LOW MOLECULAR WEIGHT IgM IN HEALTH AND DISEASE

by

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A thesis submitted for the degree of

Doctor of Medicine,

University of Adelaide

From the Department of Medicine,

University of Adelaide,

December, 1987

Awarded : March 1, 1988.

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STATEMENT OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, contains no material previously published or written by another person except where due reference is made in the text of the thesis.

Peter John Roberts-Thomson

ACKNOWLEDGEMENTS

A number of people have given me valuable assistance during the candidature of this thesis. To Prof. A. Wangel, Dept. of Medicine, Queen Elizabeth Hospital, who acted as my supervisor and to Prof. J. Bradley, Dept. of Clinical Immunology, Flinders Medical Centre, who supported and encouraged me constantly during this project I give my special thanks. I am grateful for the advice and help from Prof. Morris Ziff, Rheumatic Diseases Unit, University of Texas, South Western Medical School, with whom I spent one year during my tenure as a Fulbright Scholar. I am also grateful to the Fulbright Foundation for their financial support during this period. Two medical students, Neil Jones and Richard Harries spent elective periods working on this project and learnt and performed several of the technical procedures. Many members of the Dept. of Clinical Immunology, Flinders Medical Centre contributed to useful discussions and gave technical assistance. Special thanks to Dr. S. Neoh, Dr. I. Beckman, Mrs. L. Koh, Ms. M. Thomas and Misses A. Kennedy and K. Shepherd. Dr. A. Kwitko donated many of the sera from patients with selective IgA deficiency and supplied the relevant clinical and serological data concerning these patients. Dr. R. Wernick and Mr. D. Edelbaum performed the radioimmunoassay measurements for IgM rheumatoid factor activity described in Chapter three. Mrs. M. Molnar, Mrs. W. Graham and Ms. D. Autio provided valuable secretarial help. Thanks to Mrs. M Brown and Miss. B. Lord for typing the thesis. The Medical Illustration Department, Flinders Medical Centre, produced many of the illustrations and prints used in this thesis.

Finally, I express my appreciation to my family in supporting me at all times in my various clinical and research activities. I dedicate this thesis to them - my parents, my wife Lesley, and my daughters Rachel, Kate and Alice.

ABSTRACT

This thesis examines the presence and role of low molecular weight (LMW) IgM in health and disease. LMW IgM is the naturally occurring monomeric subunit of pentameric IgM and has been previously observed in the blood from patients suffering from a variety of disorders but rarely in health.

In Chapter one there is a general description of the known physicochemical properties, function and role of both pentameric IgM and LMW IgM. Possible theories for the presence of LMW IgM in human disease are briefly discussed.

In Chapter two a description is given of three sensitive methods to detect and quantitate LMW IgM. One of these, viz immunoblotting, appears both sensitive and specific for LMW IgM and has revealed for the first time additional oligomers of IgM in sera containing LMW IgM.

In Chapter three sera from healthy controls and cord blood were examined for the presence of LMW IgM. This moiety was not found in sera from healthy subjects but was observed in low levels in a minority of cord sera.

Chapter four details a study of LMW IgM in sera and synovial fluid from patients with a variety of rheumatic disorders. In rheumatoid arthritis, 80% of the patients were found to have circulating LMW IgM and its levels correlated significantly with absolute IgM levels (measured nephelometrically) and with levels of rheumatoid factor and

circulating immune complexes. Separated column fractions containing LMW IgM were observed to contain IgM rheumatoid factor activity.

In Chapter five peripheral blood mononuclear cells taken from patients with active rheumatoid arthritis were found to secrete considerable quantities of LMW IgM in vitro. This did not occur with cells obtained from healthy controls. A significant correlation was found between the percentage of circulating LMW IgM and with the percentage of LMW IgM secreted in vitro. No evidence was obtained to suggest that LMW IgM occurred as a consequence of proteolytic breakdown of pentameric IgM.

In Chapters six, seven, eight and nine LMW IgM was observed in a varying proportion of patients suffering from infective endocarditis, mixed cryoglobulinaemia, selective IgA deficiency and in malignant B cell lymphoproliferative disorders but not in benign macroglobulinaemia. In 3 patients with mixed cryoglobulinaemia the LMW IgM was monoclonal and of the same light chain type (kappa) as the monoclonal pentameric IgM rheumatoid factor suggesting a common clonal origin.

In Chapter ten there is a brief discussion concerning the most likely explanations for the occurrence of LMW IgM in human disease and its possible role in the pathogenesis of these disorders. It is concluded that it is highly likely that LMW IgM has a pathogenic role in human disease. Further studies concerning this long neglected immunoglobulin are indicated as there is a distinct possibility that therapeutically reverting the disorded monomeric IgM humoral response

to a normal pentameric IgM response may result in resolution or amelioration of the disease.

Finally, the findings described in this thesis and from other observations are best accounted by postulating a defect in the assembly of the monomeric IgM subunits during pentameric IgM synthesis and secretion. Possible defects are discussed together with avenues of exploring such defects in future studies.

CHAPTER ONE

GENERAL INTRODUCTION

PENTAMERIC IgM

Introduction: A characteristic feature of the human humoral immune response is the production of specific immunoglobulin (antibody) to the eliciting immunogen (antigen). The predominant immunoglobulin observed in the initial sensitization or primary response is IgM whilst on subsequent exposures to the antigen IgG tends to dominate with the IgM response being less pronounced (secondary or anamnestic response). IgM is a polymerized macroimmunoglobulin present in all species of the vertebrate kingdom and its vital importance in host defences is illustrated by the observation that those rare individuals who selectively lack this immunogobulin are at great risk from overwhelming infections (Hobbs et al, 1967; Asherson and Webster, 1980).

Physicochemical Properties of IgM: (No attempt will be made to comprehensively review this extensive topic. For further information the reader is referred to the excellent discourses by Putman (1977); and Stanworth and Turner, (1978)). Human IgM is a cyclic planar pentameric molecule consisting of 5 covalently bound monomeric subunits associated with a single J (Joining) polypeptide chain. The sedimentation constant of this molecular complex is approximately $19S_{20W}$ and it has a molecular weight of approximately 970,000 daltons. Each monomeric subunit consists of 2 identical heavy (H) chains of μ chain specificity and 2 identical light (L) chains of either kappa or lambda type. Disulphide covalent bonds link H chain to H chain and H chain to L chain. The molecular weight of the H chain is approximately 65,000-70,000 daltons and the L chain approximately

25,000 daltons. A schematic representation of the pentameric molecule is shown in Figure 1. Each H chain has five domains (compact globular structures consisting of homology regions and can be considered as a functional unit deriving from the duplication of the primordial immunoglobulin gene) while each L chain has two domains. The first domains of the H and L chain are involved in antigen binding and are unique to each individual IgM molecule while the 4 remaining domains of the H chain (Cμ) are constant to all IgM molecules. The L chain is linked to the H chain by a disulphide bond attaching to a site between the first and second domains. Similarly a single intrachain disulphide bridge linking each H chain is located between Cμ2 and Cμ3 while a similar interchain bond linking adjacent monomeric subunit is found between the third Cμ. The J chain (molecular weight of approximately 15,000 daltons) is attached covalently to the penultimate cysteine residue of the Cμ4 of two H chains from adjacent subunits and there is one J chain per polymeric molecule. The fully assembled pentamer has a planar stellate structure when viewed in the electron microscope with the antigen binding regions of the subunits arranged around the periphery of the molecule (Figure 1). IgM is rich in carbohydrate (12% by weight) having five oligosaccharide groups on each H chain. These oligosaccharides are of two types, simple and complex. Both types contain glucosamine and the complex side chain terminates in sialic acid. The function of the oligosaccharides is unclear, perhaps increasing the solubility of the molecule and influencing its conformation.

IgM makes up approximately 10% of the circulating immunoglobulin pool with healthy adult serum levels varying between 0.4 to 2.2g/l

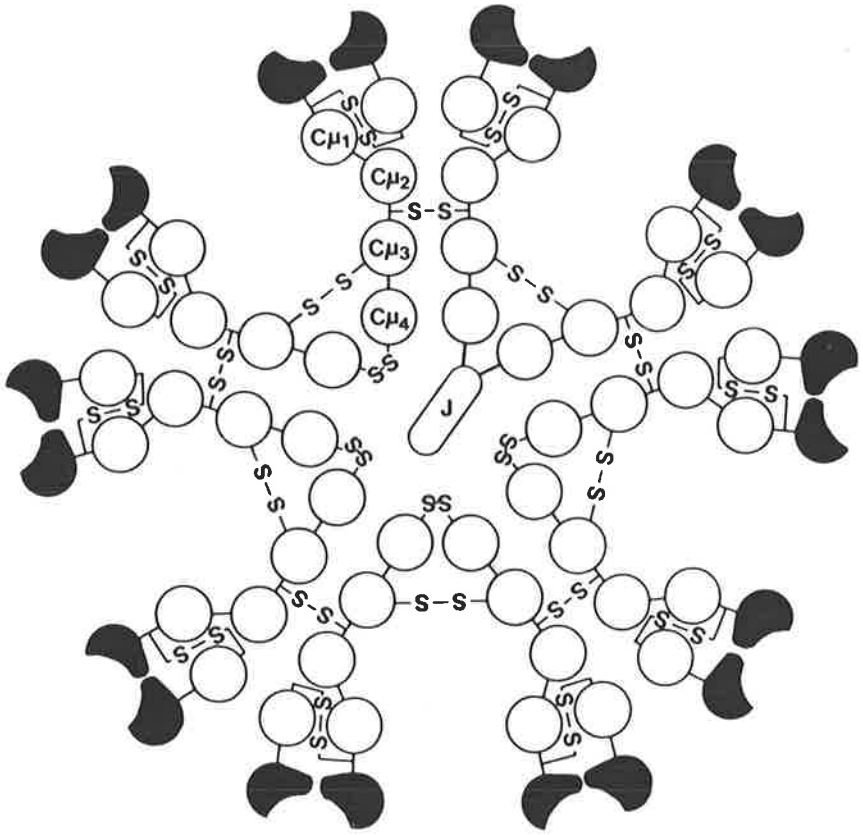

Figure 1. Schematic representation of the structure of pentameric IgM (after Stanworth and Turner, 1978). The constant homology regions in the heavy chains are designated Cμ. The light chains are covalently bound to the heavy chains by disulphide covalent bonds while disulphide bonds link Cμ3 of adjacent monomeric subunits. There is some uncertainty whether the disulphide bonds involving the penultimate cysteine residue of the heavy chain links adjacent heavy chains within each monomeric subunit or between two subunits (as demonstrated in the figure; Percy and Percy, 1975). J represents the single joining polypeptide chain linking two adjacent sub units (one J chain per polymeric molecule).

(levels are slightly higher in the female gender). Eighty percent of IgM is found in the vascular compartment. Its half life is 5-10 days and approximately 9% of the intravascular pool is catabolized per day. IgM has a synthetic rate of 3.3mg/kg/day. Electrophoretically IgM generally has a fast gamma, slow beta migratory pattern.

Biological Activities of IgM: (See Spiegelberg, 1974) Serum IgM is a polymeric immunoglobulin and, us such, it clearly illustrates the importance of polymerization as a means of expanding and improving the functions of antibodies during the course of evolution. It has already been mentioned that each heavy chain domain subserves specific functions with some modulation and amplification by interactions between neighbouring domains on the same and adjacent chains. Hence 'polymerization' of the antigen binding domains as in the IgM pentamer leads to a molecular complex which has the capacity of multivalent interactions with antigen. This leads to the formation of a strong binding interaction with the antigen. Pentameric IgM has a valency of 10 (=10 antigen binding sites) when tested with antigens of molecular weight less than 3000 daltons but a lower valency for larger antigens (valence is generally 5 for large antigens). This phenomenon is probably explicable on the steric hindrance which occurs at the antigen binding sites by large antigens; IgM tending to have a planer configuration with limited flexibility (in contrast with IgG).

As a multivalent immunoglobulin IgM is an extremely efficient agglutinating antibody and a potent activator of the classical complement pathway. This latter property results in immune lysis or immune adherence to phagocytic cells (via iC3b receptor). These

properties of strong agglutination and complement fixation together with the predominant intravascular distribution of IgM suggests that IgM is vitally important in eliminating particulate antigens and micro-organisms from the blood stream. Pentameric IgM does not cross the placenta but is found in external secretions in association with the epitheial binding and transport protein "secretory component".

Biosynthesis and Secretion of Secretory IgM: (See Chapius and Koshland, 1974; Putnam, 1977; Tartakoff and Vassalli, 1979; Roth and Koshland, 1981; Koshland, 1985; Hendershot et al, 1987). Despite some gaps in knowledge and some uncertainties, the biosynthesis and secretion of IgM is thought to proceed according to the following sequence. Activation and transcription of the genes encoding for μ, L and J chain occurs within the nucleus with the eventual formation and processing of the respective messenger RNA. Light chain protein synthesis occurs on small polysomes (190S) and heavy chain synthesis on larger polysomes (270S). These ribosomes are membrane bound and are asssociated with the cytoplasmic rough endoplasmic reticulum. Covalent assembly of μ and L chain occurs early, probably involving a half-subunit intermediate (HL) with subsequent formation of the monomeric μ_2L_2 moiety. The heavy chain binding protein (BiP) may regulate an orderly subunit assembly. This subunit assembly commences on the μ-chain polysome, is completed in the cisternae of the endoplasmic reticulum and is critically dependent on the disulphide interchange enzyme which links H to L and then H to H chains. Glycosylation of the μ chain commences while the μ chains is still on the microsome and is completed in the Golgi complex. From this complex the molecule is transferred to small secretory vesicles which

expel their contained product into the interstitial space by reverse pinocytosis. The intracellular site and mechanism for the assembly of the pentameric IgM molecule is still unclear. Polymerization appears to be dependent on both the presence of J chain which may initiate or facilitate polymerization and a membrane bound sulfhydryl oxidase which has the specific function of cross-linking monomer and J chains sulfhydryls. Absence of J chain is associated either with a non secretory state or with the secretion of limited quantities of monomeric IgM. Furthermore the fusion of J chain producing IgG-secreting or non secretory myeloma cells with B-cell non secreting lymphoma cells results in a hybrid capable of secreting pentameric IgM (i.e. as demonstrated in the process of complementation). In addition, further observations on the function of the J chain suggests that it plays a vital part in the process of secretion of IgM as this appears to be dependent on the presence of a polymeric molecule. Polymerization occurs rapidly and in man there is some evidence although somewhat dependent on which cell line is being studied, to suggest that it occurs in the rough endoplasmic reticulum well before secretion. In contrast, in studies utilizing IgM murine myelomas, polymerization appears to be a very late event occurring at or just prior to secretion. From the above sequence, although incomplete in some details, it is seen that the biosynthesis, assembly and secretion of pentameric IgM is a complex sequence of events involving many steps. Secretion of incompletely assembled subunits of IgM implies imbalance or defects in one or several of these steps and the detection and clinical associations of these secreted subunits in human disease underlies the central theme of this thesis.

Phylogenetic Antiquity of IgM: An IgM like immunoglobulin is present throughout the vertebrate kingdom and comparative immunologists suggest that it is phylogenetically the most primitive. (See Marchalonis, 1976; Benedict, 1979) This immunoglobulin is generally polymeric consisting of (2),4 or 5 monomeric subunits each subunit conforming with the structural formulae H_2L_2 (where the H chain has close antigenic and structural similarities with the mammalian μ chain) and its main distribution is the vascular compartment (Small et al, 1970; Kobayashi et al, 1982). However, in the lower vertebrates, such as the agnathans (e.g. lamprey); elasmobranchs (e.g. sharks) and bony fish the IgM like immunoglobulin also occurs in the monomeric form (together with its polymer), and not only is it distributed extravascularly and intravascularly as is the IgG in higher species, it also frequently constitutes most of the immunoglobulin present in that species (Marchalonis and Edelman, 1965; Clem and Small, 1967). Furthermore passive administration of these radiolabelled purified proteins to the homologous species reveal that the serum monomeric IgM is neither a precursor nor a degradation product of the pentameric IgM. As one ascends the vertebrate phylogenetic tree, three observations relevant to this discussion can be made. Firstly, that the predominant presence of this monomeric IgM moiety disappears although small qualities of monomeric IgM maybe observed in some species although uncommonly (Marchalonis, 1969; Marchalonis et al, 1969; Steward et al, 1969; Lebacq-Verheyden et al, 1974; Ramadass and Moriarty 1982; Warr 1983). Secondly, that in more developed vertebrates such as the lung fish or higher species additional immunoglobulin classes are found. This diversity of immunoglobulin classes reaches its apex in the mammalian kingdom (Marchalonis, 1969;

Chartrand et al, 1971; Marchalonis, 1976). Thirdly, that is certain disorders of the species e.g. infection or lymphoid neoplasia, reappearance of the monomeric IgM species may be observed (Frommel et al, 1970; McKeever et al, 1979). The relevance of these observations will be made more apparent when the theories for the presence of low molecular IgM in human disease are discussed.

Ontogeny of IgM: Synthesis of IgM can be observed in the human foetus by the 16-20 week of gestation but at birth levels are low (in the absence of intrauterine infection) (Van Oss, 1979). This early presence of an IgM humoral response is in keeping with its phylogenetic antiquity. If an intrauterine infection occurs, levels of IgM at birth can be elevated sometimes well above adult levels (Hobbs, 1971). Following birth, with exposure to normal antigenic stimuli, IgM levels rapidly rise and by 4-6 months of age the infants IgM concentrations reach or even surpass normal adult levels (Van Oss, 1979). Serum IgA and IgG levels mature less quickly.

IgM Responses in Disease: Elevated polyclonal IgM levels are seen in a wide variety of human disorders (Hobbs, 1971; Johansson, 1979). Dominant IgM responses (with normal or only slight increases in IgA and IgG) are seen in parasitic diseases including malaria, tropical splenomegaly syndrome, trypanosomiasis and filariasis; in bacterial disorders such as mycoplasma and brucella infections and in viral infections including Hepatitis A and B, cytomegalovirus, infectious monocucleosis and rubella. High levels of IgM are also found characteristically in primary biliary cirrhosis and in blood from infants suffering with intrauterine or neonatal infections. High IgM

levels may be also found in certain immunodeficiency disorders (e.g. selective IgA deficiency or immunodeficiency with hyper-IgM).

Selective depression of IgM is found in lymphoid neoplasia, chronic renal failure, and occasionally following drug exposure e.g. gold or penicillamine in rheumatoid arthritis (Stanworth, 1985). Low levels of IgM are also found as part of the general immunoparesis in primary and acquired humoral immunodeficiency syndromes such as common variable hypogammaglobulinaemia (Asherson and Webster, 1980).

Membrane IgM: (See Williams et al, 1978; Pernis and Roth 1982; Kocher et al, 1982). During the ontogeny of the B lymphocyte lineage, a series of maturation stages are identified where the non secretory B cell is characterised by the presence of monomeric IgM anchored into the surface membrane of the cell. This membrane monomeric IgM serves as an antigen receptor for the B cell and is distinguished from the secreted IgM monomeric subunits (vide infra) by being slighly heavier (by approximately 2000 daltons) and by containing μ chains which contain a 41 amino acid hydrophobic carboxy terminus adjacent to the $C\mu 4$ (in contrast to the hydrophilic carboxy terminus for the secreted μ chain). This hydrophobic tail of membrane IgM serves as the anchorage structure into the membrane lipid bilayer allowing the antigen binding first domains of the molecule to extend into the extracellular milieu and to serve as the antigen receptor. Binding of the antibody receptor by antigen (or anti-IgM) is one of the pre-requisite steps leading to B cell activation and eventual maturation and differentiation into IgM secreting B lymphocyte - plasma cells. Small quantities of this membrane IgM may be shed into

the interstitial fluid.

MONOMERIC OR LOW MOLECULAR WEIGHT IgM

Introduction: In the previous section the structure, function and kinetics of circulating pentameric IgM (macroimmunoglobulin or high molecular weight IgM) has been discussed. Whilst Killander (1963) was investigating the filtration chromatographic properties of the immunoglobulins (filtration chromatography was a new and powerful technique developed by Killander and others) he observed in a single subject the presence of small quantities of an immunoglobulin which was not excluded from the gel but like pentameric IgM still reacted with heavy chain specific anti-μ antiserum. Its molecular size was similar to IgG. This was then the first description in man of naturally occurring low molecular weight (LMW) IgM also called 7S IgM or monomeric IgM (in contrast with the pentameric immunoglobulin molecule). Shortly after this Rothfield and her colleagues (1965) confirmed the presence of LMW IgM by describing "slow sedimenting mercaptoethanol-resistant antinuclear factors related antigenically to M immunoglobulin (μ m-globulin) in patients with systemic lupus erythematosus". LMW IgM was then described in the sera of patients suffering from a wide variety of different disorders.

Physicochemical Properties of LMW IgM: Naturally occuring LMW IgM is a glycoprotein with a S_{20w} constant of approximately 8S and a molecular weight, as determined by SDS polyacrylamide electrophoresis and analytical chromatography, of 195,000 to 205,000 daltons (McDougal et al, 1975). It consists of two identical heavy chains of molecular weight of approximately 69,000 to 75,000 daltons and two identical light chains (kappa or lambda) of molecular weight 26,000 to 27,000

daltons and its structural formula is represented as H_2L_2. Carbohydrate analysis of pentameric IgM and LMW IgM has not demonstrated any differences in individual sugars or in the percentage of total carbohydrate (McDougal et al, 1975). Unlike pentameric IgM, LMW IgM does not contain J chain (Eskeland and Harboe, 1973; Parr et al, 1974; McDougal et al, 1975). Antigenically there appear to be no discernible differences between pentameric IgM and LMW IgM (Rothfield et al, 1965; Solomon, 1969; Griggs et al, 1969; Masseyeff et al, 1972; McDougal et al, 1975) although this finding is somewhat disputed by the findings of Gleich et al, (1966). LMW IgM has the capacity to bind antigen specifically although, in contrast with the pentameric molecule, it has weak or absent agglutinating and precipitating activity (Chavin and Franklin, 1969). Demonstrated antibody activity of LMW IgM includes antinuclear binding (Rothfield et al, 1965), incomplete isohaemagglutinin (Stobo and Tomasi, 1967), anti-casein (Hunter et al, 1968), anti-tetanus toxoid (Swedland et al, 1968), rheumatoid factor (Harisdangkal et al, 1975), anti-native DNA (Harisdangkal et al, 1975) and activity against determinants on various micro-organisms such as Treponema pallidum (Muller and Oelerich, 1979; Tanaka et al, 1984), hepatitis B (Sjogren and Lemon, 1983; Tsuda et al, 1984), influenza (Brown and O'Leary, 1972), cytomegalovirus (Nagington et al, 1982), and measles (Connolly et al, 1985). LMW IgM is capable of fixing complement (Caldwell, 1973; Tanaka et al, 1984) and one investigator has reported that the rate of activation and total amount of utilization of complement exceeds that of pentameric IgM when compared on a weight basis (Caldwell, 1973). However, other studies involving murine LMW IgM suggest that it is less efficient in complement activation (Shulman et al, 1982).

Results of several in vitro and in vivo studies have indicated that LMW IgM is neither a breakdown nor catabolic product of pentameric IgM. LMW IgM has not been detected after repeated freezing or thawing, or prolonged incubation of isolated pentameric IgM preparations, nor after similar treatment of sera containing only the pentameric form of IgM (Stobo and Tomasi, 1967; Klein et al, 1967; Bush et al, 1969; Solomon 1969; Dammacco et al, 1970). The intravenous injection of radiolabelled homologous pentameric IgM into a patient whose serum contained both molecular forms of IgM did not result in the appearance of labelled protein corresponding with LMW IgM (Solomon and Kunkel, 1967). Furthermore in macroglobulinaemia the presence of LMW IgM in sera is not directly related to the absolute concentrations of IgM, suggesting that the occurrence of LMW IgM has no dependence on the concentration of the IgM paraprotein (Stobo and Tomasi, 1967; Bush et al, 1969; Solomon, 1969).

In vitro culture experiments have revealed a synthetic origin for LMW IgM. Solomon and McLaughlin, (1970) studied the bone-marrow cells in vitro obtained from three patients with macroglobulinaemia whose sera contained both LMW IgM and pentameric IgM. After addition of C^{14} radiolabelled amino-acids to the culture, labelled LMW IgM and pentameric IgM were both identified in the culture supernatants. Cycloheximide, an inhibitor of protein synthesis, resulted in suppression of IgM secretion. Furthermore the analysis of culture fluids harvested at timed intervals after the addition of cycloheximide revealed not only the stability of the pentameric IgM to intracellular proteolysis but provided evidence for a possible precursor-product relationship between the LMW IgM and the pentameric

protein. Thus the above study provides strong support for a separate synthetic origin of LMW IgM.

Methods of Detection and Measurement of LMW IgM: A variety of techniques of differing sensitivities and specificities have been described to detect and quantitate LMW IgM. In general these are all designed to physically separate the monomeric IgM from the pentameric molecule with the subsequent identification or quantification of the isolated monomeric IgM fraction. Techniques used to separate the two molecular moieties include selective sieving in agar, agarose or acrylamide, gel filtration chromatography, gradient ultracentrifugation, polyethylene glycol precipitation and PHA selective electrophoresis. Techniques used to identify or quantify LMW IgM in the separated fractions include double diffusion, radial immunodiffusion, haemagglutination inhibition, radio-immunoassay and immunodetection. Examples of these methods and quoted threshold sensitivities are given in Table 1.

All these techniques have limitations. Some are insensitive or non quantitative. Others are time consuming, require expensive equipment or considerable laboratory skills. Some methods require up to 2ml of serum for analysis. There is a need for a quick, sensitive, specific and cost effective method to distinguish the two molecular forms of IgM. Development of existing methods and a new method will be described in Chapter two.

Table II. METHODS FOR DETECTION AND QUANTIFICATION OF LMW IgM

AUTHOR	SEPARATION METHOD	IDENTIFICATION OF LMW IgM	THRESHOLD SENSITIVITY	COMMENT
Killander, 1963	filtration chroma-tography	double immuno-diffusion	ND©	Time consuming expensive equipment,
Rothfield et al, 1965	preperative ultracentrifugation	immunofluorescence	ND	large volume of test serum required, non quantitative
Klein et al, 1967	sucrose gradient ultracentrifugation	double immuno-diffusion	ND	
Stobo and Tomasi, 1967	sucrose gradient ultracentrifugation	radial immuno-diffusion	ND	
	filtration chroma-tography, 4% polyacrylamide gel sieving	screening test; problems	500ug/ml+	rapid insensitive screening test; problems with precision
Swedlund et al,1968	filtration chromatography	radial immuno-diffusion	ND	
Solomon 1969	sucrose gradient ultra-centrifugation	double immuno-diffusion	ND	non quantitative
	filtration chroma-tography, immunogel filtration, 7% agarose gel sieving		ND ND 15-70ug/ml	rapid screening test
Bush et al, 1969	sucrose gradient ultra-centrifugation	radial immuno-diffusion haemagglutination inhibition	50ug/ml	

AUTHOR	SEPARATION METHOD	IDENTIFICATION OF LMW IgM	THRESHOLD SENSITIVITY	COMMENT
Carter and Hobbs 1971	thin layer dextran gel chromatography	immunodiffusion	ND	non quantitative
Houba and Lambert, 1974	10-12% polyethylene glycol precipitation		ND	incomplete separation
Starita-Geribaldi and Masseyeff, 1975	Sucrose gradient ultracentrifugation	Radioimmunoassay	1ug/ml	potential hazard of radio-activity
Romero et al, 1978	7% agar gel sieving	radial immuno-diffusion	10ug/ml	precision, sensitivity and specificity are suspect
Spengler and Weber 1980	selective electrophoresis in PHA-containing agar	immunodiffusion	ND	non quantitative
Pontet and Rousselet 1984	electro-immuno transfer in acrylamide gel	immunodetection	3.2 ug	non quantitative, time consuming

+ quoted by Dammacco et al, 1970

© ND - not determined

Occurrence and Clinical Significance of LMW IgM in Human Disease: Circulating LMW IgM occurs rarely in healthy adults but frequently in certain autoimmune, infective, immune deficient and lymphoproliferative disorders (Rothfield et al, 1965; Stobo and Tomasi, 1967; Bush et al, 1969; Dammacco et al, 1970; Stage and Mannik, 1971; Harisdangkul et al, 1975; Fakunle et al, 1979; Tsuda et al, 1984). The explanation for the occurrence of LMW IgM in these disorders and the clinical significance is still obscure. We will discuss some of these disorders in more detail.

