HOST FACTORS IN CHRONIC IMMUNE COMPLEX GLOMERULONEPHRITIS

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CONTENTS

SUMMARY

Most forms of glomerulonephritis (GN) in man are thought to be mediated by immune complexes (IC). The inability to detect circulating IC in many cases of chronic IC GN suggests that host immune defects may result in "physiologic" amounts of circulating IC becoming nephritogenic. Host disorders of immune regulation may also lead to the induction of auto-immunity, abnormal antibody responses to ubiquitous antigens and nephritogenic IC formation. It is suggested that the quality or quantity of exogenous antigen is less important than the state of the host immune system. This thesis has explored this concept and examined the following host parameters:

i) Immunoregulation

The response of peripheral blood mononuclear cells (PBMC) from a group of patients with membranous nephropathy (MN) to the mitogen phytohaemagglutinin (PHA) was no different to controls. However, 2 MN patients with reduced lymphocyte transformation responded to the immuno-modulating agent levamisole with normalization of PHA responses and a decrease in proteinuria. Studies using monoclonal antibodies to T cell subsets demonstrated a deficiency of a suppressor cell subpopulation in patients with primary MN and mesangial IgA nephropathy (IgA GN). In MN this abnormality correlated with defective Concanavalin A (Con A) inducible suppression of *in vitro* IgG synthesis. Patients with IgA GN, Henoch Schonlein purpura (HSP) and lupus nephritis (SLE) showed B cell hyper-activity *in vitro* with variable degrees of functional suppressor cell defects, most marked in HSP.

These results suggest disturbances of immune regulation in these forms of IC GN similar to those seen in the auto-immune IC disease, SLE.

ii) The role of auto-antibodies in IC GN

Auto-antibodies were neither found in the sera of a large group of primary MN patients nor in the renal eluates of 4 patients with MN. However, evidence of anti-DNA antibody activity was found in polyethylene glycol precipitates from 4/14 MN sera. Highly purified anti-DNA antibody was utilized to show the presence of extranuclear glomerular DNA in 2/6 SLE renal biopsies but this auto-antigen could not be found in 10 MN biopsies. Finally patients with MN had an increased frequency of the auto-immunity associated HLA DR3 antigen.

iii) IgG subclasses in glomerular immune deposits

IgG_3 was found to be the predominant subclass in glomerular deposits from patients with MN, SLE and anti-glomerular basement membrane antibody induced GN and to be virtually the only subtype of IgG in renal biopsies from patients with mesangiocapillary GN. This study demonstrates a difference between the distribution of IgG subclasses in normal plasma and in glomerular deposits and may be a genetically determined response of the host.

iv) Fc specific reticulo-endothelial clearance

Defective clearance of autologous, chromium labelled, IgG sensitized erythrocytes was found in HLA B8/DR3+ normal controls, 6/11 patients with IgA GN and 4/11 MN patients. Marked defects were present in 8/10 SLE patients. It is possible that this defective immune clearance may facilitate glomerular IC deposition in these diseases.

These studies indicate that multiple host defects appear to exist in individuals with IC GN. Whether these defects are primary or secondary to the disease state is unknown. Studies of the families of patients with these disorders and serial studies of individual patients may help answer this question.

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

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FEBRUARY 1983

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CHAPTER 1.

INTRODUCTION

GENERAL

Most forms of glomerulonephritis (GN) are immunologically mediated (Wilson and Dixon 1981). The evidence for this has been provided by the detection of immune reactants (immunoglobulin and complement) in glomeruli by immunofluorescence in the majority of cases of human GN and by the resemblance of human disease to experimental models in which immunological mechanisms are known to be important. In the presence of soluble antigen, immune complexes (IC) are formed in the circulation and may deposit in glomeruli and other vascular areas. If soluble antibody reacts with antigen outside the vascular space an Arthus-like localized reaction results, as in autoimmune thyroiditis or vasectomy induced orchitis (Bigazzi et al 1976). Antibodies which combine with insoluble, tissue fixed antigens result in immune damage at the site of antigen-antibody interaction. These target antigens may involve normal structural components of the kidney such as the glomerular basement membrane (GBM), with immune damage then resulting in classical anti-GBM antibody induced disease (Wilson and Dixon 1979). Antibody may also combine with non-basement membrane antigen or with antigens which are "planted" in the glomerulus (Wilson and Dixon 1979). The linear, ribbon like deposits of immunoglobulin along the GBM in anti-GBM antibody induced GN can be easily distinguished by immunofluorescence from the granular, discontinuous deposits, seen in IC mediated GN. More than 70% of human GN appears to be mediated by IC (Wilson and Dixon 1974), whereas a much smaller percentage (5%)

involves linear deposition of immunoglobulin secondary to anti-GBM antibodies.

In the following pages the relevant experimental models of IC GN from which much of our knowledge of the immunopathogenesis of the human disease has been derived will be reviewed. The traditional and more recent concepts of glomerular IC localization and tissue damage will then be discussed. Recently new ideas of the pathogenesis of IC GN have been formulated which have resulted in a more complicated picture and a revision of some previously held notions. These ideas will be reviewed followed by a discussion of the part played by the host in GN, a concept which will be expanded in the remainder of this thesis. Finally 3 of the commonest forms of IC GN in man - membranous nephropathy (MN), mesangial IgA nephropathy (IgA GN) and lupus nephritis (SLE) will be summarized, as patients with these particular forms of nephritis have provided the basis for most of the experimental work reported in the following chapters.

ANIMAL MODELS

The term serum sickness was used by Von Pirquet in 1903 when describing the host's immune response to a foreign serum protein. The experiments of Germuth (Germuth 1953, Germuth et al 1957, Germuth and McKinnon 1957) and Dixon (Dixon et al 1958, Dixon et al 1961, Dixon 1962, Dixon 1963) demonstrated that this was an IC mediated reaction using an experimental model in which serum sickness was induced in rabbits by the injection of purified bovine serum albumin (BSA).

Acute serum sickness

In this model injected BSA disappears in 3 phases. Firstly up to three quarters of the BSA leaves the circulation in equilibration with the intra and extravascular spaces (24-48

hours). Next, the BSA is catabolized with a half disappearance time of 4.2 days. At day 4 or 5, antibodies against BSA can be detected marking the third phase of antigen elimination. BSA - anti-BSA complexes are formed, which initially are in antigen excess, are too small to be removed by the reticulo-endothelial (R-E) system and continue to circulate. As the amount of antibody increases, the complexes become larger and less soluble and are phagocytosed by the R-E system resulting in rapid elimination of BSA between day 10 and 14. During the third phase, small amounts of BSA - anti-BSA complexes and complement become deposited within vascular structures and cause tissue injury, resulting in acute serum sickness. Acute GN is the most prominent manifestation of this disease although it is self limiting and disappears leaving no trace. Immunofluorescence shows the presence of finely granular deposits containing BSA, rabbit IgG and complement along glomerular capillary walls. As clinical manifestations subside the deposits increase in size as circulating free anti-BSA antibodies react with BSA - anti-BSA complexes, at the same time making the BSA more difficult to identify in the deposits.

This form of GN in rabbits closely resembles that of post infectious GN in man. Similarly models of chronic serum sickness have provided a parallel with some form of chronic GN in man.

Chronic serum sickness

To induce chronic serum sickness in rabbits, BSA is injected daily into the animals. This may result in the development of chronic IC mediated GN after 6-8 weeks. (Dixon et al 1961). Rabbits which are poor antibody responders or which are not given enough antigen to balance antibody production do not develop GN. When the correct amount of BSA is given to balance antibody production nephritogenic IC results with proteinuric

GN. The development of the renal lesion is critically dependent therefore, on the antigen-antibody ratio created in the circulation. A spectrum of GN is produced with membranous GN occurring in rabbits with a poor antibody response and proliferative GN in high responders, but with a mixed picture most commonly. The glomerular IC are in dynamic equilibrium with antigen and antibody from the circulation and it has been possible to dissolve deposits by the presence of excess antigen in the circulation (Wilson and Dixon 1971). Resolution of tissue damage has been shown to accompany the disappearance of the IC (Wilson 1974).

OTHER ANIMAL MODELS OF IC GN WHICH MAY BE RELEVANT TO HUMAN GN AND TO THE STUDIES REPORTED IN THIS THESIS.

Heymann's Nephritis

This model was first described by Heymann and colleagues (1959) and was induced by intra-peritoneal injection of homologous kidney in complete Freund's adjuvant. A crude renal tubular antigen extract (FxIA) and a more purified 28S lipoprotein (RTE-alpha 5) were shown to be antigenically important (Edgington et al 1968). Antibody could be recovered by elution from the affected kidneys which reacted with the renal tubular brush border. It was felt that the disease was mediated by circulating IC containing renal tubular antigens and their antibodies. Morphologically the renal lesion closely resembled MN in man. However, the passive administration of heterologous anti-renal tubule antigen antibodies results in a similar glomerular lesion, with these antibodies reacting directly with the glomerular capillary wall (Van Damme et al 1978, Couser et al 1978). Subsequently, eluates from kidneys of rats with Heymann's nephritis have been shown to react with antigen present at discrete sites at the subepithelial aspect of the GBM

(Neale and Wilson 1982). Evidence therefore now favours a role for auto-antibodies binding with fixed glomerular antigen in this model of MN.

Host genetic factors are also involved as different inbred rat strains are either very susceptible, weakly susceptible or resistant to the induction of GN and this suceptibility is associated with major histo-compatability haplotypes (Stenglein et al 1978).

Murine lupus-like GN induced by polyclonal B cell activators

In mice, after a single intraperitoneal injection of bacterial lipopolysaccharide (LPS), deoxyribonucleic acid (DNA) appeared in the circulation after a few hours, followed in 3 days by the appearance of anti-DNA antibodies in the serum and glomerular deposition of immunoglobulin along capillary walls and in the mesangium (Fournié et al 1974, Izui et al 1977). Anti-DNA antibodies made up almost half of the immunoglobulin eluted from these kidneys, thus indicating that DNA - anti-DNA complexes are involved in the glomerular deposits (Izui et al 1977). It was proposed that LPS releases autologous DNA into the circulation which may then bind to renal glomeruli because of its affinity for the GBM (Izui et al 1976). The release of DNA induces the production of anti-DNA antibodies which then form in situ IC with glomerular bound DNA.

Similar findings have been reported in mice after intraperitoneal infection with E.coli (Fournié et al 1980). It is suggested that polyclonal B cell activators (e.g. certain infections) may cause the release of autoantigens (e.g. DNA) and later auto-antibodies and therefore act as triggers for autoimmune tissue injury. This model may be relevant in human SLE and also in certain forms of primary GN in man.

Mercury induced glomerulopathy in the rat

Low doses of inorganic mercury can induce an IC glomerulopathy in rats similar morphologically to MN in man. This is associated with circulating anti-nuclear antibodies against non-histone nucleoprotein. (Weening et al 1978, Weening et al 1980). These antibodies could also be eluted from the kidneys but it is not clear whether localization of these antibodies in the glomeruli is caused by deposition of IC from the circulation, or by binding to a "planted" antigen, e.g. DNA.

Recently, an impairment of general T cell reactivity to mitogenic stimulation and a decrease of suppressor T cell function has been shown in this model (Weening et al 1981). Neonatal thymectomy accelerated the anti-nuclear antibody response and the development of the glomerular lesion, suggesting that impairment of effector and regulatory T cell functions (presumably mercury induced) is of pathogenetic significance.

Spontaneous murine lupus GN

This disease which closely resembles human SLE was first studied in (NZB x NZW) F_1 hybrid mice (Heyler and Howie 1963, Lambert and Dixon 1968). Two new strains of inbred mice (MRL/1 and BXSB) also develop the same disease, characterized by an IC GN, polyclonal B cell hyperactivity and formation of a variety of auto-antibodies including antibodies to nuclear antigen and to retroviral gp70 (Dixon et al 1980). Glomerular eluates of immunoglobulin from these mice are made up of 50% anti-nuclear antibody and 20% anti-retroviral antibody (Dixon et al 1971). From the study of murine SLE in these strains it has become apparent that multiple genetic backgrounds can result in the development of severe SLE. Although

polyclonal B cell hyperactivity appears early and persists, there is no demonstrable T cell regulatory abnormality early in the course of this disease. Functional measures of T cell subsets are generally normal with occasional abnormalities in some strains of mice. Some older mice tend to have demonstrable T suppressor cell defects. Finally, the disease appears to result from accelerating factors (e.g. female gender in (NZB x NZW) F_1) acting upon a genetically determined autoimmune background (Dixon 1982). This may be relevant to the aetiology of human SLE via the dual gene hypothesis (Schwartz 1981).

GLOMERULAR LOCALIZATION OF COMPLEXES

Most circulating IC do not deposit in glomeruli or cause damage. Normally the formation of IC is a physiologic event leading to rapid removal of antigenic material from the circulation. Glomerular deposition depends on i) properties of IC and ii) host factors which favour glomerular localization.

i) Properties of IC promoting glomerular localization

Extensive studies of the chronic serum sickness model in rabbits have attempted to correlate circulating IC with the development of the glomerular lesion and to identify the nature of complexes depositing in glomeruli (Dixon et al 1961, Germuth and Rodriguez 1973). IC size seems to be of major importance in determining clearance from the circulation and sites of tissue deposition. Small circulating IC (5-7 x 10^5 daltons) localize in the glomerular capillary walls, while intermediate sized IC (1 x 10^6 daltons) localize in the mesangium and the subendothelial region of the glomerular capillaries. Rabbits with larger circulating IC (> 4 x 10^6 daltons) do not develop nephritis (Germuth et al 1972). The size of IC is mainly

determined by the ratio of antigen to antibody but other factors (Germuth and Rodriguez 1973) are important including antigen characteristics (size and valency), the immunoglobulin class of the antibody and antibody avidity (Soothill and Steward 1971) (at the same antigen-antibody ratio, antibodies with low avidity will form smaller complexes than antibodies of high avidity). Analysis of circulating IC and glomerular deposition in serum sickness models has been criticized from the point of view that circulating IC may not be representative of those that deposit in glomeruli, as IC which will deposit may be rapidly removed from the circulation. Experiments have therefore been performed using pre-formed IC which have been injected into animals and the distribution of the antigen and antibodies studied. Problems associated with this approach include the non-uniformity of the injected complexes, with IC depositing in glomeruli not necessarily being representative of the bulk of the pre-formed IC, the short duration of the experiments which does not allow for the possibility of prolonged deposition of IC, modification of the host response by injected complexes which may then alter the deposition of the IC and finally the possibility that the pre-formed IC may dissociate in vivo resulting in independent antigen and antibody deposition. Nevertheless conclusions from these sorts of experiments include the following:

a) Complexes with lattice structure > Ag_2 Ab_2 are quickly removed from the circulation by the R-E system (McCluskey et al 1962, Mannik et al 1971).

b) A very small percentage of injected IC deposits in glomeruli (Arend and Mannik 1971).

c) The inability of IC to react with Fc or C3 receptors

(e.g. alkylated antibodies) increases glomerular localization (Haakenstad et al 1976).

d) Complexes with antibody of high avidity or antigen of high valency are more likely to deposit in mesangial areas than are IC with low affinity antibody or low valency antigen (Koyamo et al 1978, Germuth et al 1979).

e) Epimembranous deposits are unusual in these studies. However, a proliferative nephritis with subepithelial deposits can be produced in mice by the injection of IC containing poorly avid antibody (Germuth et al 1979). These complexes have a slower clearance from the circulation than IC with highly avid antibody and are smaller. The low avidity complexes tended to dissociate to a large extent *in vivo* raising the possibility of an *in situ* mechanism of IC formation (Germuth et al 1982). Subendothelial deposits have followed the injection of IC containing moderate affinity antibodies or antigens of low valence while IC containing antibodies of very low affinity or very low valence antigen do not deposit in glomeruli (Koyamo et al 1978).

Factors such as the binding of rheumatoid factor, complement components or anti-idiotypes may alter the nature of the IC *in vivo* so that it is difficult to draw conclusions about the nature of antibodies in the complexes which initially formed glomerular deposits in human nephritis, or to draw definite conclusions from the animal experiments described.

ii) Host factors influencing glomerular deposition of IC

Glomerular structure

The structural arrangement of the glomerulus and the selective permeability of glomerular capillaries are possibly

the most important of the host factors involved in glomerular IC deposition. This high pressure system in association with an effective barrier to filtration leads to accummulation of IC in a subendothelial position, followed later by movement into mesangial areas. Experimentally induced hypertension enhances the effects of acute serum sickness in the rabbit (Fisher and Bark 1961) and conversely impairment of glomerular filtration decreases glomerular IC deposition (Germuth et al 1967).

C3b Receptors

These have been identified on human glomerular epithelial cells (Gelfand et al 1975, Shin et al 1977, Moran et al 1977) but not in glomeruli from experimental animals. The role of these receptors in glomerular IC localization is unknown, as is the role of glomerular Fc receptors (Mizoguchi et al 1978).

Vaso-active substances

The release of substances such as histamine and serotonin leads to changes in glomerular capillary wall permeability and increased IC deposition (Kniker and Cochrane 1968, Henson and Cochrane 1968, Cochrane and Koffler 1973). Platelets are a major source of vasoactive substances and platelet depletion decreases glomerular IC deposition in rabbit serum sickness (Cochrane and Koffler 1973).

Mononuclear phagocytic system (MPS)

The rate of clearance of circulating IC influences glomerular IC deposition. The larger the IC, the faster it is cleared by the MPS. This process is enhanced by the reaction between Fc receptors on mononuclear phagocytic cells and Fc fragments of IgG containing IC. C3b receptors present on Kupffer cells probably aid in the clearance of IgM complexes while IgA complexes seem not to be efficiently removed from the circula-

tion (Normann 1972). These complexes are probably cleared by binding to secretory component present on hepatocytes (Kleinman et al 1982). Saturation of the MPS by high levels of circulating IC in animals leads to increased tissue deposition of pre-formed IC (Haakenstad and Mannik 1974). Conceivably in man failure of the MPS, either primarily through Fc receptor defects, or secondarily through IC saturation could lead to increased IC circulation and glomerular deposition. The mesangial system may also be important in clearing potentially nephritogenic material. However, the accummulation of monocytes in this area in response to deposited IC may be mainly responsible for phagocytosis.

REMOVAL OF GLOMERULAR IC DEPOSITS

Administration of excess antigen to animals with mesangial IC deposits leads to complete disappearance of the deposits if antigen is infused early enough (Mannik and Striker 1980). Solubilization of IC deposits by complement may also be important (Takahashi et al 1977, Bartolotti and Peters 1978) and has been reported to be impaired in patients with SLE (Aguado et al 1981) and mesangial IgA GN (Tomino et al 1982).

MECHANISMS LEADING TO TISSUE DAMAGE IN IC GN

Secondary mechanisms set in motion by deposited or locally formed IC include activation of the complement cascade (Schreiber and Müller-Eberhard 1979). The exact relationship of complement activation to tissue damage is not clear. An important complement effect may be the attraction of neutrophils into the area by C5a with resultant tissue damage through release of biologically active substances, including tissue oxygen products and leukocytic proteins. However, depletion of complement or neutrophils does not prevent nephritis in acute serum sickness

in rabbits (Henson and Cochrane 1971). Although complement is frequently found in glomerular deposits in human IC GN, its participation in immune injury is largely based on indirect evidence.

There is increasing evidence that the macrophage-monocyte plays a role in the development of glomerular injury in IC mediated GN. A macrophage-type cell is normally present in the glomerular mesangium and expresses the Ia (DR) antigen and C3b and Fc receptors (Schreiner et al 1981). The possibility of local immune control within the glomerulus related to this cell is a distinct possibility. The contribution of circulating monocytes to glomerular hyper-cellularity has been shown in experimental GN (Holdsworth et al 1978, Striker et al 1979) and human GN (Atkins et al 1976). In acute serum sickness in rabbits, depletion of macrophages using an anti-macrophage serum prevented clinical and histologic changes (Holdsworth et al 1981). How monocytes cause tissue injury is still not clear. They carry a range of biologically active mediators and have the ability to produce high levels of oxygen derived free radicals which may be an important factor.

Finally other cell mediated immune mechanisms may play some part in the pathogenesis of glomerular disease in man (Rocklin et al 1970) but little evidence so far exists.

PROBLEMS AND NEW CONCEPTS IN IC MEDIATED GN

1. Immune complexes, antigens and auto-immunity

The detection of circulating IC by a variety of different assays has been used as further confirmatory evidence of the role of circulating IC in the pathogenesis of human GN with granular deposits of immunoglobulin and complement in glomeruli. Many assay systems have been extensively reviewed (Wilson

1979, Theofilopoulos 1980, Williams 1981). These assays rely on the physico-chemical or immunologic properties of IC, but it has become apparent that there is no single definitive method of detecting circulating IC. Certain forms of GN associated with active systemic IC disease such as SLE or bacterial endo-carditis regularly have high levels of circulating IC in most cases, whereas in chronic idiopathic GN they are frequently not detected (Woodroffe et al 1977, Couser and Salant 1980, Theofilopoulos and Dixon 1980). In particular most investigators of primary MN find circulating IC in low incidence or not at all (Border 1979, Woodroffe et al 1979, Theofilopoulos and Dixon 1980). Possible reasons for the failure of detection of circulating IC in glomerular disease presumed to be caused by IC, include the following:

a) Lack of sensitivity of IC assays.

   Related to this is the fact that different IC assays detect different kinds of complexes, i.e. IgA class complexes which activate complement via the alternate pathway may not be detected by assays relying on the binding of CIq. IC formation is a normal physiologic event for antigen clearance and IC assays may not be able to detect these minute amounts of IC which an individual who develops GN may handle in a nephritogenic manner. In summary, most IC assay systems will detect large complexes which avidly bind complement while small IC or those not activating complement may not be detected.

b) Intermittent presence of circulating IC.

c) Circulating IC is not present because IC are formed <u>in situ</u> rather than deposited from the circulation.

In addition, certain conditions such as primary biliary cirrhosis and malignancy are associated with circulating IC in

the absence of overt glomerular disease. (Theofilopoulos and Dixon 1980). On the other hand, circulating IC has been detected in the sera of patients with minimal change GN (Levinsky et al 1978), a disease which does not have granular deposits of immune reactants in glomeruli and which is thought not to be mediated by IC.

Even greater difficulties have been encountered in attempting to demonstrate specific antigen-antibody systems in human, presumed IC mediated GN. Although a large number of exogenous aetiological agents (infectious agents, drugs, inoculations) have been implicated in IC GN by the demonstration of these antigens in association with immunoglobulin in glomerular immune deposits, the number of actual cases of GN accounted for is small. The obvious difficulty is in screening for antigens for which there are no clinical clues regarding their nature. Additionally the antigen may be masked by excess immunoglobulin and complement, making its identification difficult.

Finally, exogenous agents may not be detected because they are not involved in the majority of chronic IC GN. The possibility exists, that as in SLE, autologous host antigens are involved with corresponding antibody and the induction of autoimmunity. Endotoxin which induces polyclonal B cell activation in mice also results in anti-DNA - antibodies and DNA - anti-DNA complexes and IC GN (Izui et al 1977). Similarly mice infected with malaria or Schistosomiasis develop anti-DNA antibodies (Poels et al 1978, Fischer et al 1981). In man there is recent evidence of circulating DNA - anti-DNA complexes in conditions other than SLE (Lewis and Roberts 1980). IgG is also another possible auto-antigen. This immunoglobulin could be exogenously altered (e.g. by a viral or bacterial infection) to become immunogenic. Certain infections are associated with IC made up

of autologous IgG and rheumatoid factor, and anti-globulin antibody and IgM-IgG or IgG-IgG complexes are present in patients with idiopathic cryoglobulinemia and nephritis. Finally idiotype-anti-idiotype complexes may be involved in glomerular deposits (Rose and Lambert 1980). This process of induction of auto-immunity must involve some failure of the host regulatory mechanisms which normally limit the duration and extent of immune reactions. A failure of inhibitors of auto-reactive lymphocytes may theoretically be due to receptor blockade by antigens, to suppressor T cell defects or to failure of anti-idiotype antibody regulation of the immune response. The production of autoantibodies specific for regulatory T cell subsets could then allow the continuation of the disease process by maintaining a deficit of regulatory cells. Evidence for deregulation of antibody-producing cells through selective loss of suppressor T cells has been reported in SLE but not definitively examined in chronic idiopathic GN.

2. In situ IC formation and role of charge in IC localization

The experimental models of GN featuring deposition of circulating IC in glomeruli do not seem to explain the pathogeesis of certain types of nephritis, in particular MN. Intravenous injection of pre-formed IC does not generally lead to subepithelial deposition except when low avidity complexes were used, raising the possibility of *in vivo* dissociation of antigen and antibody, followed by *in situ* IC formation (Germuth 1979). Studies in passive Heymann's nephritis have shown that epimembranous deposits can be produced *in situ* by perfusing free antibody into isolated rat kidney under conditions where the formation of circulating IC could be excluded (Couser and Salent 1980). There is also evidence that free (non-glomerular)

antigen can localize in the glomerulus. Concanavalin A (Con A) when perfused, appears to localize in the GBM and subsequent perfusion with antibody to Con A resulted in GN (Golbus and Wilson 1979). Foreign antigens therefore may deposit in the kidney depending on factors such as size and charge, subsequently followed by localization of antibody and in situ IC formation. The high net negative charge of the GBM due to the presence of sialoglycoprotein and proteoglycans may be important in antigen and IC localization. Cationized ferritin localizes in a subepithelial site in rats, whereas ferritin normally deposits in the mesangium (Batsford et al 1980). Similarly cationized BSA induced a more severe nephritis in rabbits than neutral BSA (Border et al 1982) and the cationized protein localized only in a subepithelial location compared to neutral BSA, which rarely was found in a subepithelial site. The subepithelial deposits of human MN may therefore result from antigens which can bind to the GBM through charge or a particular affinity and specific low avidity antibody with resultant in situ IC formation.

3. Insoluble IC formation

Insoluble complexes are formed in antibody excess, increasing in amount as antigen concentration increases and reaching a maximum at a zone of equivalence. Beyond this point as antigen excess is reached soluble IC results and insoluble complexes decrease. Multivalent antigens which can form large latticed IC and high avidity antibodies increase the rate of insoluble IC formation. Recently, Cameron and Clark (1982) have suggested that poorly soluble or insoluble IC were the pathogenic IC in the chronic serum sickness model in rabbits given BSA that developed mesangiopathic lesions.

In man, studies in SLE have suggested that the association of high avidity anti-DNA antibodies and the multivalent antigen, double stranded DNA, leads to IC formation at equivalence or in antibody excess with the production of insoluble IC (Dorsch and Barnett 1974, Taylor et al 1981). Anti double stranded DNA antibody eluted from the kidneys of patients with lupus nephritis has been shown to be of higher avidity than that in the circulation or in cryoprecipitates at the time of active nephritis (Winfield et al 1977), suggesting glomerular deposition of large latticed, insoluble IC.

Finally R-E system dysfunction, which has been shown in SLE (Frank et al 1979), would increase renal exposure to insoluble complexes which normally would be cleared rapidly. The role of insoluble IC in other forms of chronic IC GN in man is yet to be determined.

4. The role of non-immunologic mechanisms of chronic glomerular damage.

These factors have recently been reviewed (Baldwin 1982) and evidence suggests that loss of nephrons results in further glomerular damage through high flows and high pressures within surviving glomerular capillaries. Hypertension further increases glomerular damage, presumably through transmission of elevated pressures. Finally, phosphorus restriction appears to preserve renal function and dietary protein accelerates glomerular sclerosis in animals with experimental nephritis.

Thus, although immunologic mechanisms may initiate chronic IC GN in man progression may be determined by haemodynamic factors.

