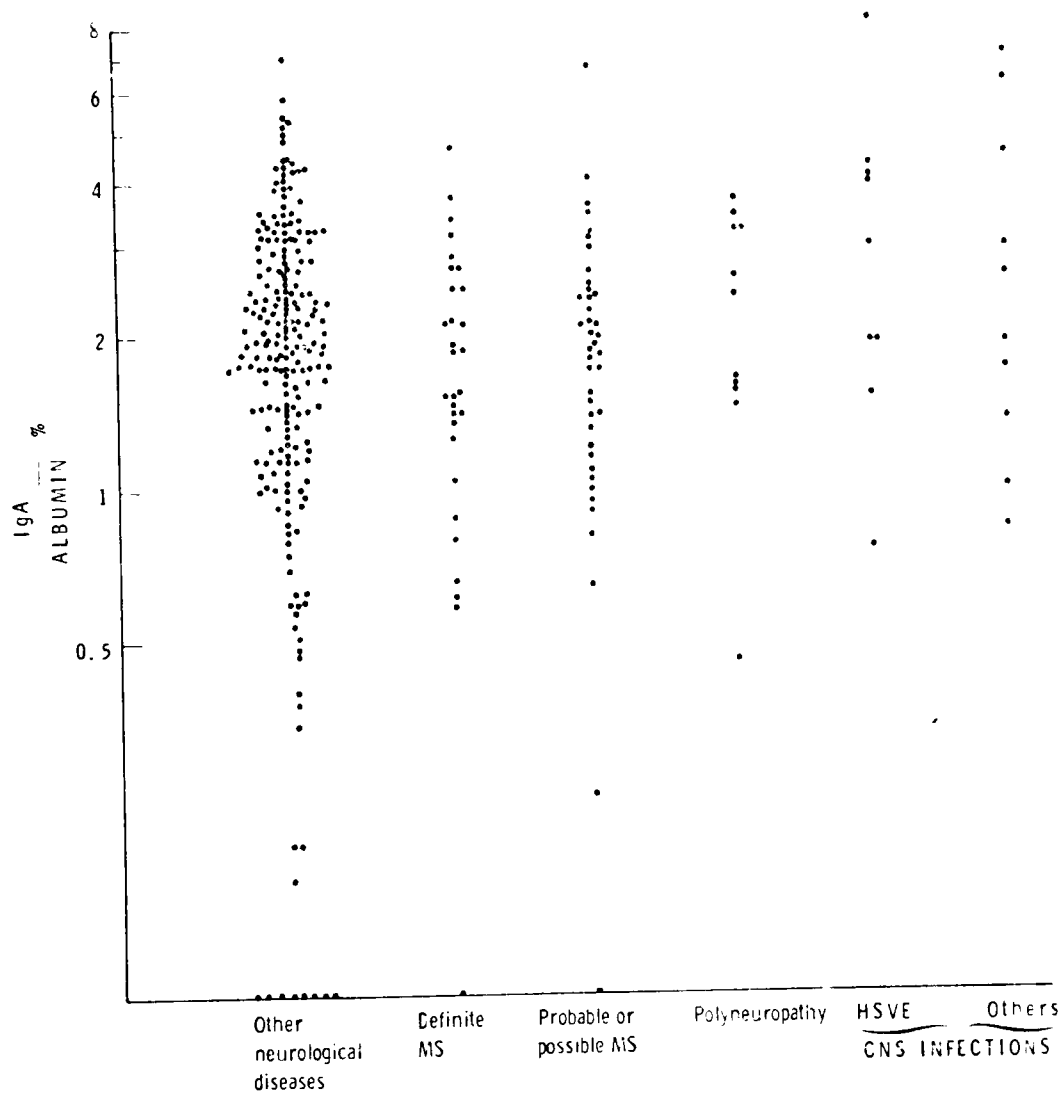


Figure 8,5 CSF IgA/albumin quotients obtained for the various diagnostic groups. These values imply that measurement of CSF IgA is of little value in distinguishing between the different groups.

Patient	Diagnosis	CSF total protein mg%	IgG/albumin %	IgA/albumin %	IgM i.u/ml	K/λ
1	HSVE	132	55	4.4	1.0	1.05
2	HSVE	175	66.1	4.04	0.1	1.51
3	HSVE	224	133	8.49	1.6	1.66
4	HSVE	319	17.3	1.53	0.15	1.51
5	Neurosyphylis	85	95.6	6.43	0.1	1.81
6	T.B. Meningitis	367	18.6	3.0	3.5	1.64
7	Polyneuropathy	570	20.3	3.3	1.0	-
8	Polyneuropathy	330	18.4	1.58	1.5	1.3

Table 8,1 Patients who had IgG, IgA and IgM detected in the CSF

Assessment of viral antibodies and a comparison with the CSF immunoglobulin quotients

A number of CSF and serum specimens were tested for antibodies against three viruses. These results will now be described and a comparison made with the corresponding CSF IgG/albumin quotient and the kappa/lambda ratio.

Antibody against measles virus

Thirty-three paired serum and CSF specimens from patients with MS or other neurological disorders and an additional 37 CSF specimens from MS patients and 10 from patients with other neurological disorders (and all of these 47 latter specimens had elevated IgG/albumin quotients) were examined for measles antibody.

The histogram of the serum measles antibody titre is shown in Fig. 8,6. The median for the serum measles titre for the MS group was significantly higher than that for the other neurological disease group; 1:64 compared with $1 < : 16$, $0.02 < P < 0.05$ (Wilcoxon sum of ranks statistical test). Seven of these MS CSF specimens had measles antibody with a titre varying from 1:8 to $1 : < 2$ (Fig. 8,6). Three specimens in the other neurological disease group also had antibody but with titres less than 1:2. In general patients with detectable CSF measles antibody also had high serum titres; this relationship and the mean age and sex ratio for both groups is shown in Fig. 8,6. The CSF total proteins were comparable in both groups.

