Complement Activation and Regulation in Human Glomerulonephritis

by

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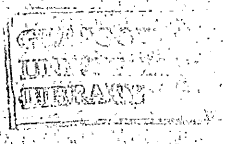

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The following publications contain work described in this thesis:

1. Evidence of glomerular modulation of complement activation. Moseley, H.L., Whaley, K.: Journal of Clinical and Laboratory Immunology, 2: 9-13, 1979.

2. Intraglomerular modulation of complement activation. Moseley, H.L., Whaley, K. In Weissman, G., Samuelsson, B., Paoletti, R. (editors): Advances in Inflammation Research. Volume I, New York, Raven Press, pp 303-310, 1979.

3. Control of complement activation in membranous and membranoproliferative glomerulonephritis. Moseley, H.L., Whaley, K.: Kidney International, 17: 535-544, 1980.

SUMMARY

This study was designed to examine closely the extent and means of activation of the complement system in human GN. In order to do this, renal biopsy material was obtained from 104 patients. Each of these biopsies fell into one of the following distinct pathological categories of glomerulonephritis (GN); membranous GN (MGN), membranoproliferative GN (MPGN), focal GN (GN), Henoch-Schönlein nephritis (HSN), systemic lupus erythematosus (SLE) and minimal change nephrotic syndrome (MCNS). The biopsies were examined for the presence of immunoglobulins, classical and alternative pathway components, C3 and C5 and the serum concentrations of the complement proteins, at the time of the biopsy, were also measured.

There was evidence of extensive complement activation in all disease groups with the exception of MCNS and deposition of C3 was apparent in all MPGN and SLE biopsies and at least 89% of MGN, FGN and HSN biopsies.

The evidence presented in this study suggests that, in MGN and SLE, the complement system was activated primarily by immune complexes involving IgG antibodies via the classical pathway since more biopsies were positive for classical than alternative pathway components and there were significant correlations between the intensities of deposition of C3 and

IgG. Properdin deposition was frequently present in SLE and was also found in a small number of MGN biopsies suggesting that activation of the alternative pathway may also be involved in these two diseases. The intensity of activation was greater in SLE than in MGN with high intensities of deposition and reduced serum concentrations of complement proteins.

In MPGN, FGN and HSN both pathways of activation were involved and although in MPGN and HSN more biopsies were positive for alternative rather than classical pathway components and in FGN the converse was true, correlation studies, comparing the intensities of deposition of classical and alternative pathway components with those of C3, were unable to distinguish which pathway was primarily responsible for C3 catabolism.

MCNS was included in this study to represent a control group and evidence of activation of complement was found, only weakly, in two biopsies.

Activation of the complement system is known to be under the control of several plasma proteins and the role of these proteins had not been previously assessed in GN. It was considered possible that the complement activation apparent in almost all types of GN and intense in some may have been the result of a breakdown in the mechanism for controlling complement activation. In order to examine this possibility,

the deposition of three control proteins of complement activation, $C\bar{1}$-inhibitor ($C\bar{1}$-INH), C3b inactivator (C3bINA) and β1H globulin was studied and the serum concentrations of these proteins measured.

The serum concentrations of control proteins were seldom reduced with low serum levels of C3bINA in the serum from four patients and low β1H levels in the serum from only one patient and although the functional activity of the proteins was not measured there is no evidence to suggest that complement activation was in general due to an acquired or a genetic deficiency of control proteins. Also the significant correlations between concentrations of C1s and $C\bar{1}$-INH in FGN and between both C3bINA and β1H and C3 in MPGN and SLE suggests that activation in the circulation was under the control of these proteins in these disease groups.

There is strong evidence to suggest that β1H was present in the tissues in response to deposition of C3 and as such was regulating the activities of C3b; β1H was present in 94% of C3 positive biopsies and was never found in the absence of C3, the intensities of deposition of C3 correlated with those of β1H in MGN, MPGN and FGN and the distribution patterns of these two proteins in the glomeruli were very similar. β1H was present even in the kidneys of three MPGN patients with circulating nephritic factor.

The other control protein of C3b, C3bINA, was present in

only 11 biopsies and therefore this study offers little evidence for the role of C3bINA in the regulation of C3b in GN. Absence of C3bINA need not mean a breakdown in regulation since the mode of action of C3bINA is to enzymatically inactivate C3b.

The role of $C\bar{1}$-INH is harder to assess since the concordance between biopsies positive for C1s and $C\bar{1}$-INH was generally not close and a significant correlation between the intensities of these two proteins was only found in MPGN. The distribution patterns of C1s and $C\bar{1}$-INH, however, where both were found together, were very similar and it is possible that where C1s and $C\bar{1}$-INH were present in the same biopsies that $C\bar{1}$-INH was acting as a regulator of complement activation and, where C1-INH was found alone, it may be acting as a regulator in one of the other humeral mediator systems in which it is known to be involved. Other possibilities have been suggested.

From the results presented in this study there is therefore little evidence to suggest that activation of the complement system in human GN is the result of a deficiency in regulation of complement.

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ABBREVIATIONS

GN	Glomerulonephritis
PAF	Platelet activating factor
MGN	Membranous glomerulonephritis
MCNS	Minimal change nephrotic syndrome
GBM	Glomerular basement membrane
NTN	Nephrotoxic nephritis
PMN	Polymorphonuclear leucocyte
FGN	Focal glomerulonephritis
SLE	Systemic lupus erythematosus
BSA	Bovine serum albumin
CVF	Cobra venom factor
RES	Reticuloendothelial system
MIF	Migration inhibition factor
C3bINA	C3b inactivator
C1-INH	C1-inhibitor
C4-bp	C4 binding protein
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FITC	Fluorescein isothiocyanate
NF	Nephritic factor
MPGN	Membranoproliferative glomerulonephritis
HSN	Henoch-Schönlein nephritis

INTRODUCTION

Glomerulonephritis

Glomerulonephritis (GN) is a disease involving a destructive inflammatory lesion of the glomerulus which is often accompanied by tubular, vascular and interstitial abnormalities (1). Classification was initially based primarily on clinical findings with histopathological information being available only following post mortem examination (2-5). After the introduction of the percutaneous renal biopsy technique (6), it became possible to employ histological criteria. Now morphological and immunological data are combined to give a more useful classification.

It is generally believed that immunological mechanisms play a major role in the pathogenesis of most types of GN. This belief was based originally on the fact that GN often followed an infection. More recently, the use of various immunological techniques has confirmed the involvement of immunological mechanisms in most types of GN.

In order to understand immunologically-mediated glomerular injury, it is important to understand how immunological processes produce injury. Gell and Coombs (7) have divided the specific immunological mechanisms into four categories of hypersensitivity, designated types I to IV and these will be considered in turn with particular reference

to the part each plays in the pathogenesis of GN.