HOST FACTORS IN IC GN

As can be seen from the previous pages, research in IC GN

has primarily involved the elucidation of pathogenetic mechanisms through experimental models and a search for antigen-antibody systems in human GN. However, only recently has the question of why only certain individuals develop IC GN been addressed. Rather than the quality or quantity of antigen load determining the development of GN, more important may be the effectiveness of the immune response in the host, in allowing the nephritic process to develop (Peters and Lachman 1974). The large and varied number of identified antigens in human IC GN supports this view. In addition the association of some forms of IC GN with HLA antigens (Garavoy 1982) points to yet undefined host genetic factors being involved in the development of nephritis. In animals evidence has been provided for specific major histocompatability linked immune response genes but this evidence is lacking in man. However, the associations between diseases and HLA determinants can probably be regarded as possible evidence for the existence of such immune response genes. The mechanisms leading to disease expression secondary to genetic susceptibility is not yet clear. However, these host factors may include:

i) Abnormalities of immune regulation

This could determine the induction of autoimmunity as previously discussed and may result in abnormal antibody responses to ubiquitous antigens with nephritogenic IC formation. This is a very complex area and may involve all facets of the immune response including antigen processing by macrophages, T cell regulation and interleukin production, idiotypic networks and B cell activity.

ii) Qualitative antibody responses

From animal models low affinity antibody appears to result

in less effective antigen elimination and pre-disposition to IC GN (Petty et al 1972). In addition, the class and subtype of the host antibody response will be important e.g. in SLE the antinuclear antibodies associated with lupus nephritis are IgG_1, and IgG_3, the subclasses that fix complement well.

iii) Complement deficiency

Inherited complement deficiency is a striking example of host immune deficiency associated with GN. The most common form of complement deficiency is that of C2. This deficiency has been associated with SLE, renal disease and vasculitis (Schur and Carpenter 1979). Deficiencies of other early complement components (Agnello 1978) have also been associated with GN. This association may be related to defective clearance of IC by complement solubilization (Schifferli et al 1981) or to a pre-disposition to infection leading to IC GN (Peters and Williams 1974), perhaps as a result of inadequate lysing of virally infected cells (Sissons and Oldstone 1980). However, because some complement components are coded for in the same area on the sixth chromosome as HLA antigens, the association between certain disease states and complement deficiency may reflect linkage of different immune deficiencies on the same chromosome.

iv) R-E clearance

As previously discussed, this host factor is of importance in the glomerular deposition of IC. Defective immune clearance by the spleen has been demonstrated in SLE (Frank et al 1979, Lockwood et al 1979) but not in other forms of primary GN. Conceivably defects in R-E function could be due to saturation by IC or secondary to perhaps a genetically determined receptor defect.

The aim of the present study is to assess the contribution of host factors in patients with chronic IC GN. In particular, immune regulation, the role of auto-antibodies, the subtypes of glomerular IgG and Fc specific R-E function have been examined. Patient material has been drawn from 3 groups. The first 2, MN and IgA GN are common forms of chronic IC GN and the third, lupus nephritis, has been used as an example of IC mediated disease with primary derangements of the host immune response.

i) Membranous nephropathy

This form of chronic IC GN is well characterized histologically with granular deposits of IgG and complement along glomerular capillary walls and initially subepithelial deposits on ultrastructural examination (Spargo et al 1980). Although originally thought to be mediated by circulating IC depositing in glomeruli, circulating antigen-antibody complexes cannot usually be detected by IC assays and *in situ* complex formation may be more likely. Although antigens have been demonstrated in glomerular deposits in some cases, the majority of MN is idiopathic. Well recognized genetic associations exist with HLA DR3 (Klouda et al 1979, Garavoy 1980) and the B cell antigen MT2 (Garavoy 1980, Muller 1981). Clinically the disease is characterized by heavy proteinuria and an indolent course. Fifty percent of adult patients with MN will be alive without endstage renal failure 10 years after diagnosis, with many of these undergoing spontaneous remission of proteinuria (Glassock 1979).

ii) Mesangial IgA nephropathy (including Henoch Schonlein purpura (HSP)).

Both disorders characteristically have IgA deposits in

the mesangium of the glomerulus and many patients have IgA deposits in the capillaries of skin and muscle. Characteristic electron dense deposits are found in the mesangium (Spargo et al 1980). Serum IgA levels are elevated in 50% of patients and with IgA specific assays circulating IC are present in up to 68% of patients (Lesavre et al 1982). Systemic abnormalities consisting of arthritis, cutaneous vasculitis and visceral involvement occur in HSP, with recurring bouts of haematuria without systemic involvement, occurring in IgA GN. These 2 disorders probably represent ends of a clinical spectrum with a similar pathogenesis. The mesangial deposits are thought to be derived from circulating IgA class antibody complexes possibly of a mucosal origin (Woodroffe et al 1982). There seems to be a primary association between HLA DR4 (Hiki et al 1982, Kashiwabara et al 1982) and secondarily with BW35 (Berthoux et al 1978) or B12 (Richman et al 1979). These latter antigens are in linkeage disequilibrium with DR4. Clinically the disease is generally benign but about 10% of patients progress to end-stage renal failure.

iii) Lupus nephritis

This form of nephritis is a prototype of chronic immune complex renal disease (Koffler et al 1971). The presence of IC in the circulation and the role of IC in the pathogenesis of the nephritis is well documented. The disease is characterized by auto-antibodies against a variety of nuclear components and membrane antigens (e.g. T cell antigens). It is an extremely heterogeneous disease and may have a number of differing aetiologies leading to a final common pathway of expression. Factors involved include immunodeficiency and defective immune regulation, major histo-compatability assoc-

iations (particularly with haplotype B8 and DR3 (Reinertson et al 1978, Celada et al 1980, Griffing et al 1980)) and female sex. Two general patterns of renal disease may be seen - proliferative GN with mesangial and subendothelial deposits of IC and membranous GN with subepithelial deposition. Clinically 30% of patients have severe GN but steroid therapy can halt the progress of the disease in virtually all cases.

CHAPTER 2.

IMMUNOREGULATION IN CHRONIC IMMUNE COMPLEX GLOMERULONEPHRITIS

INTRODUCTION

In this chapter possible host defects in immunoregulation have been examined in patients with primary IC GN and with lupus nephritis. The study is based on the premise that IC formation is a normal physiologic event for antigen clearance and that nephritogenic IC formation may reflect defective cellular immune control of antibody production by the host, leading to abnormal antibody responses (± auto-antibodies) to ubiquitous antigens.

Initial studies assessed cellular immunity in patients with primary MN using phytohaemagglutinin (PHA) induced lymphocyte transformation. Selected MN patients were treated with the immuno-modulating agent, levamisole. Later, the availability of monoclonal antibodies to T cell subpopulations allowed antigenic identification of T helper and T suppressor subgroups in patients with MN, IgA GN, HSP and SLE. Finally, functional studies were used to assess *in vitro* immunoglobulin synthesis and Con A inducible suppression of immunoglobulin and DNA synthesis (tritiated thymidine uptake) in these patients.

PHA induced lymphocyte transformation

PHA induced lymphocyte transformation is the simplest form of functional test for T cells. A normal proliferative response of lymphocytes to PHA implies only that a patient has T cells which can proliferate and a separate population of cells which can make interleukin 2. Abnormal lymphocyte responses to PHA have been shown in SLE (Utsinger and Yount 1977) and Carney

et al (1980) reported decreased maximal PHA transformation of peripheral lymphocytes from 6 patients with MN. There appears to be an inhibitor of PHA-induced lymphocyte proliferation in the sera of patients with minimal change nephrotic syndrome in relapse (Moorthy et al 1976) and similar inhibitors have been reported using sera from patients with nephrotic syndrome due to other glomerulopathies (Martini et al 1981).

In the present study PHA induced lymphocyte transformation in autologous sera was assessed in patients with primary MN. Two patients with reduced PHA response and 2 control patients with normal PHA responses were treated with the immunostimulating drug levamisole.

Levamisole

This drug is a synthetic, low molecular weight substance with anti-helminthic activity and potent immunoregulatory properties (Symoens and Rosenthal 1977, Renoux 1980). It potentiates macrophage, polymorphonuclear leukocyte and T cell activities in vitro and in vivo and can act as an immunostimulant or as an immunosuppressive agent. Although its mode of action is not yet clear, there is evidence that it mimics the thymic hormone thymopoietin in its effect on T cells. (Goldstein 1978). Levamisole enhances E rosette formation (Ramot et al 1976, Ivanyi 1979) and mitogen and antigen induced transformation (Lichtenfield et al 1974, Hadden et al 1975, Golding et al 1976). Miyawaki et al (1980) reported that levamisole stimulated suppressor T cells in pokeweed mitogen (PWM) stimulated cultures, while Hersey et al (1981) found inhibition of suppressor cells in a similar system. These and other conflicting reports of the effect of levamisole, may result from differences in the dose administered, timing of administration,

experimental assay used and host/genetic factors. Overall, levamisole appears to enhance the immune response but only when this is deficient, i.e. it does not seem to have a major effect on the normal immune system. Levamisole has been shown to increase cyclic GMP (cGMP) and to decrease cyclic AMP (cAMP) in lymphocytes. Antigen induced synthesis or secretion of nucleic acids, proteins or lymphokines by lymphocytes improves when intracellular cGMP increases and decreases when cAMP increases (Anderson et al 1976). Levamisole may therefore correct lymphocyte hyporesponsiveness by increasing the ratio of cGMP:cAMP intracellularly. In summary, levamisole seems to selectively stimulate regulatory T cell subsets to restore homeostasis in a disturbed immune system.

Clinically it has been used successfully in aphthous ulceration and herpes, recurrent infection, chronic persistent hepatitis B antigen hepatitis, rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease and cancer (Symoens 1976). Seven nephrotic children with minimal lesion GN who had not responded to conventional therapy of prednisolone or cyclophosphamide, showed complete remission of the nephrotic syndrome when treated with levamisole. E rosetting capacity, which was depressed during relapse in these children, returned to normal on treatment with levamisole (Tanphaichitr 1980).

In the present study levamisole was used to treat patients with primary MN found to have a deficient PHA response and an attempt was made to correlate serial PHA transformation responses with clinical parameters of disease activity.

Identification of T cell subpopulations

T lymphocytes include immunoregulatory cells controlling humoral and cellular immune responses. Dysfunction of these

cells may lead to disease states in man; in particular a lack of suppression may lead to an auto-immune state (Strelkauskas 1978) which may be involved in the pathogenesis of some forms of IC mediated GN. Methods for identifying T cells and immuno-regulatory subsets in man initially depended on an assessment of sheep erythrocytes (E) rosette formation for total T cells, and rosetting tests dependent on Fc receptor status to identify T cell subgroups (Moretta et al 1977). T cells with an Fc receptor for IgM (Tμ cells) could facilitate immunoglobulin synthesis by B cells, whilst T cells with IgG Fc receptors (Tγ cells) seemed to act as suppressor cells.

More recently, the identification of T cell subsets has been revolutionized by the development of monoclonal antibodies against T cell membrane antigens defining functional subdivision of T lymphocytes. Kohler and Milstein (1975) first described the technique of taking antibody producing cells from an animal immunized with a particular antigen, e.g. human lymphocytes and fusing these cells to a myeloma cell line. The resultant hybridoma cells retain the properties of the myeloma cells - continuous antibody synthesis and frequent cell division, but will synthesize the particular immunoglobulin coded for by the genes of the plasma cells taken from the immunized animal. The hybridoma clones are screened for specific antibody secretion and colonies producing only antibody against the antigen of interest selected.

Ortho Pharmaceutical Corporation has produced the most extensively studied range of T cell subset monoclonal antibodies which have been defined functionally (Reinherz et al 1979 a, 1979 b, 1980 a). OKT4 and OKT8 reagents divide peripheral human T cells into two major subsets which have been shown functionally to provide help and suppression respectively.

OKT4 antibody reacts with 46 ± 8% peripheral lymphocytes and these cells have been shown to provide help for B cell immunoglobulin production in PWM stimulated cultures (Reinherz 1979 b), increase the generation of cytotoxic T cells (Reinherz 1979 c) and make soluble T cell factors in response to soluble antigens (Reinherz 1980 b). OKT8 antibody binds with 32 ± 8% peripheral lymphocytes and these cells include both cytotoxic and suppressor T cells (Reinherz 1980 c). The use of the ratio OKT4/OKT8 circumvents to some extent the problem of monocyte contamination with passive binding of the fluoroscein conjugated second reagent and variations in total lymphocyte counts which made interpretation of absolute subset values difficult. Indirect immunofluorescence is used to identify the OKT+ cells with a fluorescein conjugated anti-mouse antibody. Cells may be counted using either fluorescent microscopy or a fluorescence activated cell sorter (FACS), the main advantage of the latter being that a greater number of cells can be rapidly and accurately counted.

The evaluation of normal controls has highlighted several problems in the use of monoclonal anti T cell antibodies. Most workers have found a significant variation in T4/T8 ratios which is even greater in older people. When using fluorescence activated cell sorting the results obtained are critically dependent on the gating (low angle ± 90 degree scatter) criteria as to whether cells are included or excluded. This may account for differences in mean T4/T8 ratios between control populations of different centres. Finally drug therapy may affect T4/T8 ratios. No study has yet addressed this question definitively although cyclosporin A seems to selectively decrease OKT4+ cells (Routhier et al 1980) and prednisolone may reduce the ratio in some patients (Bach and Bach 1981).

The use of monoclonal antibodies to study changes in T cell subpopulations in various human diseases has recently been reviewed (Bach and Bach 1981). Increased T4/T8 ratios have been reported in various auto-immune disorders including auto-immune haemolytic anaemia, chronic active hepatitis, myasthenia gravis and rheumatoid arthritis. In particular Chatenoud and Bach (1981) found elevated ratios in patients with IgA GN and MN. Conflicting reports are found in SLE, the prototype of auto-immune disease. One group (Morimoto et al 1980) found elevated T4/T8 ratios in lupus patients while Chatenoud reported normal ratios (Chatenoud and Bach 1981). Smolen et al (1982) found low helper/suppressor ratios in SLE patients with severe renal disease and high ratios in patients with multisystem disease but without lupus nephritis.

In the present study the monoclonal antibodies OKT4 and OKT8 were used to examine the T4/T8 ratios in patients with primary MN, IgA GN, lupus nephritis and HSP.

Functional suppressor cell assays

Defective immune suppression may result in B cell hyperactivity with increased immunoglobulin synthesis and the production of auto-antibodies. Auto-immunity plays a central role in SLE with marked B cell activation resulting in hypergammaglobulinaemia and antibodies against a variety of auto-antigens, including lymphocytes. Lewis and Roberts (1980) suggested that auto-immunity may be a central factor in most IC mediated diseases and recently, cold reactive antinuclear antibodies have been demonstrated in the sera from patients with IgA GN and MN (Nomoto and Sakai 1979), suggesting similarities with SLE in these 2 common forms of chronic IC GN.

Based on experiments in the NZB/NZW mouse model, which simulates human SLE, impaired suppressor T cell function was proposed to be important in the pathogenesis of the auto-immune phenomena in adult mice. (Krakauer et al 1976). Many studies have suggested defects in suppressor cell function in patients with SLE using a variety of suppressor cell assays (Abdou et al 1976, Bresnihan and Jasin 1977, Sagawa and Abdou 1978, Fauci et al 1978). Most assays are based on the observation that activation by Con A of peripheral blood mononuclear cells (PBMC) from normal individuals induces cells which non-specifically suppress effector functions of other cells. However, it is not clear whether the demonstrated suppressor cell dysfunction in SLE is connected with the B cell hyperactivity or the auto-immune state of the disease. In fact, it is possible that suppressor defects are a result of, rather than a cause of auto-immunity, with anti-lymphocyte antibodies or IC resulting in a reduction of suppressor cells (Theofilopoulos and Dixon 1982).

Concanavalin A is a plant lectin extracted from the jack-bean and its effect on the activation of suppressor cells was first described by Dutton (1972). The use of Con A to study the immunoregulation of human T cells has been recently reviewed. (Dwyer and Johnson 1981). The authors point out that T cells responding to Con A are heterogeneous and the results of suppression of one indicator system may not apply to Con A inducible suppressor cells in general. The characteristics of the cells involved in Con A inducible suppression are not yet clearly defined but it seems that monocytes, in addition to T cells, are required for Con A induced suppression of DNA synthesis (Kallenberg et al 1980). Damle and Gupta

(1982) in a study of Con A inducible suppression of peripheral T lymphocytes enriched for OKT4+ or OKT8+ subsets, found that precursors of Con A induced suppressor T cells appear to reside in both OKT4+ and OKT8+ T cell subpopulations. They concluded that lectin-induced suppression is not restricted to a single distinct subpopulation of T cells but is a result of various interacting subsets. Presently there is a lack of standardization of procedures for assessment of Con A inducible suppressor function that makes comparison of results from different laboratories difficult.

With a knowledge of these limitations of Con A inducible suppressor cell assays, the present study has used 2 types of assays to assess suppressor cell function in lupus nephritis, MN, IgA GN and HSP. Age and sex matched controls were used where possible in view of the possibility that suppressor cell activity may be affected by age and sex (Hallgren and Yunis 1977, Schulof et al 1980). Although most of our patients retained normal renal function this patient variable may also be important in the interpretation of suppressor cell assays (Lortan et al 1982). The assay in which the largest number of patients in the present study was tested was performed as described by Miller and Schwartz (1979). The test measures the ability of Con A induced suppressor cells to suppress PWM stimulated immunoglobulin synthesis in cultures containing either PWM alone, or PWM and Con A. IgG and IgA synthesis was determined by a sensitive radio-immune assay. In addition, suppressor cell function was tested in a smaller number of patients using a one way mixed lymphocyte culture to which allogeneic Con A pre-treated patient lymphocytes were added. Suppression of DNA synthesis was measured by the incorporation of tritiated thymidine into lymphocytes (Shou et al 1976,

Schulof et al 1980).

PATIENTS

A. PHA transformation of lymphocytes was measured in

(i) 23 normal laboratory controls (11 males, 12 females, mean age = 32 ± 13).

(ii) 18 patients with primary MN (14 males, 4 females, mean age 47 ± 15). 5 were nephrotic (24 hour urinary protein excretion > 3.5 g/day and serum albumin < 30 G/L). None of the MN patients were taking prednisolone or cyclophosphamide at the time of testing or were lymphopenic.

(iii) 4 nephrotic MN patients before, at monthly intervals and after a 3 month course of levamisole therapy.

B. T cell subsets were measured in

(i) 28 normal controls (17 males, 11 females; mean age 35 ± 12 years).

(ii) 14 patients with primary MN (11 males, 3 females; mean age 51 ± 13 years) including 6 who were nephrotic.

(iii) 10 patients with mesangial IgA GN (7 males, 3 females; mean age 45 ± 14 years).

(iv) 15 patients with lupus nephritis (2 males, 13 females; mean age 30 ± 12 years). 11/15 of the SLE patients were receiving prednisolone therapy at the time of testing and 1 patient was nephrotic.

(v) 2 patients with HSP (B.V. female 42 years, F.T. male 59 years). Both patients were nephrotic.

(vi) serial studies were performed in selected normal controls and patients with primary MN, lupus nephritis and mesangial IgA GN.

C. Functional studies

(i) In vitro immunoglobulin synthesis and Con A inducible suppressor cell activity was assessed in

a) normal controls

(i) matched for the non SLE patient group

IgG n=23; 15 male, 8 female) mean age 47 ± 11 years

IgA n=22; 15 male, 7 female)

(ii) matched for the SLE patient group

IgG n=18; 4 male, 14 female - mean age 32 ± 10 years

IgA n=17; 4 male, 13 female - mean age 32 ± 11 years

b) 13 patients with primary MN (9 males, 4 females; mean age 50 ± 12 years) with the nephrotic syndrome present in 5 patients.

c) 10 patients with mesangial IgA GN (8 males, 2 females; mean age 46 ± 13 years) including one nephrotic patient.

d) 3 patients with HSP (B.V.: F.T.: J.W. male 49 years). All patients with HSP were receiving steroid therapy at the time of testing.

e) 10 patients with lupus nephritis (2 males, 8 females; mean age 31 ± 14 years). 9 SLE patients were receiving prednisolone therapy at the time of testing and one was nephrotic.

(ii) Suppressor cell activity measured by tritiated thymidine uptake was measured in

a) controls

(i) 22 normal individuals (10 males, 12 females; mean age 38 ± 10 years) were tested using Con A stock solution 5 µg/ml in the suppressor assay. (0.5 µg/ml final)

(ii) 10 normal individuals (4 males, 6 females; mean age 36 ± 11 years) were tested using Con A stock solution 50 µg/ml in the suppressor assay. (5 µg/ml final)

b) 9 patients with primary MN including 5 who were nephrotic (7 males, 2 females; mean age 47 ± 14 years).

c) 9 patients with mesangial IgA GN (6 males, 3 females; mean age 43 ± 15 years).

d) 9 patients with lupus nephritis (1 male, 8 females; mean age 34 ± 14 years). 8 of these patients were receiving prednisolone therapy at the time of testing.

All patients had renal biopsy documented GN. With 3 exceptions, serum creatinine concentrations were < 0.25 mmol/Litre (normal range 0.05 - 0.12 mmol/Litre): patient B.V. with HSP who required dialysis during the course of her acute illness but recovered normal renal function and two patients with mesangial IgA GN had creatinine concentrations of 0.39 mmol/Litre and 0.60 mmol/Litre at the time of testing. Serum IgA levels were measured in all group C patients with mesangial IgA GN and in 2 patients (F.T.: J.W.) with HSP.

METHODS

A. PHA transformation of PBMC

PBMC were separated from heparinized whole blood on a Ficoll Hypaque (Pharmacia, Uppsala, Sweden) density gradient under sterile conditions. The cells were resuspended in RPMI 1640 (Flow Laboratories, Virginia, U.S.A.) containing 20% autologous serum to give a final concentration of 1×10^6

viable cells/ml. 175 µl PBMC suspension and 25 µl of appropriate PHA (Burroughs Wellcome, Beckenham, England, Lot. No. K9081) concentration was placed in each of 3 wells of a microtitre tray. A range of PHA concentrations from 0.5 to 10 µg/ml was tested, each one in triplicate. Culture plates were covered, mixed and placed in a 5% CO_2 incubator at 37°C. After 68 hours the plates were removed and 25 µl (0.4 µCi) of tritiated thymidine (^{3}H-T) (Amersham, Australia, TRK .61) added to all wells. The plates were mixed and returned to the incubator for a further 4 hours before the cells were harvested onto a glass fibre filter using a Titertek cell harvester. Individual filter discs were placed in vials containing 4 ml scintillation fluid (Aquasol - 2) and thymidine incorporation was measured in a liquid scintillation counter (Packard). The mean of the triplicate counts was calculated for each PHA concentration and the dose-response curve for each patient or patient group was compared to the mean ± S.D. of the normal control group using a Canon BX - 1 computer.

Levamisole therapy

Selected MN patients were treated with levamisole (courtesy of Janssen Pharmaceutica, Ethnor Pty., Ltd.), 2.5 mg/Kg orally twice weekly for 3 months. The following parameters were measured pre and post treatment and at monthly intervals on levamisole therapy

- 24 hour urinary protein excretion.
- serum albumin.
- serum creatinine and urea.
- serum immunoglobulins.
- serum complement components (C3, C4).

- circulating immune complexes (solid phase CIq radioimmunoassay).
- haemoglobin.
- total white cell and lymphocyte count.
- PHA induced lymphocyte transformation.

IC Assay

Circulating IC were measured in the solid phase CIq radioimmunoassay using a method modified from that of Hay et al (1976). CIq was isolated from normal human serum by the method of Yonemasu and Stroud (1971). Microtitre plates (Costar) were coated with freshly isolated CIq by incubation for 20 hours at 4°C. After washing with phosphate buffered saline (PBS) the wells were coated with 1% human serum albumin (HSA) in PBS, washed with PBS and the plates stored at -70°C until used.

The assay was performed as follows:
120 µl of 0.2m ethylenediamine tetra-acetic acid (EDTA) was incubated with 60 µl of patient serum for 30 minutes at 37°C. After the addition of 3 ml PBS, 100 µl of the EDTA treated serum was added to duplicate wells of the microtitre plate. The plate was incubated at 37°C for 1 hour and at 4°C for 20 hours and then washed 3 times with PBS. 100 µl of ^{125}I goat anti-human IgG in 1% HSA-PBS was added and incubated at 37°C for 1 hour and at 4°C for 30 minutes. After washing three times with PBS radioactivity bound to individual wells was counted in a gamma counter. The result was referred to a standard curve of the binding obtained with heat aggregated human gamma globulin (AHG) in normal human serum and expressed as µg AHG per ml serum above the mean + 2SD value of healthy control sera (n = 57).

B. T cell subsets

T cell subsets were measured using monoclonal antibodies

(Ortho Pharmaceutical Corporation, Raritan, New Jersey, U.S.A.) OKT4 (identifying helper/inducer T cells) and OKT8 (identifying suppressor/cytotoxic T cells) by indirect immunofluorescence and flow cytometry (Becton-Dickinson FACS IV). In brief: PBMC were separated from whole blood using a Ficoll Hypaque gradient. After washing, PBMC were adjusted to 3 x 10^6 cells/ml and 5 µl of monoclonal antibody was added to 200 µl of cell suspension. After incubation on ice for 30 minutes with intermittent shaking, the cells were washed and 100 µl of fluorescein conjugated goat anti-mouse antibody was added. Following 30 minutes incubation on ice with intermittent shaking, the cells were washed and the number of fluorescent cells were counted by FACS using the same operator and same cell gating criteria for all tests. Cells were detected by low angle (forward) scatter and fluorescence stimulated using light excitation of 500 milliwatt output and 488 nanometre wavelength. A 530 nanometre light filter was used to remove light of the exciting wavelength. An average of 20,000 cells was counted for each sample. Experiments were performed to determine the effect of different gating positions controlling the limits of the size of cells included. The effects of including or excluding larger cells which may be contaminating monocytes could then be assessed. Gating which excluded larger cells was used for all experiments unless otherwise stated.

C. Functional Studies

In vitro immunoglobulin synthesis and Con A inducible suppressor cell activity.

PBMC were separated from whole blood using Ficoll Hypaque and sterile conditions, and were cultured at a concentration of 1 x 10^6 cells/ml in a culture medium composed of RPMI 1640

supplemented with 10% heat-inactivated foetal calf serum, (FCS) 2 mM glutamine and 100 units/ml penicillin and 100 µg/ml streptomycin. All cultures were set up in microtitre trays with 200 µl of cell suspension added to each of 5 wells together with 25 µl of either culture medium, PWM, or PWM + Con A. Cell cultures were incubated in a 5% CO_2 incubator 37°C for 8 days and the supernatant was collected and stored at -70°C until the IgG and IgA could be assayed using a solid phase radioimmunoassay sensitive to 10 ng/ml of immunoglobulin.

Mitogens

PWM (Sigma, St. Louis, Missouri, U.S.A.; Lot No. 100F-9655) was used at a final concentration of 0.1 µg/ml and Con A (Pharmacia, Uppsala, Sweden; Lot No. FK 18607) at a final concentration of 5 µg/ml. Preliminary experiments established that these concentrations of PWM and Con A induced maximal stimulation of immunoglobulin synthesis and maximal suppression respectively. Each mitogen was prepared as a stock solution in culture medium and stored at -20°C until used. The mitogens were always added at the beginning of the culture. Percentage suppression of PWM stimulated immunoglobulin (Ig) synthesis by Con A was calculated using the formula: Con A suppression =

$$\frac{\text{PWM stimulated Ig synthesis - PWM + Con A stimulated Ig synthesis}}{\text{PWM stimulated Ig synthesis}} \times 100$$

(Miller and Schwartz 1979).

Radioimmunoassay for IgG, IgA estimation in culture supernatants (performed by Mr. P.A. Drew, Department of Medicine, University of Adelaide).