From the 47 additional CSF specimens screened for measles antibody, four were found (all were patients with MS) to contain antibody and with a titre varying from 1:2 - 1:16. Two of these positive specimens also had

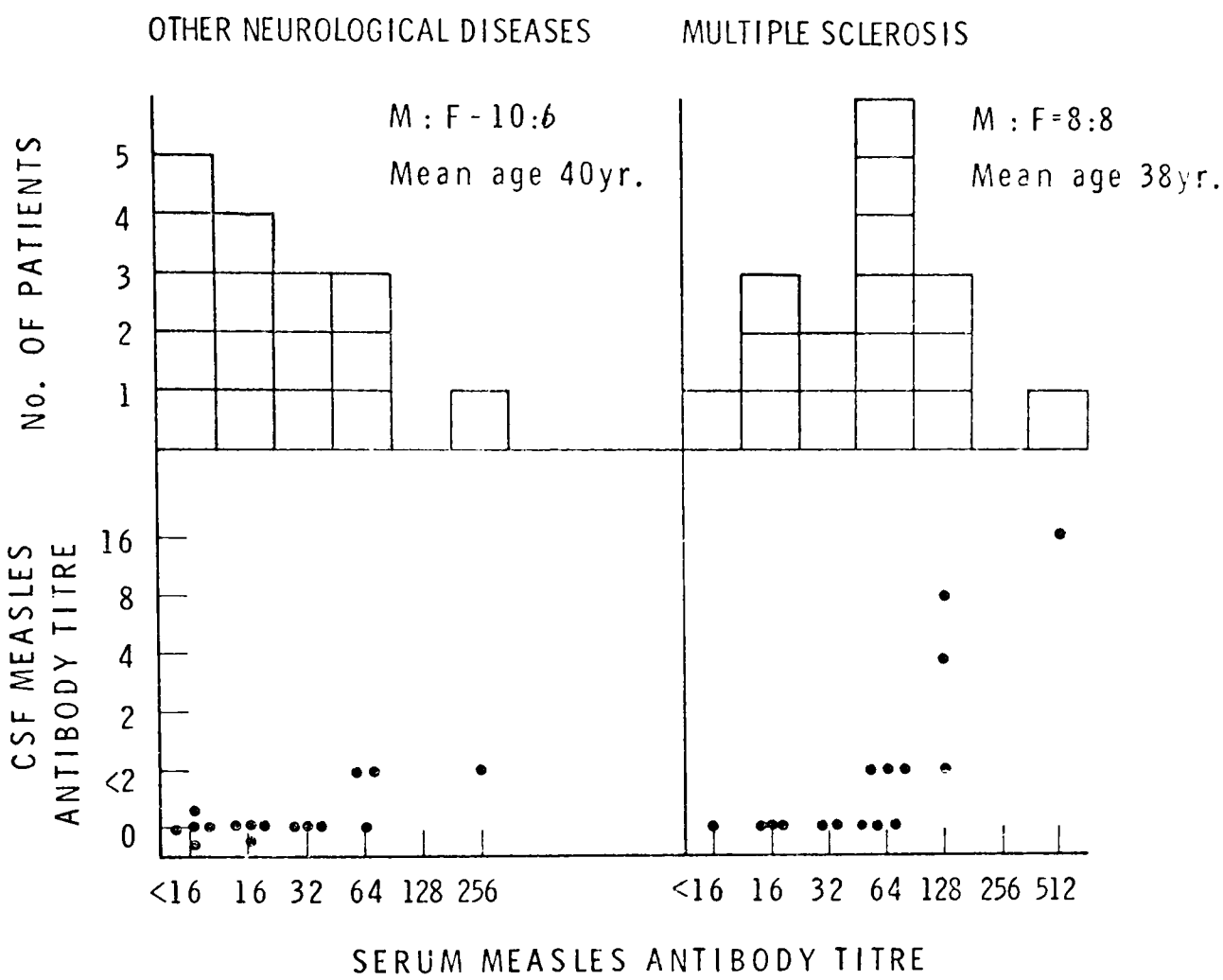


Figure 8,6 The histogram in the upper frames demonstrate the measles complement fixing antibody titre for patients with multiple sclerosis or other neurological diseases. In the lower frames the relationship between these CSF and serum titres is shown for these patients.

antibody against HSV (titre 1 : < 2) but in neither was the CSF total protein elevated and both had only slightly elevated IgG/albumin quotients (Table 8,II).

The presence of CSF measles antibody in the MS group was variably related to an elevated IgG/albumin quotient. Seven patients out of the 53 tested had detectable measles antibody associated with normal values for the IgG/albumin whereas only four patients with antibody showed an elevated IgG/albumin quotient, (Table 8,II). In particular two patients had high CSF titres of measles antibody (1 : 16, 1 : 4) and normal IgG/albumin quotients. The serum measles titre was 1 : 128 in the second patient (it was not measured in the first) and the CSF total protein was normal in the first patient and elevated to 105 mg% in the second.

The single patient with SSPE had a grossly abnormal CSF/serum measles titre ratio of 1 : 8 and a CSF total protein of 84 mg%.

Antibody against herpes simplex virus

The CSF and serum from all nine patients with HSVE and the CSF from 54 patients with MS and from 26 patients with other neurological disorders were tested for antibody against HSV.

Antibody against HSV was detected in the CSF in eight of the nine HSVE patients. In the antibody positive patients the ratio of the CSF/serum antibody titres varied between 1 : 1 to 1 : 16; grossly abnormal values (MacCallum et al., 1974). These viral studies have been reported elsewhere and will not be further discussed (MacCallum et al., 1974).

The IgG/albumin quotient was elevated in six of the antibody positive patients. In addition two of the remaining patients had

Patient	Age	Sex	Diagnosis	Cell count mm^{-3}	Total protein mg/%	IgG/albumin %	$\frac{CSF}{serum} / \frac{IgG}{albumin}$ %	K/λ	Viral antibody titre serum measles	CSF measles	CSF HSV	CSF mumps
1	31	M	definite	8	104	16.4	97	2.04	1:128	1:4	-	-
2	28	M	definite	10	42	18.9	74.4	1.5	1:64	1: <2	-	-
3	21	F	probable	8	58	23.9	90.6	2.08	1:128	1: <2	-	-
4	23	F	probable	8	51	15.1	39.7	1.12	1:64	1: <2	-	-
5	39	F	probable	5	56	19.4	ND	2.35	1:64	1: <2	-	-
6	54	F	probable	0	40	20.2	106	1.77	ND	1:16	-	ND
7	63	M	possible	ND	51	30.4	ND	1.21	ND	1:4	1: <2	ND
8	27	F	definite	14	43	31.8	ND	1.38	ND	1:4/8	1: <2	ND
9	42	F	probable	42	158	19.9	ND	1.69	ND	1:2	-	ND
10	55	F	definite	3	57.5	35	177	1.61	1:128	1:8	-	-
11	22	M	definite	83	43	42.8	189	1.5	1:512	1:16	-	-

Table 8,11 Patients with MS in which measles antibody was detected in the CSF

- = negative ND = not done

high normal quotients of 27.5% and 25.6% (see Fig. 8,1). In the former patient an abnormal total protein of 109 mg% was recorded while in the other the total protein was normal. The CSF/serum antibody titre was 1:16 in the former patient and it was not measured in the latter.

No antibody against HSV was detected in any of the CSF specimens from the neurological control group while two patients with MS had antibody against HSV (vide supra).