There have been several previous studies concerning the frequency and disease association of LMW IgM in rheumatoid arthritis (RA) (Table II). The varying frequency in each study no doubt reflects differences in sensitivities and specificities of the assay used to detect LMW IgM and in differences in patient selection. The conclusion from all of these studies suggests that LMW IgM occurs frequently in RA particularly in those patients with severe disease and with associated extra-articular manifestation such as rheumatoid vasculitis. Indeed several authors have suggested that LMW IgM may play a direct and important role in the immunopathogenesis of these extra-articular manifestations.

In systemic lupus erythematosus (SLE) LMW IgM in frequently found particularly in male patients with severe disease (Rothfield et al, 1965; Stobo and Tomasi, 1967; Harisdangkul et al, 1975). In a study of 100 SLE patients, using sucrose gradient ultra centrifugation and a radial immunodiffusion technique, Harisdangkul et al, (1984)

Table I. LMW IgM in RA

AUTHOR	METHOD	NO. PATIENTS	POSTIVE FOR LMW IgM	COMMENT
LoSpalluto, 1968	gel filtration	36	10 (28%)	Those with high SSCA titres had greatest quantities.
Dammacco et al, 1970	5% PAG	43	12 (28%)	No clinical differences between those with or without LMW IgM.
Stage and Mannik, 1971	4% PAG	264	25 (9.5%)	Those with LMW IgM had significantly more severe disease vasculitis - 12/25 had LMW IgM no vasculitis-1/25 has LMW IgM
Hunder and McDuffie, 1973	SGU & RID	14	14 (100%)	All RA patients had hypocomplemtaemia and severe disease.
Theofilopoulos et al, 1974	4% PAG	48	18 (37%)	Rheumatoid vasculitis-12/15 had LMW IgM. No vasculitis-6/33
Clark et al, 1974	SGU & RID	14	7 (50%)	
Coughlan et al, 1984	7% agarose	37	16 (43%)	Strong correlation between LMW IgM and RF

PAG = polyacrylamide gel immunodiffusion
SGU = sucrose gradient ultracentrifugation
RID = radial immunodiffusion
SSCA = sheep cell agglutination titre
RF = rheumatoid factor

detected LMW IgM in 45% of these patients and noted statistically higher levels of IgM and anti-DNA antibodies in those LMW IgM positive patients. Furthermore, from a study of 16 of their patients who died from their disease, they concluded that patients with circulating LMW IgM compared with patients without had greater mortality at an earlier age. These mortality figures therefore suggest that LMW IgM is associated with more severe disease and again raises the possibility that LMW IgM is closely associated with immunopathogenic mechanisms in SLE, perhaps participating in the formation of noxious circulating immune complexes. The same authors have also shown in an earlier study (Harisdangkul et al, 1975) that LMW IgM from SLE patients contains anti-DNA binding activity again supporting the proposition for a role in the immunopathogenesis of this disease.

LMW IgM has also been observed in another autoimmune disorder, viz primary biliary cirrhosis (Bush et al, 1969; Fakunle et al, 1979; Taal et al, 1980). Fakunle and colleagues using a polyacrylamide/agarose gel immunodiffusion technique noted LMW IgM in 33% of 69 patients with primary biliary cirrhosis but not in any of 30 healthy subjects. In the primary biliary cirrhotic group highly significant associations where observed between LMW IgM and total IgM levels and with levels of circulating immune complexes as measured by the Clq binding assay. They suggested that the presence of LMW IgM in their patients reflected a failure of complete polymerization of LMW IgM because of an increased rate of synthesis of the IgM protein. They were unable to detect, in preliminary studies, anti-mitochondrial activity in their LMW IgM fractions.

Two patients with severe haemolytic anaemia have been reported in which LMW IgM appears to have a direct role in the pathogenesis of the haemolysis (Spiva et al, 1974; Kay et al, 1975). In one patient the LMW IgM was non agglutinating but demonstrated biphasic thermal requirements for in vitro haemolysis and had anti-I specificity whilst in the seond patient the LMW IgM was monoclonal (kappa) and could be eluted from the red blood cell membrane but its specific antibody activity was not able to be characterized. These two case descriptions provide strong evidence for a direct role of LMW IgM in causing human disease.

LMW IgM also occurs frequently in patients suffering from acute and chronic infective disorders. These include bacterial diseases such us primary, secondary and tertiary syphilis and leprosy (Dammacco et al, 1970; Muller and Oelerich, 1979; Tanaka et al, 1984), parasitic diseases such as filariasis, trypanosomiasis and malaria associated disorders (Kein et al, 1967; Masseyeff et al, 1972; Fakunle and Greenwood, 1977), and viral diseases including influenza (Brown and O'Leary, 1971 and 1973), cytomegalovirus (Nagington et al, 1982), chronic hepatitis B virus carriers with or without liver disease (Sjogren and Lemon 1983; Tsuda et al, 1984), and acute measles and subacute sclerosing panencephalitis (Connolly et al, 1985). In many of these infective diseases the LMW IgM fractions have been shown to contain specific antibody activity against the infectious micro-organism and Tanaka et al, (1984) have shown that the LMW IgM anti-Treponema antibody fixes complement effectively. Of interest was the high frequency which these LMW IgM antibodies were observed; approaching 100% in many of the chronic untreated infections,

frequently in the absence of the specific pentameric IgM antibody (Tanaka et al, 1984; Tsuda et al, 1982; Sjogren and Lemon 1983). This poses new questions concerning the humoral antibody response in infectious diseases. One possible reason for the relatively late recognition of this LMW IgM antibody response in these diseases may be the poor agglutinating and precipitating properties of this class of antibody (vide supra).

A number of immune deficiency disorders have been associated with the presence of LMW IgM. Gleich et al, (1966) using both sucrose gradient ultracentrifugation and gel filtration combined with double diffusion observed LMW IgM in a single patient with dysgammaglobulinaemia of the type I (associated with low levels of IgG and IgA and very high levels of polyclonal IgM). Stobo and Tomasi, (1967) described LMW IgM in 4 of 7 patients with ataxia telangectasia (all patients having low or undetectable IgA) and chromatographic studies revealed that between 8-15% of the total IgM was of this variety. All patients with LMW IgM also contained elevated levels of serum IgM and incomplete isohemagglutination was identified in the LMW IgM fraction. McFarlin et al, (1972) have also noted LMW IgM in 14 of 18 (78%) of their patients with ataxia telangectasia and of those, 11 had elevated serum IgM levels. The association of the presence of LMW IgM and the clinical manifestations was not addressed directly in either of the two studies although in an analysis of the clinical data of the latter study it appears that LMW IgM was present in 10/11 of patients with increased frequency of infections compared with 4/7 of patients with no increase. These differences however, are not significant (Fisher's exact test).

B cell lymphoproliferative disorders are frequently associated with the presence of LMW IgM. The majority of reports have noted it occurrence in those lymphoproliferative disorders associated with circulating IgM paraproteins and when the LMW and pentameric paraproteins have been purified they appear to have the same clonal characteristics. Thus LMW IgM has been described in Waldenstroms macroglobulinaemia (Solomon and Kunkel, 1967; Stobo and Tomasi, 1967; Solomon, 1969; Bush et al 1966; Dammacco et al, 1970; Carter and Hobbs, 1971; Bigner et al, 1971; Eskeland and Harboe, 1973; Parr et al, 1974; Harisdangkul et al, 1975) and in lymphoma and chronic lymphocytic leukaemia associated with IgM paraproteins (Solomon, 1969; Carter and Hobbs, 1971; Qian et al, 1984). There appears to be no relationship between the quantity of IgM paraprotein and the presence of LMW IgM (Solomon, 1969; Bush et al, 1969). Solomon and McLaughlin, (1970) have established, using in vitro culture studies, that the LMW IgM is synthesized independently from the pentameric molecule. Solomon (1970) could find no distinctive clinical or pathological features between Waldenstroms macroglobulinaemic patients with or without LMW IgM but Carter and Hobbs, (1971) were unable to find LMW IgM in any of their 9 patients with benign macroglobulinaemia and concluded that the LMW IgM moiety was only found in patients with malignant lymphoproliferative disorders and hence could be used to help distinguish a benign macroglobulinaemic state from a malignant one.

LMW IgM has also been described in lymphoproliferative disorders associated with an acquired C1 esterase deficiency (Hauptmann et al, 1976; Gelfand et al, 1979). This uncommon but distinctive

lymphoproliferative syndrome is frequently associated with episodes of angio-oedema and serological studies reveals the presence of a paraprotein (not necessary IgM), cryoglobulins and high levels of circulating immune complexes (Gelfand et al, 1979). The acquired C1 esterase deficiency is thought to be secondary to the activation of the classical complement pathway by immune complexes and it has even been proposed that the LMW IgM is involved in this activation process (Hauptmann et al, 1976; Casali et al, 1978).

Finally LMW IgM has been described in patients having motor neurone disease or idiopathic chronic polyneuritis. Whitaker et al, (1973) using both a polyacrylamide screening test and a gel filtration technique identified LMW IgM in 3 of 13 patients with motor neurone disease and in 7 or 24 patients with idiopathic neuritis. All patients with LMW IgM had elevated serum levels of IgM. As a group those polyneuritic patients with LMW IgM were younger and had more severely impaired nerve conduction velocities than those lacking LMW IgM.

From the above it can be seen that LMW IgM occurs in a wide variety of different disorders. It is frequently associated with the more active or severe forms of the disease and there is some indirect but tantilizing evidence that it is involved in the pathogenesis of these disorders. In subsequent chapters we will examine further the relationship between LMW IgM and human disease.

Theories for the occurrence of LMW IgM in human disease: The reasons for the induction and secretion of LMW IgM in human disease are

unknown. Several theories have been proposed to account for its presence. One of the earliest, originally proposed by Stobo and Tomasi, (1967) and Solomon, (1970) was that the occurrence of LMW IgM in disease reflected a reversion of the humoral immune response to a phylogenetically and ontogenetically more primitive or immature state. Evidence quoted is support of this theory has been the observation of LMW IgM (or its equivalent) occurring naturally and in high concentration in lower vertebrates (Marchalonis and Edelman, 1965; Clem and Small, 1966), and the detection of LMW IgM in healthy neonates (Perchalski et al, 1968). A second theory favoured in the lymphoproliferative disorders is that LMW IgM represents monomeric membrane IgM released into the interstitial space during rapid cell turnover. A third theory suggests that LMW IgM occurs as a consequence of intracellular or extracellular proteolysis of pentameric IgM involved in immune complexes (Rothfield et al, 1965). This splitting of the pentameric molecule could be due to the enzymic activity of complement activation enzymes or other enzymes derived from phagocytes. This theory has some attraction in view of the common occurrence of LMW IgM in immune complex states. A fourth theory suggests that LMW IgM occurs as a consequence of defects in the assembly of monomeric IgM subunits into the completed pentameric molecule (Rothfield et al, 1965). The defect could involve one or more of the multiple steps involved in IgM polymerization.

An intriguing experimental observation has been also recorded by Manning, (1980). When healthy mice were injected with an anti-μ antiserum, high levels of circulating LMW IgM were generated. This observation would be consistent with the possibility that this anti-μ

anti-immunoglobulin interacted with membrane IgM of B lymphocytes to activate an intracellular process which leads to the secretion of LMW IgM. Anti μ immunoglobulins have also been recorded in man (MacKenzie et al, 1967).

Some of these above theories will be examined in more detail in subsequent chapters.

AIMS OF THESIS

To study the presence and role of low molecular weight IgM in health and disease. To achieve this goal the following aims have been set.

1. To develop existing methods and examine new methods for the detection and quantitation of LMW IgM in health and disease.

2. Using these methods to examine sera from healthy adults and neonates for the presence of LMW IgM.

3. To study sera, saliva and synovial fluid from patients with a variety of autoimmune, infective, immunodeficient and lymphoproliferative diseases and to relate the presence of LMW IgM with other clinical and serological indices which reflect active or severe disease.

4. To determine if peripheral blood mononuclear cell cultures prepared from patients with circulating LMW IgM secrete LMW IgM in vitro and if so to examine the variables involved in this process.

5. To determine if in vivo and ex vivo factors are concerned in the production of LMW IgM (i.e. does LMW IgM occur as a consequence of catabolic processes?).

6. To account for the presence of LMW IgM in human disease by proposing a hypothesis which best fits with the observed data.

CHAPTER TWO

PATIENTS, MATERIALS AND GENERAL METHODS

PATIENTS AND CONTROLS

The patients studied in this thesis were derived from the inpatients and outpatients of the Flinders Medical Centre, the Repatriation General Hospital, the Royal Adelaide Hospital and the Queen Elizabeth Hospital all of Adelaide and the Parkland Memorial Hospital, Dallas, Texas. Their specific diagnoses were made by the attending clinicians according to standard clinical, radiological and pathological criteria. For example the criteria of Ropes et al, (1958) were used in the diagnosis of rheumatoid arthritis. Details of specific diagnoses are given in the Method section in each chapter. In many instances the patients were personally known to this author. Blood was obtained by antecubital venepuncture, allowed to clot at room temperature (except when being drawn for cryoglobulin studies) and the serum separated by centrifugation within 4 hours of collection. Saliva from patients with selective IgA deficiency was obtained by subjects spitting repeatedly into a sterile collection pot. Synovial fluid was obtained by needle aspiration of inflammed knee joints. Blood from healthy laboratory personnel was also studied in a similar fashion to the pathological specimens. In some instances blood samples were also obtained from outpatients with minor allergic disorders. These were included as healthy subjects. The specific details of patients studied and the healthy controls are described in the respective chapters.

STORAGE OF TEST SPECIMENS

Sera and the test specimens were aliquoted into small volumes (250ul) and stored frozen at -20°C or -70°C (for immune complex estimations specimens were stored at -80°C). Aliquots were generally discarded after thawing for the first time.

IDENTIFICATION AND QUANTITATION OF LMW IgM

Three methods were used to detect and/or quantitate LMW IgM

(1) **Filtration chromatography monitored by IgM laser nephelometry**

LMW IgM was measured as previously described (Roberts-Thomson et al, 1980(a)). One or two ml of serum was applied to a column of Sepharose 6B or Sephacryl S300 (Pharmacia) (90 x 2.5 cm). Gel filtration was performed in phosphate-buffered saline (PBS), pH 7.3, at room temperature using an upward flow at a rate of 20 ml/h. The eluate was monitored with an LKB Uvicord II Recorder. Five ml fractions were collected with an LKB Ultrarac fraction collector. IgM concentrations were then determined in alternate fractions with a laser nephelometer (Behring) adapted with a single flow cell and a graph recorder (Camag W & W 1100) to record mV of light scatter; the degree of light scatter being proportional to the amount of precipitate. One vol. of anti-human IgM mu-chain specific antiserum (Dako-immunoglobulins, Copenhagen, Denmark) and 20 vols. of eluent taken from each alternate column fraction, were incubated at room temperature for 1 h. The resultant precipitate was measured by

observing the degree of light scatter (expressed in millivolts (mV)) and compared with a standard curve constructed from normal human serum of known IgM concentration (Figure 1). Control tubes, containing eluent from column fractions but without anti-IgM antibody were subtracted as background. The minimum amount of IgM detected by this method is 0.1 mg per 100 ml (1 mg/l). An IgM profile was then obtained and the detection of a second IgM peak eluting just before the IgG peak signified the presence of LMW IgM. To determine the quantity of LMW IgM the method of planimetry was used. The IgM profile was traced onto clear exposed X-ray film, the profile cut from the films, weighted and then the contribution (weight) of the LMW IgM fraction determined. The % of LMW IgM thus determined (of the total IgM profile) was then multiplied by the total serum IgM level (determined by the Beckman ICS nephelometric system). This fraction gave the absolute quantity of LMW IgM present.

It should be noted that unlike radial immunodiffusion the nephelometric technique for the determination of a specific protein concentration is not influenced by the size of that protein (Virella and Fudenberg, 1977). In addition the specificity of the anti-μ chain antiserum (Dako-immunoglobulins) used in subsequent studies was verified by immunoelectrophoretic analysis. Similar IgM elution profiles were also obtained using anti-IgM reagents obtained from two other commercial sources (Silenus Laboratories, Hoechst-Behring). Using the above chromatographic method it was found that as little as 2-4 mg/100 ml of LMW IgM could be determined. When the sera from 7 RA patients were chromatographed over the same column on two separate occasions, the mean difference between duplicate determinations was

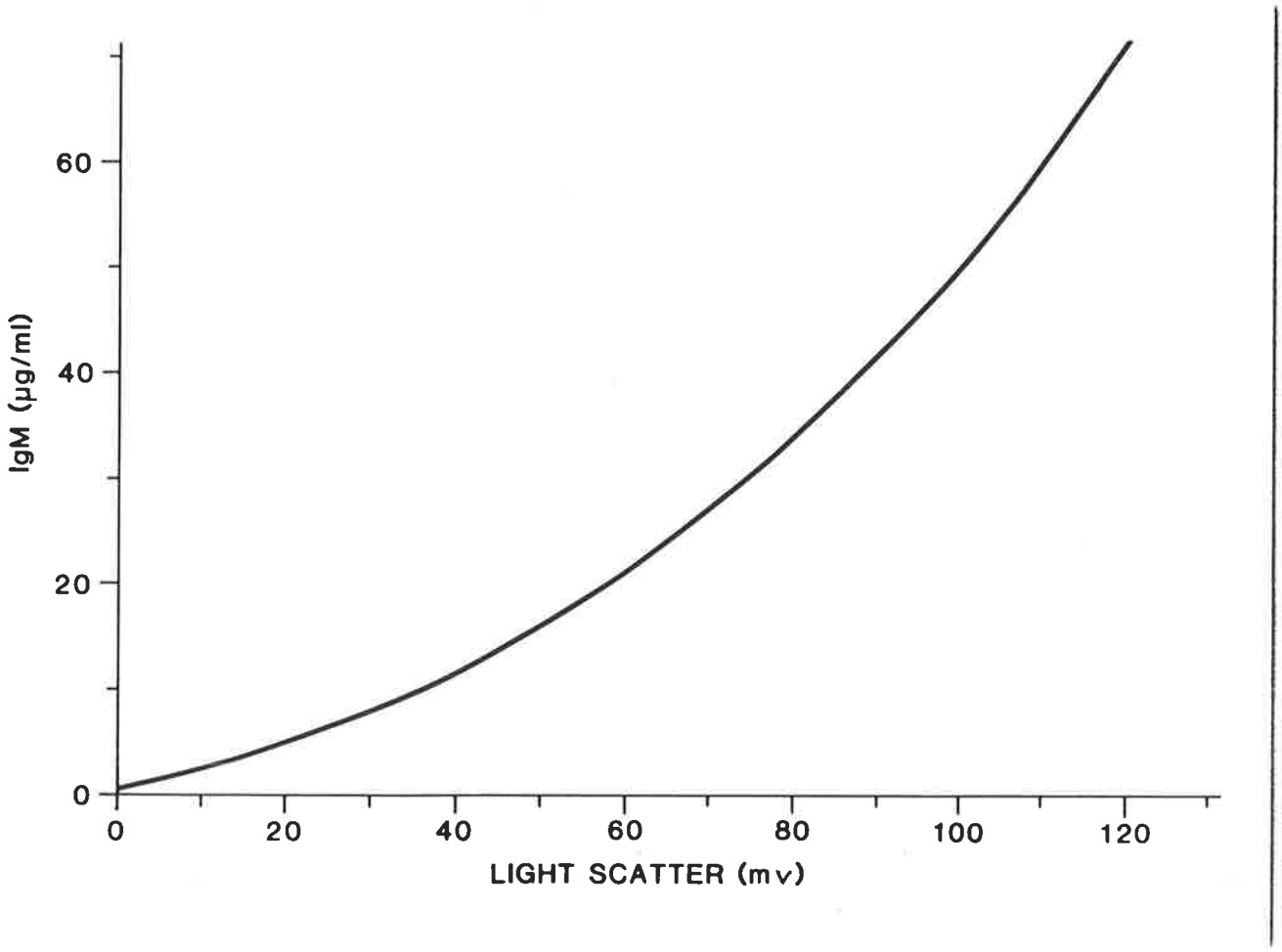

Figure 1. Standard curve for IgM measured by end point laser nephelometry. The IgM concentration is determined by the degree of light scatter (millivolts) generated by the addition of 1 volume of anti-µ antiserum and 20 volumes of column eluent.

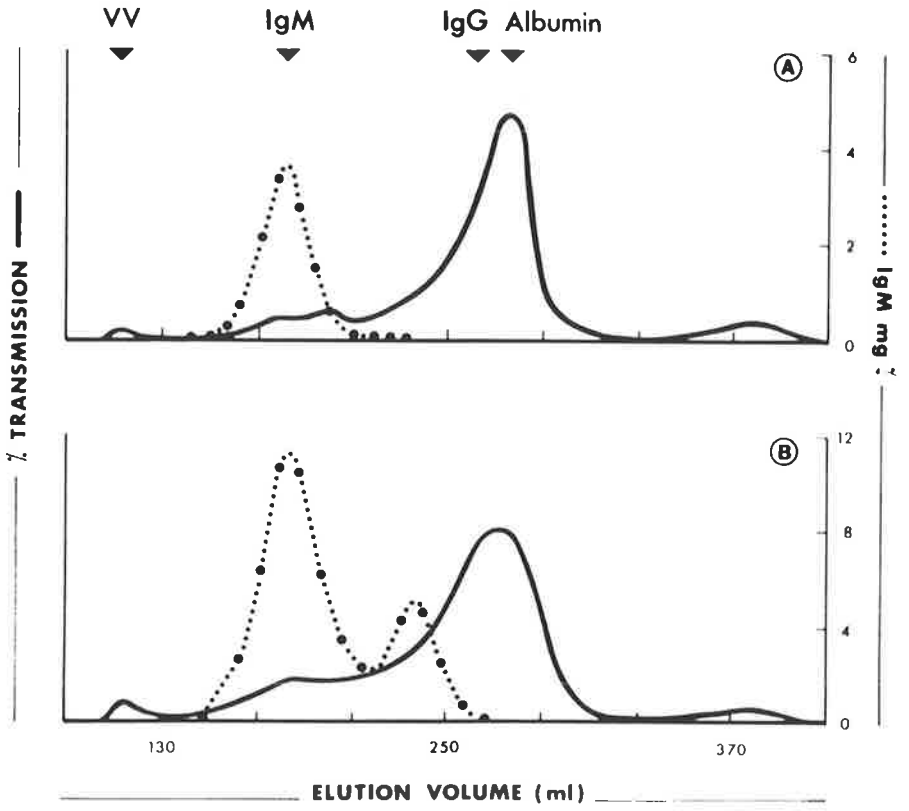

Figure 2. The percentage light transmission and IgM elution profile following Sepharose 6B filtration chromatography of 1.5 ml of serum. The eluting position of marker proteins detected in the serum are shown.
Upper panel - serum from healthy subject.
Lower panel - serum from patient with rheumatoid arthritis containing LMW IgM. This moiety accounted for 28% of the total IgM profile and was equivalent to 101 mg/100 ml i.e. 28% of the absolute serum IgM level of 360 mg/100 ml.

2.8%. When this chromatography was performed with two different columns, the mean difference was 5.3%. An example of the results obtained from a healthy subject and from a rheumatoid arthritis patient is shown in Figure 2.

(2) **Filtration chromatography monitored by IgM ELISA**

Sera, saliva or culture supernatant diluted or concentrated to contain approximately 5 x 10^4 ng of IgM were applied to a Sephacryl S300 90 x 1.5 cm column (Pharmacia) equilibrated with PBS pH 7.3 and 4 ml fractions collected as described above. IgM was determined in each fraction from the void volumes to the albumin peak by ELISA and an IgM elution profile determined. LMW IgM was identified as a second or late eluting IgM peak just prior to the IgG peak and its level determined by the planimetric technique as described above. The competitive inhibition ELISA was used exactly as described by Smart and Koh, (1983). This assay can detect as little as 10 ng/ml of IgM. It was initially determined that LMW IgM prepared from a seropositive RA patient by filtration chromatography and affinity chromatography gave a very similar binding curve to the pentameric IgM standard suggesting that the molecular size of the IgM used as the standard did not greatly influence the ELISA results (Figure 3). Dilutional experiments using sera containing LMW IgM suggested that the threshold sensitivity of this technique for the detection of LMW IgM is approximately 1-5 ug. We thank Mrs. L. Koh for performing all the ELISA IgM measurements.

(3) **Immunoblotting**

(i) Sodium dodecyl sulphate (SDS) polyacrylamide gel

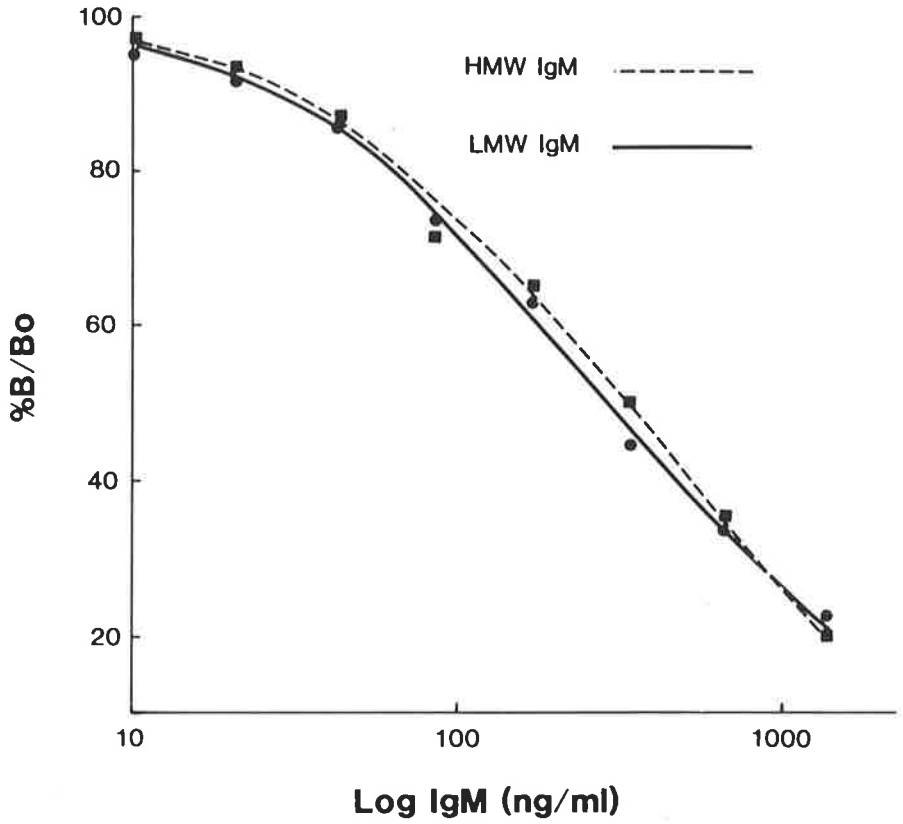

Figure 3. Binding curves for pentameric IgM (HMW IgM) and monomeric IgM (LMW IgM) in competitive inhibition enzyme immunoassay. Ordinate expresses % of conjugate bound (B) in relation to sample without IgM (Bo), and abscissa reflects the logarithm of IgM concentration. HMW IgM was prepared as previously described (Smart and Koh, 1983) and LMW IgM was prepared from the serum of a strongly seropositive rheumatoid arthritis patient by Sephacryl S300 chromatography and anti-μ affinity chromatography. The concentration of LMW IgM standard was measured by absorption at 225 nm, by nephelometry, and by the Lowry colorimetric technique. Values did not vary by more than 20% for the 3 methods.

electrophoresis. Serum electrophoresis in SDS 3.6% polyacrylamide gel slabs was peformed as previously described (Roberts-Thomson et al, 1976). Sera were diluted 1:10 in serum diluent buffer (2% SDS, 0.125 M Tris, 0.004 M iodoacetate added to normal saline at a ratio of 11:9, pH 7.2) and allowed to stand for 1 h at 37°C. A drop of bromophenol blue (0.05%) was used as the tracking dye and 0.01 ml of diluted serum applied to the gel well. Positive control sera containing LMW IgM were included in each gel run. Electrophoresis was carried out overnight at 20 mA/slab in a water cooled tank (Bio-Rad) and terminated when the tracking dye was 1-2 cm from the bottom of the gel.