Vinyl microtitre plates (Costar) were coated with heavy chain specific rabbit anti-human IgG or IgA (Hoechst-Behring,

Marburg, W.Germany) by adding 100 µl of a 1/2000 dilution of antiserum of 0.05 M carbonate - bicarbonate buffer, pH 9.6 to each well. After overnight incubation at 4°C the antiserum was aspirated and the wells were filled with 1% BSA in PBS, incubated for 3 hours at 4°C and finally washed three times with PBS. 50 µl of test serum, neat or diluted 1/5 in RPMI 1640 with 10% FCS,or standard was added to duplicate wells followed by 50 µl of radiolabelled IgG (50,000 cpm) or IgA (2000 cpm) diluted to 20 ng/ml. After overnight incubation at 4°C the wells were aspirated, washed three times with PBS and individual wells counted for 1 minute in a gamma counter. The amount of IgA or IgG in culture supernatants was calculated from standard curves prepared at the same time as the assay.

Standards

IgG: prepared from normal human serum with ammonium sulphate precipitation and DE52 chromatography (0.01 M phosphate buffer, pH 8.0). Diluted in RPMI 1640 with 10% FCS and used at 10-1000 ng/ml dilution to construct standard curves.

IgA: prepared from human colostrum (Newcomb et al 1968) with stepwise elutions from DE52 column with 0.01 M Tris HCL pH 8.0, 0.025 M, 0.05 M, 0.1 M and 2.0 M NaCl in 0.01 M Tris HCL followed by sephadex G200 chromatography of the 0.1 M fraction. Diluted in RPMI 1640 with 10% FCS and used at 10-1000 ng/ml dilutions to construct standard curves.

Radiolabelling

Radiolabelling of purified IgG and IgA with ^{125}I was performed using chloramine T for oxidation and G25 filtration to remove free ^{125}I.

Suppressor cell activity in a mitogen driven mixed lymphocyte culture with tritiated thymidine uptake measurement of DNA synthesis. (Modified from Schulof et al 1980).

PBMC were separated from whole blood on a Ficoll Hypaque density gradient under sterile conditions and after three washes were suspended in culture medium.

Induction of suppressor cells

PBMC (3×10^6 cells/ml) were cultured in a medium composed of RPMI 1640 supplemented with 2 mM glutamine, 10% heat inactivated FCS, penicillin 100 units/ml and streptomycin 100 µg/ml with a final concentration of Con A of 5.0 µg/ml in a 5% CO_2 incubator at 37°C. Control cells were incubated with culture medium alone. After 23 hours mitomycin C (25 µg/ml) was added to the cells 60 minutes before washing three times and resuspending at 1×10^6 cells/ml in culture medium. Cell viability assessed by trypan blue exclusion was greater than 90%.

Responder cells

Responder cells from the same normal control was used for all experiments and preliminary studies confirmed a normal proliferative response of these cells to Con A and PHA. Responder PBMC were separated on Ficoll Hypaque on the day of assay and adjusted to 1×10^6 cells/ml in culture medium.

Suppressor Assay

The assay was carried out in U-bottomed microtitre trays (Linbro) with each experiment performed in quadruplicate. Each well contained 100 µl of responder cells together with 100 µl of Con A pre-treated cells or control cells (no Con A pre-treatment). 25 µl of Con A stock solution (5 or 50 µg/ml)

was added to appropriate wells while 25 µl of culture medium was added to control wells. The plates were incubated at 37°C for 68 hours in a 5% CO_2 incubator and were then pulsed with 0.4 µ Ci ^{3}H-T per well. After a further 4 hours incubation cells were harvested onto glass fibre filter papers with a Titertek automatic harvester and thymidine uptake measured in a liquid scintillation counter (Beckman). The following formula based on mean values of quadruplicate cultures was used to calculate the degree of suppression induced by Con A pre-treatment.

$$\% \text{ Suppression} = \left(1 - \frac{(R+S + \text{Con A}) - (R + S)}{(R+C + \text{Con A}) - (R + C)}\right) \times 100$$

Where R = responder cells.

S = mitomycin C treated Con A induced suppressor cells.

C = mitomycin C treated control cells.

Con A = stock solution 50 µg/ml or stock solution 5 µg/ml.

Serum IgA levels

These were determined in 12 patients using laser nephelometry (Hyland).

Statistical analysis

Students t test was employed to compare the means of 2 groups. Spearmans rank correlation coefficient (r_s) was used where indicated. Results are expressed as mean ± standard error of the mean unless otherwise indicated.

RESULTS

A. PHA transformation of PBMC from patients with primary MN and controls.

The mean values for lymphocyte transformation induced by varying concentrations of PHA for the MN patients fell within the limits of mean ± S.D. of the control group (Fig. 2.1 and 2.2).

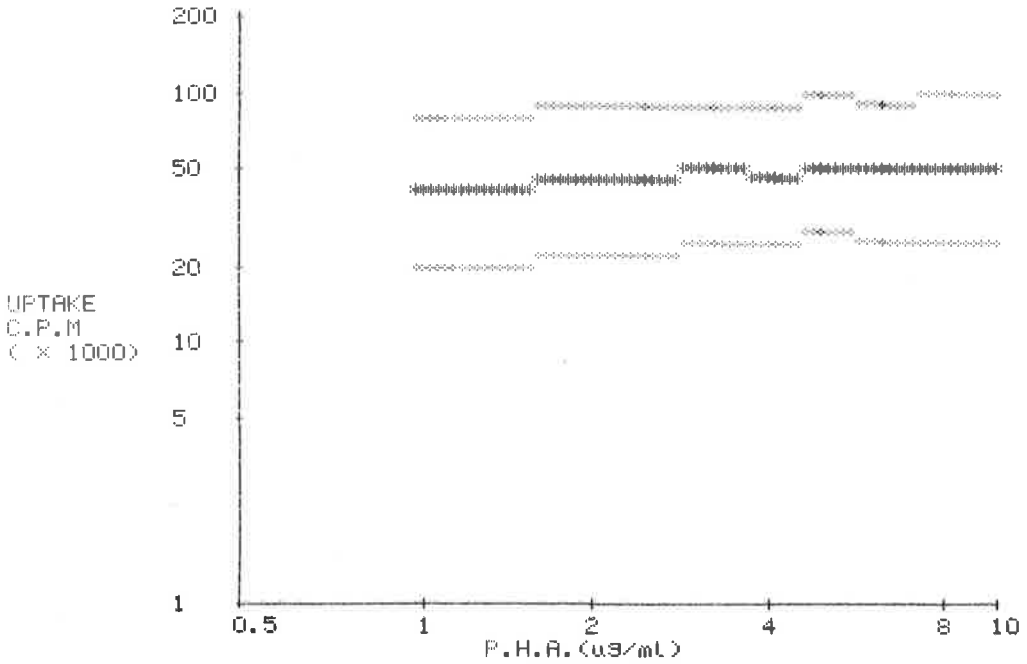

FIGURE 2.1 Mean (± SD) PBMC transformation (^{3}H-T uptake CPM x 1000) over a range of PHA concentrations (µg/ml) for normal controls (n = 23).

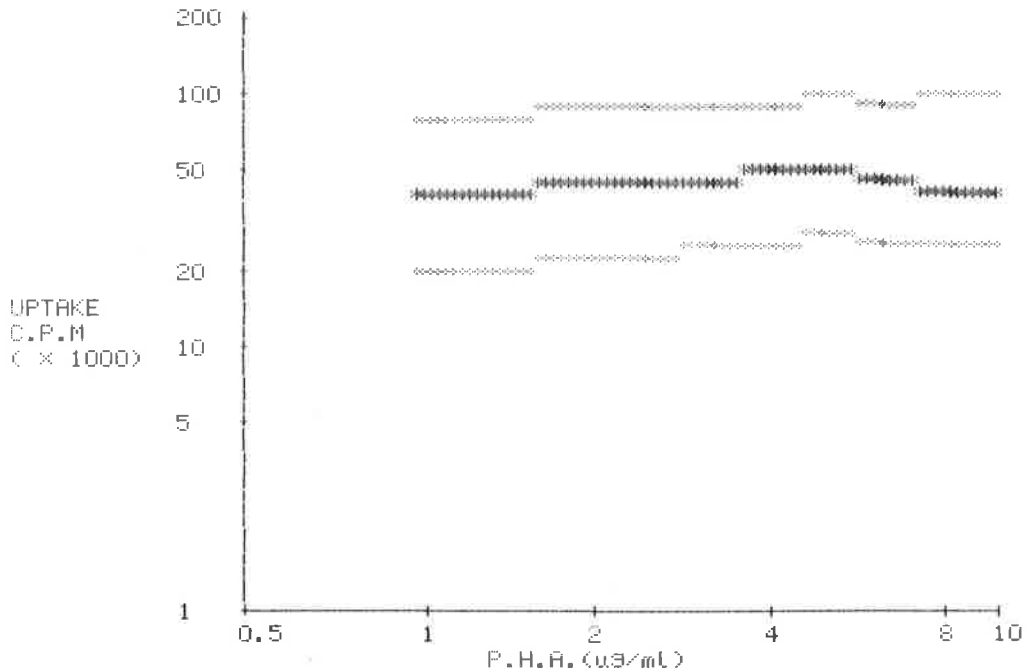

FIGURE 2.2 Mean PBMC transformation over a range of PHA concentrations for MN patients (n = 18) shown against the range mean ± SD of normal controls.

Levamisole therapy and PHA responsiveness in MN patients

Two patients (A.F. M-50; R.S. M-53) showed depressed PHA responses for all PHA concentrations on two occasions separated by a 2 month interval (Fig. 2.3 and 2.4). Both patients were nephrotic, had normal lymphocyte counts and drug therapy included frusemide and potassium supplements only. With informed consent, these patients were commenced on a 3 month course of levamisole. Two other nephrotic MN patients (N.R. M-30; L.T. M-56) with normal PHA responses were also given levamisole. The biochemical, serological and haematological status of each patient before commencing levamisole therapy and at monthly intervals thereafter is shown in Tables 2.1 - 2.4. Both patients with low PHA responses showed a return of lymphocyte responsiveness to within the normal range (mean ± SD of controls) within 1 month of starting levamisole (Fig. 2.3 and 2.4). This improved PHA response was sustained throughout the course of therapy. A decrease in urinary protein excretion occurred in 3 of the patients during the course of treatment (Fig. 2.5). Serum albumin concentrations showed an increase over pre-treatment levels in all cases and tended to increase with time on therapy (Fig. 2.6). Immunological parameters (immune complexes, complement levels and serum IgG) remained essentially unchanged, as did renal function. Total white cell counts tended to decrease in each patient with increasing duration of levamisole therapy but haemoglobin levels did not change. Patient A.F. developed pruritis while taking levamisole but this side-effect did not require withdrawal of therapy. No other side-effects were noted and all 4 patients claimed a subjective improvement in well-being and a reduction in oedema.

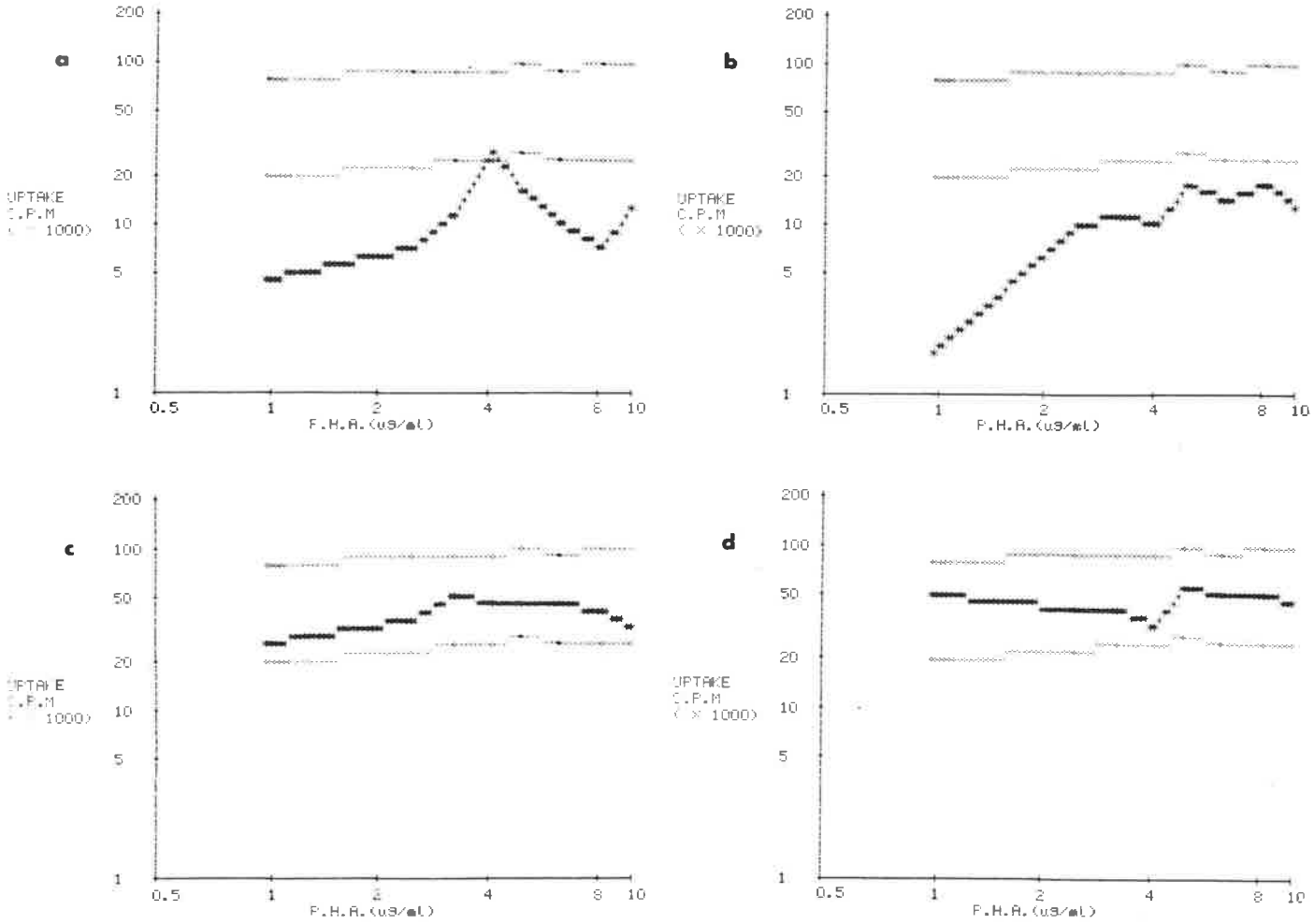

FIGURE 2.3 PHA response of PBMC from MN patient A.F. on 2 separate occasions before levamisole therapy (a,b) after 1 month on levamisole (c) and at 3 weeks post treatment (d). The mean ^{3}H-T uptake for varying concentrations of PHA for patient A.F. is shown against the range of mean ± SD of 23 normal controls.

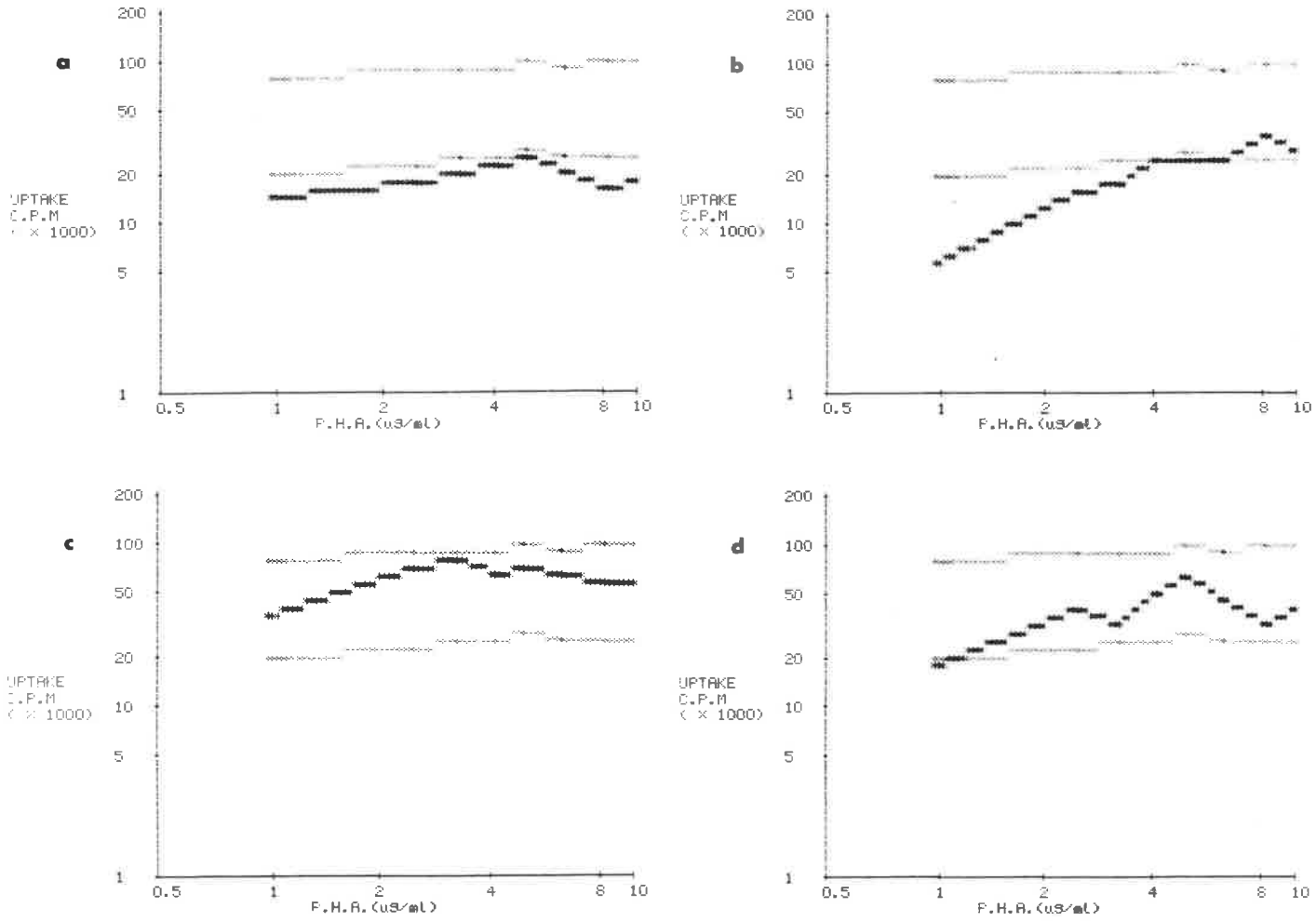

FIGURE 2.4 PHA response of PBMC from MN patient R.S. on 2 separate occasions before levamisole therapy (a,b) after 1 month of levamisole (c) and at 5 days post treatment (d). The mean ^{3}H-T uptake for varying concentrations of PHA for patient R.S. is shown against the range of mean ± SD of 23 normal controls.

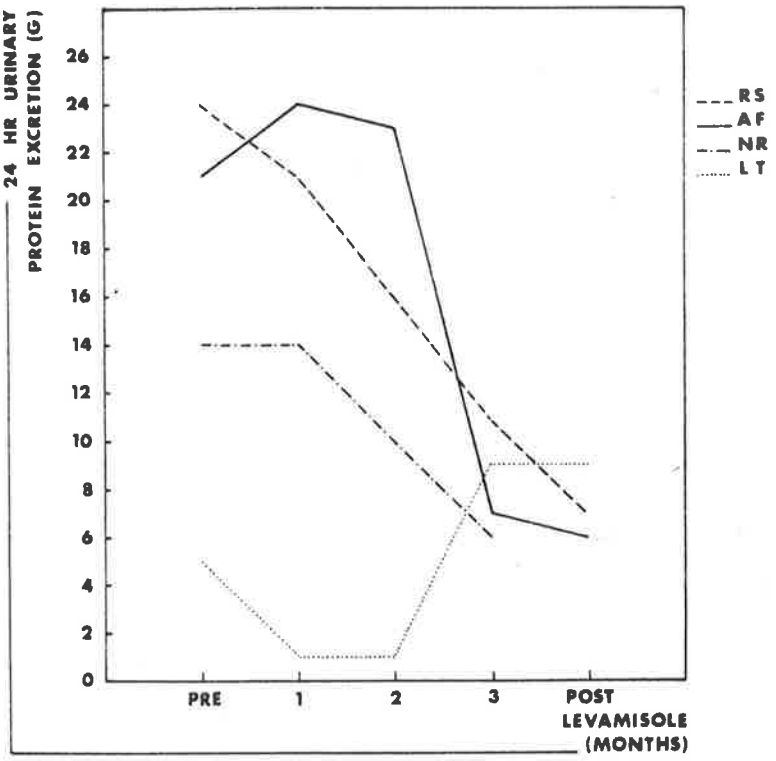

FIGURE 2.5 Urinary protein excretion (24 hour) in MN patients before, during and after treatment with levamisole for 3 months.

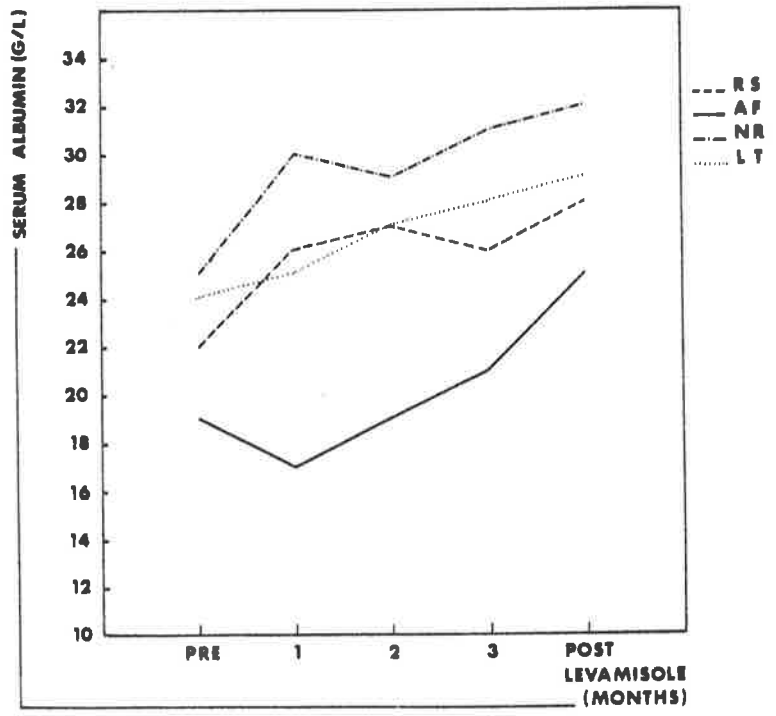

FIGURE 2.6 Serum albumin concentrations in MN patients before, during and after treatment with levamisole for 3 months.

A.F. Male 50 Levamisole 175 mg x2 weekly.

PARAMETER	NORMAL RANGE	PRE-TREATMENT	MONTH 1	MONTH 2	MONTH 3	POST-TREATMENT 1 MONTH OR 2 MONTHS
24 HOUR URINARY PROTEIN EXCRETION G/DAY	(0.00-0.12)	21	24	23	7	6
SERUM ALBUMIN G/L	(39-48)	19	17	19	21	25
SERUM CREATININE mmol/l	(0.05-0.12)	0.13	0.17	0.15	0.17	0.12
SERUM IgG G/L	(8.0 -16.0)	4.1	3.3	3.2	2.8	2.6
CIRCULATING IMMUNE COMPLEXES µgAHG/ml SERUM		0	0	0	0	0
C3 G/L	(0.65-1.25)	0.80	0.55	0.83	0.81	0.76
C4 G/L	(0.10-0.40)	0.46	0.40	0.44	0.47	0.53
WHITE CELL COUNT x 10^3/µl	(4.0 -10.0)	3.7	9.4	5.2	3.7	4.5
HAEMOGLOBIN G/100ml	(13.5-18.0)	13.6	12.4	14.1	12.4	15.0
LYMPHOCYTE COUNT/µl	(1500-3500)	1516	2444	1716	1258	1305

Table 2.1 Patient A.F. data before, during (month 1-3) and after Levamisole therapy.

R.S. MALE 53 Levamisole 175 mg x2 weekly.

PARAMETER	NORMAL RANGE	PRE-TREATMENT	MONTH 1	MONTH 2	MONTH 3	POST-TREATMENT 1 MONTH OR 2 MONTHS
24 HOUR URINARY PROTEIN EXCRETION G/DAY	(0.00-0.12)	24	21	16	11	7
SERUM ALBUMIN G/L	(39-48)	22	26	27	26	28
SERUM CREATININE mmol/l	(0.05-0.12)	0.21	0.26	0.22	0.24	0.18
SERUM IgG G/L	(8.0-16.0)	2.2	3.6	2.1	1.8	1.6
CIRCULATING IMMUNE COMPLEXES µgAHG/ml SERUM		0	0	0	0	0
C3 G/L	(0.65-1.25)	1.4	1.01	0.73	0.89	0.85
C4 G/L	(0.10-0.40)	0.2	0.28	0.18	0.31	0.39
WHITE CELL COUNT x 10^3/µl	(4.0 -10.0)	8.3	9.1	3.7	4.1	11.3
HAEMOGLOBIN G/100ml	(13.5-18.0)	13.8	14.1	13.6	13.6	13.8
LYMPHOCYTE COUNT/µl	(1500-3500)	2158	2730	1073	1394	1581

Table 2.2 Patient R.S. data before, during (month 1-3) and after Levamisole therapy.

N.R. MALE 30 Levamisole 175 mg x2 weekly.

PARAMETER	NORMAL RANGE	PRE-TREATMENT	MONTH 1	MONTH 2	MONTH 3	POST-TREATMENT 1 MONTH OR 2 MONTHS
24 HOUR URINARY PROTEIN EXCRETION G/DAY	(0.00-0.12)	14	14	9.8	6.4	-
SERUM ALBUMIN G/L	(39-48)	25	30	29	31	32
SERUM CREATININE mmol/L	(0.05-0.12)	0.17	0.20	0.20	0.22	0.19
SERUM IgG G/L	(8.0 -16.0)	2.9	3.5	2.9	3.3	-
CIRCULATING IMMUNE COMPLEXES µgAHG/ml SERUM		0	0	0	0	-
C3 G/L	(0.65-1.25)	0.72	0.76	0.64	0.86	-
C4 G/L	(0.10-0.40)	0.41	0.31	0.31	0.70	-
WHITE CELL COUNT x 10^3/µl	(4.0 -10.0)	7.5	6.0	5.0	4.3	
HAEMOGLOBIN G/100ml	(13.5-18.0)	12.2	12.1	11.6	12.2	-
LYMPHOCYTE COUNT/µl	(1500-3500)	2025	1800	1150	989	

Table 2.3 Patient N.R. data before, during (month 1-3) and after Levamisole therapy.

L.T. Male 56 Levamisole 175 mg x2 weekly.

PARAMETER	NORMAL RANGE	PRE-TREATMENT	MONTH 1	MONTH 2	MONTH 3	POST-TREATMENT 1 MONTH OR 2 MONTHS
24 HOUR URINARY PROTEIN EXCRETION G/DAY	(0.00-0.12)	5	0.9	0.8	9	9
SERUM ALBUMIN G/L	(39-48)	24	25	27	28	29
SERUM CREATININE mmol/l	(0.05-0.12)	0.08	0.09	0.11	0.12	0.10
SERUM IgG G/L	(8.0 -16.0)	5.2	2.4	3.8	2.2	2.3
CIRCULATING IMMUNE COMPLEXES µgAHG/ml SERUM		0	0	0	0	0
C3 G/L	(0.65-1.25)	1.06	0.78	0.82	0.76	0.71
C4 G/L	(0.10-0.40)	0.36	0.26	0.27	0.29	0.29
WHITE CELL COUNT x 10^3/µl	(4.0 -10.0)	10.8	11.7	6.5	7.0	7.9
HAEMOGLOBIN G/100ml	(13.5-18.0)	16.0	15.1	16.1	16.1	16.5
LYMPHOCYTE COUNT/µl	(1500-3500)	2700	3276	1755	1680	-

Table 2.4 Patient L.T. data before, during (month 1-3) and after Levamisole therapy.