Type I Hypersensitivity

In type I hypersensitivity, reaginic antibodies bind by their Fc regions to specific receptors on the membranes of basophils or mast cells. In man, these antibodies are mainly of the IgE class although IgG$_4$ antibodies may also be involved (7, 8). When antigens come into contact with their specific cell-bound antibodies, immune complexes form on the surface of the cell causing membrane changes and degranulation, with release of vasoactive substances, particularly histamine. In addition, platelet activating factor (PAF) is released which causes platelet clumping and subsequent release from the platelets of vasoactive amines (9, 10). Vasoactive amines cause contraction of smooth muscle and increased capillary permeability.

There are several reports of IgE deposition in the kidneys of patients with GN. Nagia (11) reported deposition of IgE, IgG and C3 in a patient with membranous glomerulonephritis (MGN) and asthma and significant amounts of IgE were found in 22 of 146 renal biopsies from patients with various diagnoses (12). The significance of IgE, in the kidney, where mast cells are seldom present, is uncertain but may indicate that an IgE antibody response has taken place.

It has been suggested that IgE antibody may play an important role in the deposition of immune complexes in GN (12, 13). The mechanism proposed is that antigens complex with IgE antibody on the membranes of circulating basophils and vasoactive amines are released causing increased capillary permeability and thus aiding deposition of immune complexes within the kidney. It is therefore possible that a type I hypersensitivity response may predispose to immune complex deposition. The antigen deposited may be that involved in the type I reaction or some other unrelated antigen.

An association has been noted between minimal change nephrotic syndrome (MCNS) and type I reactions in several patients (13, 14, 15), and deposition of IgE in MCNS was found in one study (13). Other workers failed to substantiate these findings (12, 16, 17). The frequent absence of immunoglobulins in the glomeruli (18) suggests that this disease is not caused by the deposition of immune complexes. It is possible however that type I hypersensitivity may play some role in the pathogenesis of MCNS, perhaps by increasing capillary permeability.

Type II Hypersensitivity

Type II hypersensitivity, or antibody mediated hypersensitivity involves the complexing of antibodies with

cellular or tissue-bound antigens. The antibodies are usually of the IgG and IgM classes and damage may result in one of three ways; a) from contact between the antibody-coated cell and a phagocytic cell via an Fc receptor, b) by direct activation of the complement system by the binding of C1q to the immune complex on the cell surface with all the biological consequences involved in complement activation, as will be discussed later, or c) by antibody-dependent cell-mediated cytotoxicity. The last mechanism involves the K cell which is a subclass of lymphocyte with the capacity to lyse antibody-coated cells by an, as yet, unknown mechanism (19).

Anti-glomerular basement membrane disease (anti-GBM disease) is known to involve a type II hypersensitivity reaction. In this, antibodies are formed with specificity for the glomerular basement membrane. They may cross-react with lung basement membrane (20, 21) and tubular basement membrane (21). The association between anti-GBM disease and the HLA antigen, DRW2 (22) suggests that genetic factors may be involved.

Various initiating mechanisms have been implicated in this antibody production; 1) streptococcal antigens share certain antigenic determinants with both lung and kidney basement membrane (23). Antibodies formed to

streptococcal antigens during an infection could therefore react against basement membrane and result in the development of an autoimmune disease. Evidence to support this is that the disease may occur in association with a raised anti-streptolysin-O titre (24), 2) anti-GBM disease has also been reported following an influenza A2 infection (25). In most cases there is no evidence of infection, 3) an association between hydrocarbon exposure and anti-GBM disease was noted in six of eight patients in one study (26) and rats made to inhale benzene developed a disease similar clinically and morphologically to anti-GBM disease (27). It has been suggested that an agent which damages lung tissue may cause the release of altered self-antigens and initiate antibody production to lung and kidney basement membrane (26), 4) glomerular basement membrane antigens are present in normal serum and urine and rabbits injected with GBM preparations from autologous urine develop anti-GBM disease (28). It has been proposed that these basement membrane fragments may be recognised as foreign perhaps during a phase of generally heightened immunological response due to an infection.

Plasmapheresis of patients with this disease over a period of time, with immunosuppression, results in the removal of antibodies (29) and the cessation of antibody

production. This suggests that the disease is self limiting, and favours the hypothesis that it is caused by damage to lung or kidney producing self-antigens. Once the lung or kidney lesion heals, the antigenic stimulus for antibody production is lost and the kidneys are not damaged further. Further evidence for the self-limiting nature of the disease lies in the fact that, after nephrectomy, the antibody titre declines slowly and, if transplantation takes place after the elimination of the antibody, recurrence of the disease is rare (21).

Once the antibody has bound to the basement membrane, all the consequences of a type II reaction occur and damage may be seen to involve infiltration of inflammatory cells, proliferation of glomerular cells, shattering and corrugation of the basement membrane (1). In rabbits, where the experimental disease is called nephrotoxic nephritis (NTN), damage may also be demonstrated by an increase of basement membrane fragments in the urine (30).

The importance of the complement system in this disease is shown when rabbits and rats, in which NTN is induced, are depleted of complement (31). The infiltration of polymorphonuclear leucocytes (PMN) is reduced and the pathological lesion is much less severe. When PMN are depleted, the results are the same although antibody deposition occurs

normally (32).

Certain anti-GBM antibodies, which do not activate complement, such as avian antibodies, are still capable of producing limited proteinuria in rats (33). Also, in the heterologous phase of guinea-pig NTN, it has been shown that proteinuria is independent of both complement activation and the actions of PMN and very little C3 is found deposited in the glomerulus (34). Some other mechanism for producing damage must therefore exist. A mononuclear cell from the bone marrow has been implicated in the pathogenesis of NTN in rats (36). If rats are depleted of bone marrow cells by irradiation before induction of the disease, glomerular hypercellularity is decreased and proteinuria is reduced compared with control animals with NTN although antibody is still present in the kidney. The effect is not altered by decomplementing the animals.

In the majority of patients with anti-GBM disease deposition of complement components occurs and the complement system is likely to play a role in pathogenesis. In some patients, however, complement components are not deposited (21, 35). Some other mechanism, perhaps involving mononuclear cells must therefore be responsible for the damage.

Six days following the induction of NTN in rabbits, fibrin deposition is found round the capillary loops and within Bowman's space. Cells of Bowman's capsule proliferate and infiltrate the fibrin resulting in crescent formation. When coagulation was prevented by the administration of arvin (37) and warfarin (38), no fibrin was deposited, fewer crescents formed and proliferation of the cells of Bowman's capsule was much reduced although immunoglobulin deposition was unaffected (38).

Thus at least two interrelated humoral effector systems, the complement and coagulation systems, and perhaps also the participation of a mononuclear cell influence the pathogenesis of experimental anti-GBM disease. Anti-GBM disease, as reported, comprises only a small proportion of GN patients (20, 21).

There is some evidence to suggest that a type II hypersensitivity reaction may be involved in the pathogenesis of focal glomerulonephritis (FGN) since IgA eluted from the kidney of an FGN patient was found to bind weakly to mesangial cells in normal kidney sections (39). This is discussed in more detail in the introduction to chapter 5.