(ii) Electrophoretic transfer. The separated proteins were transferred to nitrocellulose as described elsewhere (Towbin et al, 1984). Nitrocellulose (NC) sheets (Bio-Rad), prerinsed in transfer medium (0.025 M Tris 0.19 M glycine, 20% methanol v/v, pH 8.3) were carefully placed over the gel slab, covered with filter paper (Whatman no. 1) and Scotch-brite pad and then sandwiched between a plastic support frame. The support frame was placed in a Trans-blot cell tank (Bio-Rad) containing transfer medium, with the NC facing the positive electrode. Electrophoretic transfer of SDS protein complexes was accomplished in 4 h at 4°C at 300 mA, or 100 mA overnight at 4°C.

(iii) Immunoblotting. After electrophoretic transfer the NC sheet was removed and washed twice in PBS containing 0.05% Tween 20 at room temperature. The NC sheet was removed and washed twice in PBS containing 7% v/v bovine serum albumin to block non-specific reactive sites. The NC sheet was washed twice in PBS-Tween, and then

incubated for 1 h at room temperature on a rocking platform with a 1:500 dilution of biotinylated anti-human IgM antibody prepared by the method of Goding (1983). The NC sheet was washed in 3 changes of PBS-Tween over 1 h and then rinsed once in 0.1 M sodium bicarbonate pH 9.0. NC sheets were then incubated with avidin-D (Vector), diluted to 10 ug/ml in 0.1 M $NaHC0_3$ pH 9.0 for 30 min. The NC sheet was washed twice in the same buffer and then incubated with biotinylated alkaline phosphatase (Vector), diluted to 50 ug/ml in bicarbonate buffer for 30 min. The NC sheet was again washed thrice in PBS over 30 min and then rinsed once in 0.1 M Tris pH 8.2, before the addition of freshly prepared substrate solution. Substrate was prepared by dissolving 4 mg of naphthol As-MX or ASBI (Sigma) in 0.4 ml of dimethyl formamide in a glass tube and then adding 19.6 ml of 0.1 Tris pH 8.2. Immediately before use, Fast Red (Sigma) was added at a concentration of 1 mg/ml and the solution filtered directly onto the NC sheet. The colour reactions were usually visible within 15-20 min. Examples of sera containing LMW IgM detected by this method are shown in Figure 4. Dilutional experiments of these sera suggested that this immunoblot technique when performed optimally could detect as little as 0.10 ug of LMW IgM using an initial starting volume of 10 ul of test serum (compare 1-2 ml for the nephelometric technique). However an inherent variability in the quality and resolution of the IgM bands between different runs was observed. The reason for this variability was not determined. Blots were discarded if the resolution of the bands of the positive control serum was deficient. Many of the positive sera for LMW IgM demonstrated the presence of additional less intense IgM oligomeric bands migrating between the monomer and the pentamer (Figure 4). One of these bands migrated

with a molecular weight of approximately 4 x 10^6 daltons suggesting that it was dimeric IgM. Bands representing LMW IgM or other IgM oligomers were not observed in sera from healthy subjects. Further examples of IgM immunoblots are found in subsequent Chapters.

IgM SYNTHESIS IN VITRO

Heparinized blood (approximately 40 ml) was collected from patients and controls, diluted 1:1 in phosphate-buffered saline, layered on Ficoll-Hypaque (Pharmacia), and centrifuged at 400 g for 30 min at room temperature. The mononuclear-rich fraction was washed 4 times in PBS (centrifugation at 400 g for 10 min). The cells were then counted and adjusted to a concentration of 1 x 10^6 cell/ml in RPMI culture medium with 10% fetal calf serum, glutamine, penicillin, and streptomycin (Gibco, Grand Island. NY). Pokeweed mitogen (Sigma, St. Louis, MO) was added at a final concentration of 1 ug/ml. Comparative cultures were also incubated without this mitogen. The cell cultures were plated in 2 ml aliquots in Costar 24-well tissue culture dishes and incubated for 3 or 7 days at 37^{o}C (5% CO_2). For 7-day cultures, the medium was changed at day 3. At the completion of culturing, the cell-free supernatants were collected and stored at -20^{o}C.

To confirm active synthesis of IgM, cycloheximide (Sigma) was added to the cultures at day 0 at a final concentation of 10 ug/ml. Viability counts were performed using trypan blue exclusion.

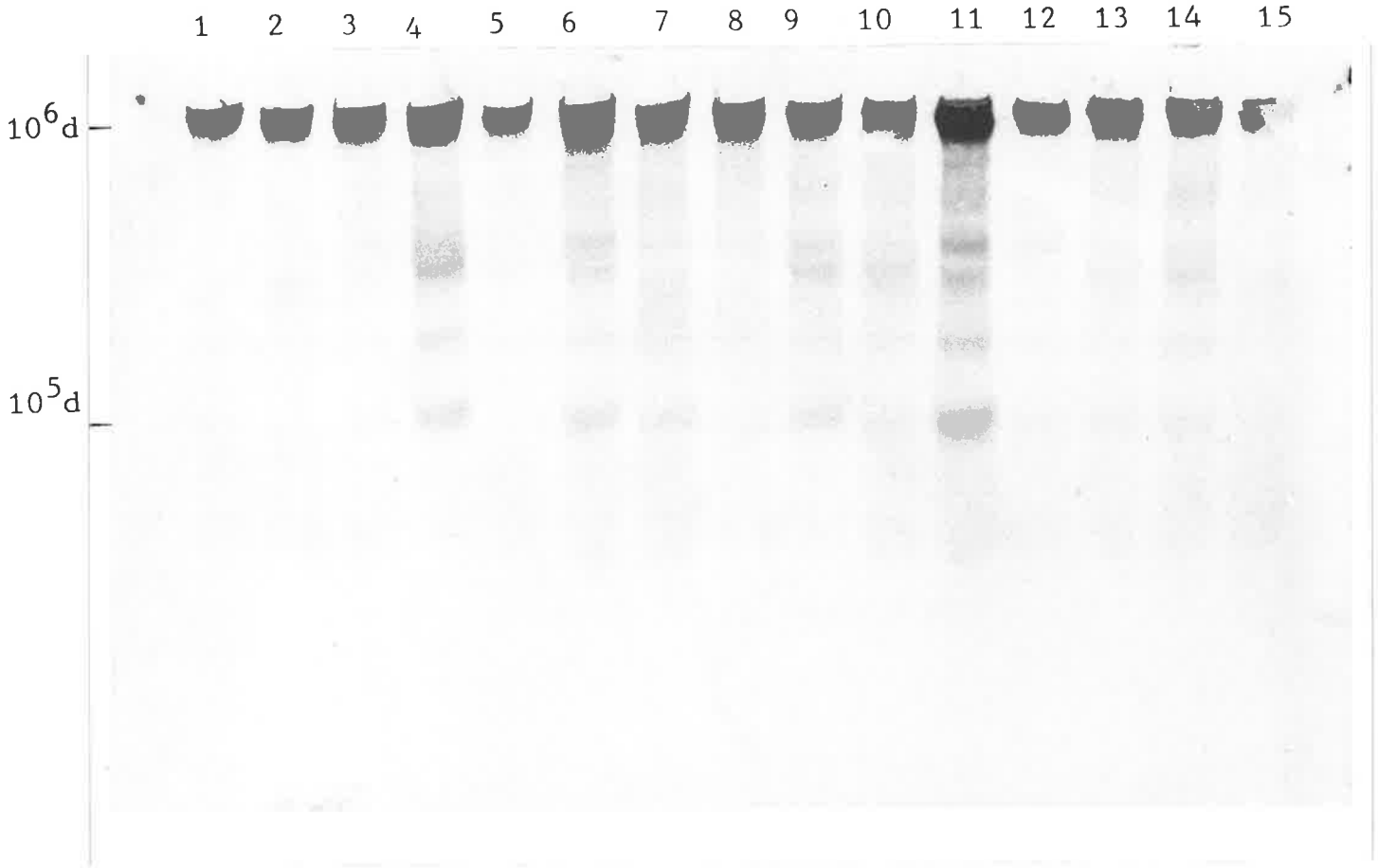

Figure 4. Immunoblot appearance of 14 RA sera and 1 serum from a healthy adult (column 5). The uppermost band represents pentameric IgM (MW approximately 10^6). The lowermost band represents monomeric IgM (MW approximately 2×10^5). Note bands migrating in between the monomer and pentamer in many of the pathological sera.

OTHER IMMUNOLOGICAL METHODS

A number of other immunochemical and general biochemical techniques were used. Each technique is described in the relevant Methods section in each Chapter. In general these methods or techniques are performed routinely in the Department of Clinical Immunology, Flinders Medical Centre, or in other laboratoreis in the Centre or elsewhere. In brief, quantitation of immunoglobulins, C reactive protein (CRP) and rheumatoid factor (RF) were measured by rate nephelometry (Beckman ICS) and immune complexes by the Clq binding method of Zubler and colleagues, (1976). These methods have been fully standardized and the author of this thesis has initiated or contributed to various quality assurance programmes or other studies to ensure the validity and precision of these techniques (Roberts-Thomson et al, 1981; Roberts-Thomson et al, 1985; Roberts-Thomson et al, 1987; Roberts-Thomson et al, 1988). Acknowledgement is made in the text when specialised immunological assays were performed by other investigators (e.g. RF radio immunoassay, Chapter four). Details of these special tests have, in general, been published elswehere and the relevant reference is quoted in the appropriate Chapter. The complete technical details of these special tests will not be described in this thesis.

STATISTICAL ANALYSIS

A number of statistical methods have been used in this thesis and

they are described in the Method section in each relevant Chapter. In general non parametric methods were used in view of the possible asymmetrical distribution of the resultant data and the small number of patients studied. A significant result has been accepted if the P value is <0.05.

CHAPTER THREE

DOES LOW MOLECULAR WEIGHT IgM OCCUR IN HEALTH?

SUMMARY

Low molecular weight IgM was not found in the sera of healthy adults using 2 different sensitive techniques. Low levels were, however, found reproducibly in 4 of 20 neonatal cord blood sera using one of these techniques. It is concluded that if low molecular weight IgM occurs in healthy adult sera its levels must be lower than the threshhold sensitivities of these assays.

INTRODUCTION

Most investigators using a variety of different techniques have not detected LMW IgM in healthy adult sera (Stobo and Tomasi, 1967; Perchalski et al, 1968; Bush et al, 1969; Romero et al, 1979). However, both Killander (1963) using a gel filtration technique and Solomon (1969) using gradient ultracentrifugation have observed detectable LMW IgM in the serum of a new delivered Rh-immunized mother, and a pregnant woman respectively (out of a combined total of 13 adult sera studied) whilst Starita and Masseyeff, (1975) using gradient ultra centrifugation and an IgM radioassay have described 'trace' quantities of LMW IgM and other molecular sized IgM moieties in healthy adult sera. In contrast to the rare occurrence of LMW IgM in healthy adult sera as reported by the preceeding investigators, Perchalski et al, 1968 recorded LMW IgM in the cord serum from 8 of 11 neonates. Similarily Starita and Masseyeff, (1975) noted small quantities of LMW IgM in 4 cord sera, a finding which has also been noted by Jol-V.D. Zijde et al, (1983).

In the present study, using two independent but sensitive techniques, we have investigated the frequency of LMW IgM in sera from healthy adults and neonates. Our findings indicate that LMW IgM occurs infrequently in cord blood but not in healthy adult sera.

SUBJECTS AND METHODS

Sera: Thirty cord bloods were obtained from newly delivered infants of gestational age 36-42 wks, the blood allowed to clot, and the sera collected and stored at -20^{o}C. In addition 5 specimens were obtained from still born infants either from cord or cardiac aspiration. Positive control sera containing LMW IgM were obtained from patients with sero positive rheumatoid arthritis (see Chapter four). Adult sera were obtained from 15 healthy subjects (8 non pregnant females and 7 males).

Measurement of total IgM: IgM was measured in each adult specimen by laser nephelometry (Beckman ICS) and in cord sera by competitive inhibition ELISA as previously described (Smart and Koh, 1983).

Detection of LMW IgM: Two methods were used to detect LMW IgM. 1) Filtration chromatography and IgM ELISA (as previously described in Chapter two). 2) Immunoblotting (as previously described in Chapter two) with the modification that increased quantities of cord sera were applied to the gel in comparison with adult sera (i.e. total IgM applied to the gel was kept constant).

RESULTS

The mean ± SD level of IgM in the 30 cord sera was 0.12 ± 0.04 g/l. Two of the five sera obtained from still born infants had elevated IgM (i.e. greater than 0.25 g/l which is the upper limit quoted in our laboratory) with levels of 0.45 and 0.60 g/l. Both sera were from infants with suspected intrauterine infections. Using the immunoblot technique LMW IgM was not detected in any of the healthy or pathological neonatal sera. Filtration chromatographic studies were performed on 20 healthy and 2 pathological neonatal sera. In four cord sera from healthy neonates small quantities of LMW IgM were detected making up 4%, 9%, 14% and 25% of the total IgM profile (Figure 1) Rechromatographic analysis of these sera confirmed the presence of LMW IgM in each instance. These sera had total IgM levels between 0.07 and 0.18 g/l. LMW IgM was not detected by either method in 15 healthy adult sera, including 5 who were selected for their high normal or high IgM levels (IgM = 2.5-3.9 g/l). It was however, detected frequently by both techniques in rheumatoid sera whereby the chromatographic technique a mean of 13% of the total IgM was LMW IgM. An example of the detection of LMW IgM by the immunoblotting technique in a positive serum, but its absence in cord sera is shown in Figure 2.

DISCUSSION

This study has revealed small quantities of LMW IgM in a minority of healthy cord sera but not adult sera and in larger quantities in

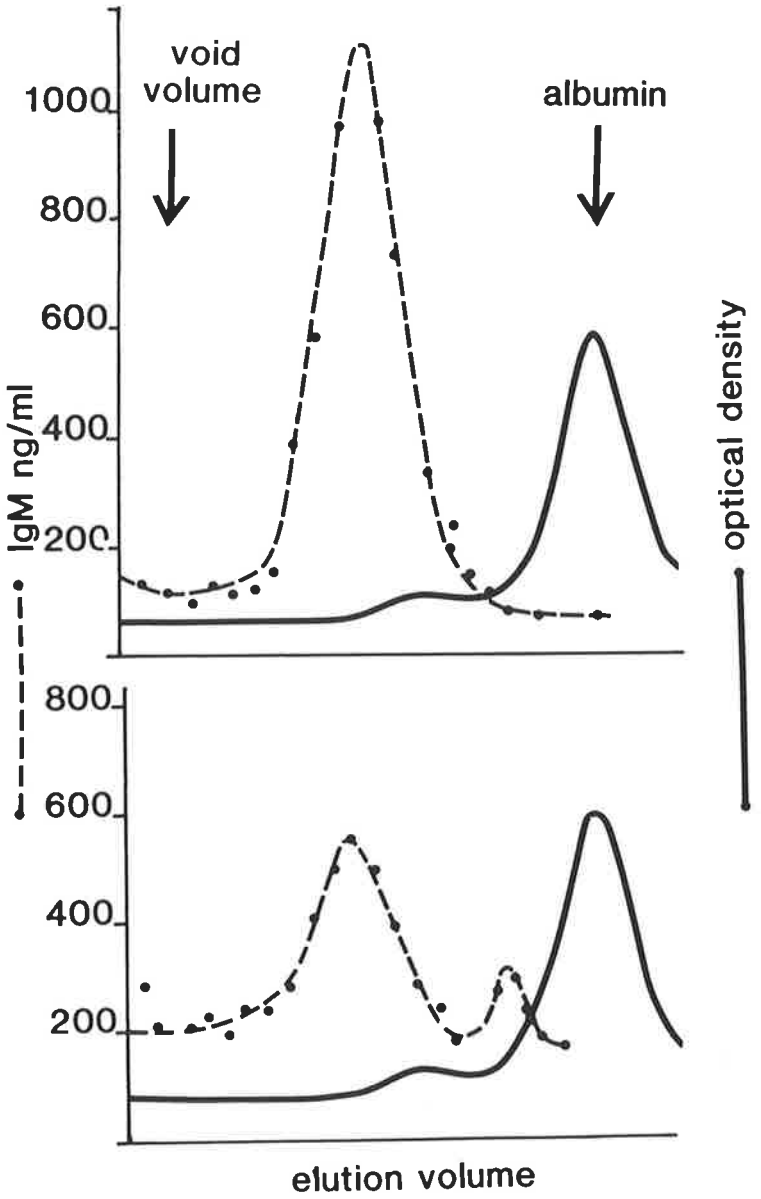

Figure 1. Column chromatographic technique for the demonstration of LMW IgM.
Upper panel - healthy cord serum showing absence of LMW IgM peak.
Lower panel - healthy cord serum showing presence of late emerging LMW IgM peak which constituted 14% of the total IgM profile. Total cord IgM level = 73 ug/ml, therefore LMW IgM = 14% of 73 ug/ml. The eluting position of albumin is shown.

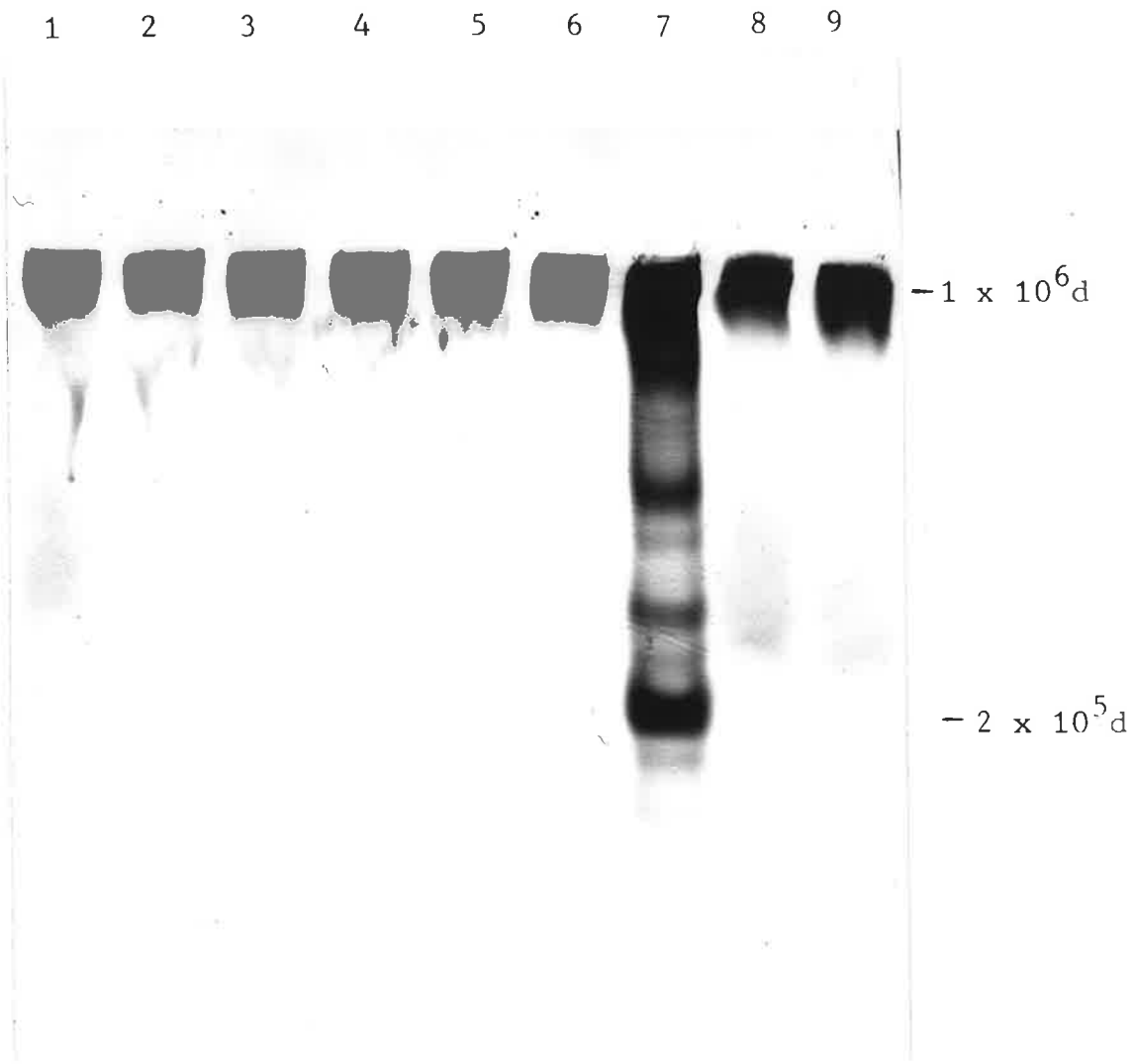

Figure 2. Immunoblotting technique for the demonstration of LMW IgM.
Lanes 1-6, 8 and 9 - cord sera with absent LMW IgM.
Lane 7 - positive control demonstrating the presence of LMW IgM and other oligomers of IgM.

rheumatoid sera which served as positive controls. Similarly Starita-Geribaldi and Masseyeff, (1975) found trace quantities of LMW IgM in all 4 healthy cord sera studied using a column chromatographic technique combined with a sensitive IgM radiometric assay. In contrast Perchalski et al, (1968) found large quantities of LMW IgM in 8 of 12 cord sera. However we believe that their assay of column chromatography combined with immunodiffusion is less specific than that of Starita-Geribaldi and of ours. To detect LMW IgM Perchalski concentrated the fractions eluting in the Sephadex G200 second peak ("7S fraction") and detected IgM by immunodiffusion. This manouvre might allow the possibility of pentameric IgM tailing into these fractions during chromatography and giving rise to a false positive. We believe that both of our LMW IgM assays are sensitive and highly specific for LMW IgM with the chromatographic method combined with the IgM ELISA being reproducible and appearing in this study to be slightly more sensitive than the immunoblot method. Both assays failed to detect LMW IgM in 15 healthy adult sera but have given consistent positive results in certain autoimmune, infective, immunodeficient and lymphoproliferative disorders (see subsequent Chapters). If LMW IgM is to be found in healthy adult sera its levels must be lower than the threshold sensitivities of our assays (approximately 1.0 ug for the chromatographic/ELISA method and 0.1 ug for the immunoblotting method (although this threshold value varies considerably depending on the quality and resolution of the IgM bands in each run - see Chapter two))

The finding of LMW IgM in 4 of the healthy cord sera but not in adult sera was of some interest. Furthermore Jol-V.D. Zijde et al,

(1983) have noted the appearance of LMW IgM during marrow reconstitution following bone marrow transplantation. An explanation for these observations might be that the origin of the LMW IgM is from 'immature' or foetal type B lymphocytes, possibly the CD5 positive subset (Lydyard et al, 1987). Indeeed the presence of LMW IgM in cord blood from healthy neonates together with the observation that LMW IgM occurs naturally in many lower vertebrates has been frequently quoted in support for the theory that the occurrence of LMW IgM in disease represents an ontogenic and phylogenic reversion of the humoral immune response to a more atavistic state. However this theorey tells us nothing about the origins, relevance and mechanisms for the secretion of LMW IgM in disease. Such topics will be explored in future Chapters.

CHAPTER FOUR

LOW MOLECULAR WEIGHT IgM IN RHEUMATOID ARTHRITIS AND OTHER RHEUMATIC DISEASES

SUMMARY

Low molecular weight (LMW) IgM has been measured in the serum and synovial fluid of patients with rheumatoid arthritis (RA) and other rheumatic diseases. High levels were seen in RA, particularly in rheumatoid vasculitis and Felty's syndrome and significant correlations occurred between LMW IgM and the rheumatoid factor (RF) and the absolute IgM level and other indices that reflected active or severe disease.

LMW IgM-RF, measured by radioimmunoassay in those column fractions containing LMW IgM, correlated significantly with circulatig LMW IgM ($P < 0.005$) and preliminary experiments suggested that in some sera a considerable proportion of the LMW IgM consisted of LMW IgM-RF. It is concluded that LMW IgM and LMW IgM-RF may have important implications in the immunopathogenesis of RA and other rheumatic diseases.

INTRODUCTION

There have been several previous studies concerning the occurrence of LMW IgM in a number of rheumatic disorders including rheumatoid arthritis and systemic lupus erythematosus (SLE) (Rothfield et al, 1965; LoSpalluto, 1968; Dammacco et al, 1970; Stage and Mannik, 1971; Anderson-Imbert et al, 1972; Theofilopoulos et al, 1974; Clark et al, 1974; Harisdangkul et al, 1975) although in none has the absolute levels of LMW IgM been assessed systematically.

In RA several authors have noted the association of LMW IgM with the presence of rheumatoid vasculitis and have suggested that LMW IgM may play an important role in the immunopathogenesis of this extraarticular manifestation (Stage and Mannik, 1971; Theofilopoulos et al, 1974). Supporting evidence for this idea has been obtained from the observation that LMW IgM manifests certain autoantibody activity including rheumatoid factor (RF) and DNA binding activity (Harisdangkul et al, 1975) and in some instances fixes complement (Caldwell, 1973). Furthermore, LMW IgM prepared from IgM by mild reduction and alkylation, although potentially bivalent, appears to function as a univalent antibody (Chavin and Franklin, 1969). If naturally occurring LMW IgM is similar in this respect, this property might enable this non-precipitating, non-agglutinating antibody to form small circulating immune complexes with a prolonged persistence in the circulation. Tissue deposition of such complexes could then lead to an immune complex state such as is seen in rheumatoid vasculitis or SLE.

The present study has been performed to quantitate the presence of LMW IgM in various rheumatic diseases and to correlate it with various clinical and laboratory variables, particularly those relating to the presence of circulating immune complexes. LMW IgM has been measured by a planimetric technique from the IgM elution profile obtained by Sepharose 6B chromatography and laser nephelometry. This technique is reproducible and more sensitive than those previously utilized (see Chapter two). In addition the various elution fractions have been measured for IgM-RF activity by solid phase radioimmunoassay. The quantities of LMW IgM-RF detected have been related to the total IgM

and to other clinical and laboratory variables.

PATIENT AND METHODS

Patients and Controls. Eighty-four patients with classical or definite RA (American Rheumatism Association (ARA) criteria (Ropes et al, 1958)) were studied. They were selected from the hospital wards (27 patients) and outpatient clinic (57). Thirty-nine were male, 45 female; the mean age was 55 years (range 14-82 years) and the mean duration of the illness was 10.8 years (range 2 months-42 years). In 75 of the patients, clinical data were available to subdivide them into 4 groups: a) 18 patients with inactive or suppressed disease defined by the absence of active synovitis; b) 41 patients showing evidence of active synovitis; c) 9 patients with active disease complicated by rheumatoid vasculitis defined by the presence, within one month of testing, of mononeuritis multiplex or a necrotizing vasculitis or the presence of skin ulceration not attributable to other causes; and d) 7 RA patients with Felty's syndrome defined by the presence of splenomegaly and leukopenia with a neutrophil count less than $2000/mm^3$. There were 26 patients with SLE (ARA criteria), 27 with selective IgA deficiency (the clinical and laboratory features of 20 of these patients have been described elsewhere (Kwitko et al, 1979), 6 with systemic sclerosis, 3 with primary Sjogren's syndrome and 12 with other diseases (2 each with idiopathic mixed cryoglobulinaemia, primary biliary cirrhosis, Reiter's disease, and fibrosing alveolitis and one each with gout, psoriasis, pneumonia and urinary tract infection). Control sera were obtained from 20 healthy

laboratory personnel.