B. T cell subsets and helper/suppressor (T4/T8) ratios

(Table 2.5)

The mean T4/T8 ratio of the normal control group was 3.0 ± 0.2 (Fig. 2.7). This is higher than has been reported in other centres and may relate to the use of fluorescent microscopy by other workers (Chatenoud and Bach 1981) or to different gating criteria for the FACS (Morimoto et al 1980). The effect of changing the gating (Fig. 2.8) to include larger cells which include contaminating monocytes is shown in Table 2.6 and resulted in an appreciable lowering of the T4/T8 ratio. No significant difference in T4/T8 ratios was found between normal controls aged less than 30 years (3.1 ± 0.2) or greater than 40 years (2.9 ± 0.5) or between males (3.0 ± 0.3) and females (3.1 ± 0.2). Patients with primary MN and IgA GN demonstrated a significantly elevated mean T4/T8 ratio ($p < 0.05$ for both) due to an absolute reduction in T8 positive cells (MN $p < 0.01$, IgA GN $p < 0.10$). In contrast, patients with SLE showed a highly significant ($p < 0.005$) mean reduction in T4/T8 ratios secondary to both a depression of mean T4 values ($p < 0.005$) and elevation of mean T8 values ($p < 0.01$). The 4 SLE patients who were not taking steroids had T4/T8 ratios greater than or equal to the mean T4/T8 ratio of the total SLE group. Two patients with HSP and severe renal involvement had markedly low T8 + cells (10.4% and 10.7%) and T4/T8 ratios of 4.0 and 2.3 respectively. There was no correlation between the presence of the nephrotic syndrome and T4/T8 ratios in any disease group.

Serial estimations of T cell subsets and T4/T8 ratios are shown in Table 2.7. The day to day variation between T4/T8 ratios in a particular subject was much less than the variation between different individuals within the normal control group. High ratios found in some patients tended to remain elevated when tested on different occasions.

	T4	T8	T4/T8
CONTROLS	52.6 ± 1.5	18.9 ± 0.96	3.0 ± 0.2
MN	51.5 ± 2.9	14.8 ± 1.3 **	4.0 ± 0.6 *
IgAGN	52.1 ± 2.6	15.9 ± 2.4	4.5 ± 1.2 *
SLE	46.0 ± 1.2 ***	24.6 ± 2.3 **	2.1 ± 0.2 ***

Table 2.5 T cell subsets (mean percentage ± SEM) and helper/suppressor (T4/T8) ratios in controls (28) and patients with primary MN (14), IgA GN (10) and lupus nephritis (15).

*p < 0.05 **p < 0.01 ***p < 0.0005

PATIENT	GATING	T4	T8	T4/T8
S.S	0	65.6	16.5	4.0
	C	61.9	13.8	4.5
J.J.	0	53.0	23.5	2.2
	C	50.2	17.0	2.9
M.H.	0	55.0	8.5	6.5
	C	51.5	7.2	7.1
S.N.	0	39.5	10.9	3.6
	C	39.6	7.7	5.1

Table 2.6 The effect on T cell subsets and T4/T8 ratios of changing FACS gating positions for forward scatter of light. With the gate open (0) larger mononuclear cells (mainly monocytes) are included. With the gate in the closed (C) position these cells are excluded from counting.

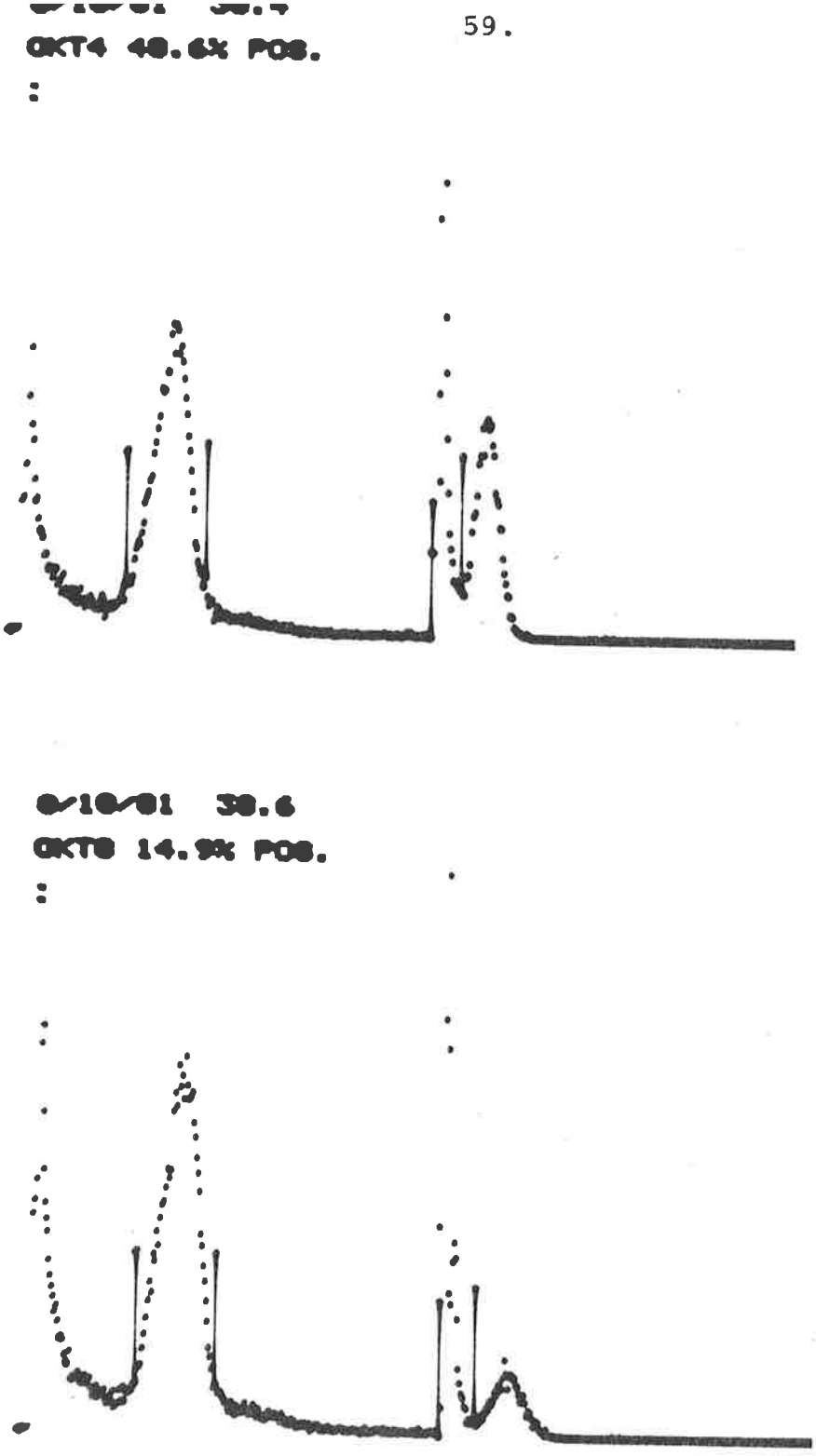

FIGURE 2.7 Examples of FACS histograms for PBMC from a normal individual. The ordinate measures cell numbers and the abscissa is divided into forward scatter (cell size) on the left and fluorescence intensity (OKT4+ or OKT8+) on the right.

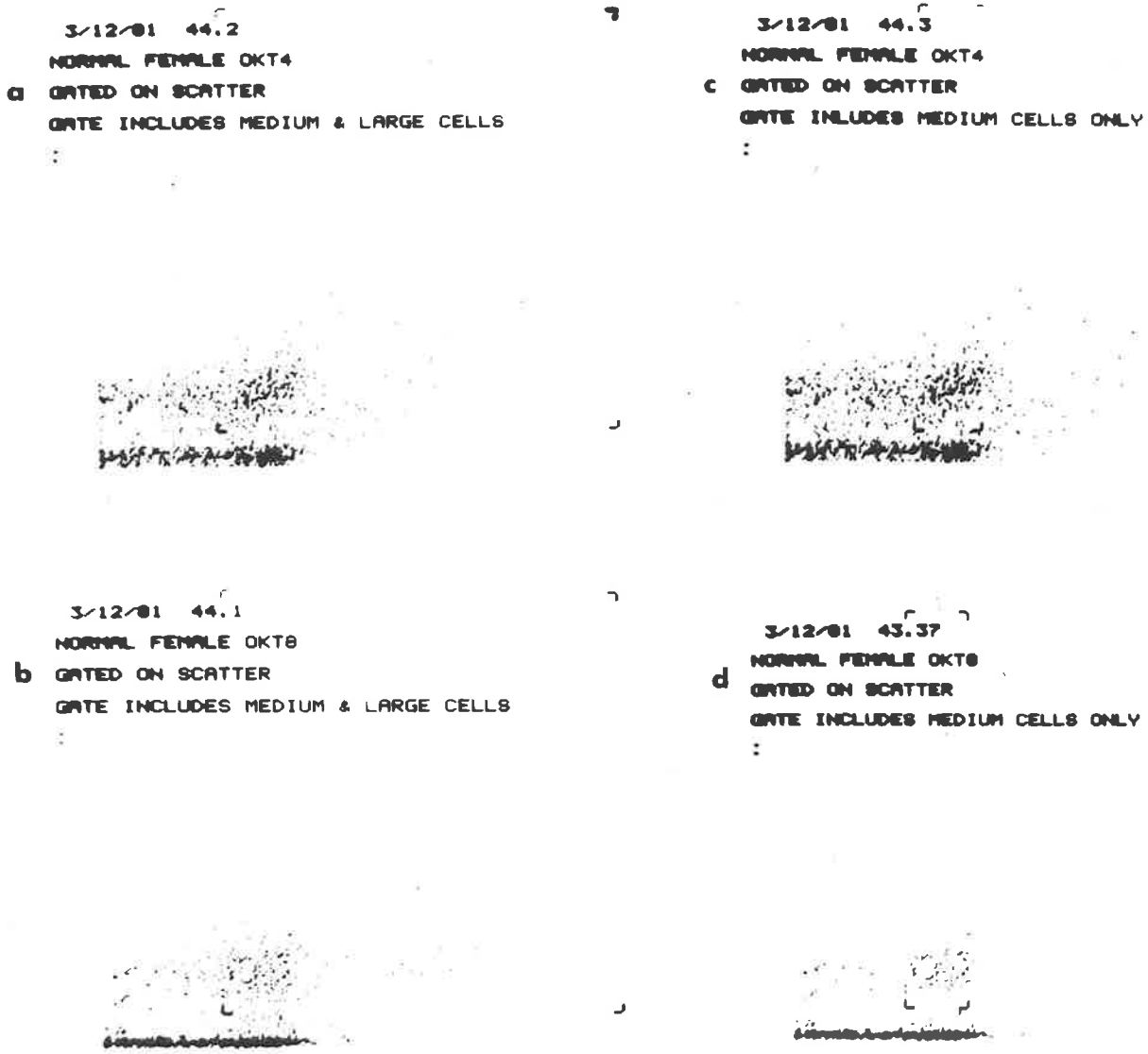

FIGURE 2.8 The effect of changing FACS gate positions for forward scatter of light to include (a, b) or exclude (c, d) large OKT4+ or OKT8+ cells. Abscissa = cell size (forward scatter). Ordinate = fluorescence intensity.

		T4	T8	T4/8
NORMAL FEMALE (26)	25/11/81	53.0	20.3	2.6
	3/12/81	48.7	20.6	2.4
	10/12/81	53.9	18.6	2.9
	17/12/81	48.7	22.9	2.1
NORMAL FEMALE (20)	19/11/81	50.2	17.0	2.9
	25/11/81	50.4	13.9	3.6
	3/12/81	53.8	15.8	3.4
	9/12/81	53.6	16.0	3.3
PATIENT M.H. (Primary MN)	18/11/81	51.5	7.2	7.1
	10/12/81	65.3	8.4	7.8
PATIENT S.K. (Secondary MN)	26/11/81	67.5	7.8	8.6
	9/12/81	66.0	7.6	8.7
PATIENT M.W. (Lupus nephritis)	2/12/81	49.0	10.0	2.4
	9/12/81	44.9	23.1	1.9
	20/ 1/82	49.2	22.9	2.1
PATIENT J.P. (Primary IgA GN)	5/11/81	41.1	32.0	1.3
	19/11/81	41.1	33.3	1.2
	24/12/81	41.2	37.8	1.1

Table 2.7 Serial studies of T cell subsets in controls and patients.

C. Functional Studies

Spontaneous, PWM stimulated and Con A induced suppression of in vitro immunoglobulin synthesis in patients with MN, IgA GN, HSP and lupus nephritis. (Fig. 2.9 and 2.10)

(a) Membranous nephropathy

No significant difference was present between MN patients and controls in the amount of IgG and IgA produced by PWM stimulated and unstimulated PBMC. However, the Con A inducible suppression of IgG synthesis differed significantly ($p < 0.05$) between the MN patients (46 ± 8%) and controls (63 ± 4%). A significant correlation ($r_s = -0.65$ $p < 0.05$) was also found between T4/T8 values in MN patients and functional IgG suppressor activity (Fig. 2.11). A difference was found in Con A inducible suppression between nephrotic (IgG 40 ± 20%; IgA 8.5 ± 15%) and non-nephrotic (IgG 54 ± 7%; IgA 17.7 ± 11%) patients but this was not statistically significant.

(b) IgA nephropathy

Patients with IgA GN had high spontaneous IgG (6/10; $p < 0.0125$) and IgA (5/10; $p < 0.05$) synthesis compared to the control group. PWM stimulation caused further significant elevations of IgG (6/10; $p < 0.025$) and IgA (6/10; $p < 0.05$) production. However, the degree of Con A induced suppression was not significantly different from the control population. Fig. 2.12 shows the relationship between serum IgA levels and spontaneous IgA synthesis in vitro in patients with IgA GN (10) and HSP (2). Half of the patients with mesangial IgA GN had raised serum IgA levels (normal range 2-4 grams/litre). Although 5/12 patients with mesangial IgA deposits had both elevated serum and in vitro IgA levels, the correlation was not significant ($r_s = 0.17$).

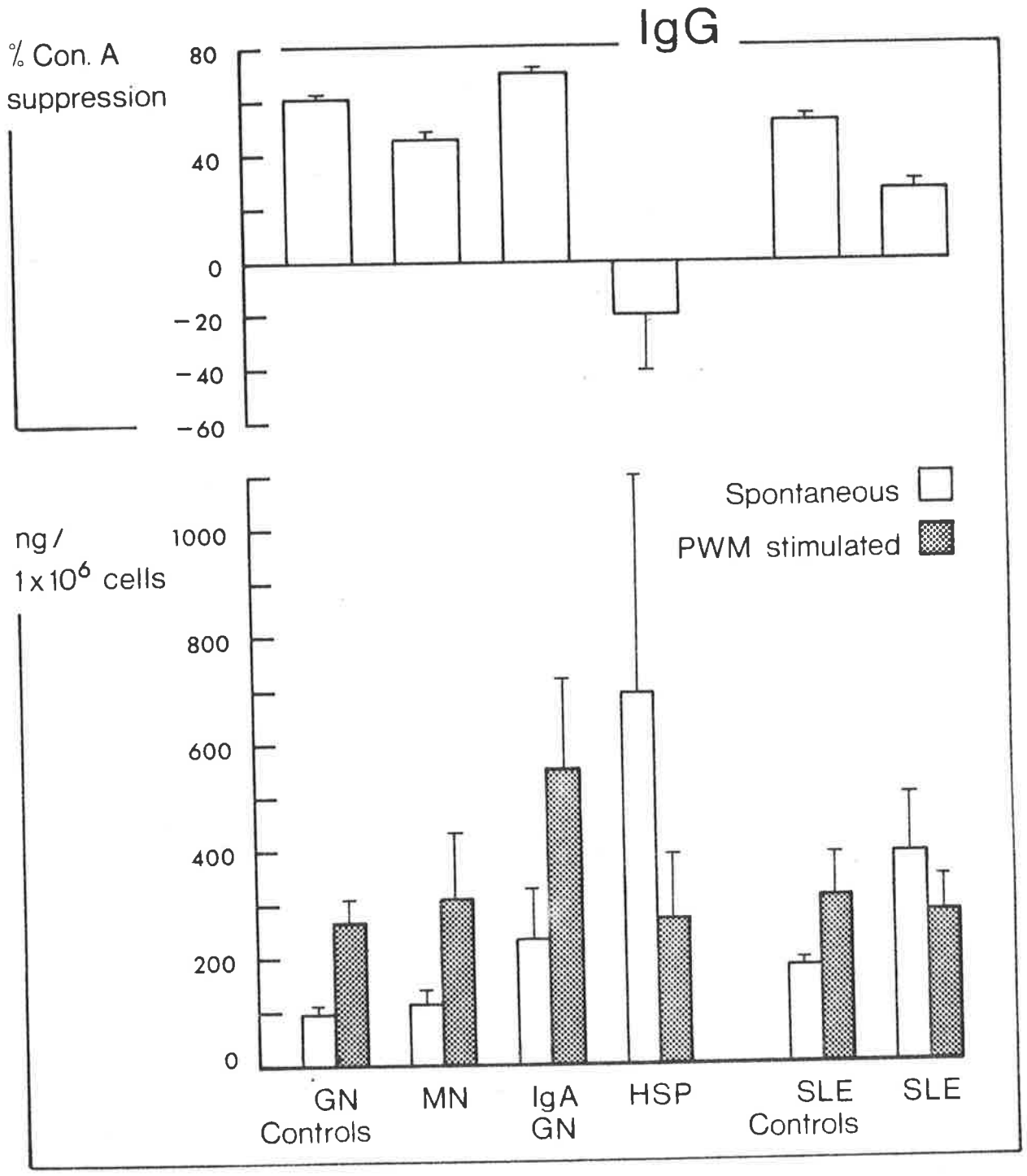

FIGURE 2.9 Mean amount (± SEM) of IgG synthesized by PBMC from controls and patients with MN (13), IgA GN (10), HSP (3) and lupus nephritis (10) spontaneously and after PWM stimulation. Also shown is the % Con A suppression of PWM stimulated IgG synthesis.

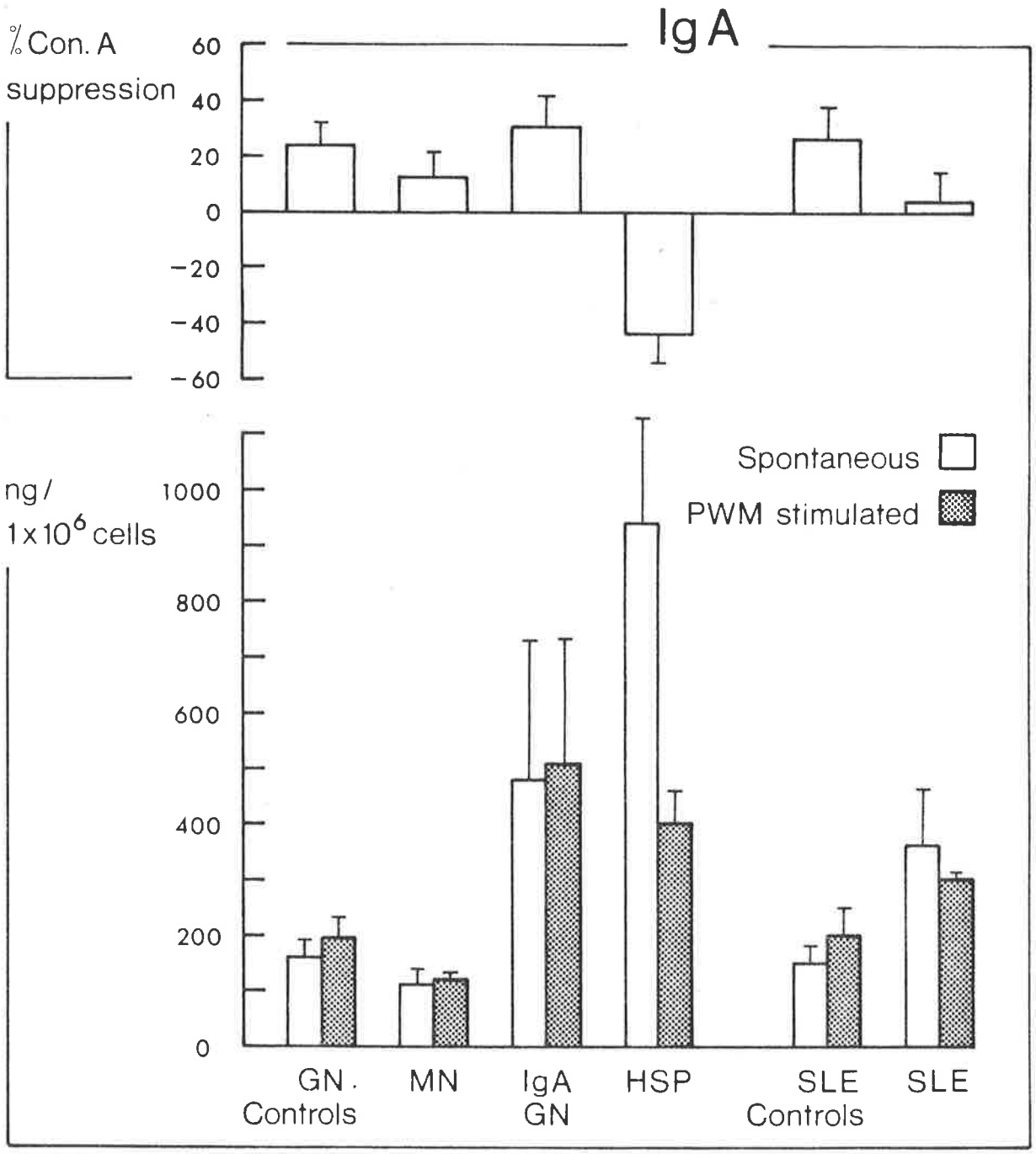

FIGURE 2.10 Mean amount (± SEM) of IgA synthesized by PBMC from controls and patients spontaneously and after PWM stimulation. Also shown is the % Con A suppression of PWM stimulated IgA synthesis.

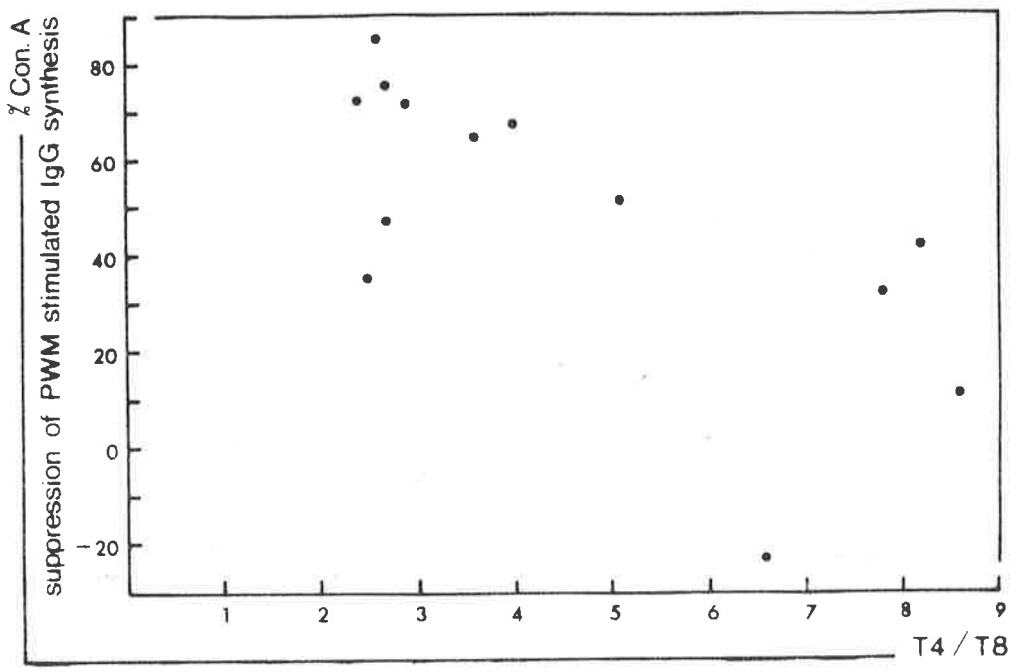

FIGURE 2.11 Correlation between T4/T8 and in vitro Con A inducible suppression of IgG synthesis in patients with primary MN (r_s = -0.65; $p < 0.05$).

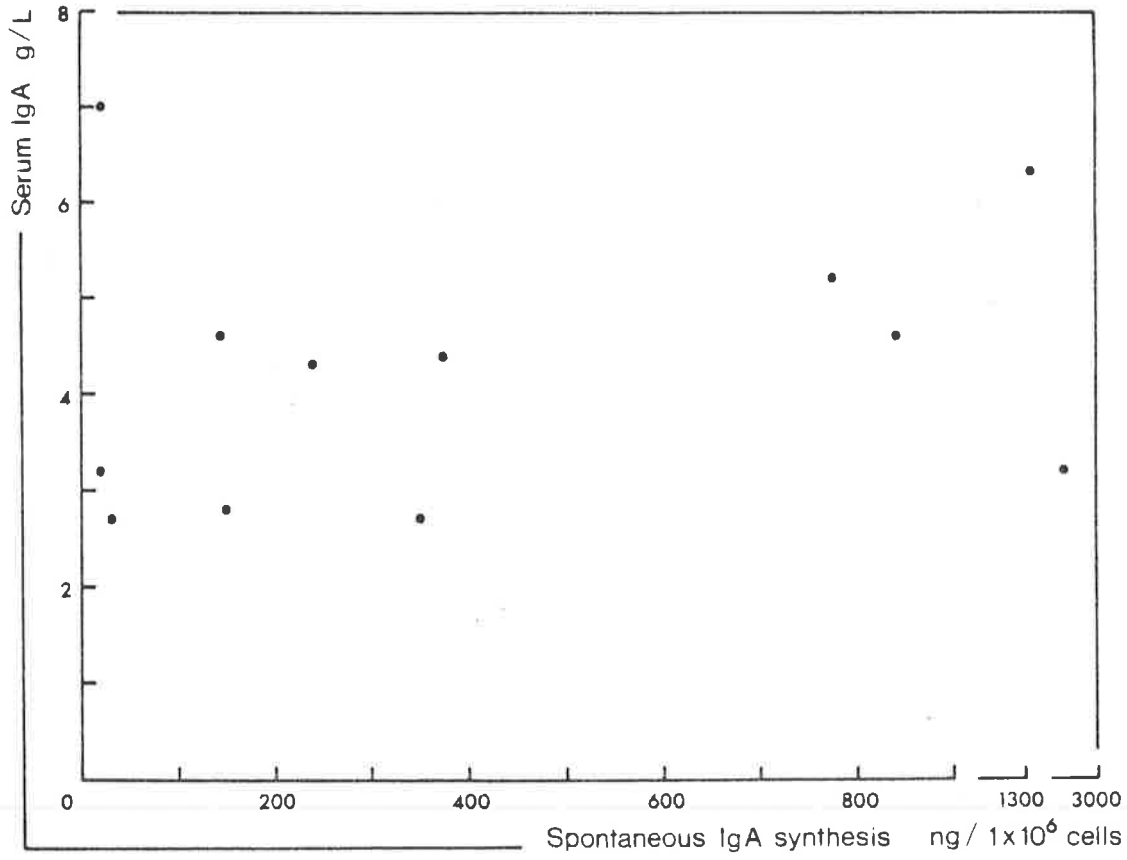

FIGURE 2.12 Correlation between serum IgA levels and spontaneous IgA synthesis by PBMC from patients with IgA GN and HSP (r_s = 0.17).

(c) Henoch Schonlein purpura

Spontaneous IgG and IgA synthesis was markedly elevated in all 3 patients with HSP (IgG 280, 1520, 280; IgA 1320, 740, 775 ng/l x 10^6 cells respectively). The addition of PWM caused a reduction rather than stimulation of both IgG and IgA synthesis in all 3 patients. Paradoxically, the addition of Con A to PWM stimulated cultures resulted in enhancement of IgG (2/3) and IgA (3/3) synthesis. Both HSP patients in whom serum IgA was measured had elevated levels. One patient (J.W.) with severe disease involving skin, joints, gut and kidneys was treated with plasma exchange therapy in combination with steroids. Fig. 2.13 shows immunoglobulin synthesis *in vitro* by PBMC from this patient collected immediately before and after the completion of 7 plasma exchanges. The return of spontaneous and PWM stimulated IgG and IgA synthesis to normal, corresponded with a dramatic and sustained improvement in his clinical status.