One patient with neurosyphylis showed a CSF antibody titre to both HSV (1:16) and to measles virus (1:4). The CSF total protein in this patient was 85 mg%, and 25% of this was IgG.

Antibody against mumps virus

The CSF from 17 patients with MS and from 16 patients with other neurological disorders was examined for mumps antibody. No antibody was detected in any of these specimens.

DISCUSSION

The results of this study have confirmed the original observation of Kabat and his colleagues (1948) and subsequent workers in that a relative rise in CSF IgG is frequently seen in demyelinating and infectious CNS disorders (see Schultze and Heremans, 1966; Chapter three for review).

Link and Müller (1971) in a recent comprehensive study noted a relative increase in CSF IgG in 73% of 64 patients with definite MS, 36% of 39 patients with CNS infections, and 16% of 81 patients with other

neurological disorders when compared with 30 'healthy' controls. However in clinical neurological practice it is often not so much a problem of distinguishing normal from abnormal but in distinguishing MS from non MS or CNS infections from non infections. I have therefore compared a group of patients with CNS infections, MS, or polyneuritis with a group comprised of patients with other neurological disorders. The results of this study have shown that measurements of the relative concentration of IgG in the CSF can be of diagnostic value and help in distinguishing MS and CNS infections from other neurological disorders.

It has also been shown that distribution of the values of the relative CSF IgG as assessed by the IgG/albumin quotient, obtained for patients with radiologically proven cervical spondylosis or lumbar disc herniation was similar to that obtained for the larger neurological control group. If this smaller group is taken as a 'normal' group, representing the situation seen in healthy individuals, this latter finding suggests that a relative increase in CSF IgG is uncommon in neurological disorders apart from MS and CNS infections. This conclusion is supported by other investigators who have found between 5.5 and 22% of patients with other neurological disorders show elevated values of CSF IgG quotients when compared with essentially healthy subjects (Harter et al., 1962; Riddock and Thompson, 1966; Schneck and Clamen, 1969; Link and Müller, 1971; Kabat et al., 1950). However it is difficult to compare or contrast individual studies due to the different diagnostic criteria and CSF quotients used in the assessment of the patients.

In many studies a small number of patients with presenile dementia, with or without extrapyramidal features, have been noted to show elevations

of the relative CSF IgG concentration (Yahr, Goldensohn and Kabat, 1954; Riddock and Thompson, 1970; Link and Müller, 1971; Skrabenek et al., 1973). In the present study two such patients were observed. The clinical features of one patient were non-specific whilst the pathological examination of the CNS in the second, showed only a cerebral infarct. In neither patient was the serum examined for the presence of a polyclonal or monoclonal increase in IgG which would also increase the CSF IgG/albumin quotient (Riddock and Thompson, 1966). It would be of interest to know whether such patients have undiagnosed MS, which occasionally gives rise to a presenile dementing process (McAlpine et al., 1972); a CNS infection such as HSV, which has been suggested to be a cause of early dementia (MacCallum, 1969); or whether these patients suffer from another disorder.

The IgG/albumin quotient was elevated in 53% of the patients with definite MS. This figure is slightly lower than those obtained in other studies but may be explained by the different control group used for comparison. The subject of CSF protein changes in MS has been critically reviewed by Tourtellotte (1970) and Link (1967).

Only 28% of the patients with suspected MS showed an elevated IgG/albumin quotient. The difference between this group and the definite MS group could be due to the inclusion in the former group of patients who will ultimately turn out to have another disease. But alternatively, since many of the CSF specimens were from patients suffering from an initial acute neurological episode, this could suggest that the IgG/albumin quotient may not be elevated in the early stages of MS to the same incidence as seen for established MS. Follow up and serial estimations of the IgG/albumin quotient

in these suspected MS patients will help to clarify this problem. However Glasner (1974) has noted a rise in the relative CSF IgG concentration in the first few months after the initial attack in the majority of 96 patients with MS. The relative IgG concentration appeared to stabilize after an interval of one year from the initial episode.

Taking both MS groups together in this study a significant correlation was observed between the IgG/albumin quotient and the CSF mononuclear leucocyte cell count, the duration of the disease and inversely with the CSF albumin level. However care is needed in the interpretation of these results since two selective groups have been incorporated for this comparison, and the possible or probable group may be a heterogeneous group. Taking the definite MS group alone the correlation between these variables did not reach significant levels.

Tourtellotte and others (see Tourtellotte, 1970 for review) have also observed a significant relationship between the CSF mononuclear cell count and the relative CSF IgG concentration but no correlation has been consistently observed with the duration of the disease or with the CSF albumin level (Yahr et al., 1954).

One third of the definite MS patients in this study had an elevated kappa/lambda ratio when compared with the neurological control group. This figure is a little lower than the 53% obtained by Link and Müller (1971) who compared 64 MS patients with a control group of 30 'healthy' subjects. These investigators also measured the same ratio in the serum and found no difference between the groups. They therefore conclude that the increase in the kappa/lambda ratio in MS CSF is probably due to a disproportionate increase

in the quantity of IgG molecules with light chain of type kappa. Why this should occur is not known.

A significant linear regression was observed in the present study between the CSF K/λ ratio and the IgG/albumin quotient for the MS group. More importantly, however, was the association in six of these patients of an abnormal K/λ ratio with a normal IgG/albumin quotient. For all other groups an abnormal K/λ ratio was associated with an elevated IgG/albumin quotient. This finding suggests that the determination of the K/λ ratio may be of occasional help in those patients with suspected MS who have normal IgG/albumin quotients.

Viral antibody studies were performed in many of the MS patients. The finding of an increased serum titre of measles antibody in a group of 16 patients with MS when compared with a group having other neurological disorders is in accord with previously reported studies (Adams and Imagawa, 1962; Haire, Fraser and Millar, 1973). Antibody to measles virus was detected in the CSF of seven of these patients and this could not be explained by blood/CSF barrier defects or unusually high serum titres. In contrast measles antibody was only detected in the CSF of 3/15 patients with other neurological disorders and all with low titres, and one of these patients had a high serum titre of 1:256.

Norrby, Link and Olsson (1974) have also studied measles titres in serum and CSF in 30 patients with definite MS and 30 patients with other neurological disorders. They found haemagglutination-inhibiting and/or nucleocapsid complement-fixing measles antibody in 13 MS CSF specimens in contrast to their neurological control group in which only two specimens were

positive. They also found a significant reduction in the serum/CSF ratio of these measles antibodies in 12 of the 30 MS patients, but did not see any reduction in their neurological disease group. These investigators conclude that antibody against measles virus is produced in the CNS of some MS patients.