Some types of GN, originally thought to involve the deposition of immune complexes formed in the circulation, may in fact be due to the binding of antigens, particularly at

the subepithelial aspect of the basement membrane and the subsequent formation of immune complexes in situ (40). This is based on the fact that the preformed immune complexes cannot be made to cross the basement membrane in experimental GN, potentially nephritogenic antigens have been detected in a subepithelial position in normal rats and workers have frequently failed to detect circulating immune complexes in diseases with glomerular subepithelial deposits particularly in membranous glomerulonephritis (MGN).

DNA is known to have a strong affinity for glomerular basement membrane (40a) and it is possible that DNA binds to basement membrane and immune complexes form in situ. The significance of this mechanism in the GN of SLE is not known but it may be important in those patients with an MGN-like lesion.

It has been shown that rat glomeruli contain renal tubular antigens in a subepithelial position and that antibody to these antigens may combine with the antigen at the basement membrane to form an MGN-type lesion (40). In this way it is possible that other antigens may bind to the basement membrane and, after induction of an antibody response, GN may develop.

Factors which may affect the binding of antigens to

basement membrane are 1) the size of the antigen, 2) the charge on the antigen and 3) factors which may alter the charge of the basement membrane.

Type III Hypersensitivity

This involves the formation of antigen/ antibody immune complexes either in the walls of blood vessels, particularly where there is antibody excess; this is called an Arthus reaction, or the formation, in the circulation, of immune complexes which may subsequently deposit in the tissues.

When immune complexes deposit in the kidney, they may cause damage in various ways; 1) the physical presence of the complexes in the membrane may disrupt the integrity of the membrane, 2) immune complexes may initiate cellular proliferation and/ or increased basement membrane production or 3) complement activation may attract inflammatory and phagocytic cells to the site of deposition with resultant damage.

Most types of GN are believed to involve type III hypersensitivity but evidence based on the detection of circulating immune complexes or the localisation of antigen along with immunoglobulin in the kidney has been found only in isolated cases.

There are a number of occasions when antigens have been found. The nephritis of SLE is known to involve the

formation of antibodies to DNA and other nuclear antigens (41). Acid eluates of kidneys from patients with SLE have been found to contain higher concentrations of DNA antibodies than was found in the serum and immunofluorescence studies using purified anti-DNA have demonstrated the presence of DNA along the capillary loops of the glomeruli along with immunoglobulins. There is therefore little doubt that SLE nephritis involves the deposition of immune complexes. Whether this is the only pathogenic mechanism is unclear and the possibility of a type II involvement has already been discussed.

Table I.1 shows a list of some antigens known to be involved in immune complex GN either because they have been demonstrated in the kidney or because specific antibody has been eluted from the kidney. Many other antigens have been implicated but not, as yet, proved to be involved.

Characterization of the antigens would prove useful for two reasons: 1) elimination of the antigen could be attempted or 2) if a pure non-toxic form of the antigen could be prepared, this could be injected into the subject in large amounts changing the ratio of antigen to antibody and causing the dissolution of the deposited complex. Using the chronic serum sickness model of GN in rabbits, a huge excess of antigen was shown to terminate antibody

Table 1.1: Antigens known to be involved in immune complex glomerulonephritis

ANTIGENS	REFERENCE	RENAL MANIFESTATIONS
EXOGENOUS		
BACTERIA:-		
STREPTOCOCCI	42	acute GN
STAPHYLOCOCCI	43	not described
SALMONELLA	44	FGN
ENTEROCOCCI	45	FGN
PNEUMOCOCCI	46	acute GN
TREPONEMA	47	MGN
VIRUSES:-		
HEPATITIS B	48	MGN
MEASLES	49	normal
ONCORNAVIRUS	50	normal
PROTOZOA:-		
PLASMODIUM	51	various
TOXOPLASMA	52	acute GN
ENDOGENOUS		
NUCLEAR ANTIGENS	53	various
TUMOUR ANTIGENS	54	MGN
RENAL TUBULAR ANTIGEN	55	MGN

production and shortened the half life of the antigen in the intraglomerular complexes (56).

It is probable that, while numerous and diverse stimuli cause GN, the host's immune response is an important factor in deciding whether GN will develop and what form it will take. Two facts support this; a) a single type of GN may develop as the result of various antigenic stimuli, for example, MGN may involve treponemal antigens (47), Australia antigen (48), tumour antigens (54), renal tubular antigens (55) and others and b) the same antigenic stimulus may cause the development of various types of GN. This is seen in the chronic serum sickness model of GN in rabbits where animals given daily doses of bovine serum albumin (BSA) exhibit at least three responses; a) the animal may make no antibody and GN fails to develop, b) it may make much strong affinity antibody with the formation of large immune complexes which are rapidly removed from the circulation and there is no damage to the kidney and c) it may have an intermediate antibody response resulting in immune complexes in antigenic excess which deposit in the kidney (57). The existence of such diversity within a single strain of rabbits suggests that even greater diversity could occur in man.

Much work has been done to establish the mechanism of immune complex deposition in tissues. The size of the immune

complexes formed is probably important in deciding whether deposition will take place. The size of the complex is determined by the antigen/ antibody ratio, the affinity of the antibody for the antigen, the class of antibody, the size of antigen, the number of antigenic sites on the antigen and the interaction of the complex with other factors such as complement and rheumatoid factor. In acute serum sickness in rabbits (58) and guinea-pigs (59), deposition of immune complexes within glomeruli and arteries occurs only where complexes greater than 19S are formed although these complexes are quickly removed from the circulation. When preformed BSA/ anti-BSA complexes of varying sizes were injected into rabbits, only where complexes larger than 19S were administered did deposition take place (60). Other proteins, such as keyhole limpet haemocyanin (59) and aggregated gamma globulin, similarly deposited only where the molecular size was in excess of 19S. Although this indirect evidence suggests strongly that only complexes greater than 19S deposit, no direct evidence is available to substantiate this since the size of intraglomerular complexes cannot be estimated and it is likely that the size of the complexes will alter once they have been deposited.

Evidence suggests that increased vascular permeability may also be necessary for deposition. Colloidal carbon

could be made to deposit in arteries, endocardium and glomeruli of mice by simultaneous injection of various vasoactive substances such as histamine, serotonin and epinephrine or by injection of low levels of immune complexes (61). If vasoactive amines are caused to be released from perivascular mast cells of guinea-pigs, the effect is the same (62). Antagonists of vasoactive amines such as antihistamines may inhibit the deposition of complexes in the vessel walls in acute serum sickness (63). The timing of the histamine administration is critical (59). If histamine is given to guinea-pigs within two minutes of administration of preformed immune complexes, they are deposited within the glomeruli. However, if the complexes are injected more than two minutes prior to histamine administration, deposition is markedly reduced. Since immune complexes larger than 19S are rapidly removed during this period while smaller complexes remain in the circulation, the critical timing supports the suggestion that only complexes larger than 19S may be deposited. If platelets are removed from the circulation, deposition is again suppressed (63).