(d) SLE

Increased B cell activation was present in this group of patients with highly significant elevations of spontaneous IgG (9/10; $p < 0.0025$) and IgA (7/10; $p < 0.0125$) synthesis compared with age and sex matched controls. In contrast to the control group, the addition of PWM caused a reduction in the amount of immunoglobulin produced and the addition of Con A to the PWM cultures resulted in a further small decrease in IgG and IgA production. A significant decrease ($p < 0.005$) in the mean Con A inducible suppression of IgG synthesis was found in the SLE group (27 ± 8%) compared with controls (56 ± 6%). The reduction in Con A induced suppression of IgA synthesis found in the SLE patients was not significant ($p < 0.10$).

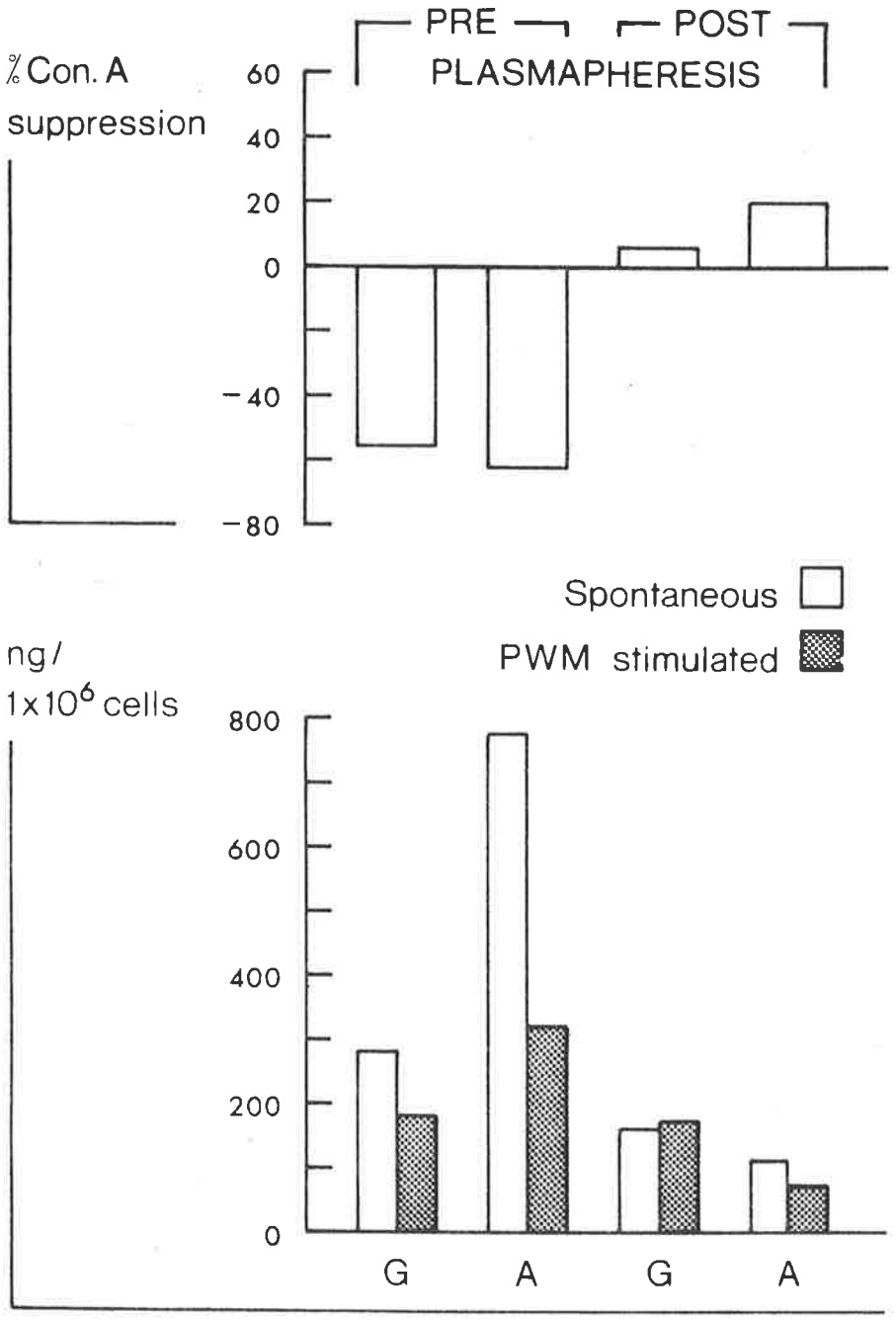

FIGURE 2.13 Spontaneous and PWM stimulated IgG and IgA synthesis by PBMC from HSP patient J.W. before and after plasma exchange therapy. Also shown is the % Con A suppression of PWM stimulated immunoglobulin synthesis.

Suppressor cell activity in patients with MN, IgA GN and lupus nephritis in a one-way mitogen driven mixed lymphocyte culture with tritiated thymidine uptake as a measure of DNA synthesis.

The results for each disease group and control subjects are shown in Fig. 2.14. Representative data of assays from 2 normal controls and 2 patients with primary MN are shown on Table 2.8.

Although suppressor activity was reduced in both the MN group (64.0 ± 6.7%) and the SLE group (62.6 ± 6.6%) compared to controls (72.3 ± 2.9%) using Con A 0.5 µg/ml (final) in the second culture the difference was not statistically significant (MN $p < 0.15$; SLE $p < 0.10$). However the mean % suppression using Con A 5 µg/ml (final) in the MN group (9.3 ± 7%) was significantly decreased compared with normal controls (34.6 ± 3.8%; $p < 0.0025$). The 5 nephrotic MN patients had a reduced % suppression (1 ± 11%) compared with non-nephrotic MN patients (20 ± 4%) but this difference was not significant ($p < 0.10$). The reduction in % suppression in the SLE group (27.1 ± 6.3%) using Con A 5 µg/ml also did not achieve statistical significance. No difference was found in mean % suppression between patients with IgA GN and controls using either of the Con A concentrations.

DISCUSSION

This study has attempted to dissect the cellular control arm of the immune response in patients with IC GN or lupus nephritis.

Cellular immunity was initially examined in 18 patients with primary MN using PHA induced transformation of peripheral lymphocytes. Although Carney et al (1980) reported a signific-

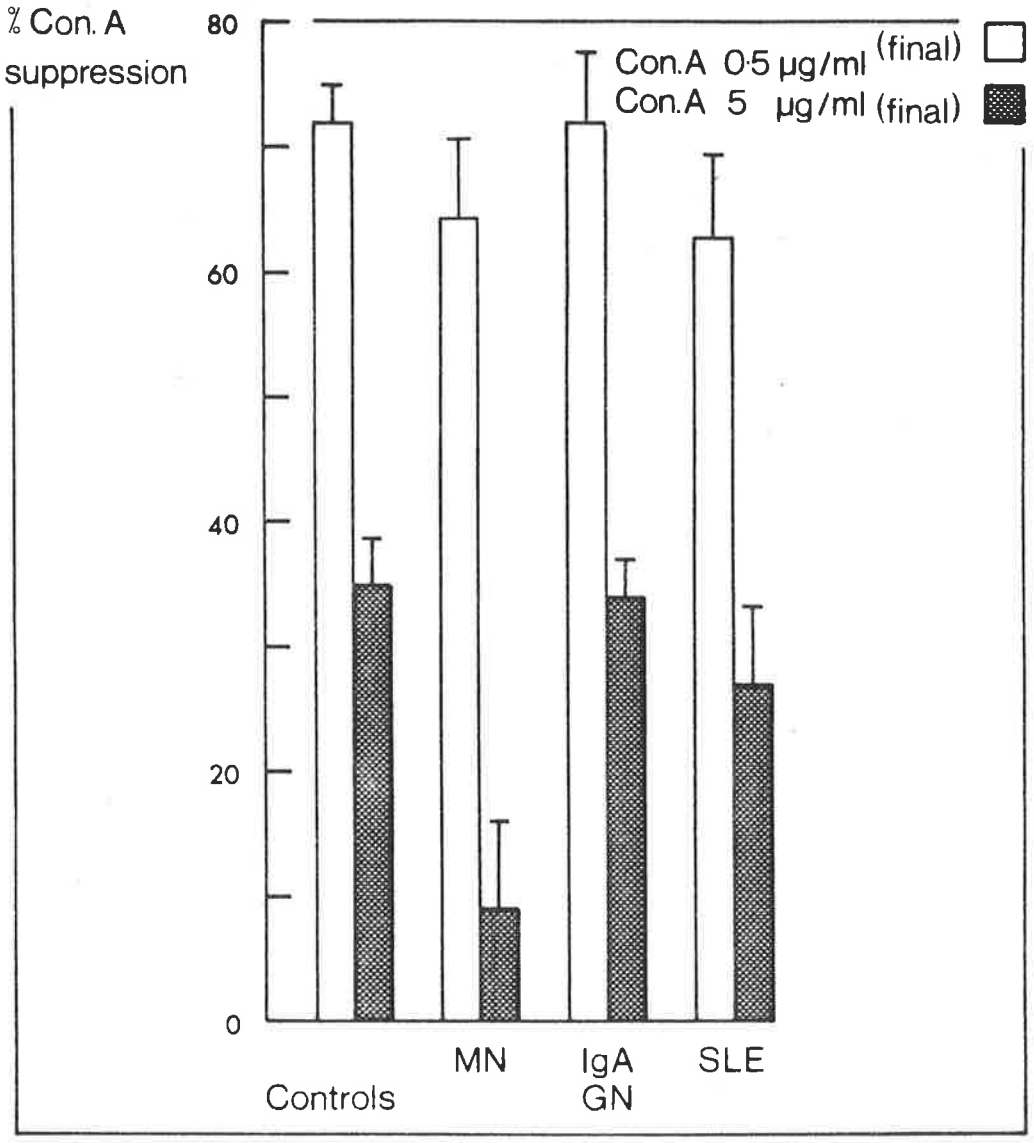

FIGURE 2.14 % suppression of normal PBMC ^{3}H-T uptake by Con A pre-treated PBMC from patients with MN (9), SLE (9), IgA GN (9) and normal controls in a one-way mitogen driven mixed lymphocyte culture.

SUBJECT	RESPONDER CELLS + CON A PRE-TREATED CELLS + CON A (5 ug/ml final)	RESPONDER CELLS + CONTROL CELLS + CON A (5 ug/ml final)	% SUPPRESSION
CONTROLS			
F, 20	15295 ± 594	24632 ± 1348	39
F, 26	14407 ± 1124	24633 ± 728	45
MN PATIENTS			
K.S.	35761 ± 1028	43497 ± 1196	16
H.D.	26511 ± 1537	23272 ± 1152	-16
	RESPONDER CELLS + CON A PRE-TREATED CELLS + CON A (0.5 ug/ml final)	RESPONDER CELLS + CONTROL CELLS + CON A (0.5 ug/ml final)	
CONTROLS			
F, 50	4152 ± 208	15655 ± 507	79
F, 26	5626 ± 197	18939 ± 290	81
MN PATIENTS			
K.S.	3694 ± 166	15890 ± 175	82
H.D.	4425 ± 228	13482 ± 726	75

Table 2.8 Examples of ^{3}H-T uptake (CPM) and % suppression of ^{3}H-T uptake by normal PBMC co-cultured with Con A pretreated PBMC from controls and MN patients.

ant difference in maximum PHA transformation between 6 patients with primary MN and controls, in the present study the mean PHA responses (for a range of PHA concentrations) of the MN group fell within a normal range (mean ± SD) established in 23 normal controls. Sasdelli et al (1981) reported similar findings in 35 patients with MN, with or without the nephrotic syndrome, using lymphocyte response to PHA in autologous plasma. In our study 2 nephrotic MN patients showed depressed PHA responses on 2 consecutive testings 2 months apart and were treated with levamisole. Within one month of treatment PHA responsiveness had returned to within the normal range and 3 of the 4 MN patients treated with levamisole showed a reduction in urinary protein excretion. Changes in clinical parameters in response to specific therapy, however, are difficult to interpret in patients with MN, in view of the natural fluctuations of the disease state. Nevertheless, the return of PHA responsiveness is encouraging since the therapeutic usefulness of levamisole in other conditions parallels improvement in tests of cellular immunity (Renoux 1980). Russell (1976) found that levamisole delayed the development of anti-nuclear antibodies and proteinuria in NZB/W mice and increased suppressor T cells, induced by levamisole, could favourably modify murine SLE (Zulman et al 1978). These effects of levamisole may also be relevant in MN and other forms of primary GN, in view of defects in suppressor cell activity in these disorders.

Two further approaches were employed to provide a more detailed assessment of possible disturbances of immune regulation in MN and also in patients with mesangial IgA GN, HSP and lupus nephritis. The use of monoclonal antibodies against T cell subsets, with or without flow cytometry, has been used by other workers (Bach and Bach 1981) to define alterations in T

cell subpopulations in immune disorders. The use of a fluorescence activated cell sorter in this study offered greater accuracy and considerable time saving. Functional studies of cultured mononuclear cells from our patients relied on established methods of tissue culture and the ability of Con A to induce suppressor cells (Dwyer and Johnson 1981), in both a system dependent on immunoglobulin synthesis and in an alternative suppressor cell assay in which DNA synthesis is measured by ^{3}H-T uptake. Certain limitations, mentioned in the introduction to this study, apply to such assays however, and these must be borne in mind when interpreting the results of suppressor cell assays in general.

In patients with primary MN a significant reduction in T8 positive cells suggested a deficiency of suppressor T cells and caused an elevated helper to suppressor T cell ratio. Similar T4/T8 findings have been reported by others (Chatenoud and Bach 1981; Short et al 1982). In addition, our studies of Con A suppression of IgG synthesis in vitro showed suppressor defects in MN which correlated with the elevation of the T4/T8 ratio. No significant difference was found in the amount of IgG or IgA synthesized by PBMC from MN patients compared to controls. This is in contrast to the findings of Ooi et al (1980) showing diminished IgG and IgM production in MN patients and to Heslan et al (1982) who reported impaired IgG synthesis in patients with the nephrotic syndrome secondary to a variety of glomerulopathies. We did not find significant differences in spontaneous or PWM stimulated IgG or IgA synthesis between MN patients with and without the nephrotic syndrome although Con A suppression was slightly decreased in nephrotic MN patients compared with non-nephrotic

patients with MN. The mean % suppression of ^{3}H-T uptake in the mitogen driven mixed lymphocyte cultures by Con A pre-treated cells from patients with MN was significantly reduced compared to controls when a final Con A concentration of 5 µg/ml was used to stimulate the mixed lymphocyte cultures. The 5 nephrotic MN patients also had slightly decreased suppression compared with the other MN patients in this system. In contrast to the report of Cagnoli et al (1982) no significant difference of the T4/T8 ratio was found between nephrotic and non-nephrotic MN patients.

These data raise the possibility that defective immune suppression may be important in the pathogenesis of MN. In some cases this disturbance may lead to auto-antibody production. We have found anti-DNA antibodies in polyethylene glycol precipitates from some patients with primary MN (Chapter 3) and Roberts and Lewis (1978) demonstrated the presence of anti-DNA antibodies in cryoprecipitates from patients with lupus and non-lupus GN, including one patient with MN. They suggested that auto-immunity may be a common denominator in IC diseases (Lewis and Roberts 1980). In addition, Nomoto and Sakai (1979) demonstrated the presence of cold reactive IgG antibody to extractable nuclear antigen (ENA) in 5/6 patients with primary MN using an immunofluorescent absorption study, and anti-ENA in 6/6 with an haemagglutination assay.

A disturbance of T4/T8 ratios was also present in patients with mesangial IgA GN. A decreased T8 positive subset again resulted in an elevated ratio. Chatenoud and Bach (1981) also found a significant elevation of the T4/T8 ratio in 5/9 patients with IgA GN although Cagnoli et al (1982) did not. In

the present study, functional tests showed B cell hyperactivity with high spontaneous and PWM stimulated IgG and IgA synthesis by PBMC from patients with IgA GN, suggesting defective suppressor cell activity. However, Con A inducible suppression of IgG and IgA synthesis was similar to controls. Serum IgA levels did not show a significant correlation with in vitro production of IgA. Egido et al (1982) have also shown increased IgA production by PWM stimulated lymphocytes from patients with IgA GN, although there was no difference in spontaneous production of IgA between patients and controls. Cosio et al (1982) found elevated in vitro IgA production in only 2/15 patients with IgA GN and increased IgG in 3/15 patients, although 6/13 patients had elevated serum IgA levels. Nomoto et al (1979) reported an increase in IgA bearing peripheral B lymphocytes in 12/12 patients with IgA GN. They were also able to show a decrease in IgA specific T suppressor cell activity in these patients (Sakai et al, 1979). The same workers demonstrated the presence of cold reactive IgM antibody to ENA in 27/33 patients with IgA GN, suggesting a disturbance of immune regulation with auto antibody production (Nomoto and Sakai, 1979). Decreased Con A induced suppressor function has also been reported in patients with mesangial IgA GN using assays to measure DNA synthesis by incorporation of tritiated thymidine (Woo et al, 1982). However, in the present study no significant difference in the suppression of ^{3}H-T incorporation was found between normal controls and patients with IgA GN.

Henoch Schonlein purpura is a disease closely allied with IgA GN with mesangial deposition of IgA and C3 (Spargo et al 1980) and frequent elevations of serum IgA and circulating IC (Levinsky and Barratt 1979). Marked disturbance of immuno-

globulin synthesis was found in 3 patients with HSP in the present study. All 3 patients had high levels of spontaneous IgG and IgA synthesis. The addition of PWM to the cultures caused a significant decrease in immunoglobulin production in each patient. This pattern was also typically observed in the SLE patients. The presence of Con A in the PWM cultures enhanced immunoglobulin production suggesting a marked suppressor defect in the patients with HSP. This finding was supported by low percentages of T8 positive cells in 2 of the patients with HSP and severe renal involvement. Beale et al (1982) found marked increases in IgA particularly, but also IgG and IgM in unstimulated cultures of PMBC from 5 patients with HSP. The addition of PWM caused a sharp decline in the synthesis of all classes of immunoglobulins. Similar findings occurred in cultures of PBMC from 8 patients with SLE. After T cell depletion, B cells from HSP and SLE patients maintained high IgG and IgA synthesis. Autologous T cell addition failed to suppress the high immunoglobulin synthesis in both HSP and SLE cultures but the addition of allogeneic normal T cells resulted in a decline in spontaneous immunoglobulin synthesis. These data (Beale et al 1982) support our findings of marked suppressor defects in patients with HSP and SLE. The reduction of spontaneous IgG and IgA synthesis and improvement of the suppressor defect in patient J.W. following plasma exchange therapy is unexplained, but may reflect removal during plasma exchange of soluble factors (e.g. anti T cell antibodies or immune complexes) which modulate lymphocyte function.

Studies in patients with SLE by other workers have demonstrated reduced suppressor cell function. Our results of Con A inducible suppression of IgG synthesis in patients with lupus nephritis confirm these findings. In addition highly signif-

icant alterations in T cell subsets with elevated T8 fractions and decreased OKT4+ cells resulting in low T4/T8 ratios were found in patients with lupus nephritis. Other workers (Chatenoud and Bach 1981, Smolen et al 1982) have reported similar findings. The reason for a low T4/T8 ratio is not clear. Although the low ratio may perhaps be related to steroid therapy, as suggested by Chatenoud and Bach (1981), the pre-incubation of Fc defined lymphocyte subpopulations in vitro with corticosteroids did not affect the relative proportions of Tγ and Tμ subsets (Haynes and Fauci 1978). In addition Smolen et al (1982) were unable to find a correlation between corticosteroid therapy and helper/suppressor ratios in patients with SLE. More subtle alterations of cell populations necessary for suppression within the OKT4+ and the OKT8+ subpopulations (Yachie et al 1982; Thomas et al 1982) may be more important in producing the low ratio in SLE patients. The high spontaneous IgG and IgA production in cultures of PBMC from patients with SLE was suppressed by the addition of PWM suggesting activation of suppressor cells. However, as suggested by Beale et al (1982), T suppressor cells inhibiting spontaneous immunoglobulin production may be a different subpopulation from those activated by PWM.

CONCLUSION

Although further work using separated T cell subpopulations and coculture experiments needs to be performed in MN, IgA GN, and HSP, the alterations in T cell subsets and the functional defects of in vitro immunoglobulin synthesis suggest that these disorders and SLE share similar disturbances of immune regulation and that defective suppression and/or autoimmunity may be important in the pathogenesis of these disorders. Miller and

Schwartz (1979) confirmed other reports of suppressor defects in SLE and through family studies suggested these defects were associated with genetic predisposition. Similar studies need to be performed in relatives of patients with primary GN and HSP to determine whether the observed defects are primary or secondary events.

CHAPTER 3.

AUTOIMMUNITY AND MEMBRANOUS NEPHROPATHY

INTRODUCTION

This study has attempted to address the hypothesis proposed by Lewis and Roberts (1980) that auto-immunity may be a central factor in IC mediated GN. MN is a relatively common form of GN in man, thought to be mediated by IC, either deposited from the circulation or formed *in situ* on the sub-epithelial aspect of the GBM. Several lines of reported evidence suggest that, as in SLE, auto-antibodies may be important in the pathogenesis of MN. In particular, cold reactive anti-nuclear antibodies have been found in the sera from patients with MN (Nomoto and Sakai 1979) and anti-native DNA antibodies were demonstrated in acid pre-treated cryoprecipitable material from a patient with MN (Roberts and Lewis 1980). In the previous chapter reduced OKT8+ cells and elevated helper/suppressor peripheral T lymphocyte ratios suggested a deficiency of suppressor T cells, while defective Con A suppression of *in vitro* IgG synthesis suggested similarities between MN and lupus nephritis. Finally, the association between HLA DR3 and MN (Klouda et al 1979) suggests a possible auto-immune predisposition in these patients.

This chapter has included a search for auto-antibodies in sera, polyethylene glycol (PEG) serum precipitates and kidney eluates from MN patients. In addition, highly purified anti-DNA antibody has been used to attempt to identify DNA in the glomerular deposits in MN. Finally, the association of MN with HLA DR3 was re-assessed in our patients.

METHODS AND PATIENTS

DNA binding activity of PEG precipitates from MN sera

(i) Preparation of 4% PEG precipitates

200 µl of heat inactivated serum was incubated with 200 µl of 8% PEG (MW = 6000) in 0.1M borate buffer pH 8.3 for 60 minutes at 4°C. After centrifugation at 1000 x G for 60 minutes at 4°C the pellet was washed with 4% PEG in 0.1M borate buffer pH 8.3 and finally dissolved in 100 µl PBS.

(ii) Farr assay for DNA binding activity

The Amersham anti-DNA kit (IM 76) was used (Pincus 1971). The PEG precipitate was diluted 1/10 with buffer A and 50 µl of ^{125}I DNA was added to 50 µl of diluted PEG precipitate. After vortex mixing the solution was incubated for 60 minutes at 37°C followed by a 24 hour incubation at 4°C. 100 µl cold saturated ammonium sulphate was added and immediately vortex mixed. The precipitate was centrifuged at 1000 x G for 15 minutes at 4°C and then counted in a gamma counter.

(iii) Patients and controls

(a) normal human serum from 9 healthy controls.

(b) sera from 6 patients with lupus nephritis (circulating IC (spCIq assay) levels from 22 - 220 µg AHG/ml). All SLE patients were taking prednisolone at the time of testing.

(c) sera from 13 patients with primary MN (no specific treatment) and 1 patient with MN associated with a renal cell carcinoma.

Preparation of fluorescein conjugated purified anti-DNA antibody

A. Purification of anti-DNA antibodies from SLE serum (Fishbein and Alarcón-Segovia 1980).

(i) Column preparation and elution of anti-DNA antibody.

A 350 x 160 mm jacketed column (LKB) was packed with hydroxyapatite (Biorad Laboratories, Richmond, California) suspended

in 0.005 M phosphate buffer pH 6.8 heated to 50°C. This temperature was maintained in the column by circulating water in the jacket at 50°C. A constant minimum flow of 30 ml buffer/hour/square cm (60 ml/hour) was maintained. Calf thymus DNA (BDH Chemicals, Poole, England) dissolved in 0.005M phosphate buffer (30 mg/30 ml) was layered on top of the column and flushed into it with the same buffer. 30 ml of SLE serum (24 year old female with active SLE; DNA binding activity (Amersham anti-DNA kit) > 96 units (N.R. 0-25 units)) was heat inactivated (56°C for 30 minutes) and layered on top of the column. After 100 ml of 0.005M phosphate buffer had run into the column a continuous gradient of phosphate buffer with an initial molarity of 0.005M and a final molarity of 0.5M, pH 6.8 was run. Deoxyribonuclease I, (Millipore Corp., Freehold, New Jersey) 100 mg, and magnesium chloride, 0.203 mg was added to pooled fractions from both peaks of the elution and after incubation at 37°C for 60 minutes and at room temperature for 60 minutes was inactivated with EDTA at a final concentration of 20 mM. After dialysis against 0.01M phosphate buffer overnight the eluate was concentrated to 12 ml and after passage through a di-ethylaminoethyl cellulose (DE52, Whatman) column and concentration to 6 ml (12 mg/ml), purified anti-DNA IgG, containing both double stranded and single stranded DNA antibodies, was obtained.

(ii) Testing of anti-DNA IgG

This antibody was tested by indirect immunofluorescence (IIF) on a normal human kidney (NHK) target with a fluorescein conjugated goat anti-human IgG second antibody.

B. Fluorescein conjugation of purified anti-DNA antibody

The anti-DNA IgG was diluted to 10 mg/ml and 3.6 ml of antibody was dialysed overnight against 36 ml of carbonate buffer,

0.025M, pH 9.4 containing fluorescein isothyocyanate (BDH Chemicals, Poole, England) at a concentration of 0.1 mg/ml buffer. After dialysis against 0.01M phosphate buffer, pH 7.5 for 48 hours the fluorescein conjugate (FITC) was concentrated and tested on a NHK target. (Fig. 3.2)

Examination of renal biopsy material by direct immunofluorescence using FITC - purified anti-DNA IgG (Koffler et al 1967)

Renal biopsies from 9 patients with primary MN and 1 patient with MN secondary to gold therapy for rheumatoid arthritis were examined. Six biopsies from patients with lupus nephritis were also examined. All biopsies had unequivocal histological appearances of the particular disease and each biopsy had extensive granular deposits of immunoglobulin and complement. Sections were acetone fixed for 10 minutes and after washing three times with PBS were incubated with 2M NaCl at 37°C for 90 seconds to remove nuclear DNA. After 3 further washes with PBS, FITC - anti-DNA IgG (½ dilution) was incubated for 30 minutes and after washing the sections were mounted and examined on a Leitz fluorescence microscope.

Elution of kidneys

Glomeruli isolation (Portis et al 1979)

Renal cortex was minced using scissors and an Ultra Tarax mincer and the tissue pressed through a 180 μm stainless steel sieve. The pressings were washed six times in cold PBS by centrifuging at 250 x G for 2 minutes. The sediment was resuspended in a sucrose solution (S.G. 1.21 - 1.24), dispersed by shaking and centrifuged at 2000 x G for 5 minutes. The pellet was then washed in PBS twice, centrifuging at 2000 x G for 5 minutes and the glomerular preparation weighed.

Elution of glomeruli

Purified glomeruli were suspended in citrate buffer 0.02M pH 3.0 (20 mls citrate buffer per gram glomeruli) for 2 hours at 37°C with gentle stirring. After centrifugation at 2000 x G for 10 minutes the supernatant was removed and the eluted immunoglobulin precipitated with 33% ammonium sulphate, dissolved in 10 ml PBS and dialysed for 48 hours against PBS. After concentration by negative pressure ultrafiltration the IgG content was measured by radial immunodiffusion.