Venous blood was allowed to clot at room temperature, and serum, obtained within 4 hours, was aliquoted and stored at -70°C. Joint fluid was aspirated from the knees of 15 RA patients and patients with gout (2), pseudogout (1) and psoriasis (1), spun at 100 g and the supernatant aliquoted and stored at -70°C.

Other Immunological Measurements. Serum immunoglobulins, C3 and C4 were measured using an automated immune precipitation nephelometer (Technicon). Rheumatoid factor was measured by two methods: a) the Rose-Waaler sheep cell agglutination (SSCA) test or b) nephelometrically, by observing the degree of precipitation in the laser nephelometer between 100 ug of heat aggregated human IgG and test serum diluted 25-fold in phosphate-buffered saline (PBS) to give a final volume of 500 ul. There was an excellent correlation between this method and the Behring latex agglutination titer ($r = 0.95$, $p < 0.001$). Immune complexes were measured by the Clq binding assay of Zubler et al, (1976) and by the nephelometric monoclonal RF (mRF) assay of Roberts-Thomson and Bradley, (1979).

Determination of LMW IgM. LMW IgM was measured in serum and synovial fluid by the planimetric technique from the IgM profile obtained by Sepharose 6B chromatography and laser nephelometry (as described in Chapter two).

IgM Rheumatoid Factor (IgM-RF) Radioimmunoassay. IgM was measured using a modification of the solid phase radioimmunoassay of Carson et

al, (1976) as described by Wernick et al, (1981). We thank Dr. Wernick and Mr. D. Edelbaum for performing these measurements. Column fractions were diluted 1:100, 1:200 or 1:500 in 1% bovine serum albumin in PBS according to the undiluted serum rheumatoid factor level and the IgM-RF determined. Results were expressed in counts per min (cpm) of labelled rabbit antibody bound as compared with a standard preparation of rheumatoid factor isolated from the serum of a Felty's syndrome patient as previously described (Wernick et al, 1981). In some experiments a LMW IgM-RF standard was also included. This was obtained from a patient with a lymphoproliferative disorder whose serum contained both a monoclonal 19S IgM kappa-RF and a LMW IgM-RF. The LMW IgM-RF was isolated by Sepharose 6B and Sephadex G200 chromatography and the IgM content measured by laser nephelometry.

Statistical Analysis. Comparison between variables was calculated by linear regression whilst comparison between groups was performed by the Wilcoxon sum of ranks method. Differences in proportions between the two groups were tested using a 2 x 2 contingency table.

RESULTS

LMW IgM in Serum and Synovial Fluid. LMW IgM was not found in any of the 20 control sera. In contrast 67 of 84 (80%) RA sera, 12 of 26 (46%) SLE sera, 3 of 3 Sjogren's sera, 3 of 6 systemic sclerosis sera, 8 of 27 (30%) selective IgA deficiency sera and 3 sera from 12 patients with other diseases were found to contain LMW IgM (Figure 1).

The positive sera in the latter group were from 2 patients with idiopathic mixed cryoglobulinemia and one with primary biliary cirrhosis. The mean ± SD LMW IgM for the RA group was 18.8 ± 21.6 mg/dl, for the SLE group 7.7 ± 10.4 mg/dl and for the IgA deficiency group 4.9 ± 8.5 mg/dl.

When the RA patients were divided into 4 groups according to their clinical features (Figure 2 and Table I), all patients with rheumatoid vasculitis and Felty's syndrome had LMW IgM, some with considerable quantities. The LMW IgM comprised a mean of 19% of the total IgM, in the latter two conditions (Table I). As a group these patients had significantly more LMW IgM than RA patients without these complications ($p<0.01$). In addition these patients with rheumatoid vasculitis and Felty's syndrome tended to have higher serum IgM levels although the increases could only partially be attributed to the higher levels of LMW IgM (Table I).

LMW IgM was found in 13 of 16 (81%) RA synovial fluids with a mean ± SD of 8.8 ± 12.4 mg/dl. It was not found in any of the 4 inflammatory fluids from non-RA patients. In 10 RA patients LMW IgM was determined in paired serum and synovial fluid samples (Figure 3). The mean LMW IgM in the synovial fluid (8.8 mg/dl) was lower than the mean for the corresponding serum (12.7 mg/dl). However in two of these fluids both LMW IgM and rheumatoid factor measured by nephelometry were found to be higher in concentration than in the paired sera, although the total IgM was lower, suggesting local synovial synthesis of both of these constituents. Significant correlations occurred between synovial fluid LMW IgM and the paired

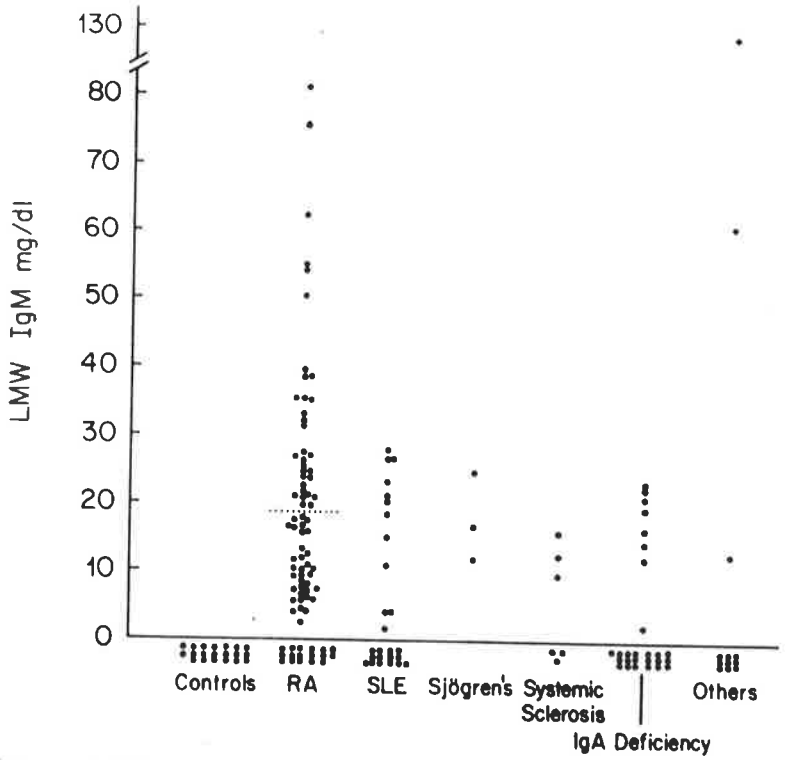

Figure 1. LMW IgM in sera from patients with various rheumatic diseases. The three positive sera in the other diseases group were from 2 patients with idiopathic mixed cryoglobulinemia and 1 patient with primary biliary cirrhosis.

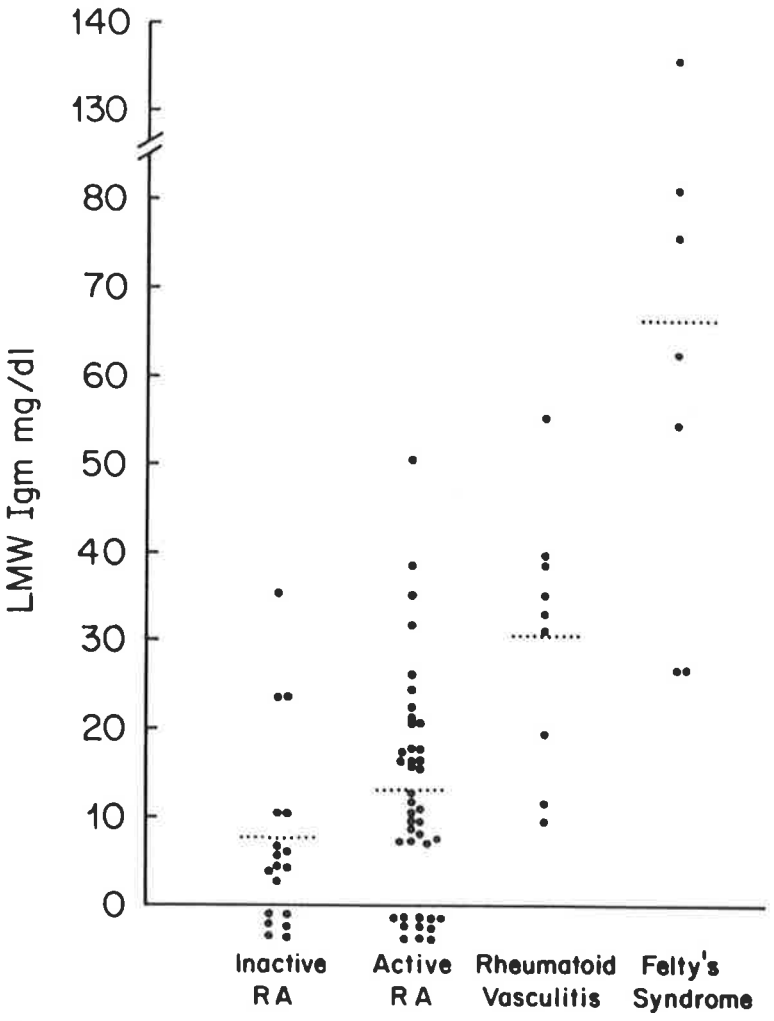

Figure 2. LMW IgM in inactive and active RA and in RA complicated by vasculitis or Felty's syndrome.

Table 1. Low molecular weight IgM in rheumatoid arthritis

Diagnosis, no. of patients	No. with LMW IgM (%)	LMW IgM*		Serum IgM mg/100 ml*
		% total IgM	mg/100 ml	
Inactive RA, 18	12 (67)	6.3 ± 7.3	7.7 ± 10.1	113 ± 41
Active RA, 41	30 (73)	9.3 ± 6.9	13.1 ± 11.9	148 ± 81.5
Rheumatoid vasculitis, 9	9 (100)	18.6 ± 8.5	30.6 ± 14.6	171 ± 181
Felty's syndrome, 7	7 (100)	18.9 ± 13	66.3 ± 37.4	279 ± 194

* Mean ± SD.

Table 2. Correlation of LMW IgM level with other clinical and laboratory variables

Variables	r	*P**	No. of patients
Age	0.28	NS	36
Disease duration	0.34	0.05	34
ESR	0.24	NS	33
SSCA titer	0.74	<0.01	35
RF†	0.76	<0.01	36
IgG	−0.31	NS	36
IgA	0.13	NS	35¶
IgM	0.65	<0.01	36
C3	−0.16	NS	35
C4	−0.36	0.05	35
C1q binding‡	0.59	<0.01	36
mRF binding§	−0.19	NS	36

* NS = not significant.
† Rheumatoid factor level measured by nephelometry.
‡ C1q binding assay for immune complexes.
§ Monoclonal rheumatoid factor assay for immune complexes.
¶ One patient with IgA deficiency has been excluded.

serum LMW IgM measurements ($r = 0.83$, $p < 0.01$) and between the synovial fluid levels of LMW IgM and the rheumatoid factor ($r = 0.80$ $p < 0.01$) and C_4 ($r = -0.72$ $p < 0.05$).

Correlation of LMW IgM with other Clinical and Laboratory Variables. Serum LMW IgM concentrations were compared with other clinical and laboratory variables in 36 patients with RA. This group included 5 patients with rheumatoid vasculitis but no patients with Felty's syndrome. LMW IgM correlated significantly (Table 2) with SSCA titer ($p < 0.01$), rheumatoid factor level as measured by nephelometry ($p < 0.01$), serum IgM level ($p < 0.01$), Clq binding level ($p < 0.01$), disease duration ($p = 0.05$) and inversely with the C_4 level ($p = 0.05$). In contrast the serum total IgM correlated significantly (Table 3) only with the rheumatoid factor ($p < 0.01$), Clq binding ($p < 0.01$) and disease duration ($p < 0.05$). When LMW IgM was expressed as a percentage of the total IgM (Table 4) strong significant correlations remained both with the SSCA titer and rheumatoid factor level ($p < 0.01$ in both) suggesting that the association with RF was a consistent one.

In the non-RA group of patients formal comparison between LMW IgM and other variables was not undertaken because of the limited number of patients available. It appeared, however, that in SLE, LMW IgM occurred more commonly in those patients with active disease; and there was also a correlation with ANA titer. For example LMW IgM occurred in all 6 SLE patients with ANF titers $\geq$ 1:320 but was observed in only one patient of 6 whose ANF titer was <1:40. Three of 8 patients with isolated IgA deficiency and LMW IgM had associated

Table 3. Correlation of serum IgM level with other clinical and laboratory variables

Variables	r	*P**	No. of patients
Age	0.05	NS	36
Disease duration	0.42	<0.05	34
ESR	0.29	NS	33
SSCA titer	0.32	NS	35
RF†	0.51	<0.01	36
IgG	−0.27	NS	36
IgA	0.01	NS	35
C3	−0.15	NS	35
C4	0.25	NS	35
C1q binding‡	0.50	<0.01	36
mRF binding§	−0.26	NS	36
LMW IgM	0.65	<0.01	36

* NS = not significant.
† Rheumatoid factor level measured by nephelometry.
‡ C1q binding assay for immune complexes.
§ Monoclonal rheumatoid factor assay for immune complexes.

Table 4. Correlation of LMW IgM expressed as percent of total IgM with other clinical and laboratory variables

Variables	r	*P**	No. of patients
Age	0.32	NS	36
Disease duration	0.17	NS	34
ESR	0.03	NS	33
SSCA titer	0.72	<0.01	35
RF†	0.52	<0.01	36
IgG	−0.22	NS	36
IgA	0.10	NS	35
IgM	0.09	NS	36
C3	−0.05	NS	35
C4	−0.10	NS	35
C1q binding‡	0.32	NS	36
mRF binding§	−0.10	NS	36

* NS = not significant.
† Rheumatoid factor level measured by nephelometry.
‡ C1q binding assay for immune complexes.
§ Monoclonal rheumatoid factor assay for immune complexes.

disease including polyarthritis, asthma and glomerulonephritis (see Chapter eight).

Rheumatoid factor status was assessed in 71 of the non-RA patients by the SSCA and the nephelometric rheumatoid factor tests using aggregated human IgG. Twenty-seven of these patients had LMW IgM and 14 of these also had rheumatoid factor by one or both methods. Only 3 patients were seen with a positive rheumatoid factor test without detectable LMW IgM. The association between LMW IgM and the occurrence of rheumatoid factor was highly significant ($p < 0.001$).

Determination of LMW IgM Rheumatoid Factor. Sera from patients and controls were chromatographed on Sepharose 6B and the elution fractions measured for IgM by nephelometry and IgM-RF by radioimmunoassay. Eight illustrative chromatographic profiles are shown in Figure 4. In normal human serum, (NHS) a symmetrical high molecular weight IgM profile was always obtained and no significant IgM-RF binding was found in any of the fractions tested (panel a, Figure 4). In contrast, in most of the pathological sera a bi-modal IgM profile was obtained, in which the later emerging smaller peak represented LMW IgM. The IgM-RF binding profile was variable. In some patients it reflected the absolute IgM profile (panels c and d, Figure 4), in others similar amounts were found in both the heavier and lighter molecular weight IgM fractions (panel e) and in one serum most of the binding occurred in the LMW IgM fraction (panel f). In still others the IgM-RF profile was skewed to the right (panel g and h). These latter profiles were interpreted as representing the summation of overlapping HMW and LMW IgM-RF profiles.

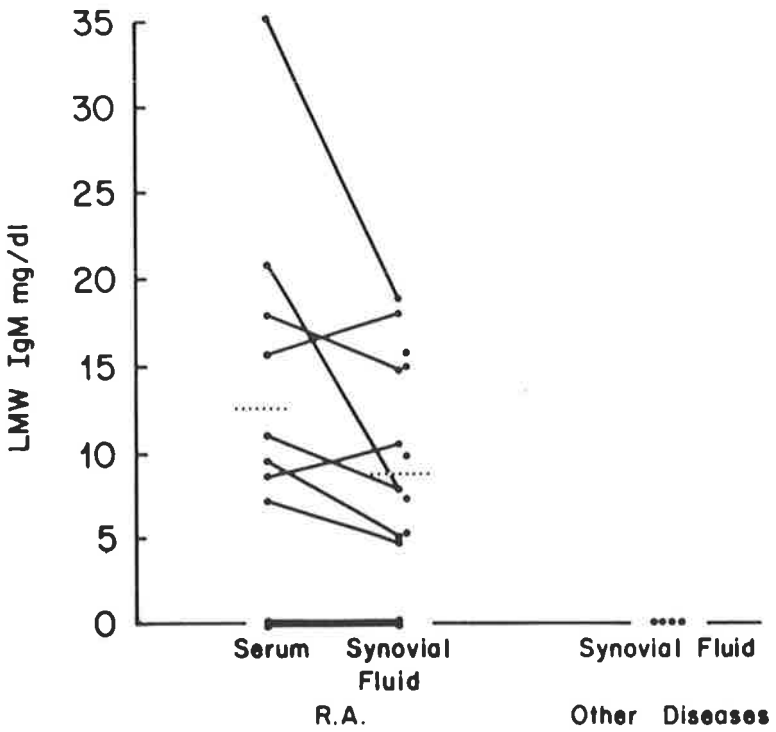

Figure 3. LMW IgM in paired serum and synovial fluid from 10 RA patients and in synovial fluid from patients with RA and other rheumatic diseases.

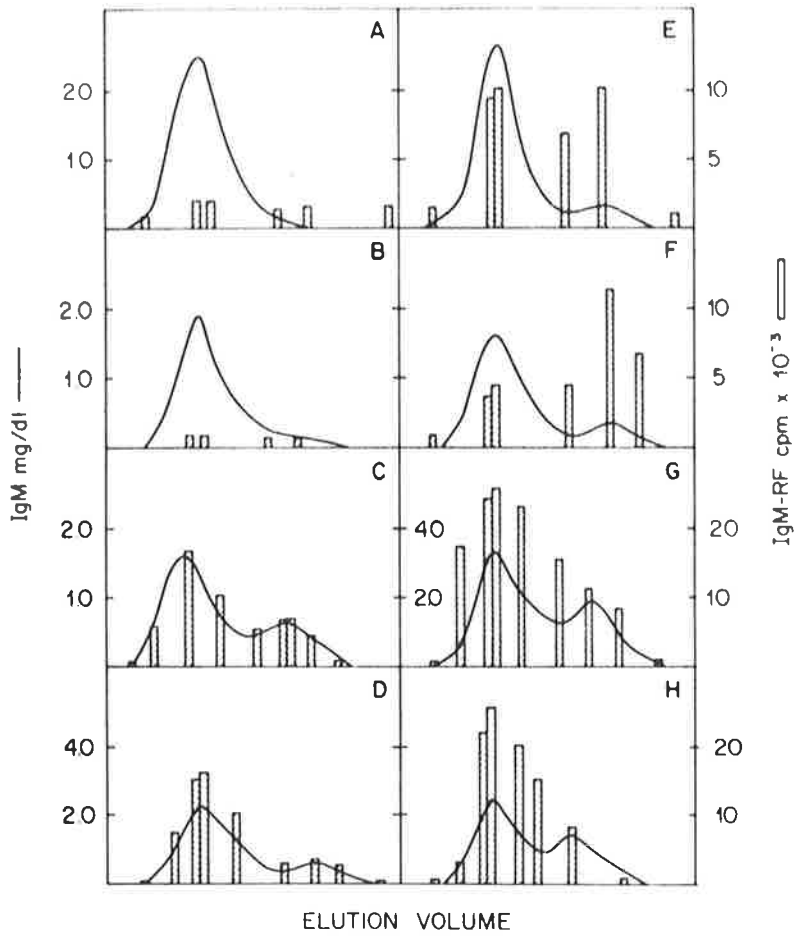

Figure 4. IgM elution profiles and simultaneous IgM-RF binding after filtration chromatography of a serum from a healthy control (**A**), 6 RA sera (**B-G**), and a patient with mixed cryoglobulinemia (**H**). **B** represents a seronegative RA patient, **D** and **G** represent Felty's syndrome patients, while **E** represents a rheumatoid vasculitis patient. The column fractions were diluted 1:100 in **A** and **E**, 1:200 in **B** and **F**, and 1:500 in panels **C**, **D**, **G**, and **H**. The lower dilution factor explains the higher non-specific background binding seen in **A** (NHS)

To confirm this interpretation, i.e. that the IgM-RF binding seen in the LMW IgM fractions was truly low molecular weight IgM-RF and did not merely represent a trailing of the HMW IgM-RF profile, LMW IgM containing fractions were pooled and rechromatographed over a Sephadex G200 Column (2.5 x 90 cm) and the IgM and IgM-RF determined. This experiment was performed using 3 different sera. In each case after rechromatography, IgM eluted as a single peak just before the IgG marker with a molecular weight of approximtely 200,000 daltons, and the IgM-RF profile could be superimposed on the LMW IgM profile, confirming the presence of LMW IgM-RF (data not shown).

Significance of LMW IgM-RF and Correlation with LMW IgM and HMW IgM-RF

Because the separation of HMW and LMW IgM-RF by chromatography and radioimmunoassay was in some sera less complete than that of HMW and LMW IgM, quantitation of LMW IgM-RF was carried out by an alternative procedure. The binding of IgM-RF (in cpm of rabbit antibody bound) measured at the peak of the LMW IgM profile was taken to represent the LMW IgM-RF level. Using this method LMW IgM-RF was quantitated in 20 pathological sera (19 RA sera and 1 mixed cryoglobulinemia). The results are shown in Figure 5. High levels of LMW IgM-RF were found for all of 3 patients with rheumatoid vasculitis, 3 of 7 patients with Felty's syndrome and the single patient with mixed cryoglobulinemia. A significant correlation was observed between LMW IgM-RF and the independently measured LMW IgM ($r = 0.74$, $p < 0.005$). In addition a strong correlation was found between LMW IgM-RF and HMW or 19S IgM-RF, also measured as cpm of rabbit antibody bound at the peak of the HMW IgM-RF profile, ($r = 0.76$, $p < 0.005$).

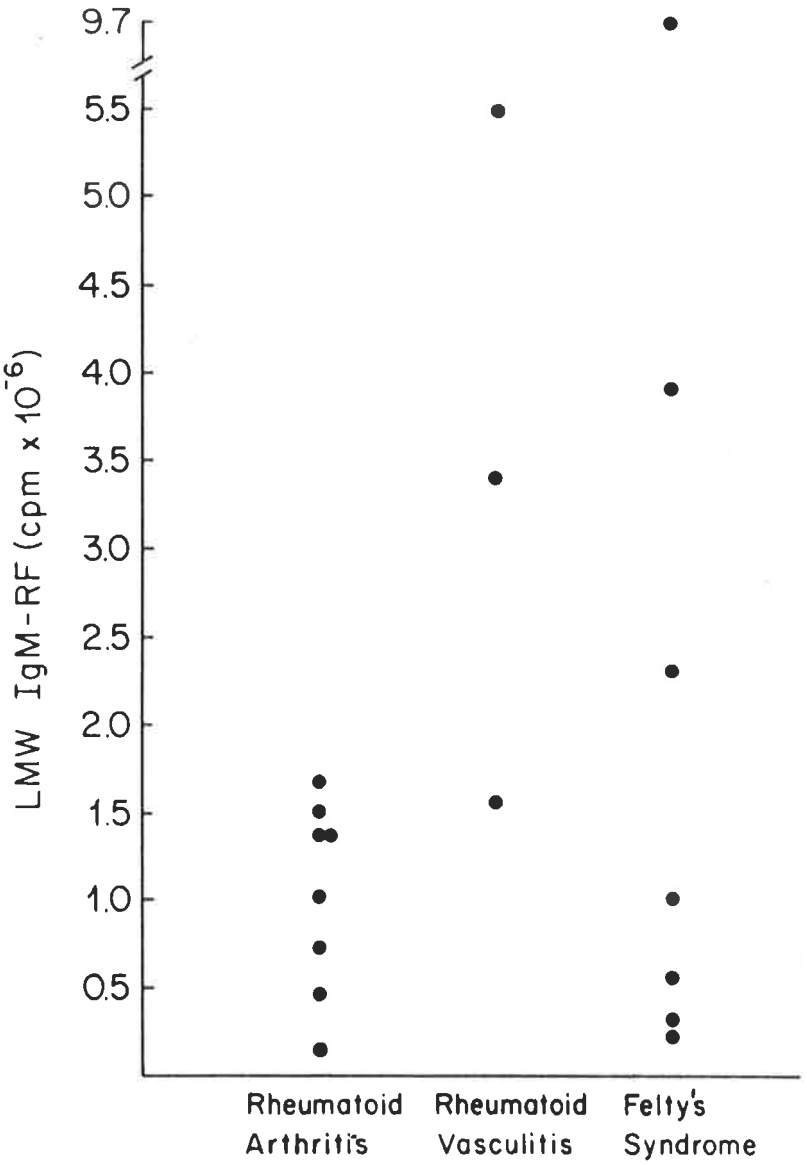

Figure 5. LMW IgM-RF in uncomplicated RA, rheumatoid vasculitis, and Felty's syndrome. LMW IgM-RF (expressed as cpm of labeled rabbit $F(ab')_2$ anti-μ, corrected for the column dilution factor), was measured at the peak of the LMW IgM profile after Sepharose 6B chromatography. Normal sera gave values less than the lowest value obtained for any RA serum, while the single patient with mixed cryoglobulinemia gave a high value of 8.2×10^6 cpm.

Preliminary experiments were performed to construct a standard curve for the binding of HMW and LMW IgM-RF to IgG substrate. Polyclonal IgM-RF, monoclonal HMW IgM-RF and LMW IgM-RF (the latter 2 reagents being obtained from a patient with a lymphoproliferative disorder) were isolated by recycling chromatography over Sepharose 6B and Sephadex G200 and the IgM concentration determined by nephelometry. The binding of these preparations to solid phase human IgG was then measured and a standard curve plotted to calculate the absolute HMW and LMW IgM-RF binding in ng/ml. In this way the IgM-RF profile measured as cpm of labelled rabbit antibody obtained after chromatography of pathological sera could then be converted to absolute values. The absolute HMW and LMW IgM-RF values were then compared with the HMW and LMW IgM measured by nephelometry. Using this method it appeared that in certain sera both HMW and LMW IgM-RF could constitute up to 40% of the total HMW and LMW IgM respectively.

DISCUSSION

Filtration chromatography combined with nephelometry is a reproducible method for the estimation of absolute levels of LMW IgM and is 5-10 times more sensitive than the gel diffusion methods previously described (Harisdangkul et al, 1975; Dammacco et al, 1970). However the disadvantages of this method are that it requires up to 2 ml of serum, is time consuming and requires sophisticated and expensive laboratory equipment. Despite these limitations it can give valuable information and has been used to measure and assess the significance of LMW IgM in RA and other rheumatic diseases.

LMW IgM was found in 80% of 84 RA patients and this figure is similar to the 71% positivity reported by us in a previous but smaller study (Roberts-Thomson et al, 1980 (a)). Several early workers, using a variety of techniques, have reported a prevalence of 10 - 50% LMW IgM in RA (LoSpalluto, 1968; Dammaco et al, 1970; Stage and Mannik, 1970; Theofilopoulos et al, 1974; Hunder and McDuffie, 1973). The higher prevalance found in the present study is attributed to the enhanced sensitivity of the nephelometric method.