In the present study the examination of 53 CSF specimens from patients with definite or suspected MS revealed antibody in 11. Seven of these antibody positive patients had a normal IgG/albumin quotient. In the study of Norrby et al. (1974) four of their MS patients had a normal IgG/total protein quotient and one of these had a significantly reduced serum/CSF ratio of measles antibody. Both these findings therefore suggest that some patients with MS may have CNS production of measles antibody without any elevation of the relative concentration of CSF IgG. The assessment of CSF measles antibody, or more importantly, the serum/CSF measles antibody titre, may be useful in detecting some patients with MS who have normal IgG/albumin quotients.

Rosenthal and Soothill (1962) have shown that the concentration of CSF IgG and albumin is dependent on the concentration of these proteins in the plasma (see Chapter seven). To account for variations in the levels of these plasma proteins the quotient CSF/serum IgG, expressed as a percentage of the CSF/serum albumin, was measured in MS and other neurological patients and compared with the IgG/albumin and IgG/total protein quotient. It was observed that an increased proportion of patients with definite or suspected MS had elevated values of this former quotient when compared with the latter two quotients. Furthermore, in two patients the IgG/albumin and IgG/total protein quotients were normal, but were associated with an elevated CSF/serum quotient and with an abnormal K/λ ratio and with detectable measles antibody

in the CSF. These findings suggested that this latter quotient is more discriminating in detecting patients with MS than the more generally used IgG/albumin or IgG/total protein quotients.

It was of particular interest that 6/9 patients with herpetic encephalitis had marked elevations of the IgG/albumin quotient. These six specimens of CSF were all obtained between the 12th - 200th day of the illness. Normal quotients were seen in one patient at the second day of the illness and in another who had survived for longer than one year. A rising quotient was also observed in one patient in the early stages of the disease and a falling quotient in another (over a five month period). These observations are reminiscent of the rise seen in the CSF titre of antibody to HSV in the first two weeks of the disease and the slow decline over the following months (MacCallum et al., 1974). This fluctuating change in HSVE may be contrasted with the stationary levels of the IgG/albumin quotient observed for three patients with definite MS who were studied sequentially over a 11 month period. I have not had the opportunity to follow serially the IgG/albumin quotients in either SSPE or neurosyphylis, but two treated patients with this latter disease had lower quotients than two untreated patients.

The relative increase in CSF IgG in HSVE has been previously noted by Welsum and Helm (1970), Rappel et al. (1971) and Link and Müller (1971). In the first study the CSF of five patients was studied by agar electrophoresis and immunoelectrophoresis. In three cases no electrophoretic abnormality was seen and in each case the illness was of short duration, death occurring within three weeks. The remaining two patients had a marked increase in the CSF gamma globulin with 3 - 5 distinct bands being observed in the gamma

region and increases in IgG and IgA being seen immunoelectrophoretically. In both cases the CSF was obtained between the 19th - 159th day of the illness. In one patient at day 68 the CSF gamma globulin composed 45% of the total protein.

Link and Müller (1971) studied the CSF from two patients with HSVE at days 19 and 65 after the onset of symptoms. CSF IgG/total protein quotients of 29.5 and 23.8% (normal < 10.5%) were found and in one, a relative increase in the CSF IgA was also seen. Both CSF specimens demonstrated a gamma oligoclonal pattern on electrophoresis but neither had abnormal K/λ ratios.

Elevation of the IgG/albumin quotient was also found in the present study in cases of neurosyphylis, SSPE, cerebral abscess and meningitis as has been previously described by others (Yahr et al., 1954; Link and Müller, 1971).

A relative increase in the CSF IgG has also been described in congenital neurosyphylis (Menninger, 1936) and in vaccinia encephalitis (Adams et al., 1973). Recently a patient with rubella panencephalitis was described in which rubella virus has been isolated from the CNS (Weil et al., 1975). In this patient, and four similar patients, large elevations of the relative CSF IgG were noted (Editorial, New England Journal of Medicine, 1975).

In all the CNS infections mentioned above the actual presence of an infectious agent in the CNS was documented or strongly implicated. This was in contrast to two cases of 'encephalitis' noted in this study in which no infectious agents were found, nor was the IgG/albumin quotient elevated. This suggests that different pathologic mechanisms may operate for these latter

types of "encephalopathies" in contrast to the infectious encephalidities.

In CNS infections there is some suggestive evidence that the relative concentration of CSF IgG (and IgM in trypanosomiasis) rises and falls according to the clinical course of the infection (vide supra). This is not the case in established MS where IgG remains at similar levels during remissions and exacerbations of the disease (Olsson and Link, 1973). It should be emphasized however that in both CNS infections and in MS much individual variation occurs to whether the CSF IgG rises, and to what degree. Other intrinsic factors must be of importance in these diseases and a study of the relationship between CSF immunoglobulin and the HLA phenotype might be rewarding.

Patients with polyneuropathies, including those with the characteristic features of the Guillain-Barré syndrome have been recorded in which a relative increase in the CSF IgG or gamma globulin has occurred (Harter et al., 1962; Laterre et al, 1970; Link, 1973). In general the frequency of occurrence is low and compares with the 18% seen in the present study. The nature or cause of the selective increase of CSF IgG is unknown. Link (1973) has also described monoclonal bands occurring in the serum of 8 out of 10 patients with the Guillain-Barré syndrome similar to that which was seen for one patient in the present study. The origin and function of this immunoglobulin is unknown.

The measurement of CSF IgA and IgM was not of great use in the comparison of the various groups. IgA and IgM were relatively increased in some patients with CNS infection but there were too few numbers in these groups to be able to decide whether this was a constant feature. It appeared that IgA and IgM were not included in the CNS antibody response seen in MS and this finding is in keeping with the conclusion of other investigators (Link

and Müller, 1971).

Five patients out of 16 tested with CNS infections showed an elevated CSF K/λ ratio while 2/5 patients with polyneuropathy also showed this increase. This is in contrast to the findings of Link and Müller (1971) where none of the 39 patients with CNS infections showed any change in this ratio. The present results show that alterations in the K/λ ratio are not specific for MS.

In conclusion it is considered that the estimation of the relative concentration of CSF immunoglobulin together with other methods of CSF immunoglobulin analysis (including K/λ ratio, viral antibody studies and electrophoresis) will supplement each other in the endeavour to detect pathological production of immunoglobulin in the CNS in certain neurological conditions.