Testing of eluates from MN kidneys

The eluates were tested for anti-nuclear reactivity using indirect immunofluorescence (IIF) with NHK and rat liver targets and FITC - anti-human IgG. Other auto-antibodies were also sought with IIF using NHK target for mitochondrial antibodies and rat stomach for gastric parietal and smooth muscle antibodies. Thyroid microsomal antibodies and thyroglobulin antibodies were tested in an haemagglutination assay (Thymune - M or Thymune - T Haemagglutination kit) by the Department of Medicine, The Queen Elizabeth Hospital. In addition:

1) one eluate (case 1) was conjugated with fluorescein isothyocyanate and tested by direct immunofluorescence on sections of the autopsy kidney from case 1.
2) eluates (cases 2 and 4) were tested by IIF on NHK targets which had been pre-treated with citrate buffered saline 0.02M, pH 3.2 for 30 minutes.

Patient data

Case 1. Mr. D.C. Age 76.

Presented with a left temporal haemorrhage in April 1979. Following discovery of proteinuria, a renal biopsy showed MN with granular deposits of IgG and C3 on immunofluorescence.

Biochemical parameters

Serum creatinine	0.20	mmol/L	(N.R. 0.05 - 0.12)
Serum albumin	34	G/L	(N.R. 34 - 45)
Serum C3	1.4	G/L	(N.R. 0.65 - 1.25)
Serum C4	0.64	G/L	(N.R. 0.10 - 0.40)

Urinary 24 hour protein excretion 9.5 G/day (N.R. 0.00 - 0.12)

Cryoglobulin present, circulating immune complexes (solid phase CIq radioimmunoassay) negative.

Anti-nuclear factor negative.

Rheumatoid factor (latex) weakly positive but Rose-Waaler titre < 1/64.

Progress

Mild neurological deficits secondary to cerebral haemorrhage. No specific treatment for MN. Death 16/11/79 from myocardial infarction with cardiac tamponade.

Autopsy findings

1. Renal - membranous nephropathy.
2. Prostate - primary adenocarcinoma.

Comment

No tumour tissue was available for immunological studies. MN possibly secondary to prostatic carcinoma.

Case 2. Mr. K.F. Age 58.

Past history of chronic obstructive airways disease (COAD) secondary to heavy smoking. MN diagnosed on renal biopsy, August 1977 with granular deposits of IgG and C3.

Biochemical parameters

Serum creatinine	0.07	mmol/L
Serum albumin	24	G/L
Serum C3	1.4	G/L
Serum C4	0.58	G/L

Urinary 24 hour protein excretion 3.7 G/day.

Antinuclear factor negative, circulating immune complexes negative.

Other investigations

Bronchoscopy, pleural biopsy and liver biopsy negative for malignancy.

Progress

Treated with cyclophosphamide, warfarin and dipyridamole for 12 months. Progress renal biopsy showed stage II MN with advanced glomerular changes and focal interstitial scarring. Right heart failure secondary to COAD progressed and patient died 7/4/81 with cor pulmonale.

Autopsy findings

1. Renal - MN stage II.
2. Lung - 8 mm L apical scar containing well differentiated squamous cell carcinoma.

Comment

1. No tumour available for immunological studies.
2. In view of minimal size of tumour ? relationship to MN.

Case 3. Mr. J.T. Age 66.

Stage II MN with granular capillary wall IgG and C3 diagnosed 28/2/80 when R. nephrectomy was performed for a renal cell carcinoma.

Biochemical parameters

Serum creatinine	0.30	mmol/L
Serum albumin	22	G/L
Serum C3	0.94	G/L
Serum C4	0.44	G/L

Urinary 24 hour protein excretion 7.5 G/day.

Anti-nuclear factor negative, circulating immune complexes negative.

Progress

Renal function deteriorated and heavy proteinuria continued. 2/12/80, serum creatinine 0.45 mmol/L, urinary 24 hour protein excretion 6.1 G/day. Death 28/5/82.

Autopsy findings

1. No evidence of metastatic tumour.
2. Renal - advanced MN stage III-IV with glomerular sclerosis and scarring.

Comment

1. Unfortunately the original tumour was unavailable for immunological studies and at autopsy no metastatic or local tumour was found.
2. Despite tumour removal the nephrotic syndrome persisted.

Case 4. Mrs. L.D. Age 60.

Chronic renal failure of unknown aetiology.

December 1979 - cadaveric renal transplant.

August 1980 - proteinuria and deteriorating renal function; transplant biopsy showed moderate cellular and humoral acute rejection with glomerulomegaly, mild mesangial swelling and scattered segmental lesions. Immunofluorescence showed only patchy, segmental reactions for IgM and C1.

May 1981 - continued deterioration in renal function associated with the nephrotic syndrome; transplant biopsy showed membranous nephropathy and also focal segmental glomerulosclerosis affecting 60% of glomeruli. Moderate cellular rejection was also present. Immunofluorescence showed granular capillary wall reactions for IgG, IgM, C1 and C3. Ultrastructural examination revealed sub-epithelial electron dense deposits and loss of foot processes.

Other investigations

Circulating immune complexes negative.

Anti-nuclear factor negative. Complement studies normal.

27/5/81 - haemodialysis recommenced.

7/6/81 - transplant nephrectomy performed.

Comment

Presumably this case represents primary MN developing "de novo" in a renal allograft.

Testing of MN sera for autoantibodies

(This was part of a collaborative study of a large group of patients with MN from the Royal Adelaide Hospital in association with Dr. P.E. McKenzie and Dr. I.K.P. Cheng).

i) Anti-nuclear factor was sought in 43 patients with MN (mean age 44 years; M 28, F 15) by IIF using rat liver targets.

ii) Anti-globulin activity (rheumatoid factor) was tested in 36 patients with R.A. latex screening (CSL) or the Rose-Waaler reaction.

iii) Screening for other autoantibodies, including anti-thyroid, gastric parietal and smooth muscle antibodies as previously described, was performed in 35 patients.

iv) Sera from 17 patients was tested by IIF on NHK targets for anti-glomerular basement membrane (GBM) and anti-renal tubular epithelial (RTE) antibodies.

v) Antibodies against RTE were also sought in 16 patients by immunodiffusion.

In brief:

a) Preparation of renal tubular antigen. (Naruse et al 1975)

100G of normal human renal cortices were homogenized in 0.02 Tris HCL pH 7.8 containing 0.8% NaCl. After centrifug-

ation at 400 x G for 10 minutes the supernatant was ultracentrifuged at 78,000 x G for 30 minutes to collect the sediment representing insoluble renal tubular epithelial segments. The RTE preparation was suspended in 60 ml of the same buffer and incubated with pronase (0.5 mg/ml) at 37°C for 12 hours. After centrifugation at 100,000 x G for 60 minutes the clear supernatant was concentrated to OD^{280} = 12.3 (4.5 ml). After testing by immunoelectropheresis and in gel, the pronase extract was found to be contaminated by proteolytic fragments of IgG. The preparation was then purified by chromatography on a Bio-Gel A column. The eluted fraction, uncontaminated by IgG (OD^{280} = 1.9; 1.5ml) was used to immunize a rabbit, together with incomplete Freund's adjuvant. On IIF the rabbit antisera gave bright brush border staining of NHK with FITC - anti-rabbit IgG.

b) Immunodiffusion to detect anti-RTE antibodies in MN sera.

This was performed in Petri dishes containing 1% agarose in PBS with 5 mm wells 3 mm apart. Diffusion took place at room temperature for 24 hours followed by 24 hours at 4°C.

vi) Antibodies against ubiquitous tissue antigen (UTA) were sought in 17 patients.

In brief:

a) Preparation of UTA (Palosuo and Milgrom 1977)

100 G of normal human kidney tissue was minced, washed with PBS and homogenized in PBS containing 0.44M sucrose. After centrifugation at 20,000 x G at 4°C the supernatant was removed and centrifuged at 105,000 x G for 60 minutes at 4°C. The supernatant was dialysed against PBS at 4°C and concentrated by dialysis against PEG (MN = 6000). After further PBS dialysis

the concentrate was centrifuged at 105,000 x G for 30 minutes at 4°C.

b) Immunodiffusion to detect antibodies to UTA in MN sera

Diffusion took place in 0.5% agarose in PBS in Petri dishes at room temperature for 24 hours followed by 24 hours at 4°C.

HLA DR typing

This was performed by the Department of Immunology, Flinders Medical Centre, Adelaide. An enriched population of B lymphocytes was obtained and typed by microcytotoxicity testing using DR anti-sera characterized in the Australian Tissue Typing Workshop. Twenty nine patients with primary MN and 4 patients with secondary MN were typed. A random panel of 41 controls was used to establish normal antigen frequencies.

Statistical analysis

A 2 x 2 contingency table was used to test the difference in DR3 antigen frequencies.

RESULTS

DNA binding activity of PEG precipitates (Fig. 3.1)

Four of 13 primary MN patients and 3/6 patients with SLE had DNA binding activity greater than the mean +2 SD of the normal control group. Of the 4 MN patients with high DNA binding 3 were DR typed and 2 were DR3 positive. 4/9 MN patients with normal DNA binding were DR3+.

Preparation of purified anti-DNA antibodies

After elution of anti-DNA antibodies from the serum of an SLE patient on an hydroxyapatite column and DE52 purification the eluate/serum ratio per µg IgG tested by IIF on a NHK to end-titre was 3:1.

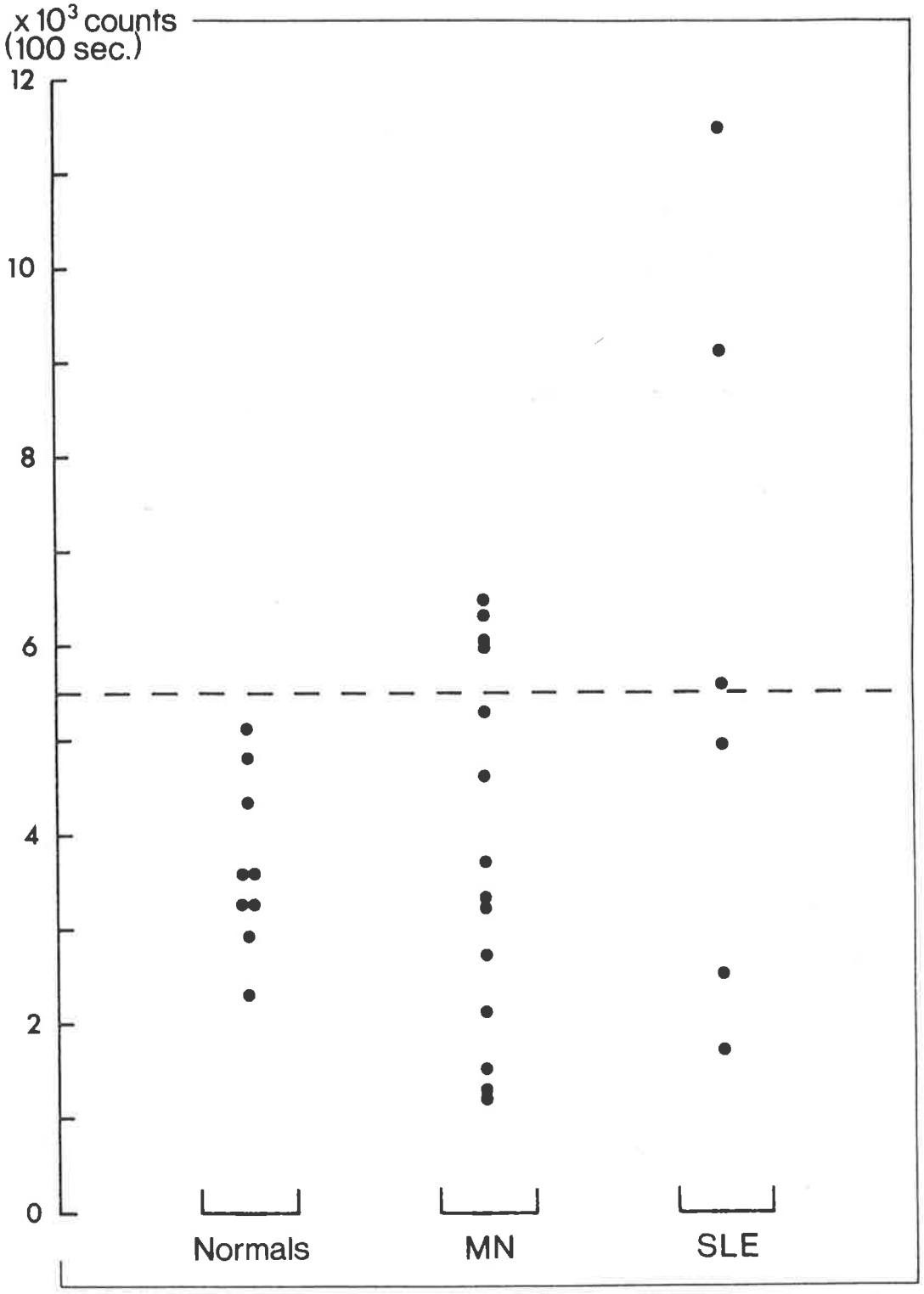

FIGURE 3.1 ^{125}I-DNA binding (CPM) in 4% PEG precipitates of sera from normal controls (9) and patients with MN (14) and lupus nephritis (6). The mean +2SD of controls is also shown.

Examination of renal biopsy material by direct immunofluorescence using FITC - anti-DNA antibody (Fig. 3.3)

In 2 of 6 biopsies from patients with lupus nephritis glomerular deposits of DNA were identified after prior brief treatment of the tissue sections with 2M NaCl. No deposits of DNA were found on untreated or saline pre-treated biopsies from 10 MN patients.

Studies on eluted immunoglobulin from kidneys of patients with MN

i) Eluted immunoglobulin (IgG) (Table 3.1)

Case	Total IgG eluted (µg)	Wt glomeruli (G)	µg IgG/G glomeruli
1	1200	7.6	158
2	60	7.5	8
3	341	19.8	17
4	12	11.6	1

TABLE 3.1 IgG content of eluates from MN kidneys.

ii) Anti-nuclear antibodies

None of the eluates showed reactivity on untreated NHK or rat liver targets nor on acid pre-treated NHK targets to expose antigenic sites. In addition there was no reactivity with renal tubular epithelium.

iii) Other autoantibodies

IIF was negative for gastric parietal cell antibody, smooth muscle antibody and mitochondrial antibody. Haemagglutination tests for thyroid microsomal and thyroglobulin antibodies were also negative in all 4 eluates.

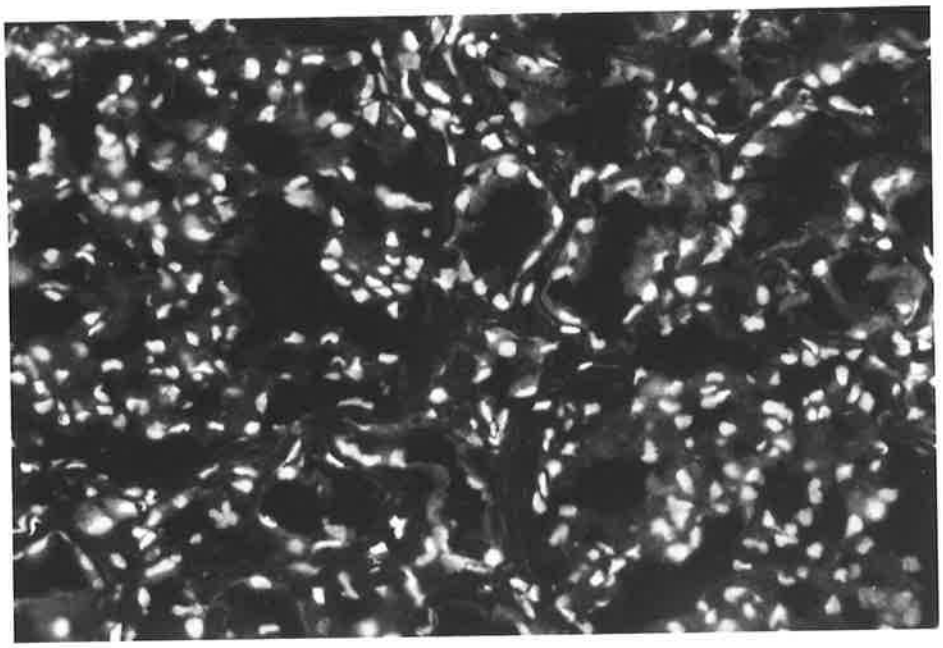

FIGURE 3.2 Nuclear staining of NHK target (direct fluorescence: FITC - highly purified anti-DNA IgG).

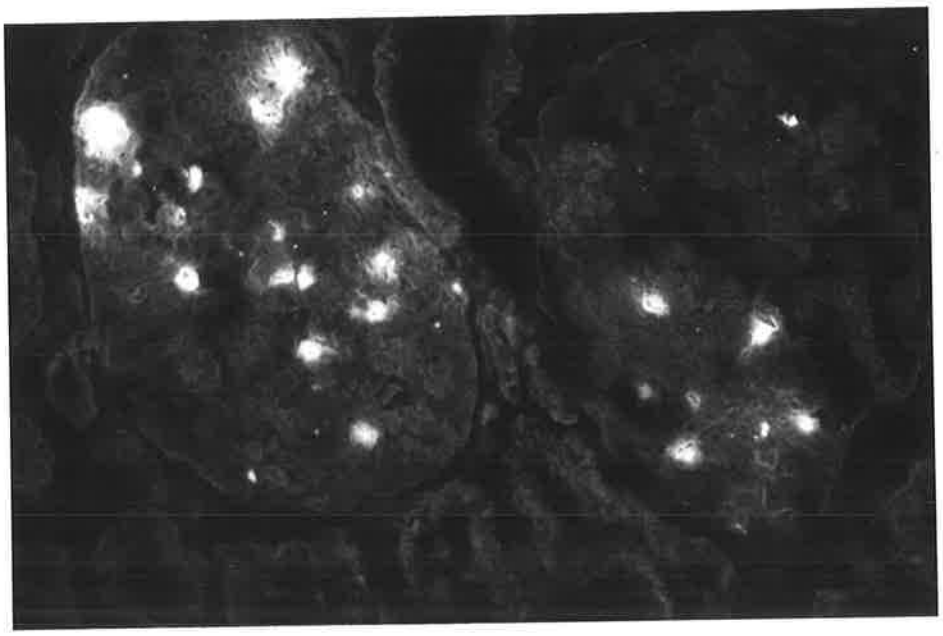

FIGURE 3.3 Extra-nuclear glomerular DNA deposits in an SLE biopsy. (Direct fluorescence: 2 M NaCl followed by FITC-anti-DNA IgG).

iv) Direct immunofluorescence using FITC - eluate (Case 1) on Case 1 renal biopsy was negative.

Autoantibodies in sera from patients with MN

i) Anti-nuclear factor was present in only the 2 patients with MN secondary to SLE and in 1 of 3 patients with MN secondary to gold therapy.

ii) Rheumatoid factor was detected in 2 patients with gold nephropathy complicating rheumatoid arthritis, in 1 patient with SLE and in 2 of 31 patients with primary MN.

iii) Screening for other auto-antibodies was performed in 35 patients with either primary (30) or secondary (5) MN. Anti-thyroid (3), gastric parietal (2) and smooth muscle (1) antibodies were demonstrated in patients with primary MN with an incidence no different from that in the normal population. In contrast, 3 of the 5 patients with secondary MN had anti-thyroid or smooth muscle antibodies.

iv) Nuclear staining was demonstrated in 2 patients with IIF on NHK targets, while 2 blood group 0 patients showed vascular staining which was completely abolished by prior absorption with group AB cells. No anti-tubular or anti-glomerular basement membrane staining was detected.

v) The serum from one patient with primary MN was positive for anti-RTE antibody on immunodiffusion. However, the specificity of this reaction is in question because there was no line of identity with purified RTE-anti-RTE antibody.

vi) One patient with gold induced nephropathy was positive for anti-UTA antibody.

HLA DR typing (Table 3.2)

Eighteen of 29 (62%) patients with primary MN were DR3 positive and 3 of 4 with secondary MN also had the DR3 antigen.

The frequency of this antigen in the random panel was 39% and the difference was not significant ($p < 0.10$).

HLA	MN (n = 29)	PANEL (n = 41)
1	3 (10%)	4 (10%)
2	2 (7%)	11 (27%)
3	18 (62%)	16 (39%)
4	6 (21%)	10 (24%)
5	4 (14%)	2 (5%)
6	9 (31%)	10 (24%)
7	5 (17%)	11 (27%)

TABLE 3.2 HLA DR antigen frequency in patients with primary MN and in a random panel ($\chi 2 = 3.61$; $p < 0.10$ for DR3)

DISCUSSION

For many years MN has been regarded as the prototype example of chronic IC mediated GN. This is supported by experimental models (Cochrane and Koffler 1973) and by secondary forms of MN in which antigen can be identified in the glomerar immune deposits. In contrast, circulating IC are unusual in patients with primary MN and antigens have not been conclusively identified. Nevertheless the granular deposits of immunoglobulin seen on immunofluorescence are felt to be due to either IC deposited from the circulation or formed *in situ*. This chapter has examined the possibility that auto-antibody - antigen complexes are involved in the pathogenesis of MN and has re-assessed the likelihood of genetic predisposition.

Four % PEG precipitation concentrates IC material from serum (Creighton et al 1973) but the percentage of monomeric IgG precipitated is small (Chia et al 1979). PEG precipitation of serum from patients with SLE also enriches anti-DNA antibodies present as soluble complexes (Chia et al 1979). In the present study 4 of 13 primary membranous sera bound ^{125}I DNA to a significantly greater extent than normals indicating the presence of anti-DNA antibody activity. Roberts and Lewis (1980) also reported the presence of anti-DNA anti-bodies in cryoprecipitate material from the serum of a patient with MN, but others, although demonstrating the presence of DNA in cryoglobulins from membranous patients, were unable to detect anti-DNA activity (Adu et al 1981). Despite anti-DNA activity in PEG precipitates from some MN patients, anti-nuclear antibodies were only detected in the sera from 3 patients with secondary MN (SLE or gold nephropathy). However, cold reactive antinuclear antibodies have been demonstrated in 5/6 patients with primary MN using immunofluorescence and in 6/6 using an haemagglutination assay (Nomoto and Sakai 1979).

To examine the possibility that anti-DNA - DNA complexes may also be involved in the glomerular immune deposits of MN patients, a fluorescein conjugated, highly purified anti-DNA (double and single stranded) antibody was prepared. Although DNA could be demonstrated in the glomeruli of some patients with lupus, as previously reported (Koffler et al 1967), biopsies from 10 patients with MN were negative. In addition 4 kidneys from patients with MN became available for elution over the course of this study and anti-nuclear activity was tested in these eluates. In 2 of the 4 cases clinically inapparent extra-renal tumour was found at autopsy. In the

third case of secondary MN associated with a renal cell carcinoma of the opposite kidney, no tumour was apparent at autopsy, despite features of ongoing MN, histologically. Possible antigen-antibody systems which may be involved in these patients include tumour associated antigens, re-expressed foetal antigens, viral antigens and autologous non-tumour antigens and their respective antibodies (Eagen and Lewis 1977). Unfortunately tumour tissue was unavailable in all 3 cases to test the first hypothesis. We were particularly interested in the possibility that auto-antibodies may be involved in these cases. Antibodies to DNA (Higgins et al 1974) and to RTE (Ozawa 1975) have been found in renal eluates and cryoprecipitates respectively from patients with metastatic oat cell carcinoma of the lung and renal cell carcinoma associated with GN. We were however, unable to find anti-nuclear antibodies, anti-RTE antibodies or other organ specific auto-antibodies in the glomerular eluates from the 3 cases of secondary MN, or from one case of "de novo" MN developing in a renal allograft. The extreme scarcity of membranous kidneys for elution studies however, makes this negative data valuable and implies that auto-antibodies are not playing a significant pathogenetic role in the glomerular deposits in these cases. No other glomerular staining was observed on IIF using untreated or acid pre-treated NHK targets. A mechanism dependent on the binding of an auto-antibody to non-classical GBM antigens as described in the spontaneous nephritis of New Zealand white rabbits (Woodroffe 1977) and Heymann's nephritis in rats (Neale and Wilson 1982) does not therefore appear to be operating in these cases of human MN.

An ubiquitous tissue antigen and/or antibody have been

found in the sera of 25% of patients with idiopathic MN (Palosuo et al 1975). One patient with gold nephropathy and rheumatoid arthritis was also found to have auto-antibodies to UTA (Palosuo et al 1976). Forty seven percent of rheumatoid arthritis patients developing proteinuria during therapy with gold or penicillamine were also found to have anti-UTA or more rarely, UTA in their sera. (Palosuo et al 1978). It was felt that gold and penicillamine may have facilitated the development of this auto-antibody. However, anti-UTA - UTA complexes have not been demonstrated in glomerular deposits in patients with anti-UTA antibodies so that the relationship of this antibody to the pathogenesis of the glomerular lesion remains speculative. We were unable to detect anti-UTA antibodies in our patients with primary MN but one patient with rheumatoid arthritis and biopsy proven MN secondary to gold therapy was positive for this auto-antibody.

Antibodies to RTE could not be detected in patients with MN using either IIF or immunodiffusion. In addition, renal eluates were negative for anti-RTE antibody. These results are supported by the negative findings of other workers (Whitworth et al 1976; Zager et al 1979; Collins et al 1981) using fluorescence or radioimmunoassay to detect antibodies to RTE. In contrast, a single group reported RTE in glomerular immune deposits in 4 of 9 patients with MN (Naruse et al 1973, 1974).

DR typing in the present study, although not achieving statistical significance ($p < 0.10$), did show an increased incidence of the DR3 antigen in patients with primary and secondary MN. Klouda et al (1979) showed a highly significant association between this antigen and primary MN and the same

group has provided some evidence that the presence of the DR3 antigen may influence clinical outcome (Cairns et al 1980). The relative risk of developing the disease is 12:1 in the presence of this antigen. In patients with nephropathy secondary to gold and penicillamine the relative risk of proteinuria developing during treatment with these agents was increased 32 times in patients who were DR3 positive (Wooley et al 1980). The DR3 antigen is also associated with auto-immune disorders including SLE, myasthenia gravis, Graves disease, Addison's disease, Sjögren's syndrome and chronic active hepatitis. The meaning of the association between this antigen and these disease states is still not clear. One possible explanation is that HLA linked immune response genes may predispose to the disease state (Fathman 1978). Griffing et al (1980) found a statistically significant association between HLA DR3 and the presence of antibodies to native DNA, both in patients with SLE and also in association with non-SLE disorders. The author concludes that an HLA-DR linked immune response gene may control the production of antibodies that are aetiologically involved in the pathogenesis of these diseases.

SUMMARY

Although the association of primary MN with the HLA DR3 antigen suggests an auto-immune predisposition to this disease, auto-antibodies were not detected in the sera or kidney eluates from patients with MN. In contrast to SLE, neither could DNA be found in glomerular deposits in renal biopsies from MN patients. However, evidence of anti-DNA antibody activity was demonstrated in PEG precipitates of 4/14 MN sera. Auto-antibodies therefore appear to have a limited role in a subgroup of MN patients. Confirmation of the finding of cold reactive

auto-antibodies in MN sera (Nomoto and Sakai 1979) may expand this group.

CHAPTER 4.

GLOMERULAR IgG SUBCLASS DISTRIBUTION IN GLOMERULONEPHRITIS

INTRODUCTION

In human forms of GN glomerular deposits of immunoglobulin and complement may be present in a granular or linear fashion indicating mediation by IC or anti-glomerular basement membrane antibodies respectively. (Dixon and Wilson 1979). Although the pattern of IgG deposition in individual forms of GN is well documented, the distribution of the four heavy chain subgroups of IgG has not been accurately studied in well-defined disease groups. The concentration of IgG subclasses is genetically controlled (Schanfield 1980) and the host response to certain antigens in individuals who develop GN may result in the preferential deposition of certain subclasses in glomerular immune deposits. Each IgG subclass has distinctive biological properties (Capra and Kunkel 1970; Stanworth and Turner 1978) which could then influence the development of the glomerular lesions. The availability of monospecific antisera to human IgG subclasses has enabled the glomerular distribution of these subgroups to be determined accurately in patients with carefully categorized forms of GN.