In RA previous investigators have observed an association between LMW IgM and the severity of the disease, particularly in those patients with multiple joint subluxations, subcutaneous nodules, high ESR, high IgM levels, the presence of ANA and the presence of rheumatoid vasculitis (Stage and Mannik, 1970; Theofilopoulos et al, 1974). Many of these associations have been confirmed in the present study. In particular, large quantities of LMW IgM were found in those patients with either rheumatoid vasculitis or Felty's syndrome. Significant correlations were also observed between LMW IgM and various indices reflecting severe and/or active disease. These indices included rheumatoid factor, Clq binding, IgM, and C_4 levels and confirm the correlations noted in our earlier independent study (Roberts-Thomson et al, 1980 (a)). All these indices are known risk factors for severe and/or active disease (Roberts-Thomson et al, 1980 (b); Schmid et al, 1961). LMW IgM was also detected in the synovial fluid of 13 RA patients. Unlike Anderson-Imbert et al, (1972) who found that the concentration of LMW IgM in synovial fluid always exceeded that of the corresponding serum, and in some fluids was as high as 150 mg/dl, we found only 2 fluids out of 10 in which the

levels were higher. Interestingly in both of these fluids the rheumatoid factor levels and Clq binding activity were also higher than in the corresponding serum although the total IgM was lower. Thus in these two fluids indirect evidence was obtained for the local synthesis of LMW IgM and RF. LMW IgM was not found in any of the non-RA inflammatory fluids suggesting that acute inflammation per se does not cause LMW IgM to be derived from HMW IgM by catabolic processes.

LMW IgM was also commonly found in other rheumatic diseases beside RA. This immunoglobulin has been described before in SLE (Rothfield et al, 1965), primary biliary cirrhosis (Fakunle et al, 1979), and in one patient with systemic sclerosis (Stage and Mannik, 1971) but to our knowledge has not been described in primary Sjogren's syndrome, or idiopathic mixed cryoglobulinemia (see Chapter seven). In SLE 40% of our patients had LMW IgM and this prevalence is greater than the 15% reported both by Dammacco et al, (1970) and Rothfield et al, (1965) and the 17% reported by Stobo and Tomasi, (1967), but similar to the 45% recorded recently by Harisdangkul et al, (1984). In our study patients with LMW IgM tended to have active disease and high ANF titers, an association previously noted by Rothfield et al, (1965). Two of the male SLE patients had LMW IgM. The high prevalence of LMW IgM in male lupus patients was initially described by Rothfield et al, (1965) and confirmed by Stobo and Tomasi, (1967), and more recently by Harrisdangkul et al, (1977).

By filtration chromatography over Sepharose 6B and Sephadex G200 it was shown in the present study that column fractions containing LMW

IgM but no HMW IgM, contained RF activity. As the rabbit $F(ab')_2$ antiserum used in the radioimmunoassay was mu chain specific, this meant that a portion of the LMW IgM found in these sera was LMW IgM-RF, and preliminary experiments revealed that in certain sera this could be as much as 40% of the LMW IgM. It should be noted that this LMW IgM-RF, measured as cpm of labelled rabbit antibody bound in fractions obtained at the peak of the LMW IgM profile, was found to correlate strongly with LMW IgM measured independently by the nephelometric technique; and the highest values were found in patients with rheumatoid vasculitis and Felty's syndrome, diseases in which circulating immune complexes have been strongly implicated (Roberts-Thomson et al, 1980 (b); Andreis et al, 1978). Furthermore, in the present investigation, a significant correlation was seen between LMW IgM-RF and HMW IgM-RF the latter being a known risk factor for the development of severe disease and rheumatoid vasculitis (Schmit et al, 1961). It is therefore possible that an immune complex of LMW IgM-RF bound to IgG may have an etiological role in the production of rheumatoid vasculitis and this possibility is supported by the previously reported association between LMW IgM and rheumatoid vasculitis (Stage and Mannik, 1971; Theofilopoulos et al, 1974).

There has been only one previously published report showing that LMW IgM isolated from a single RA serum had RF activity (Harisdangkul et al, 1975). Other antibody activities including anti-DNA (Harisdangkul et al, 1975), anti-casein (Hunter et al, 1968), anti-tetanus toxoid (Swedlund et al, 1968), and anti-isohemagglutinin (Stobo and Tomasi, 1967) have been ascribed to LMW IgM and in all instances these were non-agglutinating, non-precipitating antibodies.

LMW IgM was also shown to correlate strongly with serum rheumatoid factor level measured by two methods (the SSCA and nephelometric rheumatoid factor test) in the RA group and by one or both of these methods in the non RA group. In addition the observation that in RA sera a significant proportion of LMW IgM is LMW IgM-RF and the significant correlation between LMW IgM-RF and HMW IgM-RF suggests that the serum levels of LMW IgM (including LMW IgM-RF) reflects the same immunological inciting stimulus as that responsible for rheumatoid factor production. Although this stimulus cannot be identified at this time, it is pertinent to note that in the majority of the pathological conditions associated with the production of LMW IgM, high levels of serum IgM, and circulating immune complexes and rheumatoid factor production have also been described.

Unanswered questions relating to LMW IgM are numerous. What is its significance in the immunopathogenesis of the rheumatic diseases? What is the stimulus that initiates the secretion of LMW IgM? What are its kinetics? We conclude that despite our limitation of knowledge concerning LMW IgM it appears that LMW IgM and LMW IgM-RF commonly occur in RA and other rheumatic diseases and are closely associated with clinical and laboratory indices that reflect severe and/or active disease. It is likely that these immunoglobulins may play an important role in the immunopathogenesis of these diseases.

CHAPTER FIVE

IN VITRO SYNTHESIS OF LOW MOLECULAR WEIGHT IgM IN RHEUMATOID ARTHRITIS

SUMMARY

No evidence was found to suggest that LMW IgM occurred as a consequence of proteolytic breakdown of immune complex-bound pentameric IgM. However, in contrast, peripheral blood mononuclear cells (PBMC) from seropositive RA patients stimulated with pokeweed mitogen secreted considerable quantities of LMW IgM in vitro. This did not occur with PBMC obtained from healthy controls. Pokeweed stimulation was necessary for the phenomenon to be observed since culture supernatants from unstimulated cells contained insufficient IgM for chromatographic analysis. Cycloheximide, a potent protein synthesis inhibitor, suppressed the phenomenon, illustrating that the production of LMW IgM was dependent on active protein synthesis. Furthermore, the study revealed a significant correlation between percent LMW IgM produced in vitro and percent circulating LMW IgM.

INTRODUCTION

The factors concerned in the production of LMW IgM are largely unknown. Four hypotheses have been suggested to explain its presence (Chapter one). First, in vivo breakdown of immune complex-bound pentameric IgM may release monomeric IgM. This could possibly explain the frequent occurrence of LMW IgM in immune complex states (Chapter four). Second, a defect during IgM polymerization could result in the liberation of monomeric IgM in circulation. This is the favored postulate in B cell lymphoproliferative disorders associated with IgM paraproteins (Carter and Hobbs, 1971). Third,

LMW IgM exists in small quantities as a membrane-bound immunoglobulin on certain B cells, and its release into the circulation could theoretically occur during rapid cell proliferation. However, membrane-bound LMW IgM has different physicochemical properties and low synthetic rates compared with secretory pentameric IgM, and its release into the circulation in quantities comparable with those found is not considered possible (Vogler, 1982). Last, the presence of LMW IgM may represent a phylogenetic reversion of the immune response (Solomon and McLaughlin, 1970), as LMW IgM exists naturally in lower vertebrates (Marchalonis and Edelman, 1965). This may explain its occurrence in a number of chronic immune stimulated states such as RA and chronic infective disorders and its close association with RF, which is also frequently found in these conditions.

This study explores the first, second and fourth of these hypotheses. It documents the active secretion of LMW IgM by pokeweed-stimulated peripheral blood mononuclear cells (PBMC) obtained from RA patients in vitro, but not from healthy control subjects. A correlation is described between the levels of LMW IgM synthesized in vitro and circulating LMW IgM.

PATIENTS AND METHODS

Patients and controls. Seven patients with seropositive, classic RA according to the American Rheumatism Association criteria (Ropes et al, 1958) were studied. Patients 2 and 7 (Table I) also fulfilled the criteria for Felty's syndrome, and patient 1 had diffuse

interstitial pulmonary fibrosis. Four of the patients were women, 3 men; their mean age was 59 years (range 41-69) (Table 1). All patients were taking nonsteroidal antiinflammatory drugs. One (patient 6) was taking D-penicillamine, 500 mg/day, and patient 1 was taking prednisolone, 10 mg/day. Five healthy laboratory employees (4 males, 1 female; mean age 29 years, range 23-42) were used as controls. Blood was collected by venepuncture, allowed to clot at room temperature, and serum separated by centrifugation and stored at -20°C (-80°C for immune complex determination).

Immunological investigations. Circulating immune complexes were measured by the fluid-phase Clq binding method (Zubler et al, 1976) and total IgM and rheumatoid factor were determined by rate nephelometry (Beckman, ICS).

Quantitation of circulating LMW IgM. Serum LMW IgM was measured as previously described using Sephacryl S300 (90 x 2.5 cm) column chromatography and laser nephelometry (see Chapter two).

Peripheral blood mononuclear cell culture in vitro. This was performed as described in Chapter two.

Quantitation of LMW IgM in culture supernatant. For each subject, approximately 20 ml of culture supernatant was concentrated in an Amicon concentrator to a volume of 1 ml and applied to a 90 x 1.6 cm Sephacryl S300 column. Downward filtration was at 20 ml/h, and 2 ml fractions were collected and assayed for IgM by enzyme-linked immunosorbent assay (ELISA). From the IgM profile obtained, it was

possible to calculate the relative percentage of LMW IgM and pentameric IgM.

Enzyme-linked immunosorbent assay for IgM. IgM was measured in culture supernatants and column fractions using a competitive inhibition ELISA as previously described (Chapter two).

Radioiodination of purified IgM. A monoclonal IgM rheumatoid factor was purified by repeated cryoprecipitation and filtration chromatography in 0.1 M acetic acid buffer at pH 4.0, as previously described (Roberts-Thomson, 1982). The purity and pentameric size of the IgM were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This reagent was then radiolabelled with ^{125}I, using the lactoperoxidase method (Hesser et al, 1973).

Preparation of polymorphonuclear leucocytes. Neutrophils from a healthy donor (PJR-T) were prepared from heparinized blood by Ficoll-paque (Pharmacia) centrifugation and by 2% dextran sedimentation as previously described (Gale et al, 1985).

Preparation of heat aggregated human IgG (HAGG). HAGG was prepared by heating Cohn fraction II, CSL gammaglobulin (20 mg/ml) at 63^{o} for 45 minutes and diluting in phosphate-buffered saline as previously described (Roberts-Thomson, 1982).

Statistical analysis. Variables were compared using rank correlation coefficients.

RESULTS

Circulating LMW IgM was not found in any of the 5 control subjects, and chromatographic analysis of the culture supernatants revealed that all the IgM produced by day 7 of culture was in the pentameric form (Figure 1). In contrast, LMW IgM was found in all 7 serum specimens from the RA patients and contributed from 16 to 35% of the total IgM (Table 1). Furthermore, all pokeweed mitogen-stimulated cell culture supernatants of patients contained LMW IgM, and at day 7, the monomeric IgM contributed from 19 to 100% of the total IgM (Table 1 and Figure 1). In the cultures without pokeweed, insufficient IgM was obtained for chromatographic analysis. Viability counts at the completion of cultures of mitogen-stimulated cells revealed counts $\geq$87% in all instances. Significant correlations were observed between percent LMW IgM synthesized in vitro at day 7 and percent circulating LMW IgM ($r = 0.98$, $P < 0.01$) and between circulating LMW IgM and RF ($r = 1.0$, $P < 0.01$).

Analysis of the RA cell supernatants at day 3 of culture revealed that high levels of LMW IgM were seen in 6 of 7 patients, and LMW IgM continued to be secreted over the next 4 days (Figure 1). In contrast, the levels of pentameric IgM found at day 3 were low and were similar to the levels seen in control cultures (Table 1 and Figure 2).

Addition of cycloheximide to the cultures at day 0 resulted in an almost complete inhibition (>95%) of both pentameric and LMW IgM synthesis at day 3 compared with the control cultures. Viability

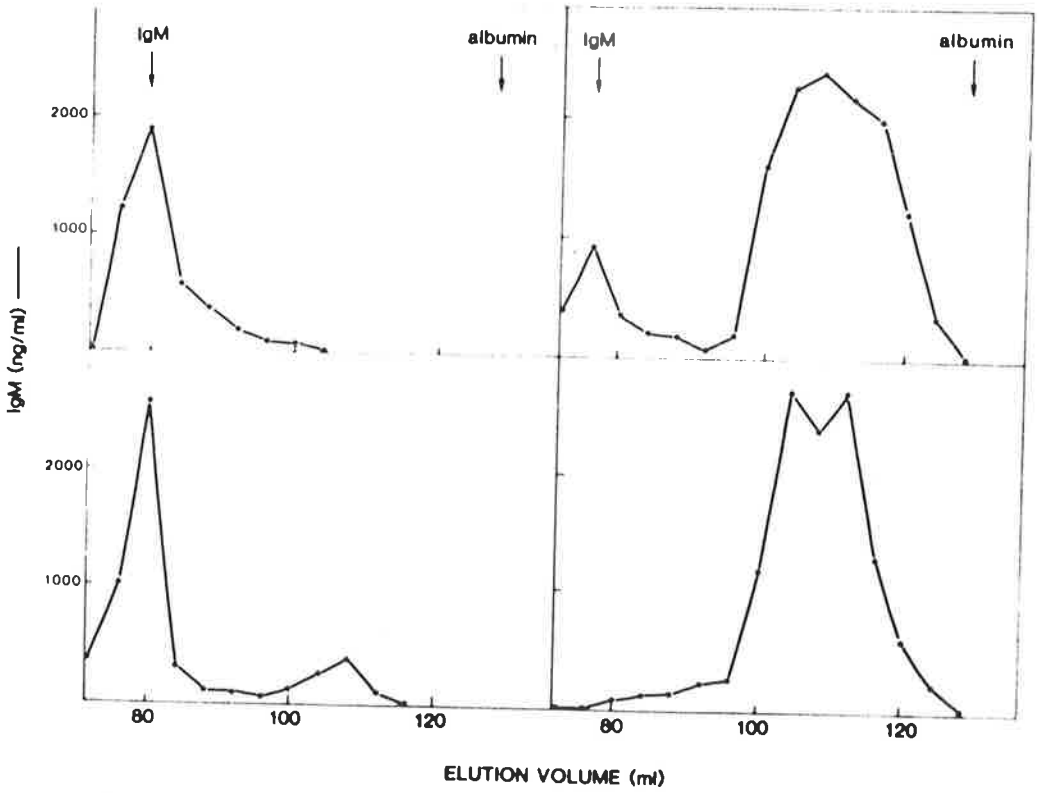

Figure 1. Enzyme-linked immunosorbent assay IgM profiles following column chromatography of cell culture supernatants at day 7. The elution positions of albumin and pentameric IgM are indicated. **Upper left**, Healthy control showing complete absence of monomeric IgM (LMW IgM). **Lower left**, Rheumatoid arthritis patient showing moderate quantities of LMW IgM. **Upper right**, Patient with Felty's syndrome showing large quantities of LMW IgM. **Lower right**, Rheumatoid arthritis patient with pulmonary fibrosis showing large quantities of LMW IgM and no pentameric IgM.

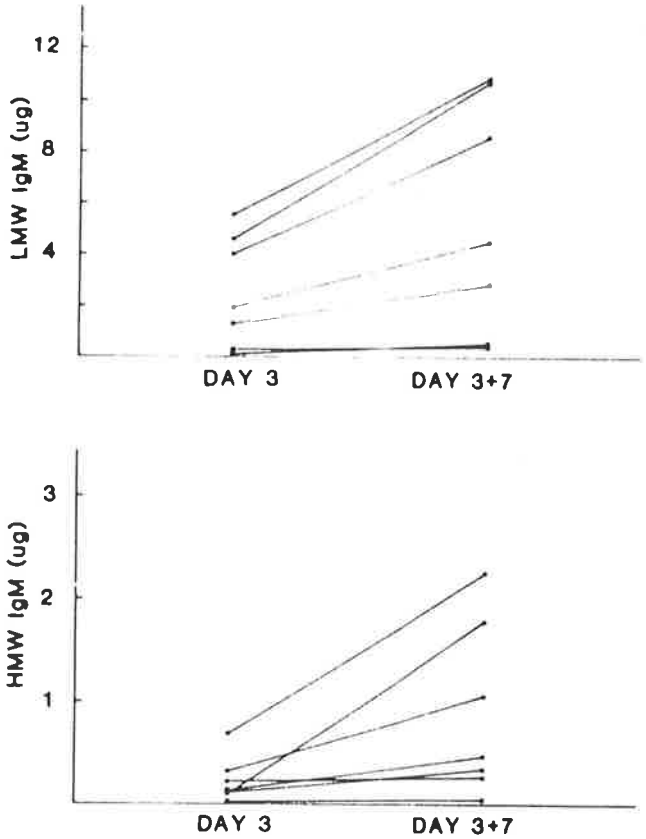

Figure 2. Quantities of monomeric IgM (LMW IgM) and pentameric IgM (HMW IgM) obtained at day 3 and day 3 + 7 cell culture. The lines join values for individual subjects.

Table 1. Clinical and laboratory data of rheumatoid arthritis patients secreting LMW IgM in vitro*

			Serum					Day 3			Day 7		
Patient	Sex	Age	RF IU/ml	IC units/ml	IgM gm/liter	% LMW IgM	LMW IgM gm/liter	IgM μg/culture†	% LMW IgM	LMW IgM μg/culture	IgM μg/culture	% LMW IgM	LMW IgM μg/culture
1	F	66	1.280	8	1.05	36	0.38	4.59	100	4.59	6.12	100	6.12
2	F	58	>9.999	41	7.7	35	2.7	2.08	94	1.96	2.84	87	2.47
3	M	69	4.000	41	3.24	26	0.84	6.27	89	5.58	6.79	83	5.64
4	F	67	270	4.1	0.64	16	0.10	0.22	51	0.11	2.06	19	0.39
5	M	41	>9.999	>75	5.79	35	2.03	1.39	92	1.29	1.77	87	1.53
6	M	59	1.260	>75	1.48	25	0.37	0.42	51	0.21	0.24	78	0.19
7	F	51	8.810	>75	6.75	26	1.76	4.31	93	4.00	5.26	86	4.52
Normal (n = 5)			<60	<2	0.5–2.2	0	0	0‡	0	0	0.88§	0	0

* LMW IgM = low molecular weight IgM; RF = rheumatoid factor; IC = immune complex.
† Culture = 2×10^6 peripheral blood mononuclear cells.
‡ <0.04 μg IgM/culture.
§ Mean of 5 healthy control subjects.

counts on both sets of cultures were similar (83-90%).

To exclude the possibility that LMW IgM resulted from the breakdown of secreted pentameric IgM during the period of culture, ^{125}I-radiolabelled pentameric IgM was added at day 0 to 2 cultures which were secreting considerable quantities of LMW IgM. At day 4, these culture supernatants were pooled and the ^{125}I column chromatographic profile was compared with the profile of the uncultured ^{125}I-IgM (Figure 4). The profiles were very similar and, in particular, no additional counts were observed in the LMW IgM region after culture, thereby refuting the possibility of spontaneous breakdown of pentameric IgM. Following culture a new peak which made up to 3% of the total counts was observed eluting at the position of the albumin marker, suggesting some transfer of ^{125}I to fetal calf albumin during the culture period (Figure 4, Panel D).

To investigate the possiblity that LMW IgM occurred as a consequence of breakdown of immune complex-bound pentameric IgM, radiolabelled monoclonal IgM-RF diluted in 10 ul of purified monoclonal RF (4.1 mg/ml) was incubated with 100 ug of HAGG and 100 ul of fresh normal human serum (final volume 1 ml) and incubated at 37^{o} overnight. One ml of 0.1 M acetic acid (pH 4.0) was then added to the solution, centrifuged at 1000 g for 20 min and the radioactive elution profile of the resultant supernatant determined following Sephacryl S300 chromatography (Figure 4, Panel B). This was then compared with the profile of the original I^{125} IgM-RF. In addition a similar experiment was performed with the addition of 10^{6} freshly prepared neutrophils to the reaction mixture. The resultant I^{125}

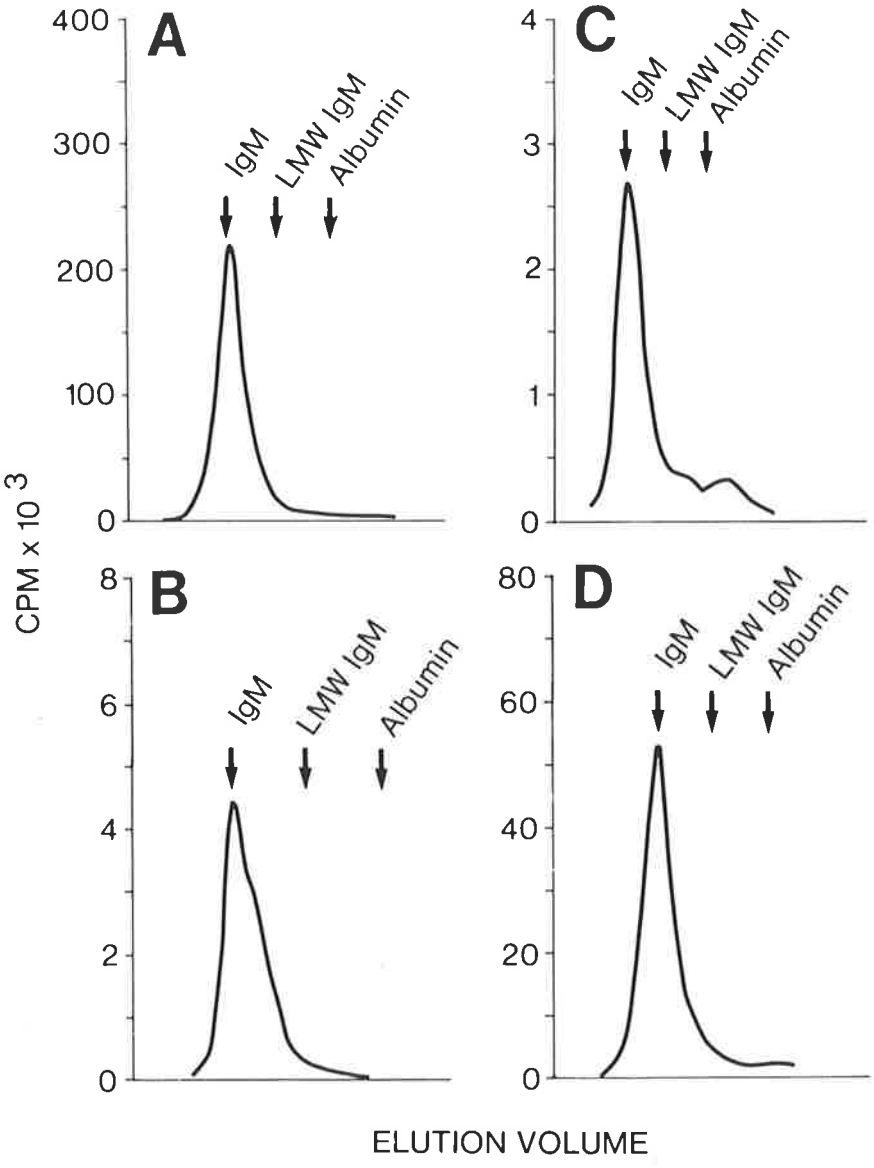

Figure 4. Panel A. Radiolabelled elution profile (cpm) of purified I^{125} pentameric IgM-RF (used in subsequent experiments) following Sephacryl S300 chromatography.
Panel B. Elution profile following incubation of I^{125} IgM-RF with HAGG and fresh normal human serum (NHS).
Panel C. Elution profile following incubation of I^{125} IgM-RF with HAGG, NHS and neutrophils.
Panel D. Elution profile following 4 day incubation of I^{125} IgM-RF with PBMC culture stimulated with pokeweed mitogen.

profile following incubation of I^{125} IgM-RF with HAGG and fresh human serum (as a source of complement) with and without neutrophils was similar to the original I^{125} IgM-RF profile and in particular there was no development of a new radioactive peak in the LMW IgM region (Figure 4, Panel C).

DISCUSSION

It has been demonstrated that PBMC from seropositive RA patients can be stimulated with pokeweed mitogen to actively secrete considerable quantities of LMW IgM in vitro. This did not occur with PBMC obtained from healthy controls. Furthermore, the study revealed a significant correlation between percent LMW IgM produced in vitro and percent circulating LMW IgM. No evidence was obtained to suggest that LMW IgM arose from catabolism or breakdown of immune complex-bound pentameric IgM.

This study has raised a number of questions related to LMW IgM. In the RA patients the percentage of LMW IgM contribution to the total IgM is greater for that produced by PBMC in vitro than that found in the serum. Whether this reflects an enhancement of the mechanism for LMW IgM production by the mitogen used or whether there is a variation between different sites of origins of IgM production (e.g. bone marrow, spleen, synovium) will need further analysis.

The correlation between LMW IgM synthesis in vitro and circulating RF raises questions about the relationship between these 2 entities.

Does their presence suggest a closely-related biologic phenomenon, e.g. phylogenetic reversion of the IgM immune response? We would predict from our studies described in Chapter three that a proportion of the LMW IgM secreted in vitro would have rheumatoid factor activity, but our initial attempts to demonstrate this have been unsuccessful, probably because of the large dilution that occurs following chromatography of culture supernatants.

The presence of IgM in the early stages (namely, day 3) of culture in the rheumatoid arthritis patients in contrast with its absence at the same time in the control subjects and the predominance of LMW IgM, particularly at day 3, over pentameric IgM raises the possibility of a B cell maturation disorder in RA. Does this suggest that the B cells are already primed and have some IgM polymerizing defect, or are the B cells programmed to secrete LMW IgM? It should be noted, however, that the quantity of IgM produced in culture at day 7 by the rheumatoid PBMC was considerably more than that produced by the controls. This is in contrast to most published studies where, in general, IgM synthesis by rheumatoid PBMC in response to pokeweed mitogen is depressed in comparison with that of normal individuals (Alarcon et al, 1982; Olsen and Jasin, 1985). The explanation of this difference is unclear, but possibly is due to the selection of our rheumatoid arthritis patients. Although they were selected because of their gross serologic abnormalities, 6 of 7 were not taking any specific disease-suppressive medications and three were taking nonsteroidal antiinflammatory drugs infrequenttly. Further synthetic studies will be required to clarify the difference. Nevertheless, having described the secretion of LMW IgM from RA PBMC in vitro, it is now

possible to study many of these questions using this technique. It is hoped that this approach will increase our understanding of the significance of LMW IgM in the rheumatic diseases.

CHAPTER SIX

APPEARANCE OF LOW MOLECULAR WEIGHT IGM DURING COURSE OF INFECTIVE ENDOCARDITIS

SUMMARY

Low molecular weight IgM has not been described before in infective endocarditis (IE). Eighteen patients with IE were studied; 16 with subacute bacterial endocarditis (SBE) and 2 with acute endocarditis. LMW IgM was detected in the sera of 6 patients, all having SBE in association with circulating rheumatoid factor (RF). Of the remaining 12 patients without LMW IgM only three had RF in low quantities. Sequential studies revealed that LMW IgM appeared during the later stages of the illness at or following the peak RF and IgM response. LMW IgM was not detected in any of 20 control sera. Immunoblot analysis of sera containing LMW IgM revealed the presence of small quantities of dimeric and oligomeric IgM in addition to monomeric IgM.

It is concluded that LMW IgM occurs predominantly in those patients with IE who have associated RF. Immunoblot analysis suggests that the presence of monomeric and oligomeric LMW IgM reflects a disorder of IgM polymerization occuring in those patients.

INTRODUCTION

In Chapter four the prevalence of LMW IgM in a number of rheumatic disorders was studied and a close association with RF and circulating immune complexes was observed. Infective endocarditis is a recognized immune complex disorder (Bayer et al, 1976) and it was therefore predicted that this disease would be

associated with the presence of LMW IgM. Moreover, in IE RF is found in approximately one third to one half of patients and usually levels peak after the peak levels of circulating immune complexes (Carson et al, 1978). Therefore both cross sectional and sequential studies were performed in patients with IE as one possible explanation for the occurrence of LMW IgM is that it results from the degradation and solubilization of immune complex bound pentameric IgM. The results suggest that this mechanism is unlikely to be the explanation for the occurrence of LMW IgM in IE and we suggest that it is more likely to be due to a disorder of monomeric assembly during IgM synthesis and secretion.