CONCLUSION

A description has been given of cerebrospinal fluid (CSF) immunoglobulins in 355 patients with demyelinating, infectious, neuropathic and other neurological disorders. An increase in the CSF IgG/albumin quotient was observed in 19/36 (53%) cases of definite multiple sclerosis (MS), in 13/47 (28%) cases of probable or possible MS, in 6/9 (67%) cases of proven herpes simplex viral encephalitis (HSVE), in 3/4 (75%) cases of neurosyphylis, in 1/1 case of subacute sclerosing panencephalitis (SSPE), in 2/9 (22%) cases of other central nervous system infections and in 2/12 (18%) cases of polyneuritis when compared with the group of 236 patients having other neurological disorders. This group of other neurological disorders had only slightly higher

values of the IgG/Alb quotient than a subgroup of patients with structural defects of the spine.

In contrast a relative increase in the CSF IgA or IgM was only observed in a small number of these patients and apart from a relative increase of both these immunoglobulins in some patients with central nervous system infections no consistent pattern was apparent.

In previously reported studies several CSF immunoglobulin quotients have been described in an attempt to detect pathological production of immunoglobulin in the central nervous system; e.g. CSF IgG/total protein, CSF IgG/albumin, CSF K/λ ratio and CSF/serum titre of antibody.

It was found that the quotient CSF/serum IgG, expressed as a percentage of the CSF/serum albumin was more discriminatory in distinguishing patients with definite or suspected MS from those with other neurological disorders, than the quotients IgG/albumin or IgG/total protein.

The CSF K/λ ratio and the CSF and serum complement fixing antibody titre to measles and herpes simplex virus was measured in many of the patients. A varying relationship was observed between these values and the IgG/albumin quotient. An interesting finding was the occurrence of a normal IgG/albumin quotient in eight patients with definite or suspected MS and two patients with HSVE in association with an abnormal CSF titre of measles antibody (7 cases), herpes antibody (2 cases), or an abnormal CSF K/λ ratio (7 cases).

These findings suggest that the measurement of the relative concentration of CSF immunoglobulin in combination with the K/λ ratio and antibody titre to various viruses may supplement each other in the endeavour to detect central nervous system immunoglobulin production in neurological diseases.

CHAPTER NINE

GENERAL CONCLUSION

CHAPTER NINE

GENERAL CONCLUSION

In the introductory chapter of this thesis it was mentioned that there are several methods currently available for the routine examination of the soluble proteins of blood. These include the simple techniques of inspection of serum for its appearance, the measurement of the erythrocyte sedimentation rate and the detection of cryoglobulins. Other slightly more technical methods used are serum electrophoresis on cellulose acetate, agarose or other electrophoretic media, and immunoelectrophoresis. Both these electrophoretic methods separate serum proteins according to their charge and give useful information about quantitative and qualitative changes in these proteins. More precise quantitation of specific serum proteins is generally obtained using single radial immunodiffusion. By using these methods a vast amount of information has accumulated about serum proteins in health and disease (described briefly in Chapter one) and it is now apparent that the assessment of these proteins is vitally important in the diagnosis and management of many human diseases.

In this thesis two other analytical methods have been applied to the examination of serum proteins. These methods are gel filtration chromatography and SDSPAGE. The importance of these methods are several-fold. Firstly, and most importantly, they separate serum proteins according to their molecular size and, as such, differ from the traditional electrophoretic methods. Secondly, they are technically simple and require inexpensive apparatus and readily available materials. This is in contrast to the costly analytical ultracentrifuge. Thirdly they give good resolution of serum proteins (again in contrast to the

analytical ultracentrifuge). Finally the method of SDSPAGE is sensitive requiring only 10 μl of serum. Consequently the aim of this thesis was to examine these methods and see if they could be profitably applied to the examination of serum protein and, in particular, to use these methods in the assessment of the molecular size of certain serum proteins.

In Chapter two both these methods were described and normal serum was characterized. In addition, various pathological sera were examined and it was noted that, in some patients with multiple myeloma, immunoglobulin fragments were present in the circulation. In Chapter three the size heterogeneity of IgA paraproteins was clearly demonstrated and the clinical significance examined. It was concluded that both these methods gave useful clinical information which supplemented that obtained by standard analytical techniques.

As a result of these conclusions both methods have been included in the proformat of a prospective study of the serum and urine of myeloma patients in the Medical Research Council's myeloma trial and the results of these studies will be compared with the relevant clinical findings. It is hoped that the examination of the size of paraproteins (and their variation) will provide a further impetus to the understanding of this fascinating and common disease.

In Chapters five and six gel chromatography was used to detect high molecular weight IgG in the serum of patients with rheumatoid arthritis and cutaneous vasculitis. This high molecular weight IgG was not present in healthy controls or in the majority of patients with either other joint diseases or acute myeloid leukaemia. Further evidence using other biological assays

suggested that this high molecular weight IgG was present in the form of immune complexes and this conclusion was consistent with the literature. It was concluded that gel filtration chromatography was useful in detecting small circulating immune complexes containing IgG.

This method together with several others will now be used in a World Health Organisation collaborative study in which sera (collected from patients with suspected immune complex disorders) will be examined for immune complexes. The results using these different methods will be compared, with the object of defining the biological significance and clinical relevance of these complexes. In addition it is hoped that this study will contribute towards solving that urgent need in clinical medicine viz, the availability of a simple method for detecting circulating complexes.

In Chapter seven the method of SDSPAGE was used in an examination of the sieving effect of the blood/CSF barrier. It was concluded that this barrier could be simply assessed using SDSPAGE and, moreover, that this method will possibly be of value in assessing other molecular sieves such as the renal glomerulus.

From the examples given above it can be seen that the assessment of the molecular size of serum proteins can give additional information to that obtained using the current analytical methods and is useful in the diagnosis and management of certain disorders. Now that simple techniques are available, it is hoped that this assessment will be more frequently performed in the analysis of serum proteins.

The topic of Chapter eight deviated from the main theme. In this chapter an examination was undertaken of the CSF immunoglobulins in various

disorders. The CSF immunoglobulins of patients with multiple sclerosis, neurological infection or polyneuritis were compared with those of a control group consisting of patients with other neurological disorders. Many of the patients in the former diagnostic groups had relative increases in CSF IgG and this finding was considered to reflect a CNS production of IgG. This conclusion has been stressed in other studies and strongly enforces the view that the assessment of CSF IgG has important diagnostic relevance.

Appendix 1

SERUM PROTEINS IN RHEUMATOID ARTHRITIS

There have been several previous studies of serum proteins in rheumatoid arthritis (Pruzanski et al., 1973). In some rheumatoid patients depressed albumin levels are found and this may be associated with elevated levels of immunoglobulins and haptoglobin. However no consistent relationship has been found between these changes and other clinical and laboratory features of the disease (Pruzanski et al., 1973).

In the present study the serum proteins of 35 patients with rheumatoid arthritis have been studied by Sepharose 6B chromatography, and SDSPAGE, and six specific proteins have been quantitated using single radial immunodiffusion. This study was performed on the same patients as described in Chapter five and forms an addendum to that study.