Therefore increased capillary permeability probably plays a major role in the deposition of complexes in the animal models studied and platelets are likely to be involved

in this mechanism.

Work on the longer term autologous immune complex disease in rats (64) showed that vasoactive amine antagonists had no effect on the deposition of immune complexes after the disease was established. It is possible therefore that increased capillary permeability induced by vasoactive amines is only important at the outset of the disease or in certain types of disease.

The role of complement in the deposition of immune complexes has also been considered. In the acute serum sickness model of GN, the pathogenesis appears to be complement independent. In rabbits, decomplemented by cobra venom factor (CVF), immune complexes are deposited normally and the renal lesion is morphologically identical to that observed in animals which were not decomplemented (65), although necrotising arteritis is not seen in decomplemented animals. Because CVF cannot be used over a prolonged period of time, the role of the complement system in chronic experimental GN cannot be assessed. In most types of human GN, however, there is evidence of complement activation and one of the results of activation is the release of C3a and C5a (66) which may cause increased capillary permeability by triggering the release of vasoactive amines from basophils. The complement system therefore has the

capacity to aid deposition of immune complexes.

Hydrodynamic forces may play a role in immune complex deposition. This is reflected in areas where deposition occurs, that is, generally at filtering membranes or at places of turbulence. In the kidney, in particular, the arteriolar pressure is high. If this is artificially increased, by induction of experimental hypertension, deposition of immune complexes is correspondingly increased (63).

Cochrane and Hawkins (59) found no correlation between charge and the ability of various proteins to be deposited in tissues. This work was done using proteins of different molecular weights and charges in the presence of increased vascular permeability. Other authors (68) found that when the charge on ferritin was altered, cationic ferritin penetrated further than native ferritin. Similarly, when horse radish peroxidase was considered the cationic molecules were excreted more quickly than those which were anionic (67). Charge may therefore be a significant factor in immune complex deposition.

It has been shown that immune complex saturation of the hepatic reticuloendothelial system (RES) may occur in the acute serum sickness model of GN in mice and that this occurs at levels of complexes in the same range as those required for the formation of GN (69). The RES may therefore be important

in preventing deposition of immune complexes normally and deposition may occur only when the RES is in some way deficient, has reached saturation because of a previous infection or because of the presence of a large number of circulating immune complexes.

Gelfand (70) has demonstrated the presence of C3b receptors in the glomeruli of human kidneys and has suggested that immune complexes with bound C3b may be deposited in the kidneys by means of these receptors. In kidneys from patients with GN with C3 present in the glomeruli, the number of receptors is reduced. Since these receptors are not present in various animal species which may develop spontaneous or experimental GN, the significance of the receptors is unknown.

Thus several factors involved in the deposition of immune complexes have been, to some extent, elucidated. Most of the work has involved the acute serum sickness model or the administration of preformed immune complexes. The situation in the chronic models of GN which are probably more analogous to human disease is by no means so well defined.

In the various types of human GN involving immune complexes, it is therefore likely that deposition occurs due to a complex set of factors; a) the size of the complex, b) the number of complexes in the plasma, c) the extent of

vascular permeability, d) capillary blood pressure, e) complement activation while apparently not involved in the acute serum sickness model has the capacity to cause increased capillary permeability, f) the state of the RES, g) hydrodynamic forces, h) charge and i) C3b receptors.

Type IV Hypersensitivity

Type IV hypersensitivity reactions involve cellular rather than antibody mediated immunity. When thymus-dependent lymphocytes (T-lymphocytes) come into contact with antigens with which they are specifically sensitised, they bind to the antigen and then undergo a complex series of interactions including cell transformation and mitosis. During this, soluble factors called lymphokines are secreted which have various effects (71), they may; a) cause the inhibition of migration of macrophages and PMN in vitro, b) attract PMN and mononuclear phagocytes, c) cause lymphocyte transformation and d) release cytotoxic factors. Antigens therefore act as a focus for inflammatory and phagocytic cells.

There is evidence for the existence of a type IV reaction in various types of human GN. The migration inhibition assay has been most often used to test for delayed hypersensitivity. Rocklin (72) and Dardenne (73) found that lymphocytes from patients with anti-GBM disease, on being

incubated with GBM antigens produced migration inhibition factor (MIF). Since anti-GBM disease may be induced by injection of antibody alone, type IV hypersensitivity is not necessarily an important pathogenic mechanism in this disease. While in one study, the reaction was found to be specific for anti-GBM disease (72) in the other, lymphocytes from patients with various types of GN reacted in a similar way to the antigen (73).

Sensitivity to foetal kidney antigens in all patients with MCNS and in some with a proliferative GN has been found using the MIF assay (74, 75). Eyres, using a cytotoxicity assay (76) found that lymphocytes from patients with MCNS had an enhanced ability to kill foetal kidney target cells while peripheral blood lymphocytes from patients with other types of GN did not differ significantly from normal. The hypothesis proposed was that foetal proteins are retained in some children and become antigenic, resulting in a type IV reaction. Since the antigens involved are tubular antigens and the tubular cells appear to suffer no damage, the pathogenic significance of the reaction is unclear. The fact that lymphocytes are not generally present in the kidney in MCNS casts further doubt on the importance of the mechanism.

Delayed hypersensitivity to streptococcal antigens (73) has been demonstrated in various types of GN but there is

no evidence that this contributes to the pathogenesis. Recent work has been done using a model of GN in which a subnephritogenic dose of rabbit anti-rat GBM antibody is administered to rats which have been injected with lymph node cells from rats previously immunised with rabbit immunoglobulin (77). If the lymph node cells were partially depleted of B cells (22% reduced to 5%), the proliferation of glomerular cells and the infiltration of circulating mononuclear cells was unaffected. It was suggested that the disease involved a type IV reaction. Since 5% of B cells remained and no titration of cells was done to define minimum dose and, since production of antibody to rabbit immunoglobulin would be likely to be enhanced by the introduction of sensitised T-cells, this work is not conclusive evidence of the requirement for a type IV response.

More recently, it has been shown that the hypercellularity found in acute serum sickness of rabbits is primarily the result of a mononuclear cell infiltration (78). The authors suggested that there were three possible explanations for this infiltration; a) that it was a non-specific event following tissue damage, b) that the monocytes bound via their Fc receptor to immune complexes in the glomeruli, or c) that the monocytes were present as the result of a T cell response. Since monocytes were present at the outset of the disease

and since proteinuria related to the number of monocytes but not to the extent of immune complex deposition, the first two explanations were considered unlikely. The frequent presence of lymphocytes in the vicinity of the glomerular monocytes suggests that a type IV reaction may be involved in the hypercellularity and thus in the pathogenesis of acute serum sickness of rabbits.