PATIENTS AND METHODS

Patients and Controls. Eighteen patients with IE were studied. Sixteen had SBE, 2 acute endocarditis and the diagnosis was made according to clinical, microbiological, serological and echocardiographic criteria. In three instances a specific microbiological organism was not identified. Twelve of the patients were male, 6 female and the mean age was 61 years (range 28-78yr). Serum was obtained on 59 occasions from these 18 patients and 3 or more serial specimens were studied in 13 patients. Control sera was obtained from 20 healthy laboratory personnel. All sera were stored at $-20^{o}C$ for serological testing and at $-80^{o}C$ for immune complex determinations.

Detection and Quantitation of LMW IgM. LMW IgM was detected in sera by two methods.

a) Filtration chromatography method (as described in Chapter two). In brief, serum was separated on either Sepharose 6B or Sephacryl S300 (Pharmacia, Uppsala) and IgM quantitated in the eluate fraction by laser nephelometry or ELISA. An IgM profile was then obtained and the detection of a second IgM peak eluting just before the IgG peak, signified the presence of LMW IgM.

b) Immunoblot analysis (as described in Chapter two).

Other serological investigations. C-reactive protein (CRP), serum IgM and RF were measured by rate nephelometry (Beckman ICS). Circulating immune complexes (CIC) were measured by the liquid phase Clq binding assay of Zubler et al, (1976).

RESULTS

LMW IgM was detected in 6 patients, all with the subacute variety of IE and 4 of these had extracardiac manifestation (splenomegaly in three, nephritis in two, polyarthritis in two, vaculitis in one). All 6 patients with LMW IgM had circulating RF. LMW IgM was not detected in the remaining 12 patients of whom 3 had low levels of RF or in any of the control sera. The IgM chromatographic elution profile from a patient with LMW IgM and a healthy control is shown in Figure 1. Sequential serum analysis (on 3 or more samples) was performed in 13 of the patients and the results obtained in 5/6 of those with

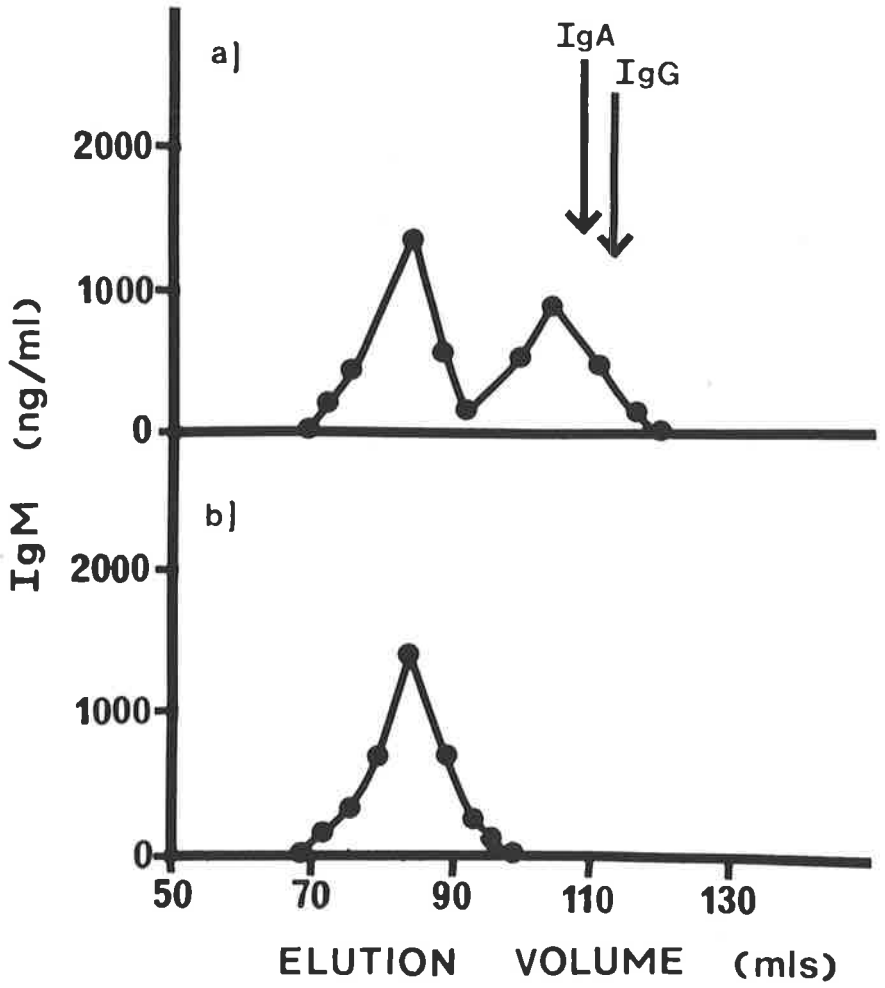

Fig. 1. IgM chromatographic prolifes following Sephacryl S300 filtration chromatography. (a) IgM profile from a patient with infective endocarditis; note the late eluting IgM peak reflecting LMW IgM. The elution positions of IgG and IgA are designated as shown. (b) Healthy controls.

Table 1. Sequential clinical and serological data obtained for five patients with circulating LMW IgM

Patient	Gender	Age (years)	Duration* illness (days)	Blood culture	CRP N < 6 mg/l	CIC N < 3 u/ml	IgM g/l	RF N < 80 iu/ml	LMW IgM	Treatment† and Outcome
1	M	60	1	*Strep. sanguis*	81	30	1·9	309	–	P & G
			3		49	22	2·85	377	–	Responded
			10		15	36	2·87	233	+	
			33		9	6·3	1·42	119	+	
			61		< 6	2·2	1·15	63	–	
			90			2·7	1·20		–	
2	M	74	1	*Strep. bovis*	> 82	6·5	1·44	304	–	P & G
			18		98	28	nd	nd	–	Responded
			29		59	11·5	1·72	nd	–	
			36		8	9	1·73	464	–	
			45		< 6	10	1·9	369	+	
			65		< 6	3·5	1·36	221	+	
			87		< 6	5·0	1·34	231	+	
3	M	60	1	*Strep. sanguis*	nd	14·5	nd	nd	nd	P & G
			7		96	16·5	2·18	383	–	died
			12		66	12·0	1·64	243	+	day 32
			15		104	9·6	1·78	243	+	
4	F	69	1		38	4·1	0·97	125	–	P & G
			9		16	4·5	0·94	141	–	died
			16	neg.	63	5·5	1·46	164	+	day 49
			24		29	32	1·92	92	–	
			37		34	15				
			41		53	17				
5	F	52	1	*Strep. faecalis*	160	3·5	3·24	201	–	A, G & Am
			5		86	5·6	3·82	228	+	Responded
			97		< 6	nd	3·77	313	+	

* From day of admission.
† (P) penicillin; (G) gentamycin; (A) ampicillin; (AM) amoxycillin.
nd Not done.

circulating LMW IgM are shown in Table I, (only 1 sample was available for study in the remaining sixth patient). It is seen that LMW IgM appeared during the evolution of the illness and was present in 4/5 patients by the third week after presentation and persisted for a variable period of time. In general its time course coincided with or followed slightly behind the peak IgM and RF response but was delayed in comparison with the peak levels of CRP and CIC's. These later serological indices fell to normal values by 4 weeks in 3 patients with successful treatment, but remained elevated in two patients who succumbed to their infection. Immunoblot analysis of sera containing LMW IgM revealed additional IgM bands migrating between the monomeric and pentameric bands. One of these bands migrated with a molecular weight of approximately 410,000 daltons suggesting the presence of dimeric IgM. Faint bands above and below this dimeric moiety were also evident.

DISCUSSION

At the onset of this study it was predicted that one would observe LMW IgM in IE because of its known close association with CIC and RF in RA (Chapter five and four). Moreover a sequential study of LMW IgM in IE might provide some information concerning its likely mode of production. The study has confirmed the presence of LMW IgM in IE, demonstrated a close association with extracardiac manifestations and RF, and noted its appearance late (following treatment) in the course of the disease generally after the zenith of the CIC levels and coinciding with or following the peak RF and IgM response.

As discussed in Chapters one and five there are several possible explanations for the occurrence of LMW IgM in human disease. Firstly, the presence of LMW IgM may represent a phylogenetic reversion of the immune response, as LMW IgM exists naturally in many lower vertebrates (Marchalonis and Edelman, 1965). This explanation may account for its occurrence in a number of chronic immune stimulated states such as IE and RA and explain its close association with RF which frequently occurs in these conditions as well. However this hypothesis provides no explanation for the mechanism of occurrence of LMW IgM. Secondly, LMW IgM exists in small quantities as a membrane bound immunoglobulin on certain B cells and its release into the circulation could occur during rapid B cell proliferation. However, the rate of cell turnover required to account for the LMW IgM levels in serum appear to be far in excess of that thought physiologically possible (Tartakoff and Vassalli, 1979; Johnstone, 1982). Thirdly, breakdown, in vivo, of immune complex bound pentameric IgM may release monomeric IgM. This could explain the frequent occurrence of LMW IgM in immune complex states. However the lack of concordance between the temporal profiles of CIC and LMW IgM as seen in this study, together with the in vitro findings using radiolabelled IgM antibody/antigen complexes (Chapter five) does not support this third hypothesis. Finally, LMW IgM may be released into circulation due to a defect of assembly of the monomeric subunits into the completed pentamer during the assembly of the IgM molecule. Evidence in support of this hypothesis would be 1) our previous observation of the active secretion of LMW IgM in vitro by peripheral blood mononuclear cells obtained from RA patients with circulating LMW IgM but not in healthy controls (Chapter five); 2) our current observations showing a temporal association between the

occurrence of LMW IgM and with the IgM and RF response during the course of IE; and 3) the presence of dimeric IgM and other oligomers together with monomeric IgM in patients with LMW IgM. Thus we currently consider that the most likely explanation for the presence of LMW IgM in IE and other human disorders, is due to a disorder of assembly of the monomeric IgM subunits during molecular polymerization. In summary our findings of the occurrence of LMW IgM during the course of IE, closely associated with RF and coinciding or closely following the IgM and RF response would be in support of a disorder of assembly of monomeric IgM subunits occurring during the humoral immune response to the infecting micro-organism.

CHAPTER SEVEN

LARGE QUANTITIES OF LOW MOLECULAR WEIGHT IgM IN MIXED CRYOGLOBULINAEMIA

SUMMARY

Low molecular weight IgM was not found in the sera of 20 healthy subjects but was detected in all 6 patients with mixed cryoglobulinaemia with a mean value of 1.4 g/l representing 34% of the total IgM. In 3 of 4 patients studied LMW IgM was monoclonal and of the same light chain type (kappa) as the pentameric monoclonal IgM rheumatoid factor (RF) observed in the cryoprecipitate. However LMW IgM was proportionately under represented in the cryoprecipitate compared with the corresponding serum possibly because of the lower valency of the LMW molecule. Immunoblot analysis of sera revealed the presence of other oligomers of IgM in addition to monomeric IgM suggesting that a disorder of IgM assembly was responsible for its occurrence and this was supported by the secretion of large proportions of LMW IgM in vitro by peripheral blood mononuclear cells (PBMC) from one patient with this disorder but not from healthy controls.

INTRODUCTION

Mixed cryoglobulinaemia is an uncommon clinical syndrome that consists of polyarthralgia, myalgia, recurrent palpable purpura and in many instances nephritis (Meltzer et al, 1966). The basic pathological feature is the presence of a necrotizing vasculitis occuring particularly in the skin and kidney. Mixed cryoglobulinaemia may occur as an isolated clinical entity (viz. the essential or idiopathic form) or in association with

lymphoproliferative, infective or autoimmune rheumatic disorders. The cause is unknown although some patients have evidence of past or present infection with Hepatitis B virus (Levo et al, 1977).

A large variety of immunological abnormalities have been reported in this condition particularly involving the humoral arm of the immune response (Brouet et al, 1974). These include the presence of large quantities of mixed cryoglobulins, consisting in the majority of cases of monoclonal kappa IgM with RF activity and polyclonal IgG; low levels of haemolytic complement, C3 and C4, and high levels of serum RF and circulating immune complexes. Serum immunoglobulins may be elevated, normal or depressed and elevated titres to various ubiquitous viruses are described (Fiorini et al, 1986). In the current study we report a further humoral abnormality in this disorder, viz the occurrence of considerable quantities of LMW IgM which appear to have a monoclonal origin in the majority of cases. Furthermore we present data which suggests that the LMW IgM arises due to a disorder of assembly of the monomeric IgM subunits during IgM synthesis.

PATIENTS AND METHODS

Patients and controls. There were 6 patients with mixed cryoglobulinaemia, 4 females and 2 males with a mean age of 69 years. Five patients had essential mixed cryoglobulinaemia and one patient had mixed cryoglobulinaemia secondary to a lymphoproliferative disorder (patient 1, Table I). The diagnosis of essential mixed

cryoglobulinaemia was made according to standard clinical, laboratory and histopathological criteria (Gorevic et al, 1980). Control sera were obtained from 20 healthy laboratory personnel. All sera were separated from venous blood after clotting at 37^{O}C and were stored at -80^{O}C.

Isolation of cryoglobulin. Patient's serum was allowed to stand at 4^{O}C for 72 hours, and the cryoglobulin separated by centrifugation at 1000g for 20 minutes at 4^{O}C. The cryoglobulin was then dissolved in warmed (37^{O}C) saline and the process of cryoprecipitation repeated over 72 hours. The cryoglobulin was then washed twice with iced saline.

Detection and Quantification of LMW IgM. LMW IgM was detected in sera by two methods.

Filtration chromatography method as described in Chapter two but with minor modifications. In brief, serum diluted in equal volumes of 0.1M Na acetate buffer pH 4.0 (to dissociate the components of the mixed cryoglobulin) was separated at room temperature on Sepharose 6B or Sephacryl S300 (Pharmacia, Uppsala, Sweden) equilibrated in phosphate-buffered saline pH 7.4 and the IgM quantitated in the eluate fraction by laser nephelometry or ELISA (as described in Chapter two). An IgM profile was then obtained and the detection of a second IgM peak eluting just before the IgG peak, signified the presence of LMW IgM. The method of planimetry was used to determine the quantity of LMW IgM.

Immunoblot analysis. The method used was exactly as described in Chapter two.

Other serological investigations. C-reactive protein (CRP), serum immunoglobulins and RF were measured by rate nephelometry (Beckman ICS). Circulating immune complexes (CIC) were measured by the liquid phase Clq binding assay of Zubler et al, (1976). Quantitation of cryoprotein from one ml serum was measured by the Ponceaux S dye binding method and identification of its specific proteins by zone electrophoresis and immuno electrophoresis using commercially derived antisera (Dakopatts).

Secretion of LMW IgM in vitro. (See Chapter two). Peripheral blood mononuclear cells (PBMC) were separated from heparinised blood by Ficoll-Hypaque sedimentation using standard techniques. No patient was receiving cytotoxic chemotherapy at the time of study. Replicate 2 ml cultures were established containing 2×10^6 PBMC and pokeweed mitogen at a final concentration of 1 ug/ml. The medium was changed at day three. At day seven the culture supernatants were collected (total volume 15 to 20 ml), concentrated in an Amicon concentrator unit to a final volume of 0.5 ml and applied to a 90 x 1.6 cm Sephacryl S300 column. Four ml fractions were collected and assayed for IgM using a specific IgM ELISA as described in Chapter two. To confirm active synthesis and secretion of IgM in vitro, cycloheximide was added to some of the cultures to a final concentration of 10 ug/ml.

RESULTS

The salient serological findings obtained for the 6 patients are shown in Table I. Considerable quantities of mixed cryoglobulins were found in all patients and the properties of these are shown in Table II. All patients' sera contained LMW IgM (mean level of 1.4 g/l) which contributed between 17-59% of the total IgM but LMW IgM was not detected in any of the control sera. In three patients the proportion of high molecular weight (HMW) and LMW IgM in the supernatant following cryoprecipitation was compared with the proportion in the original serum (Table III and Figure 1). An increase in the proportion of LMW IgM was seen in the supernatant suggesting that the HMW IgM was preferentially represented in the cryoprecipitate.

Column fractions containing LMW IgM from 4 patients were concentrated and analysed by zone and immunoelectrophoresis. In three patients the LMW IgM was identified as monoclonal IgM kappa whilst in the fourth it appeared to be polyclonal with both kappa and lambda light chain type.

The sera, cryoprecipitate and serum supernatant from 5 patients were also analysed by immunoblotting using IgM specific antisera (Figure 2). In addition to a band representing the monomer (MW approximately 2×10^5 d) being identified in all samples (although less evident in the cryoprecipitate specimens) additional higher molecular weight bands were also identified migrating at the dimer and trimer positions and also intermediary bands between the monomer and dimer

Table I

SEROLOGICAL FINDINGS IN PATIENTS WITH MIXED CRYOGLOBULINAEMIA

Patient	Gender	Age	IgG	IgA	IgM	LMW IgM	C3	C4	RF	CIC	Cryo-globulin	CRP
		Years	g/l	g/l	g/l	g/l	g/l	g/l	IU/ml	U/ml	g/l	mg/l
1	F	75	7.9	2.1	13.5	2.6	1.3	0.03	72,000	>75	10.4	ND
2	M	63	3.6	1.4	1.7	0.3	0.4	0.03	1,590	>100	2.6	99
3	F	64	5.9	0.7	3.8	2.2	0.6	0.08	2,290	50	+#	29
4	F	72	4.9	0.3	1.5	0.5	1.0	0.01	207	11	+	10
5	M	62	7.0	1.2	3.5	1.5	0.4	0.02	3,550	>75	4.9	ND
6	F	77	16.6	1.6	7.1	1.2	0.6	0.2	+o	ND*	4.0	ND
Normal			7-19	0.5-4.0	0.55-2.2	Nil	0.7-1.7	0.12-0.4	<80	<3	Nil	<6

o by Rose-Waaler

#+ = present but not quantitated

* ND = not done

Table II

CHARACTERISATION OF MIXED CRYOGLOBULINS

Patient	Cryoglobulin g/l[O]	Zone and immunoelectrophoretic appearance	RF activity
1	10.4	m[#] IgM kappa + p[©] IgG	+
2	2.6	m IgM kappa + p IgG	+
3	+[*]	m IgM kappa + p IgG	+
4	+	p IgM + p IgG	+
5	4.9	m IgM kappa + p IgG	+
6	4.0	Not done	+

[O] = derived from 1ml of serum

[#]m = monoclonal, [©]p = polyclonal

[*]+ = detected but not quantitated

Table III

PROPORTION OF IgM OCCURING AS THE LOW MOLECULAR WEIGHT MOEITY IN ORIGINAL SERUM AND IN SUPERNATANT FOLLOWING CRYOPRECIPITATION

Patient	Percentage LMW IgM	
	Original serum	Supernatant
1	15.9	19.6
2	17.0	45.0
4	35.0	48.0

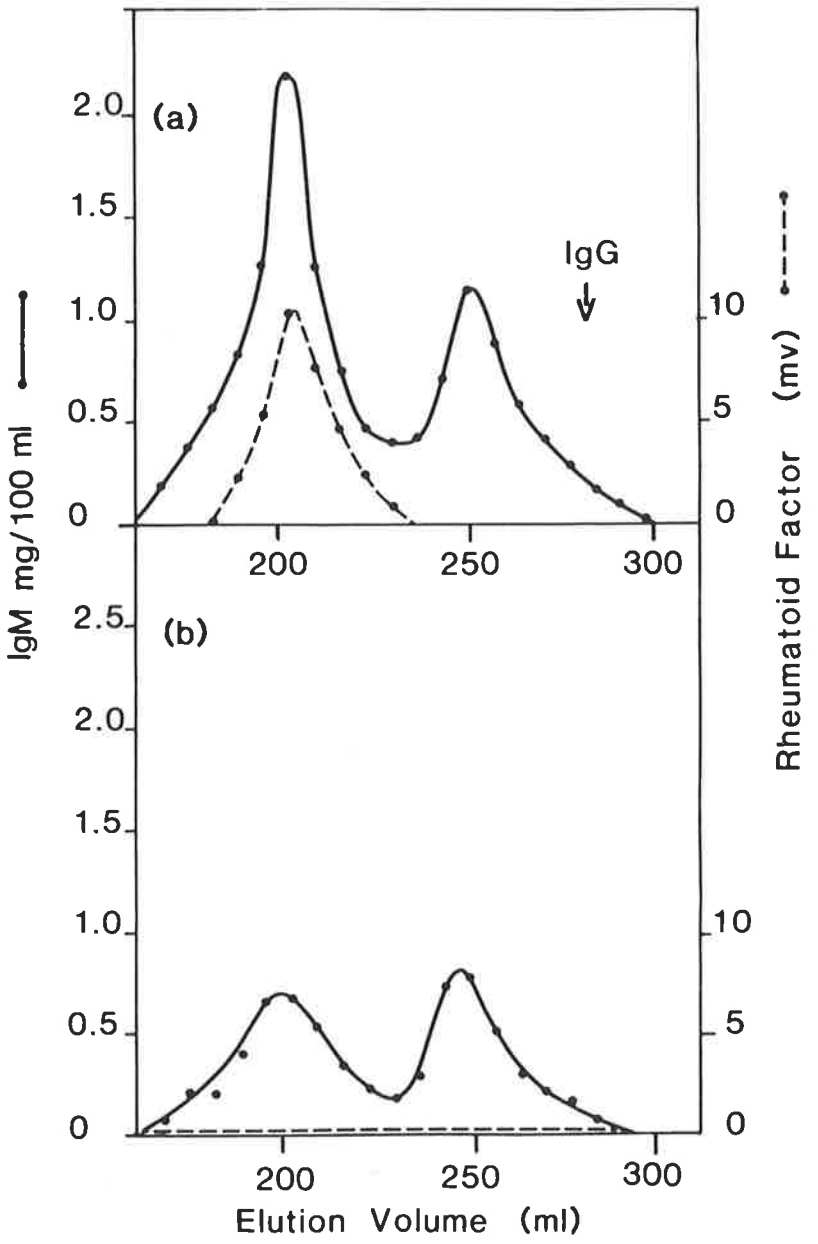

Figure 1. Elution profile of IgM and RF activity following Sepharose 6B chromatography of original serum of patient 3, Table I (upper panel a) and the corresponding serum supernatant following separation of cryoprecipitate (lower panel b). Note the loss of RF activity in the supernatant and the relative preservation of the LMW IgM. RF is measured by a nephelometric technique and expressed in millivolts (mv) (Roberts-Thomson et al, 1980).

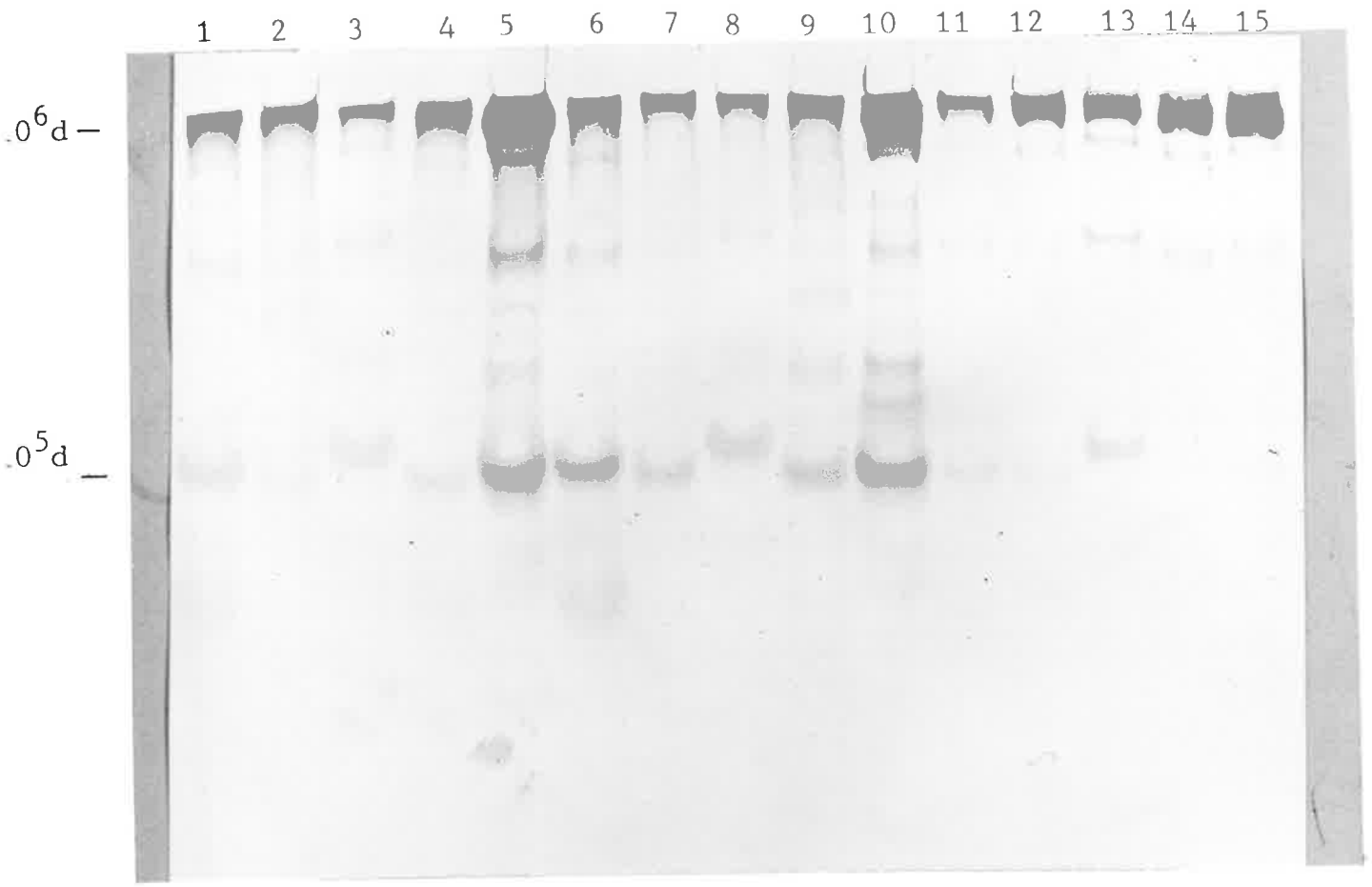

Figure 2. Immunoblot appearance of original sera (lanes 1-5) and corresponding supernatants (lanes 6-10) and cryoprecipitates (lanes 11-15) stained with an anti u-avidin, biotin, avidin-alkaline phosphatase, substrate sandwich. In normal serum (not shown) only pentameric IgM bands are observed.

bands. The molecular nature of these intermediary bands was not determined. In control sera these lower molecular weight IgM bands were not seen and only pentameric bands were observed.

PBMC from 2 patients and 3 controls were stimulated with pokeweed mitogen and cultured over 7 days. The supernatants were then concentrated and analysed by filtration chromatography for LMW IgM. LMW IgM was not detected in any of the controls but was found in large proportions (constituting 80% of the total secreted IgM) in the supernatant from pokeweed stimulated PBMC from one of the patients. Insufficient IgM was secreted in vitro in the second patient to allow satisfactory chromatographic analysis. Cycloheximide inhibited IgM synthesis by >90%.

DISCUSSION

Large quantitites of LMW IgM has been identified in the sera of all 6 patients studied with mixed cryoglobulinaemia. In 3 of 4 patients studied the LMW IgM was monoclonal and, as the light chain type was identical to the monoclonal pentameric IgM observed in the cryoglobulin, this association suggests that the origin of the LMW IgM was from the same clone of lymphoid cells that was secreting the pentameric IgM. As demonstrated in Chapter four using a specific RF radioimmunoassay it was determined that partially purified LMW IgM obtained from patient 1 of this current study had RF activity and this was also observed for LMW IgM prepared from another patient with mixed cryoglobulinaemia (and not included in this present study). It was

not possible to test the LMW IgM fractions for RF activity from the remaining patients in this study. Considering all the above observations it appears likely, therefore, that most of the LMW IgM found in mixed cryoglobulinaemia is derived from the same clone that secretes the pentameric IgM-RF which participates in the cryoglobulin.