Patients and Controls

The patients with rheumatoid arthritis have been previously described (see Chapter five). For comparative purposes two control groups were studied. The first group consisted of nine patients with other joint diseases (also described in Chapter five) and the second of 26 healthy laboratory subjects (Chapter two).

Methods

Sepharose 6B chromatography, SDSPAGE and single radial immunodiffusion have been previously described (Chapter two). Haptoglobin typing was

performed in 5% polyacrylamide gels according to the electrophoretic method of Ferris et al. (1963). In this method haemoglobin was added to serum to form haemoglobin/haptoglobin complexes. After electrophoresis the complexes were stained with a benzidine - peroxide reagent and this resulted in a characteristic pattern. This pattern belonged to one of three different haptoglobin types, Hp 1-1; Hp 2-1 or Hp 2-2 (see Chapter two).

Results

Gel filtration chromatography: The optical density elution profile (at 280 mμ) of the rheumatoid sera differed from the normal sera in that they showed a significantly greater absorption in the region between the void volume and the IgG marker (measured at two constant elution positions, $p < 0.005$ see Fig. 2,1). The major eluting peak and the final eluting peak of the rheumatoid sera were not significantly different from those of the controls. The elution profiles of the other joint disease group were of normal appearance.

SDSPAGE: The most striking difference between the rheumatoid and control sera was the prominence of the haptoglobin bands in the gels (Figure 2,5; Chapter two). In addition C_3 and IgM were particularly obvious in several of the rheumatoid sera. However no additional bands were detected in any of the pathological sera when compared with the sera obtained from healthy subjects.

Haptoglobin typing: Three of the rheumatoid sera belonged to the Hp 1-1 type, twenty were of the Hp 2-1 type and the remainder were of Hp 2-2 type (Appendix II). All haptoglobin typing obtained using the standard

benzidine method agreed with the SDSPAGE typing (see Chapter two). However in five sera haptoglobin bands were not detected in the SDSPAGE electrophoretogram due to low levels of haptoglobin and therefore it was not possible to type these sera using SDSPAGE. The electrophoretic mobilities of the various haptoglobin members in 3.6% SDSPAGE are shown in Fig. 2,6 (Chapter two).

Single radial immunodiffusion: Six proteins were measured and the results are shown graphically in Figures 1 and 2 and are tabulated in Appendix II. Six out of 35 rheumatoid patients showed a significantly elevated IgG and haptoglobin level, 13/35 showed an elevated IgA level, and 2/35 showed an elevated IgM level when compared with healthy controls. No rheumatoid patient showed a significantly depressed albumin level although the mean for the rheumatoid group was less than the mean for the control group (Fig. 2).

Clinical correlation: The values obtained for these six proteins were compared with several clinical and laboratory indices used to assess the rheumatoid disease. These results are described in Chapter five and are tabulated in Table 5,II. A significant correlation was seen between IgA and IgM levels and the age of the patient, and between IgG levels and those patients on steroids. The haptoglobin level correlated significantly with the erythrocyte sedimentation rate.

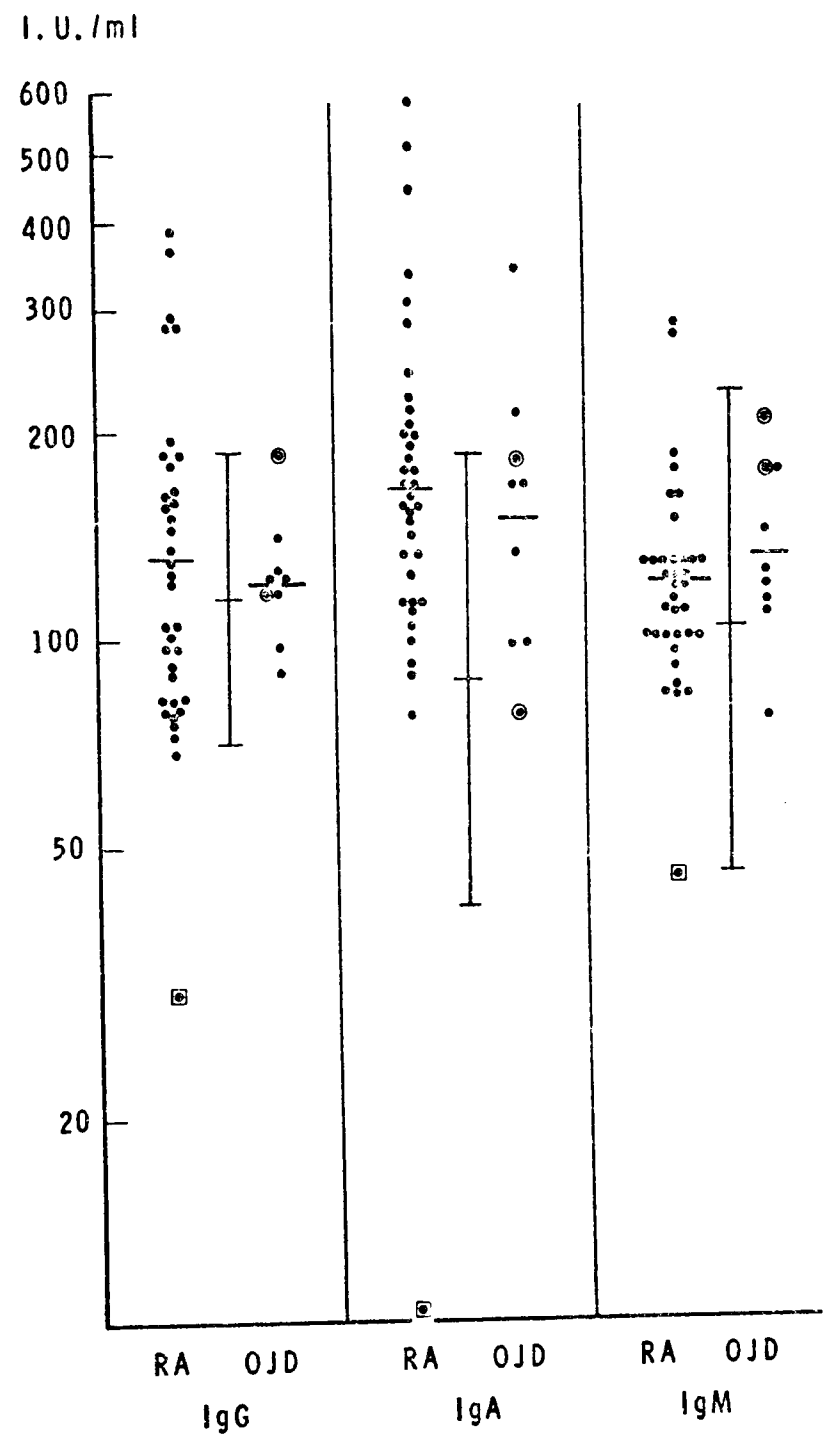


Figure 1 The immunoglobulin levels obtained for 35 patients with rheumatoid arthritis (RA) and for nine patients with other joint diseases (OJD). The mean $\pm$ 2 SD of the values obtained for 26 healthy laboratory subjects is indicated by the vertical line. One rheumatoid patient (boxed circle) was found later to have multiple myeloma and was therefore eliminated from the study. Two patients with ankylosing spondylitis have been encircled.