While there is little evidence to suggest that the type IV hypersensitivity reaction is of general importance in the pathogenesis of GN, the existence of such a response even if it is secondary to tissue injury, may affect the course of the disease.

Thus all types of hypersensitivity may be implicated, to some extent, in GN. Type III is probably the most important while anti-GBM disease and perhaps other types of GN with subepithelial immune deposits, involve primarily a type II response. No single category of GN can be ascribed to types I or IV although type I may be involved in immune complex deposition. Type IV hypersensitivity is found in most types of GN and it may be involved in the pathogenesis of acute serum sickness in rabbits. It is not clear what role it plays in the development of human GN although it is likely to result in tissue damage.

With some types of GN, it is not apparent which type or

types of hypersensitivity are involved. The pathogenesis of each of the disease groups examined in this study will be considered in the introductions to the relevant chapters.

The Complement System

Glomerulonephritis is an inflammatory lesion and one of the major effectors of inflammation is the complement system. This system has been shown to be of pathogenic significance in experimental anti-GBM disease (31) although it was not essential for the development of the acute serum sickness model of GN (58). The role of complement in chronic experimental GN and human GN is not known but its frequent presence in the glomeruli of GN patients and its potency as a mediator of inflammation suggests that complement may be of importance in human GN.

Complement was first discovered when serum was found to be capable of lysing bacteria (79). Bordet (80) noted that two substances were necessary for lysis; one was heat stable and was later called antibody and the other was a heat labile substance which he called complement.

The lytic action of complement, originally assumed to be its main function, is now known to be only one of several important biological properties which mediate inflammation. With the comparatively recent advances in knowledge, aided by the development of methods for purifying plasma proteins, many of the complex interactions and biological properties of many of the proteins which constitute the complement system have, to some extent, been elucidated.

Complement Activation

The complement system, the interactions of which are shown in diagrammatic form in figure 1.1, may be divided into two parts, a) activation and b) the attack sequence. Activation may take place by either of two pathways.

The Classical Pathway

The classical pathway, as its name suggests, was the first to be discovered and is activated when specific antibody binds to an antigen to form an immune complex. Once immunoglobulin binds to the antigen, the C1 complex, consisting of three proteins, C1q, C1r and C1s, held together in the presence of calcium ions (81), may bind to the immunoglobulin. Only certain immunoglobulin classes may activate C1, these are: IgM and three subclasses of IgG; IgG1, IgG2 and IgG3 (83). The recognition molecule is C1q and the activation of C1 requires more than one Fc fragment. C1r is then activated which in turn causes enzymatic activation of C1s (84). $\overline{C1}$s cleaves C4 into C4a and C4b (85). C4b has a labile membrane binding site and membrane bound C4b in the presence of magnesium ions binds C2. $\overline{C1}$ then cleaves C2b from C2 leaving $\overline{C4b2a}$ as an active enzyme on the membrane capable of cleaving C3 (86).

Since the activation of C1s, C4 and C2 is enzymatic, many molecules of C4b and C2a may be formed by the binding of a

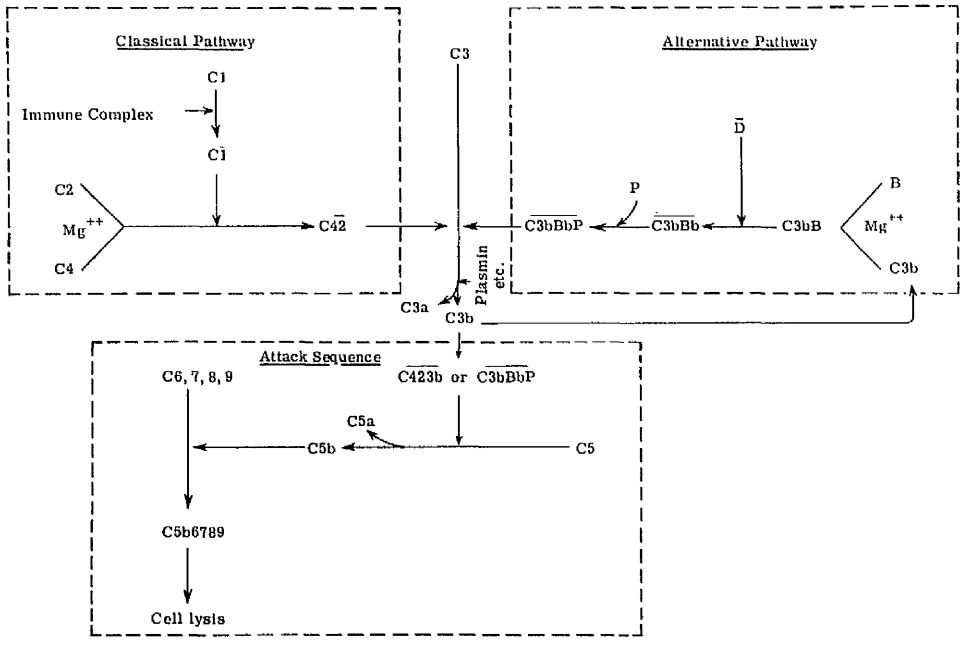


Figure 1-1 Diagram of the interactions of the complement system

single C1q molecule.

The Alternative Pathway

It was apparent that antibody, in some cases, was not necessary for the complement mediated lysis of certain micro-organisms (87) and that some other pathway must be responsible for activation of complement.

The proteins which are known to be involved in the alternative pathway are C3b, factor B, $\overline{D}$, properdin, C3b inactivator (C3bINA) and β1H (88). C3b is formed in normal serum probably as the result of low-grade continuous turnover (89). C3b binds to factor B in the presence of magnesium ions and $\overline{D}$ cleaves factor B to release a fragment, Ba, and leave the alternative pathway C3 convertase; $\overline{C3bBb}$. The binding of properdin to C3b increases the stability of the complex by retarding the decay of Bb (90). As more C3b is produced, the cleavage of C3 enters an amplification phase. This is prevented in normal serum by the actions of two regulatory proteins, C3bINA and β1H. The mechanism of this regulation will be discussed later. Where large quantities of C3b are produced by the classical pathway, amplification of the alternative pathway may result despite the presence of regulatory proteins. Also, under certain circumstances, C3b is protected from the regulatory actions of β1H and C3bINA, permitting alternative pathway activation

to progress in an uncontrolled manner. This may occur, in vivo, in the presence of bacterial endotoxins (91), nephritic factor (92) and where there is a deficiency of C3bINA (93) and in vitro in the presence of endotoxin (94), immune complexes involving 5S rabbit IgG (95), immune complexes involving guinea-pig IgG1 antibodies (96), rabbit erythrocytes (97), neuraminidase treated sheep erythrocytes (98), cobra venom factor (99) and where C3bINA (89) or βlH (100) have been depleted.

Thus the two activation pathways result in the formation of C3 cleaving enzymes; $\overline{C4b2a}$ and $\overline{C3bBbP}$ respectively.