METHODS

Patients

Renal biopsies were examined from 10 patients with MN, 6 with type I mesangiocapillary glomerulonephritis (MCGN), 4 with anti-glomerular basement membrane antibody induced glomerulonephritis (aGBM), 6 with lupus nephritis and 5 with no significant glomerular abnormalities (controls). Autopsy kidney specimens were examined from a further 2 patients with

aGBM nephritis. The 33 patients were selected on the basis of unequivocal clinico-pathological categorization and the clear-cut presence or absence of glomerular IgG deposits.

Anti-sera to IgG subclasses

Antisera to the subclasses of human IgG were obtained from the Central Laboratory of the Netherlands Red Cross Transfusion Service (KH-9540-9); anti-IgG_1 was raised in sheep and anti-IgG_2, IgG_3 and IgG_4 in rabbits. These antisera were absorbed to ensure monospecificity. (Beck 1981).

Relative potencies of subclass anti-sera (Table 4.1)

The subclass antisera were tested on spleen sections from a normal Wistar rat 60 minutes after IV injection of heat aggregated human gammaglobulin. Using the same incubation times and reagents as described below, anti IgG_1, anti IgG_2 and IgG_3 were shown to be equipotent and anti IgG_4 to be a slightly weaker reagent. However, 3 doubling dilutions (1/4, 1/8, 1/16) of each reagent did not significantly change the fluorescence reactions indicating conditions of antibody excess at the working dilution (1/4).

Dilution of Sub-class anti-sera	IgG_1	IgG_2	IgG_3	IgG_4
1/4	3.0	3.0	3.0	2.50
1/8	3.0	3.0	3.0	2.0
1/16	3.0	3.0	2.75	2.0
1/32	2.50	3.0	2.50	1.0

TABLE 4.1 Relative potencies of IgG subclass antisera. (IIF: IgG subclass antisera tested on rat spleen following IV injection of aggregated human IgG followed by FITC - goat anti-sheep (G_1) or anti-rabbit ($G_{2,3,4}$) IgG. (Fluorescence scored from 0-5).

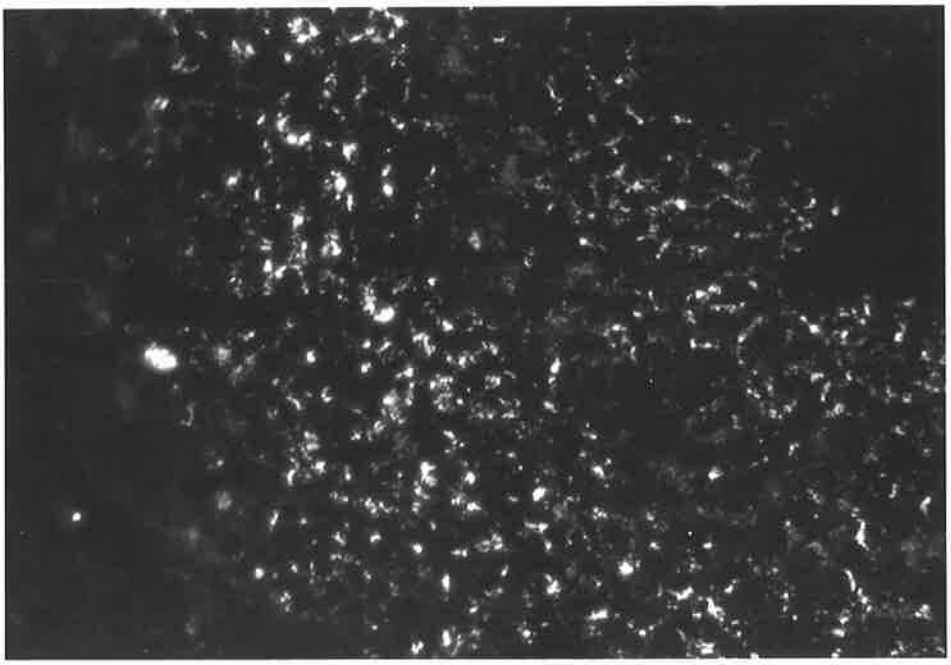

FIGURE 4.1 Aggregates of human IgG in rat spleen following IV injection of neat aggregated human IgG. (Direct fluorescence: FITC - anti-human IgG (Burroughs-Wellcome)).

Fluorescein conjugates

IgG from the sera of goats immunized with either sheep or rabbit IgG was precipitated with saturated ammonium sulphate and purified by diethylaminoethyl cellulose (DE52, Whatman) chromatography. After testing by immuno-electrophoresis the purified IgG was conjugated to fluorescein isothyocyanate (BDH Chemicals, Poole, England) and used at a 1/6 dilution. The fluorescein conjugates were absorbed with normal human serum before use and were shown to have no reactivity when used with PBS, normal sheep or normal rabbit serum as the first layer.

Renal Immunofluorescence

Tissue was examined for IgG, IgA, IgM, CIq, C3, fibrinogen and alpha 2-macroglobulin by routine direct immunofluorescence. (Clarkson et al 1977). The subclasses of IgG were sought by indirect immunofluorescence as follows:-

unfixed cryostat sections were incubated for 45 minutes with 1/4 dilution of IgG subclass anti-sera, washed and then incubated with fluorescein conjugated goat anti-sheep IgG or goat anti-rabbit IgG for 30 minutes. Immunofluorescence was scored from 0 to 5 (negative, trace, ±, 1+, 2+, 3+).

RESULTS

(Table 4.2; Fig. 4.2 and 4.3)

None of the 5 control specimens was positive for IgG or its subclasses. All patients with MN had finely granular deposits of IgG along the glomerular capillary wall. CIq was present in 6/10 and C3 in 9/10. $IgG_{1, 2, 3}$ and $_4$ were present in all 10 patients, with IgG_3 and IgG_4 predominating. The patients with MCGN all had granular glomerular deposits of

IgG, CIq and C3. All 6 of these biopsies contained large amounts of IgG_3 with very little or none of the other subclasses present. Patients with a GBM all had linear IgG glomerular deposits with segmental C3 in 5/6 and CIq in 3/6. IgG_3, and to a lesser extent IgG_1, were found in all patients. IgG_4 was present in 5/6 and very small amounts of IgG_2 in 3/6. All patients with SLE had heavy granular glomerular deposits of IgG, IgA, IgM, CIq and C3. IgG_3 and IgG_1 were present in 6/6, and predominant in 4/6 biopsies. IgG_2 was present in smaller amounts in 5/6 with IgG_4 also present in 5/6 but in even smaller quantities.

	IgG	CIq	C3	IgG_1	IgG_2	IgG_3	IgG_4
Membranous nephropathy (10)	4.5	1.4	3.4	3.5	3.0	4.7	4.3
Mesangio-capillary glomerulonephritis (6)	4.2	3.8	4.3	0.9	0.7	4.7	0.7
aGBM antibody induced nephritis (6)	4.0	1.4	2.5	2.6	0.8	3.0	2.2
Lupus glomerulonephritis (6)	4.3	3.9	3.7	3.4	2.8	4.4	2.1

TABLE 4.2 Glomerular immune deposits: mean amount scored by immunofluorescence from 0-5.

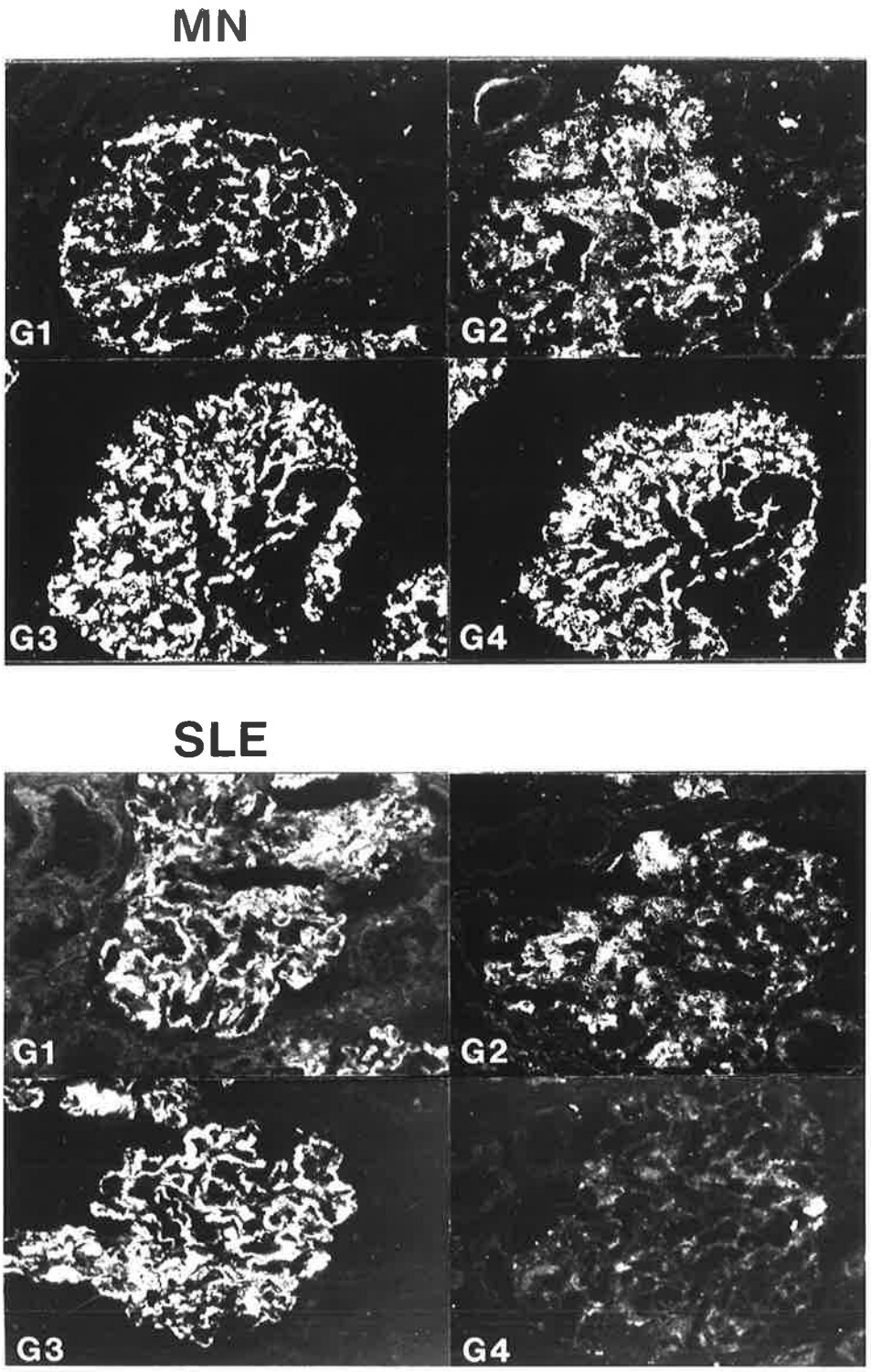

FIGURE 4.2 IgG subclasses in glomerular immune deposits from patients with MN and SLE (IIF: sheep anti-human IgG_1; rabbit anti-human $IgG_{2,3,4}$; then FITC - goat anti-sheep or anti-rabbit IgG).

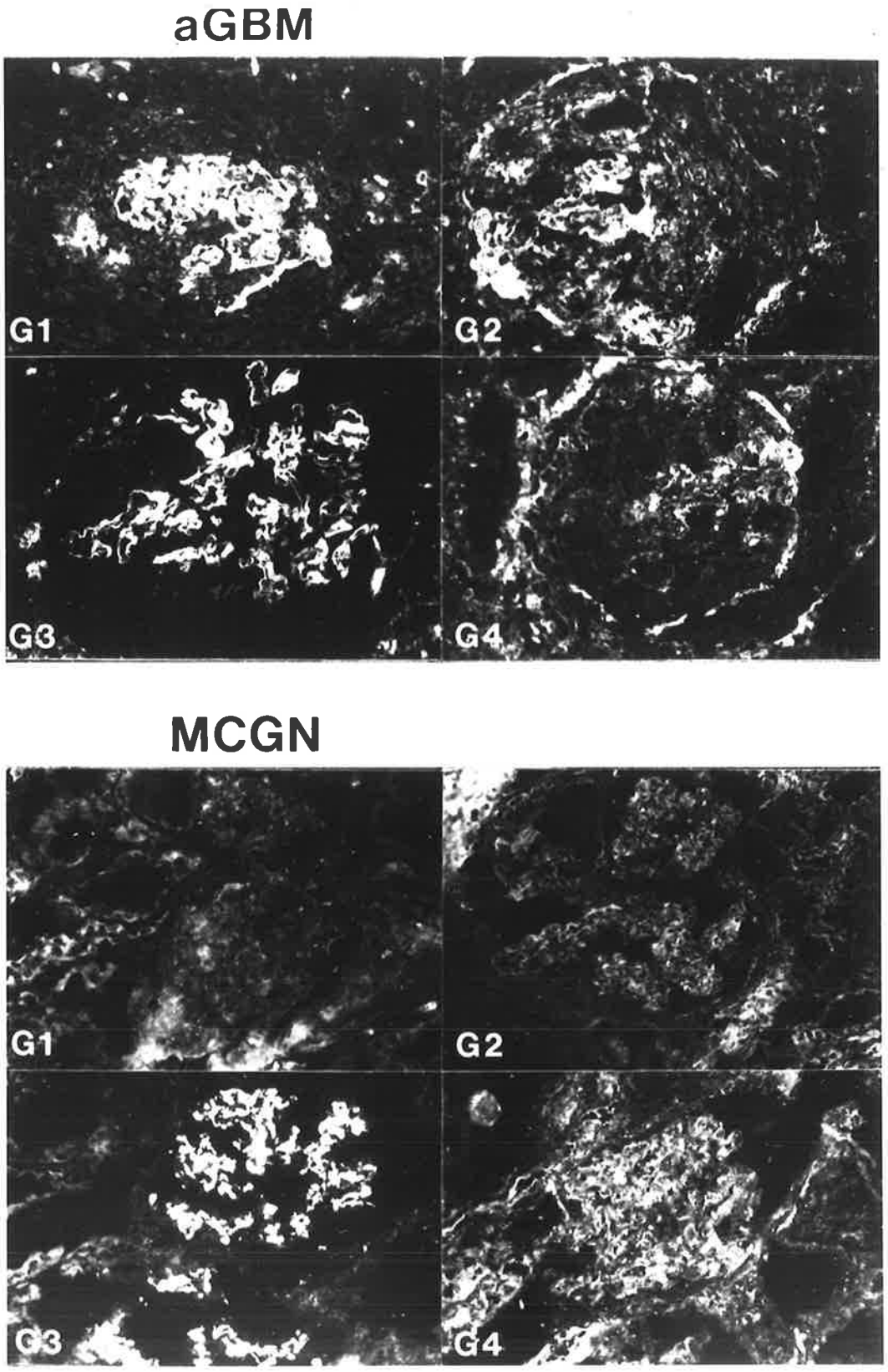

FIGURE 4.3 IgG subclasses in glomerular immune deposits from patients with aGBM and MCGN (IIF: sheep anti-human IgG_1; rabbit anti-human $IgG_{2,3,4}$; then FITC - goat anti-sheep or anti-rabbit IgG).

DISCUSSION

This study demonstrates a different distribution of IgG subclasses in glomerular immune deposits than that found in normal plasma. $IgG_{1, 2, 3}$ and $_4$ represent 66%, 23%, 7% and 4% of total serum IgG respectively (Yount et al 1970). IgG_3, making up only 7% of serum IgG, was found to be the predominant subclass in the glomerular deposits of patients with MN, MCGN, aGBM and SLE nephritis. IgG_4 was also commonly present in MN and to a lesser extent in aGBM and SLE. In addition there was a lack of correlation between glomerular IgG_3, which is a potent activator of the classical complement pathway (Table 4.3), and glomerular CIq in MN and aGBM. Lewis and others (1979), also reported a disparity between IgG subclasses in plasma and in glomerular deposits. In this earlier study, IgG_2 was the sole subclass present in glomeruli from 5/11 cases of SLE and in 4/12 cases of non-SLE nephritis with granular deposits of immunoglobulin. IgG_3 was the dominant subclass seen in one biopsy of lobular GN. Patients with linear IgG deposits generally had a selective absence of IgG_3 and often large amounts of IgG_4. However, the correlates of immunofluorescence and histopathology in individual patients limit the conclusions from this study. Differences between the two studies may reflect more formal categorization of our patients and the use of different antisera to IgG subclasses.

Perhaps IgG_3, and to a lesser extent IgG_4, are intrinsically "nephritogenic". The size of circulating antigen-antibody complexes is known to be one factor influencing glomerular deposition (Germuth and Rodriguez 1973) and this is in turn influenced by antibody avidity. However, the author is not aware of any data relating IgG antibody avidity to the various

subclasses. R-E clearance of IC is protective to the glomerulus. This is mediated by Fc (and complement) receptors. The Fc receptors on mononuclear cells bind IgG_1 and $_3$ and those on polymorphs bind IgG_1, $_2$, and $_4$ (Table 4.3), so this does not help to explain the excess of glomerular IgG_3. Finally, the negative charge of the GBM can selectively trap cationic proteins to produce an *in situ* form of glomerulonephritis. (Batsford et al 1980). However IgG_4 is anionic and the isoelectric points of the other subclasses (Table 4.3) are lower than that reportedly required for GBM binding. (Batsford et al 1980). Recently, however, evidence has been provided suggesting that there are sites on the GBM which can, in fact, bind anionic proteins *in vivo* (Melvin et al 1982). Of course, the charge of the antigen may be more critical in this respect.

It is possible, though unproven, that there is a selective increase in production of certain subclasses of IgG in various types of GN. There is some evidence for this in MCGN where Thompson (1972) has reported increased serum IgG_3 concentrations. This would be consistent with our finding of almost exclusively IgG_3 in MCGN glomeruli. It is also possible that the antibodies that contribute in the mediation of nephritis are of selected subclasses. For example, Beck (1981) has shown that antiviral antibody activity resides exclusively in the IgG_3 subclass, and more particularly C3 nephritic factor in MCGN has been shown to be an IgG_3 antibody (Fontaine et al 1980), while anti-nuclear antibodies in SLE are predominantly IgG_1 and IgG_3. (Puritz et al 1973). However, no such restrictions seem to apply to aGBM antibodies (Poskitt 1970, McPhaul and Dixon 1971). These observations correlate reasonably well with the glomerular findings in the present study.

SUBCLASS	MEAN SERUM CONC^{n}. mg/ml	SEDIM. COEFFICIENT (S)	M.W.	T½ (days)	COMPLEMENT FIXATION	ISO-ELECTRIC POINT (pI)	PROTEIN A REACT-IVITY	BINDING TO MONONUCLEAR CELLS	BINDING TO PMN
IgG_1	9	7.2-8.0	146,000	21	++	6.8-9.5	+	+	+
IgG_2	3	7.2-8.0	146,000	20	+	6.8-8.3	+	-	-
IgG_3	1	6.2-6.8	170,000	7	+++	8.2-9.0	-	+	+
IgG_4	0.5	7.2-8.0	146,000	21	-	< 6.0	+	-	+

Table 4.3 Major physico-chemical and biological properties of the 4 heavy chain subgroups of human IgG. (Stanworth and Turner, 1978, Capra and Kunkel, 1970)

Additionally the concentration of the subclasses appears to be under genetic control. (Schanfield 1980). Inherited differences on human immunoglobulin G heavy chains are referred to as Gm markers. $IgG_{1, 2}$ and $_3$ have these allotypic markers but they have not yet been confirmed on IgG_4. The serum concentrations of the subclasses appear to be related to the Gm type of the individual. (Schanfield 1980). In addition, the response to certain antigens also appears to be related to the Gm types. (Schanfield 1980). Interactions between the major histocompatability complex and these allotypes may account for the association between certain disease states, HLA haplotypes and IgG subclasses.

These studies need to be extended to examine IgG subclass distribution in other forms of GN, e.g. IgA GN and post-infectious GN. It is not yet clear which factors are of major importance in the glomerular deposition of the IgG subclasses and further work, particularly on genetic association and antigen specificity is necessary.

SUMMARY

IgG subclass distribution was determined in glomerular immune deposits found in patients with MN, MCGN, SLE and aGBM nephritis. In each disease category IgG_3 was the predominant subclass found. In MN, SLE and aGBM nephritis the other subclasses were detected in significant but lesser amounts although in aGBM nephritis IgG_2 deposition was minimal. Particularly striking was the excess of IgG_3 compared with other subclasses in MCGN and the greater amount of IgG_4 in membranous glomeruli compared to the other disease categories. These findings indicate a difference between the distribution of IgG sub-

classes in normal plasma and glomerular immune deposits and may be of importance in the pathogenesis of the types of glomerulonephritis studied.

CHAPTER 5.

Fc SPECIFIC RETICULO-ENDOTHELIAL CLEARANCE IN CHRONIC IMMUNE COMPLEX GLOMERULONEPHRITIS

INTRODUCTION

This chapter explores the hypothesis that defective Fc specific R-E clearance is present in individuals who develop IC mediated nephritis, resulting in increased circulation of IC and enhanced glomerular deposition.

While glomerular deposition of circulating IC depends on several factors, IC size (Germuth and Rodriguez 1973) and clearance from the circulation by the mononuclear phagocytic system (Mannik et al 1971) seem the most important. Complexes which have a large lattice size or fix complement are cleared more readily by the R-E system (Mannik et al 1971) and in animal models saturation of the R-E system by pre-formed IC increases vascular IC deposition (Haakenstad and Mannik 1974). IgG containing IC are thought to be cleared by attachment of the Fc portion of the immunoglobulin molecule to Fc receptors present on Kuppfer cells and splenic macrophages, while IgM complexes, after fixing complement are cleared by C3b receptors on Kuppfer cells. Although it is known that neutrophils have IgA receptors, monocytes do not (Lawrence et al 1975), and a mechanism for removal of IgA class IC from the circulation is still not clear. IgA polymers and IgA class IC can activate the alternate complement pathway (Götze and Müller-Eberhard 1971) and it is therefore possible that C3b receptors are involved. In the rat, IgA polymers are removed from the circulation by receptors (secretory component) on hepatocytes (Fisher et al 1979).

Recently, methods for assessing Fc receptor function have become available using immunologically specific techniques (Atkinson and Frank 1974; Frank et al 1977). These tests rely on measurement of the clearance *in vivo* of autologous erythrocytes sensitized with immunoglobulin and labelled with a radio-nuclide. Using this sytem Fc specific clearance defects have been demonstrated in SLE (Frank et al 1979; Lockwood et al 1979), the prototype of IC mediated disease (Koffler et al 1971). Earlier studies using the clearance of aggregated albumin, failed to demonstrate these defects (Frank et al 1979).

In the present study, Fc clearance was measured in patients with IgA GN, MN and lupus nephritis using autologous erythrocytes coated with non-complement fixing anti-D IgG. The importance of selection of controls has recently been highlighted by the demonstration that normal individuals with the HLA B8, DR3 haplotype have defective Fc specific clearance (Lawley et al 1981). Tissue typing was therefore carried out on all controls and patients.

PATIENTS

Eleven normal Rh+ individuals were studied, (8 males, 3 females, age range 20-42 years). Five were HLA B8, DR3 haplotype positive and the DR3 antigen alone was present in one subject. The remaining 5 controls were HLA B8 and DR3 negative. Studies were also performed on 2 Rh+ individuals with previous splenectomies and 2 Rh- individuals. Thirty two Rh+ patients were studied. Details of age, sex, histological diagnosis on renal biopsy, steroid therapy and HLA typing are summarized in Table 5.1 All patients had serum creatinine concentrations of less than 0.25 mmol/L (normal range 0.05 - 0.12 mmol/L). Each of the 10 patients with SLE had biopsy proven renal involve-

PATIENT	DIAGNOSIS	AGE	SEX	STEROIDS mg/day	T 1/2 (min)	CIRCULATING IMMUNE COMPLEXES (ug AHG/ml serum)	HLA TYPING
R.P	N	28	M	-	50	-	A24,28, B14,17,W4,W6 DRI,7
W.W.	N	23	F	-	145	-	A2 B14,W49,W4,W6 DR5,7
G.T.	N	23	M	-	127	-	AW24, B35,39,W6 DR6,4
H.S.	N	37	M	-	80	-	A11,29 B44,40,W4,W6 DRI,2
P.M.	N	27	M	-	85	-	A2,24 B7,W44,W4,W6 DR2,5
A.B.	N	33	M	-	130	-	A1,29 B8,W44,W4,W6 DR3,7
C.N.	N	21	F	-	350	-	A2,B7,18,W6 DR2,3
A.W.	N	29	M	-	80	-	A2,11 B8,W44,W4,W6 DR3,4
A.C.	N	42	M	-	180	-	A1,2 B8,40,W6 DR2,3
D.M.	N	31	M	-	175	-	A1,W24, B8,W35,W6 DR3
S.G.	N	24	F	-	200	-	A1,29 B8,45W6 DR3,4
P.H.	IgAGN	32	M	-	54	0	A2,3 B44,51 DR3,5
D.B.	IgAGN	31	F	-	>350	0	A1,W24 B7,17,W4 DR7
P.C.	IgAGN	30	F	-	55	0	A29,30 B21,15 DR5
E.P.	IgAGN	54	M	-	185	0	A1,2 B51,18 DR2,5
W.C.	IgAGN	49	M	-	>350	0	A2 B7,40,W6 DR2,4
J.P.	IgAGN	51	M	-	120	0	A28,3 BW44,27,W4 DR3,6
I.M.	IgAGN	65	F	-	300	0	A1,BW44,37,W4 DR4,7
D.L.	IgAGN	30	M	-	250	0	A2,B14,W6 DR4,7
R.P.	IgAGN & Cirrhosis	58	M	-	90	12	A2,W23, B7,W44,W4 DR3-ve multi-reactive
P.F.	HSP	28	M	-	162	0	A2,3 B39,W44,W4,W6 DR1
F.T.	HSP	59	M	60	>350	0	A3,32 B7,47 DR2,6
N.R.	MN	33	M	-	74	0	A2,24 B12,40 DR4
A.F.	MN	51	M	-	72	0	A1 B8,17 DR3
E.J.	MN	58	F	-	280	0	A1,B8,7 DR3,4
D.K.	MN	56	M	-	170	0	A2,11 B7,51 DR7,1
R.S.	MN	55	M	-	90	0	A2 B7 DR6
A.P.	MN	39	F	-	195	0	A2 B18,13,W4,W6 DR3,7
S.N.	MN	73	M	-	295	0	A2,28 B5 DR5,7
K.S.	MN	54	M	-	>350	33	A1,2 B8,40,W6 DR3,6
S.K.	2^{O}MN	74	M	-	130	0	A2,28 BW44,14,W4 DR4,3
M.H.	MN	52	M	-	170	130	A25,32, B44,27,W4 DR3,7
J.B.	MN	64	M	-	125	0	A2,3 B7,W35,W6 DR3,7
S.E.	SLE (Active)	32	F	-	>350	37	A3,28 B7,W22,W6 DR4,7
H.W.	SLE (Inactive)	27	F	5	>350	180	A1,11 B7,8,W6 DR3,7
S.C.	SLE	29	F	-	50	0	A32 B7,8 DR1
J.S.	SLE (Active)	28	F	12.5	>350	0	A2,W33 B15,16,W4,W6 DR2,4
Mich.M	SLE (Inactive)	22	F	12.5	220	30	A1,11 B35,8,W6 DR1,3
C.B.	SLE (Active)	19	F	70	>350	70	A1,2 B8,W44,W4,W6 DR3,7
M.M.	SLE (Inactive)	27	F	5	150	22	A1,2 BW50,W51,W6,DR3
I.O.	SLE (Active)	26	F	15	>350	64	A2,24 B7,18,W6 DR2,4
M.W.	SLE (Active)	19	F	-	>350	230	A2,29 BW44,27,W4 DR4,7
G.K.	SLE (Inactive)	25	M	-	180	0	A2,29 B7,W44,W4,W6 DR5,7

TABLE 5.1 Patient and control (N) data for Fc clearance studies.

ment and disease activity was assessed clinically and with reference to appropriate laboratory tests including DNA binding and serum complement concentrations.