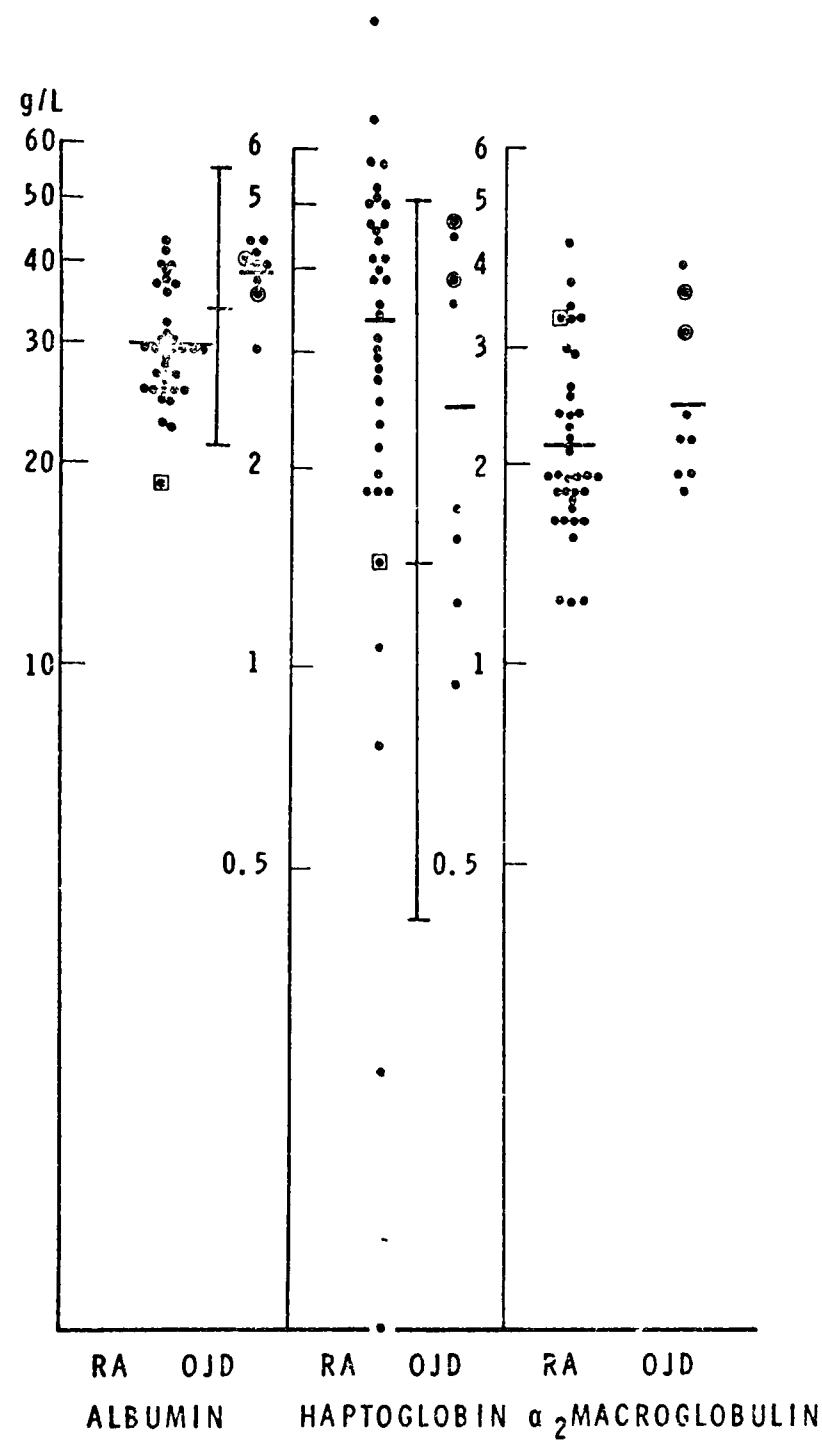


Figure 2 Protein levels of albumin, haptoglobin and α_2 macroglobulin obtained for patients and controls. For explanation of the captions see Figure 1

Discussion

A study of the serum proteins in 35 patients with rheumatoid arthritis has shown an elevation of IgG, IgA, IgM and haptoglobin, either singly or in combination, in many of these patients when compared with a group of 26 healthy laboratory subjects. These results are consistent with those obtained by other investigators and confirm that serum protein changes are a feature of rheumatoid arthritis (Pruzanski et al., 1973).

In the present study the significant elevation of the optical density profile in the region between the void volume and the IgG marker was explained by the elevation of the proteins which elute in this region, viz, IgA, haptoglobin and IgM. This was also evident after SDSPAGE where haptoglobin bands were particularly prominent in many of the patients, and for patients with haptoglobin type Hp 2-1 and 2-2 migrated between IgG and IgM.

It was necessary to determine the haptoglobin type of these patients in order to quantitate haptoglobin levels using single radial immunodiffusion. An excellent agreement in the haptoglobin type was found between the traditional method using benzidine stain and the electrophoretic mobility of haptoglobin members in 3.6% SDSPAGE. This suggests that SDSPAGE can be used with confidence, in haptoglobin typing, provided that haptoglobin bands are visible in the gels.

Two patients had very low levels of haptoglobin (Fig. 2). This could suggest that these patients were suffering from a chronic haemolytic process but alternatively other mechanisms could be involved (Pintera, 1971). The explanation for these low levels was not further explored.

No significant correlation was found between the protein changes

observed in this study and with the clinical and laboratory indices used to assess the disease. This lack of correlation has also been obtained by other investigators (Pruzanski et al., 1973), and suggests that the measurement of these proteins in rheumatoid arthritis has limited diagnostic relevance.