The Attack Sequence

The C3 convertase formed from either pathway may cause cleavage of C3 into C3a, a small fluid phase molecule, and C3b which has a temporary membrane binding site (101). C3b may bind to the converting enzyme to form a C5 convertase, $\overline{C423b}$ or $\overline{C3bBb}$ (102, 103), resulting in the formation of C5a, a fluid phase molecule, and C5b which may bind to membranes. Cleavage of C5 is the last enzymatic step in the sequence. C5b binds to C6 to form C5b6 which may then bind C7. C8 and C9 are then added to $\overline{C5b67}$ to form a complex capable of cell lysis. To form the C5b-9 cytolytic complex, a dimer is formed consisting of two sets of molecules each containing one molecule of C5b, C6, C7 and C8 and three molecules of

C9 (104). Alternatively C5b may bind C6 and then C7 in the fluid phase to form C5b67 which has the capacity to bind to membranes on adjacent cells. With the addition of C8 and C9, these cells are called "bystander" cells (105).

Control of Complement Activation

A system such as this, with an enzyme cascade, the capacity for internal amplification and a spontaneous turnover, will require regulation. This is afforded both intrinsically in the form of natural decay, and extrinsically by regulatory proteins in the plasma.

Intrinsic Control

For significant complement activation, C4b and C3b each require to be membrane bound. This ability to bind to cell membranes is transient (106, 107), and most molecules which are activated remain ineffective. In addition to this, C2 and factor B decay quickly from their respective C3 convertases (108, 109). This reduces the extent of C3 cleavage although C4b and C3b retain the ability to bind more C2 and factor B respectively. C5b also decays rapidly (102).

Extrinsic Control

C1-inhibitor: C1-inhibitor (C1-INH) binds to C1s which is a key molecule in classical pathway activation and, by

this binding, inhibits the activity of $C\bar{1}s$: preventing the activation of C4 and C2. $C\bar{1}s$ and $C\bar{1}$-INH bind in a 1:1 molar ratio to form a stable complex (110). $C\bar{1}$-INH has no effect on precursor C1s.

In 1963, the absence of a serum inhibitor of C1s was noted in a patient with hereditary angioneurotic oedema and this was designated $C\bar{1}$-INH (111). One year previous to this, Landermann (112) had recorded a deficiency of kallikrein inhibitor in the same disease. $C\bar{1}$-INH is now known to inhibit proteins in several interrelated mediator pathways, such as 1) plasmin, 2) plasma kallikrein, causing inhibition of kinin production (113), 3) Hagemann factor fragments, thus inhibiting coagulation, 4) Hagemann factor preventing kinin generation and fibrinolysis (114) and 5) C1r, part of the complex containing C1s (115). This binding does not compete effectively with the binding of $C\bar{1}$-INH to $C\bar{1}s$. There is no known regulator of the alternative pathway analogous to $C\bar{1}$-INH.

C4 binding protein: Recently a new regulator of complement activation was discovered, the C4-binding protein (C4-bp) (116). This has been shown to form a stable complex with C4b and is necessary, along with C3bINA, for the enzymatic inactivation of C4b. As the result of the combined actions of C3bINA and C4-bp on C4b, the cleavage products C4c

and C4d are formed (117).

C3b inactivator: C3bINA was first detected in guinea-pig serum (118) as a component of plasma which inhibited immune adherence and immune haemolysis of cell bound but not native C3. It was called C3-inactivator. The following year, the same component was demonstrated in rabbits (119). Its activity was found to be temperature dependent and the protein was not consumed during inactivation, suggesting an enzymatic mechanism. C3bINA was believed to cleave C3b into C3c and C3d (120), but the mechanism is now known to be at least a two stage procedure with inactivation of C3b by C3bINA and the subsequent cleavage by trypsin or a similar enzyme into C3c and C3d (121). With cell bound C3b (122), this reaction is greatly enhanced by the participation of β1H and in the fluid phase β1H appears to be an absolute requirement for the inactivation of C3b (123). Depletion of C3bINA in vitro (89) and deficiency in vivo (93) result in utilization of factor B.

The C3bINA level in normal serum may be critical since artificially increasing levels, in vitro, causes the inhibition, to varying extents, of the activation of the alternative pathway in the presence of inulin and aggregated gamma globulins (124). C3bINA may also inactivate cell bound or free C4b although ten times more C3bINA is required

for this reaction than is required for the inactivation of C3b (125, 126). This involves C4-bp and the mechanism appears to be different from the inactivation of C3b since further enzymatic digestion is not necessary to produce C4c and C4d in the fluid phase inactivation of C4b (117).

<u>βlH-globulin:</u> βlH also functions to control C3b although its mode of action differs from that of C3bINA. It was found to potentiate the inhibition of haemolysis by C3bINA by binding to C3b molecules on sheep erythrocytes (122). βlH alone had a slight inhibitory effect but when present with C3bINA, the inhibition exceeded the sum of the inhibitions attributable to C3bINA and βlH separately. A second effect of βlH was to accelerate the decay of the alternative pathway C3 convertase on sheep erythrocytes. This effect was caused by displacement of factor B by βlH from the convertase (127). βlH was ineffective if the convertase was on the surface of rabbit erythrocytes (97), on zymosan (128) or sheep erythrocytes treated with neuraminidase (98). This resistance of the C3 convertase of the alternative pathway on certain surfaces to regulation by βlH is assumed to be the means by which these surfaces trigger the amplification phase of complement activation. The capacity of certain surfaces to cause amplification is due to their low sialic acid content (97).

The binding of C3b and βIH is reversible and βIH may be displaced by factor B at high factor B concentrations (129). Properdin, which is known to stabilize the alternative pathway C3 convertase (90), also enhances binding of βIH to C3b (130). Because of the competition and the similar effects of properdin on βIH and factor B, it was assumed that factor B and βIH shared the same binding site on C3b (130). However, since under certain micro-environmental conditions βIH is unable to control C3 cleavage by the C3 convertase (97, 128), it is more probable that the binding sites are close and that the competition is caused by steric hindrance.

Inhibition of $\overline{C5b67}$: Two inhibitors of $\overline{C5b67}$ have been described. One is a very low density lipoprotein (131) and the other, a glycoprotein, called the S-protein (132). Both of these act by binding to the transient membrane binding site of $\overline{C5b67}$ and thus prevent the binding of this molecular complex to cell membranes (133).

Anaphylotoxin inactivator: C3a and C5a may be inhibited enzymatically by a carboxypeptidase B which removes the carboxyterminal arginine residue from C3a and C5a thus causing the inactivation of the anaphylotoxic properties of the molecule (134). The inactivation of C5a results in a smaller molecule $C5a_{des\ arg}$ which retains its chemotactic properties.

Biological Properties of Complement

Table 1.2 shows a list of complement components or complexes of complement components which have been shown to elicit biological effects. Membrane damage is therefore only one of a number of consequences of complement activation. Two main categories of biological activity exist: a) mediators causing increased vascular permeability and b) those causing the accumulation of inflammatory cells at the site of injury.