METHODS

IC Assay

Circulating IC were measured in the solid phase CIq radioimmunoassay (Methods, Chapter 2).

Serum IgA Levels

These were measured by laser nephelometry (Hyland) in 10 patients with IgA GN or HSP.

Tissue Typing

(Performed by Dr. Judith Hay, Blood Transfusion Service, Australian Red Cross Society, Adelaide).

HLA, A, B and DR typing was performed on all patients by standard micro-lymphocytotoxicity testing (Johnson et al 1980).

Fc Specific R-E Clearance

Sixteen ml of venous blood was drawn into a syringe containing 4 ml of acid citrate-dextrose. The volume of blood containing 5 ml of erythrocytes was centrifuged at 900 G for 5 minutes. These packed cells were incubated at 900 G for 5 minutes. These packed cells were incubated with shaking at room temperature with 10 μCi^{51} Cr for 15 minutes. The cells were washed once in normal saline and incubated with shaking at 37°C for 30 minutes with an equal volume of 1:9 dilution in normal saline of anti-D immunoglobulin (CSL anti-D, 125 µg specific anti-D). After washing twice with normal saline the cell volume was adjusted to 15 ml with saline. All incubations and washings were carried out in the original syringe with centrifugation performed using a specially adapted syringe

holder to ensure complete sterility throughout the procedure. Ten ml of the chromium labelled, IgG sensitized cells were injected into the ante-cubital vein over 60 seconds and serial blood samples at 3, 10, 20, 40, 60, 90, 120 and 180 minutes after injection were taken from the contra-lateral arm, via an indwelling venous catheter. Blood samples were lysed and the radioactivity per unit volume of blood measured overnight in a gamma counter. Clearance curves were calculated by expressing the counts as a percentage of the 3 minute values. The half-life ($T\frac{1}{2}$) of the injected cells was then calculated (Fig. 5.1).

In vitro anti-D binding experiments

Blood was taken from 3 control subjects and 2 patients with SLE. Under the same conditions described for the in vivo clearance studies, the same relative packed cell volumes were incubated with the same relative amounts and dilution of ^{125}I labelled anti-D. Using the specific activity of ^{125}I anti-D, red cell number and radioactivity of the cell pellet after washing, the number of antibody molecules per erythrocyte could be calculated.

Statistical Analysis

Students t test was used for statistical analysis unless otherwise stated.

RESULTS

In vitro Anti-D binding experiments (Table 5.2)

Differences in clearance rates were not related to differences in the amount of antibody attached to erythrocytes. In addition, in vivo clearance studies in normal subjects showed no correlation between Rh phenotypes and clearance times (different Rh phenotypes have different binding characteristics for

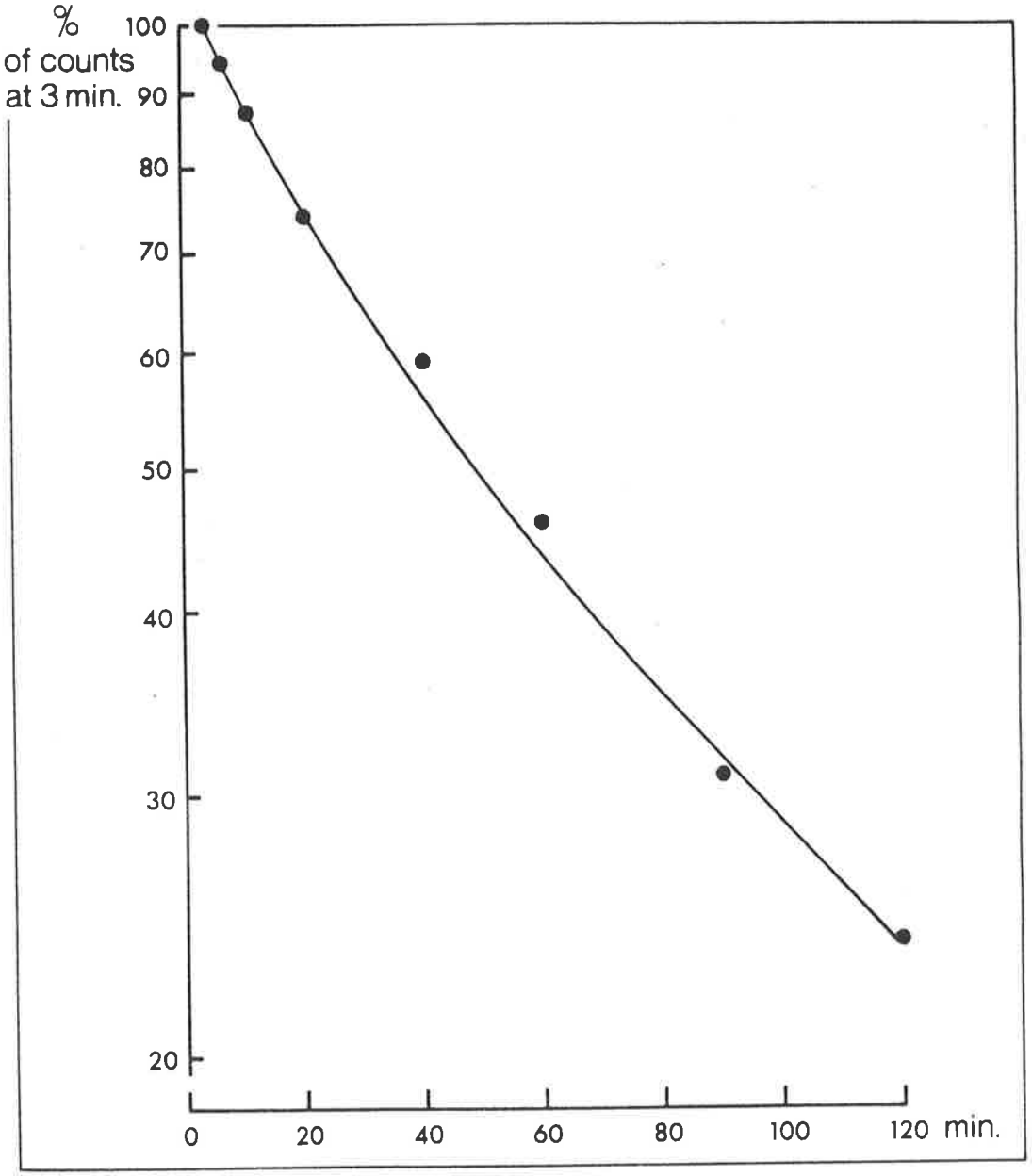

FIGURE 5.1 Example of exponential decline of radioactivity in serial blood samples from a normal (DR3/B8-) control following injection of Cr^{51} labelled, IgG sensitized erythrocytes.

anti-D (Morley 1978)).

A.			B.			% DIFFERENCE IN NUMBER OF ANTI-BODY MOLECULES PER RBC BETWEEN A & B
PATIENT	HLA	T½(MINS)	PATIENT	HLA	T½(MINS)	
N_1	B8,DR3	80	N_2	B8,DR3	130	6
N_1	B8,DR3	80	N_3	DR3	>350	3
N_1	B8,DR3	80	S.E.(SLE)	B8,DR3	>350	7
S.E.(SLE)	B8,DR3	>350	M.M.(SLE)	DR3	150	3

TABLE 5.2 In vitro antibody binding experiments

$N_{1,2,3}$ = normal controls.

Fc specific R-E clearance

Rh- and splenectomized patients

Zero clearance was found in both these groups of patients.

Normal controls (Fig. 5.2)

A significant difference ($p < 0.05$) was present between the mean T½ of controls with the HLA B8 and/or DR3 haplotype and B8, DR3 negative subjects. The mean T½ of subjects without HLA B8, DR3 antigens was 90 ± 38 minutes. The upper 95% confidence limit was 173 minutes and this was used as the upper limit of normal (Fig. 5.3).

Systemic lupus erythematosus

Eight of 10 patients with SLE had T½ greater than the upper 95% confidence limit of the control group (Fig. 5.3). It is of interest that the patient with lowest T½ (S.C. T½ 50) had splenomegaly. There was no correlation between steroid therapy and T½.

Patients with less active clinical disease tended to have

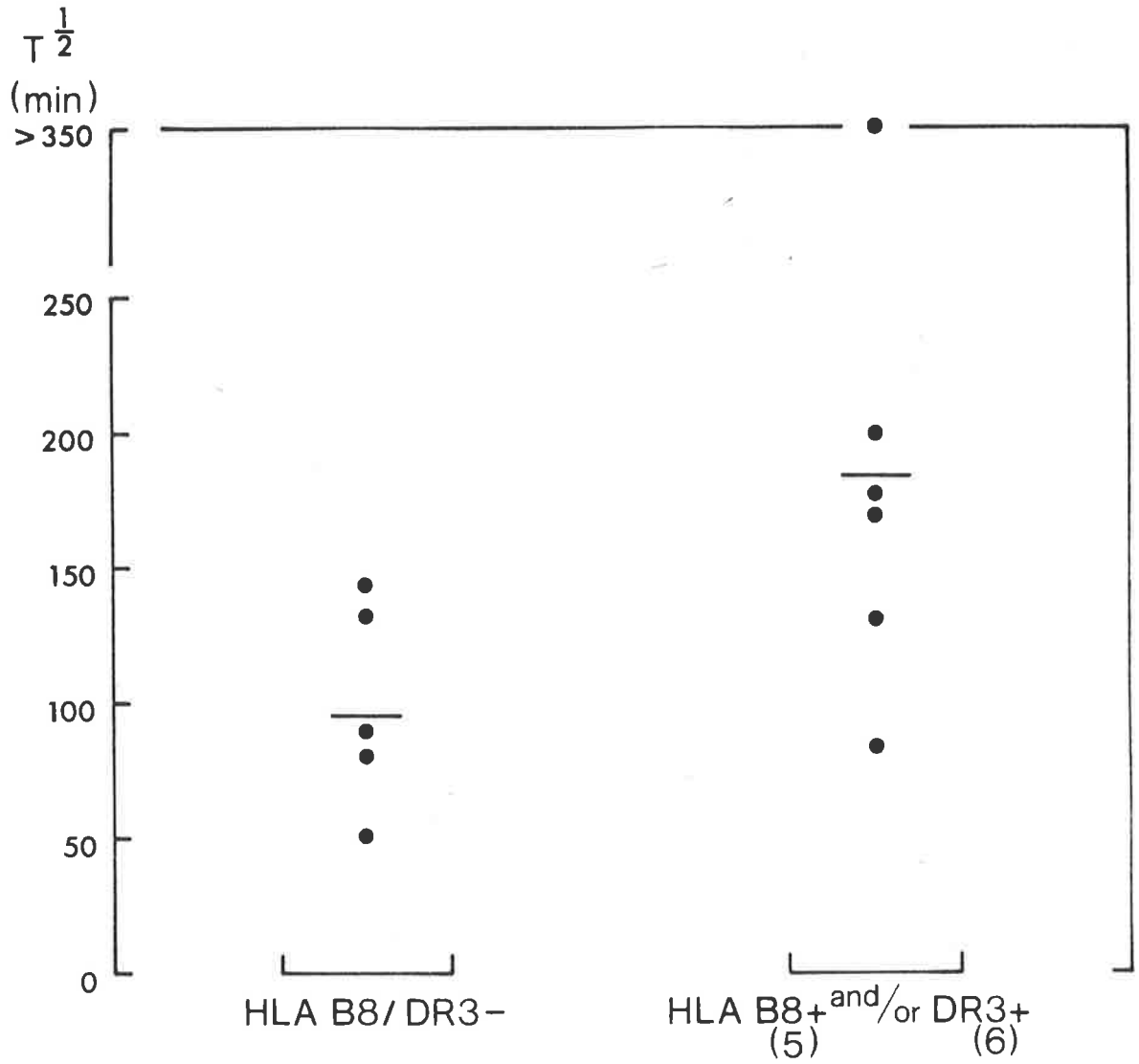

FIGURE 5.2 $T_{\frac{1}{2}}$ in normal controls with and without HLA B8 and/or DR3 antigens.

lower $T\frac{1}{2}$ values and $T\frac{1}{2}$ values correlated with the level of circulating IC ($p < 0.05$ by Spearman's rank correlation coefficient adapted for tied values) (Fig. 5.4). No correlation was found between the presence of B8 and/or DR3 and $T\frac{1}{2}$.

IgA nephropathy

Five of 8 patients with primary IgA GN demonstrated abnormal clearance (Fig. 5.3). Splenomegaly was present in one patient (R.P.) who had IgA GN secondary to alcoholic cirrhosis. In this patient $T\frac{1}{2}$ was within the normal range.

One of 2 patients with HSP and mesangial IgA deposits showed a marked clearance defect. This patient was receiving prednisolone therapy at the time of testing.

No correlation was found between serum IgA levels, circulating IC or B8 and/or DR3 positivity and $T\frac{1}{2}$ values in patients with IgA GN or HSP.

Membranous nephropathy

Four of 10 patients with primary MN had delayed clearance, although the abnormalities were not as great as those in patients with SLE or IgA GN (Fig. 5.3). One patient (S.K.) possessing the DR3 antigen, with MN secondary to gold therapy for rheumatoid arthritis had a normal clearance value. HLA B8 and/or DR3 was present in 7 MN patients but there was no correlation between $T\frac{1}{2}$ and these antigens. Similarly, no correlation was observed between circulating IC and clearance rates.

DISCUSSION

This study of Fc specific R-E clearance in patients with SLE nephritis and IC mediated GN confirms the previously reported pronounced clearance defects in SLE (Frank et al 1979) and has demonstrated a new finding of defective clearance in MN and mesangial IgA GN. Clearance defects were more frequent

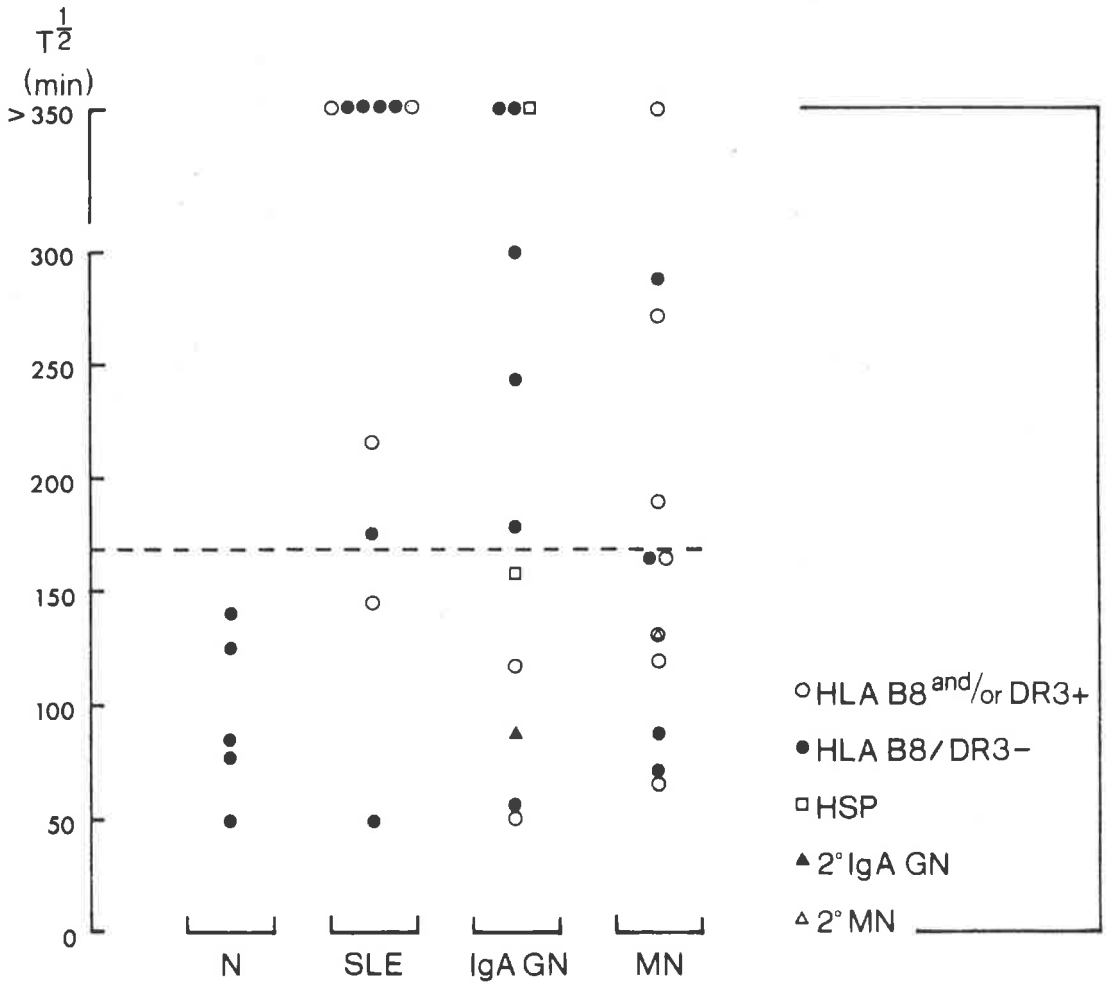

FIGURE 5.3 Fc specific clearance ($T\frac{1}{2}$) in patients with SLE, IgA GN, MN and HLA B8/DR3 negative controls. Broken line indicates upper 95% confidence limit for control group.

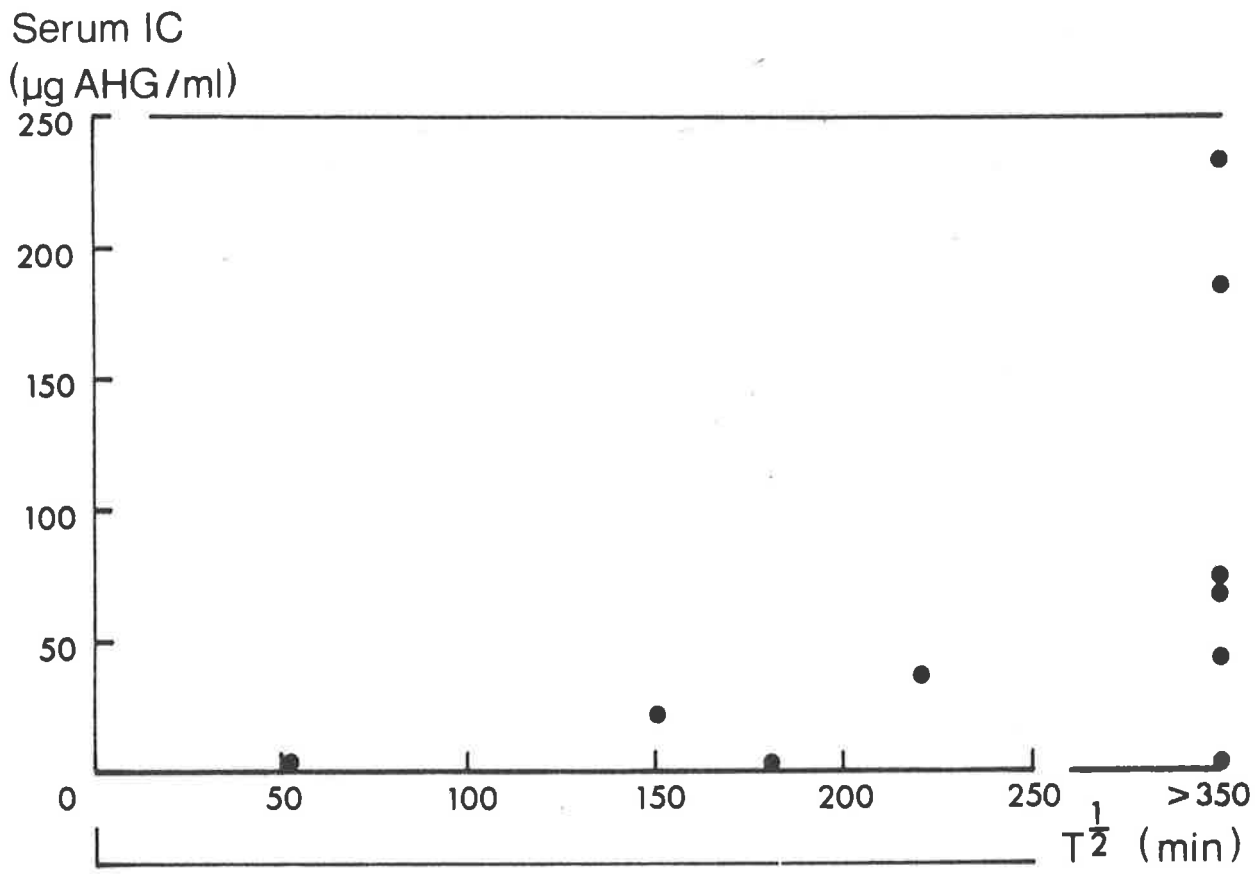

FIGURE 5.4 Correlation between serum IC concentration and $T_{\frac{1}{2}}$ in patients with SLE.

(6/11) and more profound ($T\frac{1}{2}$ > 300 minutes in 4 cases) in diseases associated with mesangial IgA deposition than those present in patients with MN (4/11 delayed clearance; $T\frac{1}{2}$ > 300 minutes in 1 case). Differences in absolute $T\frac{1}{2}$ times between the present study and previously reported studies (Frank et al 1979; Lockwood et al 1979) probably reflect the use of different preparations and concentrations of anti-D IgG. Similar considerations may explain in part why earlier studies (Solomon et al 1981) failed to find Fc specific clearance defects in mesangial proliferative GN and MN.

In contrast to patients with SLE, there was no correlation in the present study between clearance times and circulating IC levels in patients with MN, IgA GN or HSP. In fact, many of these patients did not have detectable circulating IC. This suggests that in these diseases defective clearance is not due to saturation of Fc receptors by IC.

Although MN has been thought to be mediated by circulating IC it has been difficult to demonstrate complexes in the sera of patients with this disease (Woodroffe et al 1977; Ooi et al 1977). Recently, an hypothesis has been proposed which suggests that glomerular IC are formed in situ (Couser and Salant 1980). This is yet to be confirmed in human MN. In addition, there is evidence to suggest that IgG with a high isoelectric point (> 9.5) can bind to anionic sites on the GBM (Batsford 1980). Defective Fc mediated clearance of such IgG could facilitate its glomerular deposition.

IgG Fc specific clearance was often defective in our patients with IgA GN. Wilton (1978) has demonstrated that both serum and secretory IgA inhibit neutrophil phagocytosis of candida albicans blastospores (CA) coated with specific IgG

antibody. The effect is apparently mediated by a functional inhibition of the binding of CA via Fc receptors for IgG. Perhaps the IgA class IC (Lesavre et al 1982) (which may not be detected by CIq assays) or polymeric IgA (Lopez Trascasca et al 1980) that have been associated with IgA GN impair the IgG Fc receptor function of the mononuclear phagocytic system.

A recent report (Lawley et al 1931) has demonstrated defective Fc specific clearance in normal individuals with the HLA B8, DR3 haplotype. Additional immunological studies of the lymphocytes from these subjects demonstrated decreased percentages and total numbers of T cells bearing Fc receptors for IgG. The authors postulated that a generalized genetically linked Fc receptor defect may exist in these people. The present study confirmed a difference between clearance rates in normal individuals with and without the HLA B8, DR3 haplotype. However, it was surprising that in our patients with MN only 3 out of 7 with HLA B8, DR3 or the DR3 antigen alone had defective clearance. Additionally, 1 patient without this haplotype had delayed clearance. The 2 patients with IgA GN and the DR3 antigen had normal clearance times. Indeed, none of the patients with primary IgA GN and defective clearance had B8 or DR3 antigens or circulating IC. These findings indicate that whilst the B8, DR3 haplotype and levels of IC are of importance in considering defective Fc specific R-E function, other unknown factors may play an equally important role. It is not clear whether clearance defects in patients with IgA GN or MN are primary or secondary events. A study of Fc specific clearance in members of the families of such patients may help to answer this question.

Finally, the question of improving R-E function needs to be addressed. Lockwood et al (1979) has shown that plasma - pheresis is effective in unblocking R-E function in patients with vasculitis and/or nephritis . Barcelli (1981) demonstrated reduced glomerular IC deposition in a mouse serum sickness model following immunostimulation of the R-E system with Corynebacterium parvum and zymosan stimulation of the mononuclear phagocytic system in the rat has been shown to increase the clearance of aggregated human IgG and protect the glomeruli from aggregate deposition (Raij et al 1981). However, specific stimulation of the R-E system has not been attempted in humans with defective Fc clearance.

SUMMARY

Fc specific R-E clearance was determined in control subjects (11) and in patients with IC GN (22) and lupus nephritis (10). Clearance ($T\frac{1}{2}$) depended on the removal of autologous erythrocytes labelled with ^{51}Cr and sensitized with anti-D IgG by fixed splenic macrophages bearing receptors for the Fc portion of the IgG molecule. A significant difference in clearance rates was demonstrated between normal individuals with and without the HLA B8, DR3 haplotype. Marked clearance defects were found in SLE (8/10) and $T\frac{1}{2}$ correlated with the levels of circulating IC. Delayed clearance was also observed in 6/11 patients with IgA GN or HSP and in 4/11 patients with MN. No correlation was found between circulating IC levels and $T\frac{1}{2}$ in these diseases. Clearance defects in these patients did not correlate with the presence of the B8, DR3 haplotype. This study demonstrates that some patients with IC GN have defective Fc mediated clearance which does not appear to be secondary to immune complex blockade and suggests the possib-

ility of a primary Fc receptor defect. This could impair the normal sequestration of IC and facilitate their glomerular deposition.

CONCLUSION

This study had demonstrated multiple defects of the host immune response in individuals with chronic IC GN.

In particular, similar disturbances of certain regulatory mechanisms governing *in vitro* immunoglobulin production by peripheral blood lymphocytes were found in patients with MN, IgA GN (and HSP) and lupus nephritis. It is proposed, on the basis of data from this thesis and work by others, that defective immune suppression exists in individuals who develop IC GN and that, as in SLE, auto-antibodies are produced with the consequent generation of nephritogenic IC. Lupus nephritis, MN and IgA GN may therefore be part of the same spectrum of genetically based, auto-immune disorders, with the varying nature of the glomerular lesions in these diseases depending on whether IC and formed *in situ* or deposited from the circulation, and on the class and subtype of the antibody involved in the complex.

In addition there appears to be a partial failure of IgG Fc receptors of the R-E system to remove circulating IC. Although in SLE this defect may be due to saturation by IC, the lack of correlation between circulating IC levels in patients with MN and IgA GN suggests a primary abnormality of Fc receptor function. This defect appears to be shared by normal individuals with the HLA B8 and /or DR3 antigens.

It is not clear however, whether the observed host defects of immune regulation are primary or are in fact, induced by the disease state as secondary phenomena.

Logical extensions of the research reported in this thesis would include the following:

1) a study of helper/suppressor T cell ratios, suppressor cell activity and Fc specific clearance in family members of individuals with IC GN to examine the question of primary versus secondary host defects.

2) serial studies of patients with IC GN to establish the relationship of immune defects to the clinical expression of the disease. This may also provide some insight into the question above.

3) a study of cell types involved in the actual tissue lesions of human IC GN, especially in those diseases with a tubulo-interstitial component.

4) more sophisticated in vitro cell studies using FACS separation of cell subpopulations from patients with IC GN to more clearly define abnormal function of regulatory T cell subsets.

5) examination of the role of abnormalities of fine tuning of the immune system, e.g. idiotypic networks and interleukin production in human IC GN.

6) studies of the effects of new drugs such as cyclosporin A, specific anti-T cell subset monoclonal antibodies and prostaglandin E_1 on glomerular lesions and tissue and peripheral T cell subsets in animal models of nephritis, in an attempt to restore a balance of immune help and suppression.

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