Appendix II

Patient	Age (years)	Sex	Duration (years)	Clinical activity	Morning stiffness	ESR (mm/hour)	Latex titre	Haemoglobin g%	White cell count -3 mm
1	30	F	16	40	30	40	-	10.9	9,400
2	47	F	7	42	30	22	4096	14.2	8,400
3	62	F	2	53	120	88	2048	10.7	8,400
4	14	M	0.1	0	0	16	-	13.9	8,900
5	75	F	8	44	180	55	256	11.8	6,900
6	66	F	27	80	30	33	1024	12.4	10,600
7	63	F	2	79	30	70	-	10.5	4,900
8	61	M	8	22	120	16	512	10.7	7,700
9	62	F	5	43	30	78	64	10.4	6,000
0	68	F	11	50	30	60	256	11.2	6,500
1	64	F	8	12	30	14	256	15.6	7,800
2	61	M	9	9	10	19	-	13.6	7,500
3	43	F	6	29	60	21	128	12.6	12,600
4	40	F	0.7	66	360	38	-	12.7	7,900
5	62	F	30	23	60	26	-	10.7	5,200
6	44	F	9	30	30	50	128	11.9	12,100
7	56	M	0.5	39	120	21	2	12	13,100
8	50	F	0.3	49	180	45	32	11.3	6,500
9	60	F	5	71	60	35	1024	11.5	6,100
0	59	F	12	45	0	53	4	12.1	7,200
1	63	F	11	78	120	47	2048	11.7	7,400
2	49	F	12	33	10	50	512	11.6	6,700
3	69	F	4	64	120	62	512	12	8,600
4	53	F	12	91	20	34	4096	14.4	9,300
5	64	F	32	63	30	59	512	12.4	5,600
6	59	F	43	32	120	63	512	11.6	5,400
7	37	F	7.5	21	10	32	-	10.6	5,100
8	66	M	3	52	60	97	64	6.8	7,300
9	70	F	30	54	15	3	64	15.2	8,400
0	49	F	9	36	30	11	256	13.2	3,700
1	52	F	6	83	30	47	256	11.6	10,400
2	77	F	27	41	10	104	32	9.7	4,500
3	49	M	10	64	120	56	-	11.5	7,000
4	60	F	10	39	30	34	32	13.7	5,600
5	48	F	8	80	60	65	4	11.0	5,600

Appendix II

Clinical and laboratory data obtained for 35 patients with rheumatoid arthritis (see Chapter four)

IgG i.u/ml	IgA i.u/ml	IgM i.u/ml	Albumin g%	alpha-2-macroglobulin mg %	Haptoglobin mg %	% inhibition	IgG (c) mls	Monomer : dimer ratio	Chemotactic Index	Haptoglobin type
145	115	84	3.0	183	418	45	120.1	9.4	418	2-1
79	133	101	2.8	183	184	79	122.5	4.8	ND	2-1
162	207	163	3.0	348	318	32	118	8.2	ND	1-1
91	174	130	3.9	429	504	19	123.2	5.8	ND	2-1
75	310	151	2.8	177	184	60	125.4	5.2	328	2-2
97	158	115	3.3	183	358	100	119.5	5.0	498	2-1
154	175	84	2.6	192	577	100	124.9	8.3	501	2-1
68	150	130	3.0	165	4	ND	118.5	6.6	225	2-2
186	78	101	3.0	192	459	63	116.5	6.8	ND	2-2
366	199	130	2.3	237	470	28	108.5	7.9	560	2-1
180	133	170	4.3	264	219	ND	118	7.6	ND	2-1
72	126	101	3.0	123	378	87	111.2	5.6	ND	2-1
81	99	125	3.8	333	278	ND	112.3	3.1	ND	1-1
97	91	96	2.7	294	251	32	105.2	5.9	318	2-1
283	450	188	3.0	378	199	71	109.4	2.4	166	2-1
79	106	120	2.9	255	285	100	116.5	4.0	ND	2-1
186	192	163	2.6	123	530	38	114.4	13	482	2-1
158	153	110	4.0	192	577	16	123.2	4.4	368	2-1
81	157	92	2.6	192	413	20	104	4.9	493	2-2
101	115	84	3.0	219	451	87	98	4.1	ND	2-2
151	523	130	2.6	106	390	31	109	8.4	462	2-2
384	160	101	2.8	183	184	47	121.5	6.2	557	1-1
77	600	130	2.7	165	466	100	120	4.9	ND	2-2
81	290	110	2.6	210	306	51	117.6	6.5	440	2-2
89	199	130	2.5	123	239	56	116	5.1	ND	2-1
166	111	130	3.3	294	503	47	108.1	9.5	438	2-1
194	115	101	3.0	192	298	3	120.5	6.9	601	2-1
293	168	280	2.3	192	933	38	111	3.7	ND	2-2
103	218	120	4.0	237	107	72	120	3.7	252	2-2
103	245	290	3.0	165	76	100	121	5.2	456	2-2
283	340	125	3.8	333	25	71	102.4	3.6	396	2-2
125	168	110	2.6	264	517	96	110	6.4	355	2-1
135	183	101	3.7	165	664	42	118.2	9.4	ND	2-1
130	220	125	4.3	237	391	18	120.1	7.3	445	2-1
121	141	84	3.0	174	345	66	105.6	7.1	ND	2-1

\- negative
ND not done

Patient	Age	Sex	Diagnosis	Duration (years)	Clinical activity	Morning stiffness	ESR (mm/hour)	Latex titre	Haemoglobin g%	White cell count $\frac{}{mm^3}$	IgG i.u./ml	IgA i.u./ml	IgM i.u./ml	Albumin g %	α_2 macroglobulin mg %	haptoglobin mg %	% inhibition	IgG (c) mb	monomer:dimer ratio
1	79	F	OA	15	44	90	71	-	11.8	6,700	89	168	110	3.0	219	444	4	115.6	7.2
2	70	F	OA	10	7	180	.5	-	14.1	5,600	121	99	115	4.3	393	126	28	124.3	12.2
3	75	F	OA	2	-	-	8	-	13.9	6,300	97	168	125	3.8	192	172	86	123.9	11.1
4	68	F	OA	2	8	-	28	-	12.5	7,800	121	133	120	4.3	237	375	81	126.5	16.3
5	72	F	OA	8	4	-	20	-	12.2	8,900	141	349	77	4.0	318	358	20	114.4	9.6
6	64	F	OA	2	-	-	4	-	12.6	7,000	116	214	146	4.1	219	159	77	116.7	3.3
7	54	M	OA	8	-	-	23	-	15.5	9,200	125	99	177	4.0	183	95	35	127.5	8.9
8	29	M	AS	7	25	120	43	-	12.1	3,600	116	183	177	4.1	357	384	40	125.4	4.9
9	33	M	AS	5	3	-	53	-	12.2	4,800	186	78	210	3.6	192	470	48	116.7	4.7

Appendix II

Clinical and laboratory data obtained for nine patients with other joint diseases (see Chapter four)

AS = ankylosing spondylitis, OA = osteoarthritis

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