Table 1.2: Biological properties of the complement system

EFFECTOR	BIOLOGICAL PROPERTY
C2 kinin	increased vascular permeability
C4b	immune adherence
C3a	anaphylotoxin activity
C3b	immune adherence, alternative pathway activation, solubilization of immune complexes
C5a	anaphylotoxin activity, chemotaxis
$\overline{C5b6789}$	cytolysis

C2-kinin activity: This is generated experimentally when activated $\overline{C1s}$ is injected into human skin. Swelling occurs due to increased capillary permeability (135). This swelling is not blocked by antihistamine treatment nor is the reaction reduced in patients with acquired C3 deficiency,

suggesting that only classical pathway components are used. This response may be involved in the symptoms of hereditary angioneurotic oedema.

The Immune Adherence Phenomenon: The immune adherence phenomenon involves the binding of complement coated particles to cells with complement receptors (136). The components involved are C3b (137) and to a lesser extent C4b (138). Receptors for these components are found on many cell types including erythrocytes, macrophages, lymphocytes and polymorphonuclear leucocytes (PMN) (139). The binding of complexes to erythrocytes via C3b receptors greatly enhances the phagocytosis of the complex (136) and the binding of such particles directly to phagocytic cells via C3b receptors also aids the ingestion of antigens (140).

It has been suggested that the binding of lymphocytes to antigens via complement receptors may be important in antibody production (141). The presence of normal antibody levels in a C3 deficient patient however argues against this (142). The binding of C3b to the surface of B-lymphocytes may result in the release of lymphokines which are chemotactic for monocytes (143).

When PMN bind via their C3b receptors to immune complexes, secretion of the granule contents results in the

release of various hydrolases important in the inflammatory response (144).

Solubilization of Immune Complexes: Precipitated immune complexes may be solubilized on addition of normal serum but not zymosan or heat treated serum (145). The reaction is prevented if $\bar{D}$, factor B or properdin are removed although it may proceed in the absence of calcium ions or classical pathway components. The process therefore requires an intact alternative pathway although dissolution of complexes proceeds more quickly if the classical pathway is also intact (146). The mechanism is independent of antibody class and of the antigen involved in the complex (147) and it may involve the intercalation of complement components into the immune complex forcing a break-up of the lattice. As well as dissolving precipitated immune complexes, this process may remove complexes from the surface of lymphocytes and may be the mechanism by which immune complexes are removed from the kidney in immune complex GN.

Anaphylatoxin Activity: Anaphylatoxins cause histamine to be released from mast cells with a resultant increase in vascular permeability (148). The anaphylatoxin activity resides in the smaller cleavage products of C3 and C5, that is C3a and C5a. C3a and C5a bind to different receptors on

mast cells to give an additive effect (148) and C3a has been demonstrated by immunofluorescence on the cell surface (149). C3a and C5a anaphylatoxins may be inactivated enzymatically (134).

Chemotaxis: Various products of complement activation have been suggested as being chemotactic for PMN. These are C3a (150, 151), C5a (152) and $\overline{C5b67}$ (153, 154). Recent work suggests that C3a does not exert a chemotactic effect on PMN at physiological concentrations and that C3a preparations were contaminated with C5a in previous studies (155). The chemotactic effects attributed to $\overline{C5b67}$ may also be due to C5a contamination. C5a is a powerful chemotactic agent and it retains a reduced amount of activity after treatment with carboxypeptidase B. The accumulation of PMN has various biological consequences as previously discussed. C5a may also cause the accumulation of basophils (156).

Cytolysis: The current view of complement-mediated cell lysis is based on the "doughnut hypothesis" of Mayer (157) in which he suggested that products of complement activation are inserted into the cell membrane to form a transmembrane channel. The outside of the channel is hydrophobic and thus binds with the lipid in the membrane and the inside is hydrophilic, allowing passage of fluids into

the cell and causing lysis. The functional unit is a dimer of the C5b-9 complex, each containing one molecule of each of C5b, C6, C7 and C8 and three molecules of C9 (158). Despite the fact that the structure and the molecules involved are known, doubt still exists as to the site of the channel, whether it is through the complement components, beside them or even whether a channel is necessary.

Because of the frequent presence of complement components in the glomeruli of patients with GN and the effectiveness of the complement system as an inflammatory mediator, it was considered useful to study complement activation in GN in detail. Many studies have examined biopsies for the presence of immunoglobulins and C3 and, in a few, restricted almost exclusively to diseases involving intense complement activation, e.g. membranoproliferative glomerulonephritis and acute post streptococcal glomerulonephritis, deposition of either classical or alternative pathway components and, occasionally both, have been studied. In some communications serum levels, generally only of C3, have been reported.

The purpose of this thesis was to examine complement activation with particular reference to six major types of GN in terms of the involvement of immunoglobulins, the relative importance of the classical and the alternative pathways and the extent of activation of the complement system. For this, the presence and intensity of deposition of immunoglobulins and complement components within the glomeruli and the serum concentrations of complement components were studied.

Since complement activation may result from a deficiency in the control of the complement system, the role of regulators

of complement activation in GN was also considered. Complement activation is controlled by at least four proteins, $C\bar{1}$-INH, C4-bp, C3bINA and β1H. Because of the relatively recent discovery of C4-bp, only the effects of $C\bar{1}$-INH, C3bINA and β1H were considered in this study.

MATERIALS & METHODS

Chemicals and other reagents

Chemicals and other reagents were obtained from the following companies:-

BDH Chemicals Ltd.	Sodium chloride (NaCl) Potassium dihydrogen orthophosphate (KH_2PO_4) di-Potassium hydrogen orthophosphate (K_2HPO_4) Sodium barbitone Barbitone Sodium dihydrogen orthophosphate (NaH_2PO_4) di-Sodium hydrogen orthophosphate (Na_2HPO_4) Glycine Sodium acetate Acetic acid Silica gel Calcium chloride ($CaCl_2$) Ammonium sulphate $(NH_4)_2SO_4$ Agarose Potassium thiocyanate (KCNS) Acetone
May & Baker Ltd.	Hydrochloric acid (HCl) Sodium hydroxide (NaOH) Sodium carbonate (Na_2CO_3) Sodium bicarbonate ($NaHCO_3$) Magnesium chloride ($MgCl_2$)
Sigma Chemical Co.	Trisma base (tris) Cyanogen bromide (CNBr) Fluorecein isothiocyanate (FITC) Monoethanolamine Ethyleneglycol-bis-(β-amino-ethyl ether) N, N′-tetra-acetic acid (EGTA)
Koch-Light Lab. Ltd.	Ethylenediamine tetra-acetic acid (EDTA) Bovine serum albumin (BSA) Hydrazine hydrate