The clinical significance of the circulating LMW IgM found in mixed cryoglobulinaemia is uncertain. Due to its divalent or monovalent nature (Chavin and Franklin, 1969) it has far less avidity than its corresponding pentameric pentavalent member. This factor is a possible explanation for its under representation in the cryoprecipitate which consists predominantly of pentameric IgM-RF and its corresponding antigen viz. polyclonal IgG. If one assumes that this immune complex has pathological consequences it would appear that LMW IgM because of its lower valence would have less pathogenic potential than the pentameric molecule. However on the other hand, it could be argued that LMW IgM-RF because of its lower valency and poor agglutinating activity might lead to the formation of relatively small immune complexes with a prolonged circulatory half life. Deposition of such complexes in vascular beds might result in a vasculitic reaction. Furthermore, LMW IgM might also have the potential to interfere or block the effective immune clearance mechanism of pentameric IgM and if a virus is responsible for mixed cryoglobulinaemia (e.g. Hepatitis B), this property might allow for the continuation of this infection. Regardless of the role that LMW IgM plays in the pathogenesis of this disorder it does appear that it is a marker of the clonal expansion of a subset of B lymphocytes which appears to be a fundamental abnormality in this disorder.

The reasons for the secretion of LMW IgM is unclear. As previously discussed in Chapters one, five and six, several hypotheses can be proposed. We feel the most likely explanation for the occurrence of LMW IgM is due to a defect in the assembly of monomeric IgM subunits during the polymerization of the pentameric molecule. This hypothesis would be in accordance with the observed secretion of LMW IgM from the PMBC from one patient but not controls and with the finding of additional oligomers and other intermediatory molecular forms of IgM in the sera of our patients. Whether this defect is related to a disorder of J chain availability and/or function or to deficiencies in the probable polymerising enzyme sulfhydryl oxidase (Roth and Koshland, 1981) is unclear.

In conclusion we have observed large quantities of monomeric and oligomeric IgM in patients with mixed cryoglobulinaemia. In the majority of patients this LMW IgM appeared to be monoclonal in origin and of the same light chain type as the pentameric IgM-RF which was found in the cryoglobulin. In a separate study we have identified RF activity in the LMW IgM fraction. The reason for the secretion of LMW IgM in this disorder is unknown but we postulate a disorder of assembly of monomeric IgM subunits during polymerization of the pentameric molecule.

CHAPTER EIGHT

LOW MOLECULAR WEIGHT IgM IN SELECTIVE IgA DEFICIENCY

SUMMARY

Low molecular weight IgM was sought in the serum and saliva of 28 subjects with selective IgA deficiency. LMW IgM was detected in the sera of nine (32%) of these, and of the 17 subjects discovered by population screening, five (29%) had serum LMW IgM. In the nine positive persons, LMW IgM constituted up to 17% of the total serum IgM concentration. Eight of the nine IgA deficient persons with LMW IgM, had clinical disease while associated disease in the entire IgA deficient population was less frequent. Serum immune complexes were demonstrated in five of seven subject with LMW IgM; four of these had immune complex associated disorders, three with polyarthritis and one with glomerulonephritis. Because circulating immune complexes (CIC) are frequently detected in IgA deficient persons without disease, it is proposed that the presence of serum LMW IgM in IgA deficiency may be associated with disease due to the formation of specific pathogenic immune complexes.

LMW IgM and smaller sized IgM fragments were observed in 6 of 7 saliva specimens from these IgA deficient subjects but not in 3 specimens from healthy individuals. However incubation of a radiolabelled pentametric IgM with saliva from one of these subjects resulted in the formation of smaller IgM fragments suggesting that proteolytic degradation was the probable explanation for the occurrence of LMW IgM (and smaller IgM fragments) in these subjects.

INTRODUCTION

There is only one previous report demonstrating the presence of LMW IgM in selective IgA deficiency (Ammann and Hong, 1971). In that study LMW IgM was detected in three of 30 IgA deficient persons using an acrylamide gel diffusion technique, but there was no attempt to quantitate it or relate its presence with clinical or serological parameters.

In the present study we have measured LMW IgM in 28 patients with selective IgA deficiency and have correlated its presence with other clinical and laboratory variables frequently associated with IgA deficiency. Many of the clinical and laboratory features of IgA deficient subjects have been reported in an earlier study where it was found that circulating immune complexes were present in 48% of IgA deficient subjects in the local community (Kwitko et al, 1979).

PATIENTS AND METHODS

Patient selection. Patients attending the emergency clinic of the Royal Adelaide Hospital with mild trauma were screened for selective IgA deficiency. Twenty-seven such patients were detected and in 17 of these there was sufficient serum remaining after initial serological studies for LMW IgM quantitation. In addition 12 patients with known selective IgA deficiency were included (patient 3, 12,13,23,32-39; Table 1). Control sera were obtained from healthy laboratory staff and volunteers.

Serum for immune complex determination was stored at -70°C until testing. Otherwise serum was stored at -20°C.

Saliva was obtained from 17 patients and 3 controls. It was collected by having the subject spit repeatingly into a pot until a volume of 20ml was reached. The specimen was then stored at -20°C.

Measurement of serum LMW IgM. This was performed by Sepharose 6B chromatography and laser nephelometry as previously described in Chapter two.

Measurement of salivary IgM. This was preformed by IgM Elisa.

Measurement of salivary LMW IgM. This was preformed by Sephacryl S300 chromatography and IgM ELISA as described in Chapter two. The saliva specimen was concentrated in an Amincon concentrating unit to a volume of approximately 1ml before application to the column.

Other immunological tests. Quantitation of IgG, IgA and IgM, rheumatoid factor, circulating immune complexes (C1q binding radioimmunoassay) and serum anti-IgA antibody (solid phase radioimmunoassay) were preformed as previously described (Kwitko et al. 1979; Chapter two and subsequent chapters). Purification and radiolabelling of a pentameric IgM protein was performed as described in Chapter five.

RESULTS

A typical IgM elution profile in an IgA deficient serum showing the late LMW IgM peak constituting approximately 15% of the total IgM curve is seen in Figure 1. Similar elution profiles revealing the presence of serum LMW IgM were detected in nine of the 28 (32%) IgA deficient subjects studied (Table 1) but in none of the 25 control sera. The mean concentration of LMW IgM for the positive sera was 13.2mg/dl (range 2.2-22.6 mg/dl). Eight of the nine IgA deficient subjects with LMW IgM has associated diseases (Table 1), three with arthritis, three with recurrent infections, one with glomerulonephritis and one with asthma. This increase in disease incidence was significant (P=0.04 by Fisher's exact test) when compared with 19 subjects without LMW IgM, nine of whom had associated disease. Five of seven patients with LMW IgM had circulating immune complexes detected and four of five had serum anti-IgA antibody. Serum concentrations of IgA and IgM and presence of rheumatoid factor were not significantly different between patients with and without LMW IgM.

Salivary IgM was measured in 17 of the patients and varied from being undetectable to a level >67ug/ml. In seven patients (Table 2) sufficient IgM was present in the saliva for chromatographic studies. LMW IgM and smaller fragments were found in 6 of these patients but not in any of the 3 saliva specimens from controls (Figure 2). LMW IgM was present in the serum of at least 2 of these patients (Table 1). Incubation of I^{125} labelled pentameric IgM with 5mls of fresh saliva from patient No. 8 for 120 mins at $37^{o}C$ resulted in the

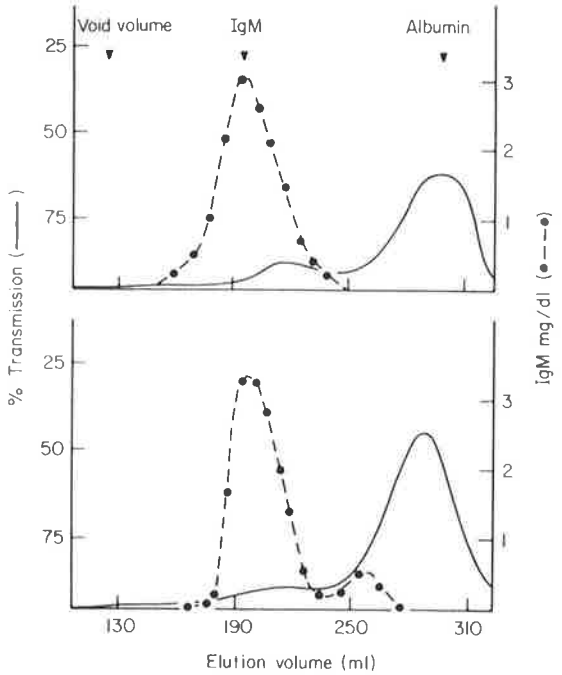

Fig. 1. The percentage transmission and IgM elution profile following Sepharose 6B filtration chromatography of 1·5 ml of serum. The eluting positions of marker proteins are indicated. (a) normal serum; (b) IgA deficient serum containing LMW IgM which accounts for approximately 15% of the total IgM profile.

Table 1. Clinical and serological findings in 28 IgA deficient subjects

No.	Sex/age	Clinical disease	Serum IgA (mg/dl) R 37–300	Serum IgM (mg/dl) R 70–210	LMW IgM (mg/dl)	Rheumatoid factor		Serum immune complexes (C1q RIA)	Serum anti-IgA antibody (SP RIA)
						Latex test	Rose Waaler test		
1	M/16	Asthma	10	70	8·6	–	–	–	+
2	F/48	Colitis	3	80	Absent	–	–	+	+
3	F/19	Eczema/R.Inf.	30	50	Absent	–	–	–	–
5	F/64	—	30	90	Absent	–	–	–	–
6	F/25	Arthritis R.Inf.	1	280	6·0	–	+	+	+
7	M/41	—	2	210	Absent	+	+	+	+
8	F/67	—	< 1	240	Absent	+	–	+	+
9	M/22	—	< 1	60	Absent	+	+	–	–
12	F/18	—	5	110	Absent	–	–	–	–
13	M/31	G.Nephritis	7	130	Absent	+	+	–	–
15	F/72	R.Inf.	5	60	Absent	–	+	–	–
17	F/21	Arthiritis	2	110	13·8	–	+	+	+
18	F/35	Coeliac	20	120	Absent	–	–	+	+
21	F/26	—	30	60	Absent	–	–	–	–
25	F/28	Eczema	2	110	Absent	–	–	+	–
27	M/41	G.Nephritis	4	140	11·0	–	–	+	+
28	F/31	—	1	60	Absent	–	–	+	–
29	F/20	—	3	110	Absent	–	+	–	–
30	F/22	—	4	40	Absent	–	–	+	+
31	M/65	—	2	160	14·4	–	–	+	–
32	M/32	R.Inf.	< 11	130	22·6	–	–	–	n.d.
33	F/22	R.Inf.	< 11	237	21·0	n.d.	n.d.	n.d.	n.d.
34	M/36	R.Inf.	12	35	2·2	–	–	n.d.	n.d.
35	F/25	SLE	10	270	Absent	–	–	n.d.	n.d.
36	F/50	R.Inf. Asthma	< 11	76	Absent	n.d.	n.d.	n.d.	n.d.
37	F/30	R.Inf.	< 5	116	Absent	–	+	n.d.	n.d.
38	M/10	—	< 5	59	Absent	n.d.	n.d.	n.d.	n.d.
39	F/32	Arthritis	5	131	19·7	+	+	+	n.d.

R.Inf. = Recurrent infection; n.d. = Not done; Values < Mean + 2 s.d. (controls) recorded as –; R = Reference range for healthy individuals.

Table 2. Salivary Immunoglobulins, Albumin and LMW IgM in 7 IgA deficient subjects

	Saliva (mg/dl)				
No+	IgG	IgA	IgM	Albumin	LMW IgM
8	1.88	3.26	2.76	<0.77	+
9	2.37	<1.1	1.51	5.54	+
10	<1.2	1.83	0.40	1.04	+
19	2.94	<1.1	2.25	7.01	+
20	1,88	1.91	<6.75	0.81	+
27	>1.2	1.72	0.32	<0.77	-
31	2.52	3.62	2.70	1.42	+
Normal*	-	12.9±0.2	-	0.66±0.66	-

+ Patient No. from Table 1
* Data from Plebani et al, 1983 (mean ± S.D.)

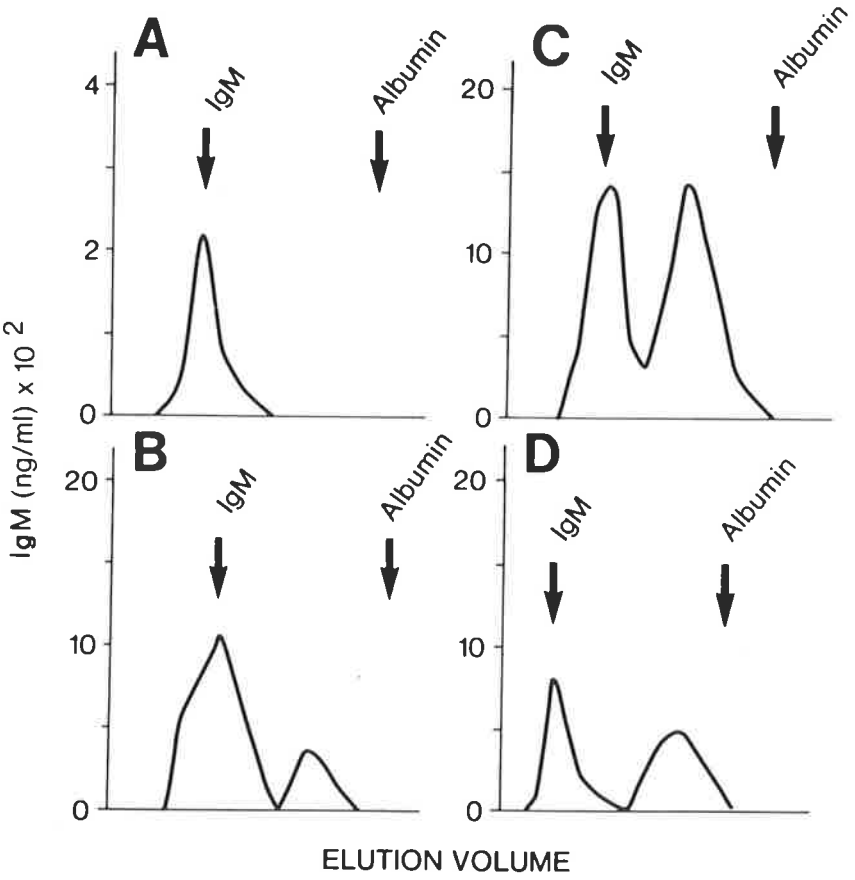

Figure 2. Salivary IgM elution profiles following Sephacryl S300 filtration chromatography.
Panel A - saliva from a healthy subject.
Panel B, C and D - saliva from 3 subjects with selective IgA deficiency. Note the presence of low molecular weight IgM in fractions eluting before the albumin marker.

appearance of a peak of radioactivity appearing between the IgG and albumin markers, but no breakdown was apparent when the I^{125} pentameric IgM was incubated under the same conditions with saliva from a healthy control (Figure 3).

DISCUSSION

We have shown the presence of small quantities of LMW IgM in nine of 28 patients with selective IgA deficiency. This figure (32%) is higher than the 10% reported by Amman and Hong (1971) but we attribute the increased prevalence in our study to the enchanced sensitivity of our assay used to measure LMW IgM. In the present study LMW IgM appeared particularly in those patients with associated disease and although the numbers were small, the majority of the patients with LMW IgM also demonstrated CIC and serum anti-IgA antibody.

The stimulus for the production of LMW IgM is unknown. We have previously observed in rheumatoid arthritis a striking correlation between levels of LMW IgM and levels of CIC and RF suggesting that the initiating stimulus for LMW IgM is closely linked to the stimulus for rheumatoid factor production (Chapter four). In the present study, although we could not confirm the association between LMW IgM and rheumatoid factor, five of the seven patients with LMW IgM had CIC and four had immune complex-mediated disorders, especially arthritis and glomerulonephritis. It is possible therefore that the synthesis of LMW IgM is associated with both the production of rheumatoid factor and with the formation of specific types of serum immune complexes

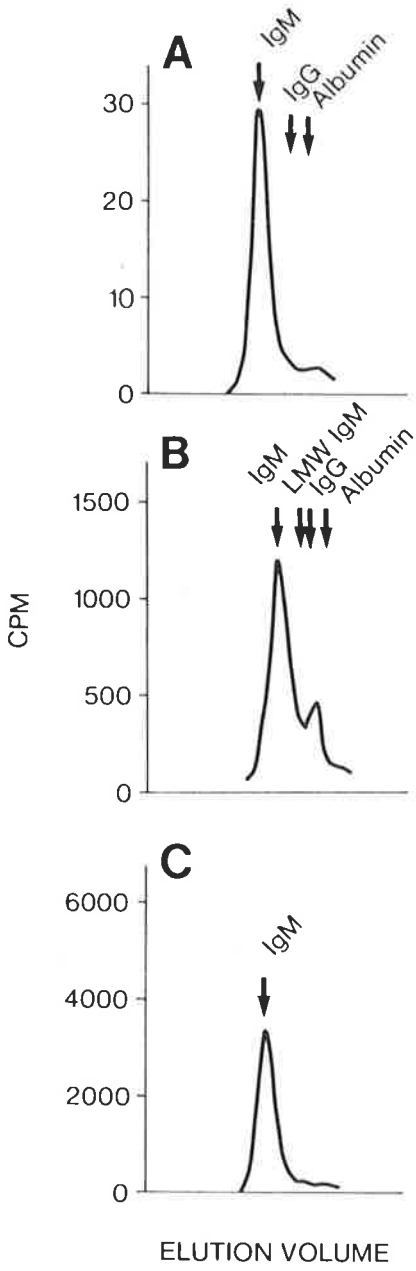

Figure 3. Panel A - I^{125} elution profile (cpm) of radiolabelled purified pentameric IgM control diluted in normal serum following Sephacryl S300 filtration chromatography. The elution position of marker proteins are indicated.

Panel B - I^{125} elution profile following incubation of radiolabelled pentameric IgM control for 120 mins at 37°C with saliva from patient No. 8. Note the appearance of a new peak eluting between IgG and albumin.

Panel C - I^{125} elution profile following incubation of radiolabelled pentameric IgM with saliva from a healthy subject.

that could be particularly pathogenic.

It has been previously shown that although serum immune complexes were detectable in IgA deficiency, they were not necessarly associated with disease (Kwitko et al, 1979). Therefore specific types of complexes are probably responsible. As suggested earlier, LMW IgM may represent a phylogenetic reversion of the immune response to a more primitive state, and in IgA deficiency the lack of normal secretory IgA secretion may favour increased production of IgM. In many of our patients increased serum IgM levels were seen but there was no apparent relationship between serum IgM level and the presence of LMW IgM. Salivary IgM levels were also increased above quoted reference ranges in 5 of our 17 patients in agreement with Plebani and colleagues (1983) conclusions in their analysis of salivary immunoglobulin levels in 14 children aged between 2 and 16 years. Analysis of the molecular size of this salivary IgM is seven of our patients revealed the presence of considerable quantities of LMW IgM or smaller sized IgM fragments in five of these patients. We feel the most likely explanation for the occurrence of this salivary LMW IgM is due to proteolytic degradation (as was suggested by the preincubation of a radiolabelled pentameric IgM protein with one of the patients saliva with the resultant formation of a low molecular weight fragment of IgM). Such a phenomenon has previously been suggested as an explanation for the presence of low molecular weight IgM identified in intestinal fluids in healthy individuals (Richman and Brown, 1977). The absence of LMW IgM in saliva from our healthy control subjects could possibly be due to the fact that these salivary specimens were rapidly frozen following collection in contrast to the

longer period at which the IgA deficient salivary specimens spent at room temperature. Delay in freezing could allow for proteolytic degradation. Alternatively, the bacterial oral flora of IgA deficient subjects might differ from healthy controls and could promote IgM degradation. Klein and colleagues, (1967) and Solomon (1969) have described breakdown of pentameric IgM by bacterial action. Further studies to explore there suggestions are required.

We can find only one other occasion which LMW IgM has been identified in concentrated saliva from a patient with ataxia-telangiectasia (McFarlin et al, 1972). LMW IgM has been sought in unconcentrated saliva by Stobo and Tomasi, (1967) but as in the study of McFarlin et al, 1972 it is likely that their methods of detection were too in-sensitive to detect the low levels present as shown in this study.

The results presented in this Chapter provide additional evidence that there is a wide spectrum of immunological dysfunction in "selective IgA deficiency". In a previous study it has been shown that specific defects of IgM and IgG class antibodies can coexist in IgA deficient persons (Heddle et al, 1981). Oxelius et al, (1981) have subsequently demonstrated a relationship between coexistent IgG_2 deficiency and disease in IgA deficiency. The demonstration of a possible relationship between LMW IgM and disease in IgA deficiency in this study further highlights the clinical heterogeneity of this disorder.

CHAPTER NINE

LOW MOLECULAR WEIGHT IgM IN B CELL LYMPHOPROLIFERATIVE DISORDERS

SUMMARY

Circulating low molecular weight (LMW) IgM was demonstrated in 5 of 38 patients with B cell lymphoproliferative disorders. These 5 patients all had malignant disease and could be subdivided into 2 groups. In the first group were 3 patients, each with an associated serum IgM paraprotein, and the diagnosis was Waldenstrom's macroglobulinemia in two, and lymphocytic lymphoma in one. The two patients of the second group did not have IgM paraproteins and their diagnosis was lymphocytic lymphoma and chronic lymphocytic leukaemia. Both these patients also had acquired C1 esterase inhibitor deficiency a previously recognized association with circulating LMW IgM. None of the 16 patients with benign IgM macroglobulinemia had circulating LMW IgM. In those positive sera with LMW IgM this moeity contributed between 10.5% and 37.5% of the total IgM. There was no apparent association between LMW IgM and total IgM levels, kappa/lambda light chain type nor the presence of Bence Jones proteinuria, but rheumatoid factor, immune complexes and cryoglobulins occurred in many of the sera which contained LMW IgM. Pokeweed mitogen stimulated peripheral blood mononuclear cells from 2 patients with circulating LMW IgM secreted considerable quantities of this moiety in vitro but this did not occur in 2 patients with benign IgM macroglobulinemia.

We conclude that LMW IgM is found in the malignant but not the benign forms of B cell lymphoproliferative disorders and is frequently associated with other serological abnormalities. However the basic abnormality causing the occurrence of LMW IgM in these disorders is obscure.

INTRODUCTION

The occurrence of LMW IgM has previously been reported in a number of B cell lymphoproliferative disorders such as multiple myeloma, Waldenstrom's macroglobulinemia and non Hodgkin's lymphoma (Stobo and Tomasi, 1967; Bush et al, 1969; Dammacco et al, 1970; Carter and Hobbs, 1971; Eskeland and Harboe, 1973; Harrisdangkul et al, 1975), the frequency varying between studies. For instance in macroglobulinemia, LMW IgM has been reported in as few as 17% of the patients (Stobo and Tomasi, 1967) while other studies have reported a frequency of 100% (Harrisdangkul et al, 1975; Eskeland and Harboe, 1973). This variation in frequency is probably due to the differences in sensitivities and specificities of the assays used to detect LMW IgM.

In B cell lymphoproliferative disorders associated with IgM paraproteins, one group has demonstrated LMW IgM only in the more aggressive or malignant forms such as Waldenstrom's macroglobulinemia and lymphoma and not in the benign variety such as benign IgM paraproteinemia and these authors have suggested that its presence reflects a polymerization defect occurring in the immature or less differentiated malignant cell (Carter and Hobbs, 1970).

In the present study we have used a sensitive technique for LMW IgM determination and have measured its frequency in 38 patients with B cell lymphoproliferative disorders of both the benign and malignant variety. In vitro studies were also performed confirming the active synthesis of LMW IgM and these findings were compared with the

respective serum. The patients were also studied for other serological abnormalities.

PATIENTS AND METHODS

Patients. Thirty eight patients with B cell lymphoproliferative disorders were studied. The diagnosis of each patient was confirmed by reviewing the clinical records. These patients fell naturally into 2 groups; those with serum IgM paraproteins and those without. Twenty seven patients had IgM paraproteins and the diagnosis was Waldenstrom's macroglobulinemia in 6, lymphocytic lymphoma in 4, cold agglutinin disease in 1 and "benign" IgM macroglobulinemia in 16. This latter category included 3 patients with associated carcinoma, 2 patients with polyneuropathy and 11 patients with a variety of other medical and surgical conditions. The latter 2 subgroups had all been followed for at least 2 years to confirm their benign nature. Of the 11 patients without IgM paraproteins 7 had chronic lymphocytic leukaemia and 4 had lymphocytic lymphoma. The mean age of the total patient group was 67 years (range 54-84 years) and there were 20 females and 18 males.

Immunological Tests. The sera from these patients were examined for paraproteins (agarose electrophoresis and immunoelectrophoresis), immunoglobulin levels (nephelometry), cryoglobulins, rheumatoid factor activity (latex and Rose Waaler titres) and for immune complexes (liquid phase Clq binding assay) using standard immunological techniques (Chapter two). When clinically indicated some sera were

examined for alterations in complement components using both functional and immunochemical assays as described elsewhere (Neoh et al, 1981). Urine was concentrated 50-100 fold using an Amicon B15 concentrator and examined for free light chains using immunoelectrophoresis.

Determination of LMW IgM. LMW IgM was measured by Sepharose 6B chromatography and laser nephelometry as previously described in Chapter two but with minor modifications. Briefly, between 0.25 to 2.0 ml of serum was applied to a 90 x 2.5 cm Sephacryl S300 column (Pharmacia, Uppsala, Sweden). Alternative fractions were collected and assayed for IgM by laser nephelometry at two different dilutions (1:25 and undiluted) using anti-IgM antiserum (Dako-immunoglobulins, Copenhagen, Denmark). An IgM profile was then obtained and the percent LMW IgM was calculated by planimetry from the relative areas of high and low molecular weight fractions.

Synthesis of LMW IgM. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood by Ficoll-Hypaque sedimentation using standard techniques. No patient was receiving cytotoxic chemotherapy at the time of study. PBMC culture was performed as previously described and the culture supernatant analysed for LMW IgM by Sephacryl S300 chromatography and IgM ELISA (Chapter two).

RESULTS

Circulating LMW IgM was demonstrated in 5 of the 38 patients; in 3

of 27 patients with associated IgM paraproteins (Figure 1) and in 2 of 11 patients who lacked this paraprotein. All 5 patients with LMW IgM had malignant disease whilst none of the 16 patients with benign IgM macroglobulinemia showed LMW IgM. The clinical and laboratory findings of the 5 patients with LMW IgM are shown in Table I. The laboratory findings of the 33 patients who did not have LMW IgM demonstrated low levels of rheumatoid factor in two but none showed cryoglobulinemia. Low levels (<10 units/ml) of immune complexes were seen in nearly half of the macroglobulinemic sera; this was attributed to non specific precipitation in 2.5% polyethylene glycol.

The 2 patients with lymphoproliferative disease who had LMW IgM in the absence of serum IgM paraproteins (patients 4 and 5, Table 1) were distinct in that they demonstrated episodes of angioedema. Both sera from these patients showed persistent and marked depression of C1, C4, C2 and C1 esterase inhibitor (Table II). In contrast in the 3 patients with LMW IgM and serum IgM paraproteins, complement studies were either normal or reflected the presence of complement fixing cryoglobulins.

Synthetic studies in vitro revealed that two patients with circulating LMW IgM (Nos. 1 and 3, Table I) produced considerable quantities of IgM by day 7 of culture and column chromatography revealed that greater than 75% was LMW IgM (Table III and Figure 2). In contrast 2 patients with benign IgM macroglobulinemia without circulating LMW IgM and 5 healthy control subjects produced no LMW IgM in culture. Addition of cycloheximide to cultures totally suppressed both pentameric IgM and LMW IgM synthesis.

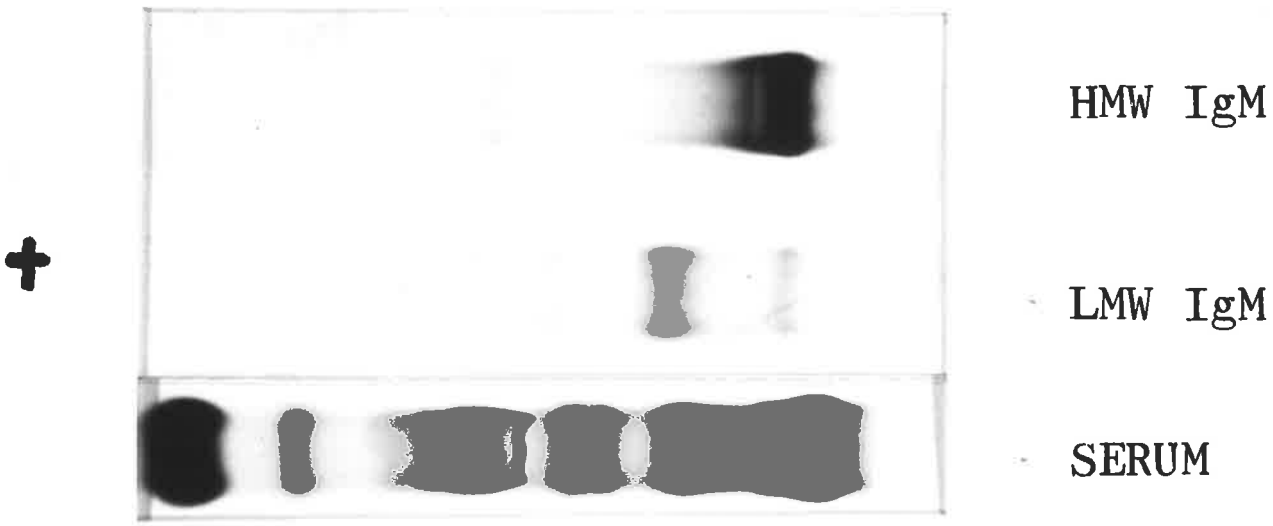

Figure 1. 1% agarose gel electrophoresis; anode on left. Lower electrophoretogram: original serum from patient with Waldenstroms macroglobulinemia showing a light and dense monoclonal band in the gamma region (immunoelectrophoresis demonstrated that these bands were both IgM (lambda) with common line of identity). Middle electrophoretogram: partially purified LMW IgM fraction (by recycling filtration chromatography) is seen to constitute the fast gamma IgM monoclonal band. Upper electrophoretogram: partially purified HMW IgM fraction (by recycling chromatography) constitutes the dense band.

TABLE 1
Clinical and Laboratory Features of Patients Containing Circulating LMW IgM

Patient	Age	Sex	Diagnosis*	IgG g/l	IgA g/l	IgM g/l	% LMW IgM	Serum paraprotein	Bence-Jones proteinuria	Serum cryoglobulin	Rheumatoid factor Rose-Waaler	Immune complexes units/ml
1	60	F	WM	3.9	<0.2	66	10.5	IgM λ	ND†	Nil	<1:32	<2.0
2	66	F	WM	9.3	0.6	20	~33‡	IgM κ	κ	5.2	<1:32	4.8
3	78	F	LL	3.5	2.2	12.4	~25‡	IgM κ	Neg	9.3	1:4096	>75
4	70	F	LL	20.4	0.3	1.2	37.5	IgG κ	ND	0.3	1:32	21.0
5	56	F	CLL	4.8	0.4	0.4	23	Nil	ND	ND	ND	<2.0
Normal				7-19	0.5-4.0	0.5-2.2	0	Nil	Nil	Nil	<1:32	<2.0

* WM = Waldenström's macroglobulinemia; LL = lymphocytic lymphoma; CLL = chronic lymphocytic leukemia.
† = Not done.
‡ = Accurate quantitation not possible because of cyroglobulins.

TABLE 2
Complement Abnormalities in 2 Patients with LMW IgM and Angioedema

Patient	Immunochemical			Functional				
	C3 g/l	C4 g/l	C1 INH g/l	CH 50 U/ml	C1 %N*	C4 %N	C2 %N	C1 INH %N
4	0.54	0.09	0.016	16.5	2	1	4	50
5	0.74	0.09	0.017	16.5	10	2	10	75
Normal	0.55-1.2	0.2-0.5	0.15-0.35	20-50	80-150	80-135	85-120	88-135

* %N = % Normal

TABLE 3
LMW IgM Synthesis *in vitro*

Patient	Diagnosis	Serum		*In vitro*	
		IgM (g/l)	% LMW IgM	IgM μg/culture‡	% LMW IgM
1	WM	66	10.5	3.4	76
3	LL	12.4	25	2.3	80
6	BM†	27.7	0	1.9	0
7	BM	21.8	0	1.8	0

† BM = Benign macroglobulinemia.
‡ /culture = 2×10^6 PBMC.

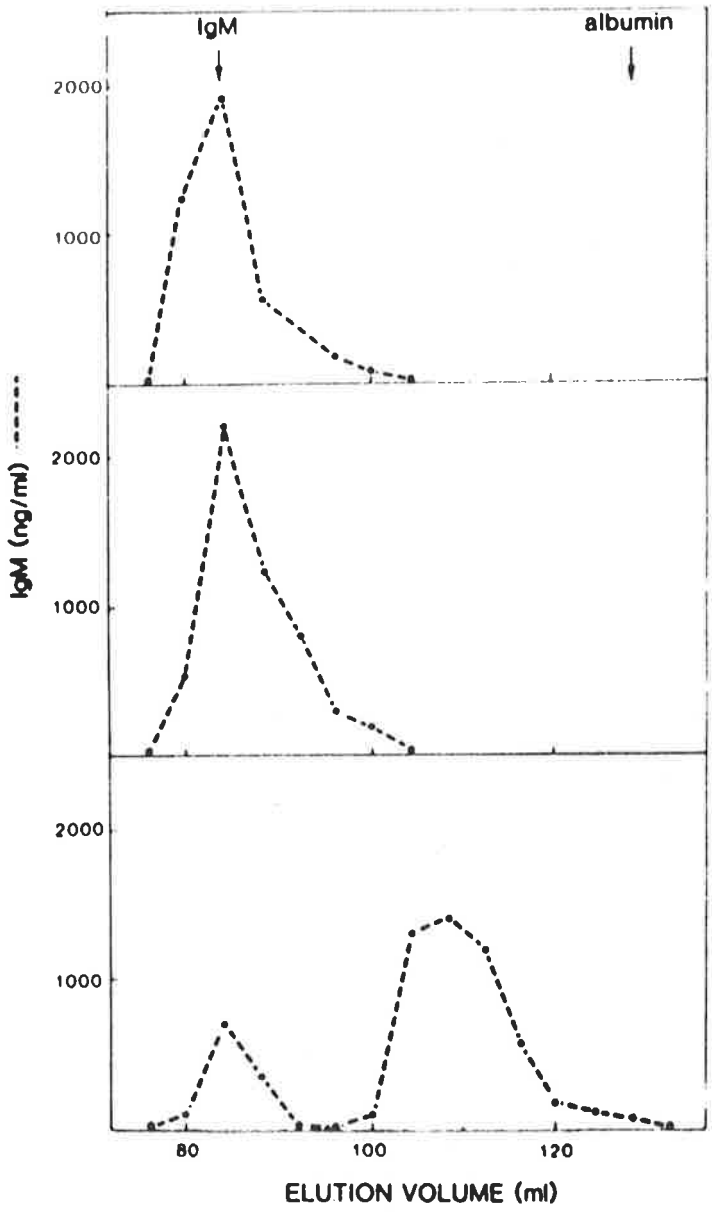

Figure 1: Sephacryl S300 chromatographic IgM profiles of PBMC culture supernatants. The elution positions of pentameric IgM and albumin are indicated. Upper panel: IgM profile from normal control — pentameric IgM present. Middle panel: IgM profile from patient with benign macroglobulinemia — pentameric IgM present. Lower panel: IgM profile from patient with Waldenstroms macroglobulinemia with circulating LMW IgM — pentameric and LMW IgM present.

DISCUSSION

There is considerable uncertainty regarding the frequency of LMW IgM in the B cell lymphoproliferative disorders, particularly macroglobulinemia. This to a large part relates to the different assays used to detect LMW IgM, each having a different sensitivity and specificity. The assay used in the present study has been shown to be reproducible and can detect lower levels of LMW IgM than those previously published techniques (Chapter two). Furthermore, studies with rechromatography of positive LMW IgM fractions has confirmed specificity (data not shown). Our frequency is considerably lower than some but compares well with Carter and Hobbs (1970) who also used a chromatographic assay. Our study has revealed that LMW IgM occurs infrequently in B cell lymphoproliferative disorders, but, if found it is generally associated with a malignant lymphoproliferative disorder. This was also the conclusion of Carter and Hobbs who found no evidence of LMW IgM in 9 patients with benign IgM macroglobulinemia. If these findings are confirmed in future studies the detection of LMW IgM may then be one means of distinguishing benign from malignant lymphoproliferative disorders.

The finding of LMW IgM in neoplastic disorders raises the possibility of an IgM polymerization defect occurring in the neoplastic cell, with spillover of monomeric subunits into the circulation. In this regard it is of interest that abnormalities of J chain synthesis have been noted in the malignant lymphoproliferative disorders (Mestecky et al, 1980; Yasuola et al, 1980; Mason and Stein, 1981) and possibly this could relate to disordered assembly of

pentameric IgM, although we should be aware of the uncertainties regarding the exact function of J chain in immunoglobulin synthesis and secretion (Mestecky et al, 1980).

Our in vitro studies revealed that PBMC from 2 patients with malignant lymphoproliferative disorders and circulating LMW IgM synthesized considerable quantities of LMW IgM in vitro under pokeweed mitogen stimulation whilst, in contrast, PBMC from 2 benign macroglobulinemic patients and 5 healthy control subjects only produced pentameric IgM. It will be necessary to enlarge this study to see if these preliminary studies are confirmed and also to examine other sources of mononuclear cells (e.g. bone marrow, spleen) to see if there are any variations in the percentage of LMW IgM secreted. This may explain the discrepancy noted between the higher percentage of LMW IgM synthesized in vitro by PBMC and that noted in the circulation.

Our patients with circulating LMW IgM also demonstrated other serological findings. The association of lymphoproliferative disease (often with angioedema) with acquired C1 inhibitor deficiency and circulating LMW IgM is well recognized and over 10 cases have been reported (Gelfand et al, 1979). Associated serological features frequently seen in this syndrome are the presence of IgG or IgM serum paraproteins, cryoglobulins and high levels of immune complexes. The LMW IgM can be monoclonal or polyclonal and, as in our 2 patients, can make up a considerable proportion of the total IgM. In one report it has even been proposed that the LMW IgM is responsible for the activation of the classical pathway with subsequent C1 inhibitor

deficiency (Hauptmann et al, 1976) although there is no definitive evidence to support this hypothesis.

High levels of a monoclonal rheumatoid factor were found in one lymphoma patient with monoclonal LMW IgM. It is of interest that LMW IgM has a strong correlation with rheumatoid factor in RA and in some patients up to 40% of the LMW IgM is LMW IgM rheumatoid factor (Chapter four). These observations suggest that there is a close relationship between the stimulus for the production of rheumatoid factor and LMW IgM. It is possible that rheumatoid factor reflects a primitive antibody with non-specific antibacterial agglutinating activity (Bokisch et al, 1973) and several authors have considered that the presence of LMW IgM reflects an atavistic reversion of the immune response (Solomon, 1969; Stage and Mannik, 1971). Thus it is conceivable that if this reversion occurs both rheumatoid factor and LMW IgM secretion would be initiated and their continual production be closely related.

We have sought LMW IgM in the sera of 38 patients with B cell lymphoproliferative disorders. Its occurrence in the malignant varieties of these disorders, but not in the benign, and its frequent association with other serological phenomena, suggest that more detailed analysis of this neglected immunoglobulin may enhance our understanding of the normal and abnormal processes that lead to IgM secretion.

CHAPTER TEN

GENERAL DISCUSSION

Occurrence of LMW IgM in Disease: From the findings described in this thesis and from the observations of others it is clear the LMW IgM is rarely found in health but commonly occurs in a variety of immunological and infective disorders. These can be conveniently subdivided into three broad clinical groups.

1. Those disorders characterised by the presence of an immunological 'stimulated' state such as is present in autoimmune and infective disorders.
2. Immunodeficiency states such as selective IgA deficiency and ataxia telangectasia.
3. B cell lymphoproliferative states.

In the first two groups there is generally a close association between the presence or quantity of LMW IgM and with levels of total IgM, rheumatoid factor and circulating immune complexes (Table 1). Thus in the current study these associations were seen in rheumatoid arthritis, infective endocarditis and mixed cryoglobulinaemia, but were not universally found e.g. the lack of correlation between LMW IgM and rheumatoid factor is selective IgA deficiency. Furthermore rheumatoid factor has a relatively low prevalence in primary biliary cirrhosis where high levels of LMW IgM, total IgM and immune complexes are found (Fakunle et al, 1979).

In general however, the close associations noted between LMW IgM and these other variables allows one to postulate that in these first two clinical groups,

a) The occurrence of LMW IgM is related to a stimulated IgM humoral response characterised by increased rates of IgM synthesis.
b) The occurrence of LMW IgM relates to clinical manifestations

characterised by the presence of circulating immune complexes. This last postulation immediately raises the corollary, does LMW IgM have a direct pathogenic role in these immune complex mediated disorders?

In the remaining third clinical group of lymphoproliferative disorders there appeared to be no correlation between the quantity of LMW IgM and with total IgM levels, and this finding is in accord with other studies (Solomon 1969; Bush et al, 1969). There did, however, appear to be a correlation with the malignant nature of the lymphoproliferative state; an association previously noted by Carter and Hobbs (1971). These observations suggest therefore, that the occurrence of LMW IgM in this latter group could have different mechanisms and consequences as compared with the first two groups.

Possible role of LMW IgM in pathogenesis of disease: In Chapter one some of the evidence implicating LMW IgM in a variety of human disorders was reviewed. Most studies have documented a varying freqency in certain autoimmune, infective, immunodeficient and lymphoproliferative disorders, the difference between the studies probably reflecting differences in assay sensitivity and specificity and patient selection. As this molecular moiety was most prevalent in patients with active or severe disease and in one study was related to an enhanced mortality (Harisdangkul et al, 1984) it was concluded by most authors that this evidence, although circumstantial, was sufficient to implicate LMW IgM in the pathogenesis of these disorders. Furthermore, in two patients with haemolytic anaemia it appeared that the LMW IgM was directly associated with the haemolytic

process although the exact mechanism was uncertain (Spiva et al, 1974; Kay et al, 1975). In this thesis this disease association has been extended and detailed studies performed between the absolute quantities of LMW IgM and other indices which reflect active or severe disease. Thus in Chapter four, high levels of LMW IgM were observed in active or severe forms of rheumatoid arthritis (e.g. rheumatoid vasculitis or Felty's syndrome) and significant correlations were observed between LMW IgM and total IgM, rheumatoid factor and circulating immune complexes. Moreover, data was presented which suggested that in certain patients, a considerable proportion of the LMW IgM contained rheumatoid factor activity. Likewise in infective endocarditis there was strong circumstantial evidence relating quantities of circulating LMW IgM with extracardiac manifestations and with levels of rheumatoid factor and circulating immune complexes while in selective IgA deficiency there was an association between LMW IgM and with clinical markers implicating immune complexes mediated manifestations, e.g. polyarthritis.

In reviewing the disease association with circulating LMW IgM one is impressed by the strong association of LMW IgM with circulating immune complexes (Table 1). In many of these diseases there is good evidence which suggests that these circulating immune complexes are pathogeneic and cause many of the clinical manifestations (Theofilopoulos and Dixon, 1979). The question, therefore arises as to whether LMW IgM is involved in these circulating immune complexes and hence directly with the disease pathogenesis.

Evidence has been previously cited in Chapter one which describes

Table 1. LMW IgM IN VARIOUS DISEASES

Disease	% Patients with LMW IgM[s]	% Patients with RF[©]	Circulating Immune complexes[o]	Increase in IgM[#]
AUTOIMMUNE				
RA	80	80	+	+
SLE	46	50	+	+
Sjogrens Syndrome	100	75	+	+
Systemic Sclerosis	50	30	±	–
Primary Biliary Cirrhosis	33	30	+	+
Mixed cryoglobulinaemia	100	100	+	+
INFECTIVE				
Hepatitis B	94	24	+	±
Syphilis	82	13		±
Tryanosomiasis	67	27	+	+
Tropical Splenomegaly	61	91	+	+
Leprosy	13	24	+	+
Infective endocarditis	37	30	+	+
IMMUNE DEFICIENT				
IgA deficiency	32	30	+	+
Ataxia Telangectasia	78	?	?	+
LYMPHOPROLIFERATIVE				
Waldenstroms Macroglobulinaemia	33	5	±	+
Lymphoma with Macroglobulinaemia	50	5	±	+
Benign Macroglobulinaemia	0	5	–	+

+ present
- absent
? unknown
s from references quoted (Chapter one) or findings in this thesis
© Bartfeld, 1969
o Theofilopoulos and Dixon, 1979
Hobbs, 1971

a number of different antigen binding activities for LMW IgM. Furthermore, this monomeric IgM antibody, in contrast with the pentameric species has poor or absent agglutinating and precipitating activity and a maximum valency of two (Stobo and Tomasi, 1967; Harisdangkal et al, 1975). Indeed evidence obtained from reduction and alkylation of pentameric IgM antibody indicates that the functional binding valence of LMW IgM is most likely one (Chavin and Franklin, 1969). The above observations would thus suggest that LMW IgM would be inefficient, as compared with pentameric IgM, in clearing soluble or particulate antigenic material from the circulation and such persistance might lead to an immune complex state with deposition of these immune complexes containing LMW IgM in susceptible vascular beds. LMW IgM can fix complement effectively (Caldwell, 1973; Tanaka et al, 1984) and if antigen/antibody immune complexes involving LMW IgM were deposited in vascular beds, subsequent complement activation by the relevant H chain domains of the molecule could initiate an inflammatory reaction. In support of this paradigm it would be of special interest to determine the pathological consequences and half lives of immune complexes containing LMW IgM. It is predicted that those immune complexes containing LMW IgM antibody would have a prolonged half life compared with those containing pentameric IgM.

The association of LMW IgM with a number of chronic or persistent bacterial, parasitic and viral infections such as infective endocarditis (Chapter six), Hepatitis B associated disease (Sjogren and Lemon 1983; Tsuda et al, 1984), leprosy (Dammacco et al, 1970), secondary and latent syphilis (Muller and Oelerich 1979; Tanaka et al, 1984) filariasis, trypanosomiasis and malaria associated disorders

(Klein et al, 1966; Masseyeff et al, 1972; Fakunle and Greenwood, 1977) and others as discussed in Chapter one suggests that LMW IgM is relatively inefficient in eliminating the microbiological organism and facilitates its persistence. Indeed in chronic infections with Hepatitis B or treponema pallidum the majority of the IgM is LMW IgM and it occurs almost universally in these conditions (Tsuda et al, 1984; Tanaka et al, 1984). This apparent relative inefficiency might be accountable by the blocking of the respective antigenic determinants on the micro-organism by LMW IgM antibody thus impeding the access of the biologically more efficient pentameric IgM antibody. Such an explanation would be consistent with the rapid decline of the dominant LMW IgM humoral immune response (as occurs in the cartilaginous fish (Marchalonis and Edelman, 1965)), as one ascends the vertebrate kingdom (See Chapter one). This observation suggests a biological disadvantaged status for LMW IgM as compared with pentameric IgM. A corollary might also be suggested that its reappearance in higher species in disease reflects a biologically compromised state.

Possible mechanisms for the occurrence of LMW IgM in disease: Several possibilities can be suggested to account for the presence of LMW IgM. Firstly, however, it is necessary to discuss the evidence refuting the possibility that circulating LMW IgM occurs as a consequence of proteolytic breakdown of pentameric IgM. Some of this evidence has been reviewed in Chapter one. Thus LMW IgM is not generated by prolonged standing or repeated freezing or thawing of sera (Stobo and Tomasi, 1967; Dammacco et al, 1970). In patients with macroglobulinaemia, LMW IgM is not related to the absolute IgM levels

(Chapter nine, Solomon, 1969; Bush et al, 1969). Infusion of radiolabelled homologous pentameric IgM into a patient containing circulating LMW IgM did not lead to the appearance of the radio tracer in LMW IgM containing fractions (Solomon and Kunkel, 1967) whilst Solomon and McLaughlin, (1970) have shown in vitro that LMW IgM is synthesized independently from pentameric IgM by marrow cells obtained from macroglobulinaemia patients. This latter finding was expanded in Chapter five where it was shown that PBMC obtained from patients with active rheumatoid arthritis secreted considerable quantities of LMW IgM in vitro and the percentage LMW IgM secreted was significantly correlated with the percentage circulating LMW IgM. Furthermore, in Chapter five no evidence was obtained to suggest that LMW IgM occurred by breakdown in vitro of pentameric IgM rheumatoid factor/IgG immune complexes either through the action of complement or by proteolytic enzymes derived from polymorphonuclear leucocytes. Finally, whilst LMW IgM was observed in rheumatoid synovial fluid it was not seen in synovial fluid from other acute arthritides such as gout where strong inflammatory processes are occurring. Hence it can be concluded that the majority of evidence suggests that circulating LMW IgM is not derived by protelytic breakdown of pentameric IgM. In contrast, LMW IgM and smaller sized fragments seen in saliva from patients with selective IgA deficiency are most likely due to breakdown of mucosal pentameric IgM but the mechanisms involved in this process are yet to be clarified (Chapter eight).

If circulating LMW IgM is not derived from pentameric IgM what is the mechanism for its occurrence? In Chapters five, six, seven and nine possible mechanisms were discussed and it was concluded that the

most likely explanation was due to a disorder(s) in the assembly of the IgM subunits during pentameric IgM polymerization with subsequent release into the extracellular milieu of monomeric IgM subunits. Evidence supporting this conclusion was the observation of an association between circulating LMW IgM and high serum IgM levels in autoimmune, infective and immunodeficient disorders; the preferential secretion in vitro of LMW IgM during the early phases of PBMC cultures obtained from patients with active rheumatoid arthritis; and the presence of other oligomers of IgM in addition to monomeric IgM as detected by immunoblotting in patients with circulating LMW IgM. What are some possible explanations for the apparent disorder of IgM assembly? Possiblities include quantitative or qualitative defects in J chain expression (Koshland, 1985); abnormalities of the sulfhydryl oxidase enzyme probably involved in polymerization of the IgM subunits (Roth and Koshland, 1981); defects in the glycosylation of the IgM subunits as suggested by Shulman et al, (1982) although McDougal and other, (1975) have not found differences in individual sugars or total carbohydrate content between monomeric and pentameric IgM; or due to mutations in amino acid residues critically involved in conformation or intermolecular association (Davis et al, 1986). To date there is little data regarding which of these possibilities is most likely to account for the apparent defective assembly. It is of interest that Shulman and colleagues (1982) have shown, using murine IgM secreting hybridomas, that structural alterations in the μ heavy chain involving partial deletion, polypeptide addition or abnormal glycosylation have all been associated with the secretion of LMW IgM in preference to the pentamer. With this in mind it is of interest to note that the LMW IgM molecule observed in one patient with mixed cyoglobulinaemia

appeared to have a slower migration on SDS polyacrylamide electrophoresis (i.e. possibly due to carbohydrate or amino acid additions) than the rest (Figure 2, Chapter seven) raising the possibility of a structural variant. To explore the role of glycosylation defects causing structural alterations culture studies involving tunicamycin (an inhibitor of glycosylation) would be of interest.

As mentioned in Chapters one and nine J chain appears to have a vital role in IgM subunit assembly and secretion. Absence of intracellular J chain has been associated with either a non secretory state or with the secretion of limited quantities of monomeric IgM (Koshland, 1985) while the incorporation of the genes involved in J chain expression into a non secretory lymphomatous B cell line has restored pentameric IgM secretion (Raschke et al, 1979). Quantitative alterations of J chain expression have also been observed in malignant B cell lymphoproliferative states (Mestecky et al, 1980; Mason and Stein, 1981). Is this then the explanation for the presence of LMW IgM in these conditions. No other data, to the authors knowledge, is available concerning defects of J chain expression in autoimmune and infective disorders. Neither is there data concerning alterations in the sulfhydryl oxidase IgM polymerizing enzyme. Indeed the exact role of this enzyme is still controversial (Roth and Koshland, 1981). In vitro studies employing oxidative stress and chelating environments should help to clarify some of these unknown areas.

While it is clear that peripheral blood mononuclear cells from selective patients or bone marrow cells from patients with

macroglobulinaemia can secrete both pentameric and LMW IgM in vitro it is still not known whether an individual cell secretes all its IgM in one molecular form or whether it secretes both forms in varying proportions. Limiting dilution studies may help to distinguish which possiblity is more likely. Furthermore the cellular origins of LMW IgM in man have not been explored in contrast with murine studies (Melchers et al, 1976). Can any B cell secrete LMW IgM under the appropriate conditions or is it only derived from a subset of B cell as is suggested from the murine studies (e.g. in man could it derive from the CD5 positive B subset previously characterised as secreting IgM natural autoantibodies (Lydyard et al, 1987))?

These and many other questions regarding the cause, origin, role and mechanisms of secretion of LMW IgM in human disease have yet to be answered. If LMW IgM has a contributing or casual role in the pathogenesis of many of these diseases, the exploration of these questions may provide information which will allow the therapeutic reversal of this apparent abnormality with possible resolution or amelioration of these diseases. The continuing study of this neglected immunoglobulin appears, to the author, to be strongly indicated.

APPENDIX

During the candidature of this thesis the following papers were published on the topic of low molecular weight IgM. All papers with the exception of No. 3 were written by PJR-T. In paper No. 3, PJR-T contributed approximately 50% of the written script and derived all the data relating to LMW IgM in the patients studied.

1. Roberts-Thomson PJ, Neoh S & Bradley J : Quantitation and evaluation of low molecular weight IgM in rheumatoid arthritis Ann Rheum Dis 39:349-353, 1980.

2. Roberts-Thomson PJ, Wernick RM & Ziff M : Low molecular weight IgM in rheumatoid arthritis and other rheumatic diseases. Arth & Rheum 24:795-802, 1981.

3. Kwitko A, Roberts-Thomson PJ & Shearman DJC : Low molecular weight IgM in selective IgA deficiency. Clin & Exp Immunol 50(1):198-202, 1983.

4. Roberts-Thomson PJ, Koh LY, Neoh SH, Thomas M & Bradley J : Low molecular weight IgM in B cell lymphoproliferative disorders. Aust & NZ J Med 14:121-125, 1984.

5. Jones SN, Koh Lin T, Bertouch JV & Roberts-Thomson PJ : Synthesis of low molecular weight IgM in vitro in rheumatoid arthritis. Arth & Rheum 28:112-117, 1985.

6. Harris R, Beckman I & Roberts-Thomson PJ : Low molecular weight

IgM: Detection using immunoblotting. J Immunol Meths 88:97-100, 1986.

7. Koh Lin Y, Jones DN & Roberts-Thomson PJ : Appearance of low molecular weight IgM during course of infective endocarditis. Clin Exp Immunol 64:471-475, 1986.

8. Roberts-Thomson PJ, Kennedy A, Koh LY & Harries RH : Frequency of low molecular weight IgM in cord blood. J Reprod Med (in press).

9. Roberts-Thomson PJ, Kennedy A & Koh LY : Large quantities of low molecular weight IgM in mixed cryoglobulinaemia. Ann Rheum Dis (in press).

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