Evans Medical Ltd.	Glycerol
Fisons Scientific Apparatus	Potassium iodide (kI) Iodine (I_2)
Difco Laboratories	Freund's complete adjuvant (FCA) Freund's incomplete adjuvant (FIA)
Pharmacia Fine Chemicals	Sephadex G25 Sepharose 4B
Kodak Ltd.	High Speed Ektachrome Ektachrome 200 professional
Macfarlan Smith	Anaesthetic ether
British Petroleum Chemicals	740P Methylated Spirits
Merck	Gelatin
Service Laboratories Ltd.	Mannitol
Taab Laboratories	Glutaraldehyde
Wellcome Reagents Ltd	Pig liver powder
Miles Laboratories Incorporated	Tissue Tek II OTC Compound
Park Davis & Co.	Gelatin capsules, size 00
British Oxygen Co.	Liquid nitrogen
Nunc Laboratories	6 cm plastic petri dishes
Gibco Europe	Sheep blood cells
Imperial Chemical Industries	Solid carbon dioxide

Buffers and other preparations

All buffers were made in distilled water except where stated and pH adjustments, where necessary, were made using 1.0N HCl or 1.0N NaOH.

Buffers used for antisera

Phosphate buffered saline (PBS), pH 7.0

8 g of NaCl
0.34 g of KH_2PO_4
1.21 g of K_2HPO_4

dissolved in one litre of distilled water

Carbonate buffer, pH 9.5

5.8 ml of 5.3% Na_2CO_3
10.0 ml of 4.2% $NaHCO_3$

adjusted to pH 9.5

Veronal buffered saline (VBS) x 5, pH 7.4

42.5 g of NaCl
1.87 g of sodium barbitone
2.87 g of barbitone

dissolved separately, added together and made up to 1 litre in distilled water

VBS 0.01M EDTA, pH 7.4

1 x VBS
0.01M tri sodium EDTA

Barbitone-HCl, pH 8.4

65 ml of 0.1N HCl
9.0 g of sodium barbitone
0.002 M EDTA tri sodium

Dissolved separately and added together, adjusted to pH 8.4 and made up to 1 litre in distilled water

0.2M phosphate buffer, pH 7.2

56 ml of 0.2M NaH_2PO_4 $2H_2O$
144 ml of 0.2M Na_2PO_4

Glycine-HCl buffer, pH 2.8

74.7 ml of 0.1M glycine in 0.1N NaCl
24.4 ml of 0.1N HCl

0.1M acetate buffer in 1.0M NaCl, pH 4.0

17 ml of 0.1M sodium acetate
83 ml of 0.1N acetic acid

Each dissolved in 1.0M NaCl and added together

Tris buffer in 1.0M NaCl, pH 8.0

25 ml of 0.2M tris
28 ml of 0.1N HCl
5.84 g of NaCl

Added dissolved and made to 100 ml in distilled water

Buffers and other preparations for immunofluorescence

Ether Alcohol

500 ml of anaesthetic ether
500 ml of absolute industrial alcohol (prepared by drying 740P methylated spirits over silica gel)

95% Alcohol

950 ml of industrial alcohol (prepared as above)
50 ml of distilled water

Tris buffered glycerol, pH 8.5

2.5 ml of 0.1M tris
1.7 ml of 0.1N HCl

Made to 10 ml in distilled water and added to 90 ml of glycerol

Buffers and other preparations for nephritic factor assay

All buffers in this section were prepared in sterile deionised water.

$GVB^{=}$, pH 7.4

400 ml of 5 x VBS
20 ml of 10% gelatin

Made to 2 litres in deionised water

0.01M EDTA $GVB^{=}$, pH 7.4

57.5 ml of 0.086M EDTA (pH to 7.4 with NaOH)

Made up to 500 ml with $GVB^{=}$

GVB^{++}, pH 7.4

400 ml of 5 x VBS
10 ml of 0.03M $CaCl_2$
20 ml of 0.1M $MgCl_2$
20 ml of 10% gelatin

Made to 2 litres in deionided water

$D5W^{++}$, pH 6.7

100 g of D glucose
10 ml of 0.03M $CaCl_2$
20 ml of 0.1M $MgCl_2$

Made to 2 litres in deionised water

$DGVB^{++}$, pH 7.4

50 ml of GVB^{++}
50 ml of $D5W^{++}$

0.04M EDTA/ $GVB^{=}$, pH 7.4

230 ml of 0.086M EDTA (pH to 7.4 with NaOH)

Made up to 500 ml with $GVB^{=}$

D50S

100 ml of 50% glucose
5.95 g of NaCl

Mannitol/ GVB^{++}, pH 7.4

4 parts of 20% Mannitol
1 part of GVB^{++}

Potassium iodide/ iodine oxidising agent

8.25 g of KI
0.3 g of I_2

dissolved in 100 ml of 0.1M phosphate buffer, pH 6.0

0.1M Phosphate buffer, pH 6.0

88 ml of 0.2M NaH_2PO_4
12 ml of 0.2M Na_2HPO_4

made up to 200 ml in deionised water

Antisera

Source: Antisera for this study were obtained from the following sources:-

FITC conjugated rabbit anti-human IgG FITC conjugated rabbit anti-human IgA FITC conjugated rabbit anti-human IgM	Wellcome Reagents Limited
FITC conjugated rabbit anti-human C1q FITC conjugated rabbit anti-human C3 FITC conjugated rabbit anti-goat immunoglobulin rabbit anti-human C1s	Hoechst Pharmaceuticals
rabbit anti-normal human serum rabbit anti-human properdin rabbit anti-human factor B rabbit anti-human C1̄-INH rabbit anti-human βIH rabbit anti-human C1q rabbit anti-human C4 rabbit anti-human C3	Dr. K. Whaley
rabbit anti-human C3bINA	Prof. P.J. Lachmann
goat anti-human C5	Dr. D.R. Schultze

FITC conjugated sheep anti-rabbit globulin - prepared as described

Preparation of FITC conjugated sheep anti-rabbit globulin

Preparation of rabbit globulin: The globulin fraction of 200 ml of rabbit serum was precipitated with 33% saturated ammonium sulphate for one hour and centrifuged for 15 minutes at 1000 g at room temperature. This precipitate was washed twice in 50 ml of 40% saturated ammonium sulphate at room temperature, redissolved in 50 ml of phosphate buffered saline (PBS) and recentrifuged to remove undissolved material. The precipitation was repeated

twice before redissolving the globulin fraction in 20 ml of PBS. This solution was dialysed for 48 hours against two changes of two litres of PBS at 4°C, lyophilised and stored at -20°C. Goat globulin, for characterization of anti-goat globulin, was prepared in this way also.

Preparation of Antibody: A sheep was injected intra-muscularly and subcutaneously with 1 mg of rabbit globulin, dissolved in 1 ml of PBS and emulsified in 1 ml of Freund's complete adjuvant (FCA). The sheep was boosted after six and eight weeks with 1 mg of antigen dissolved in 1 ml of PBS and emulsified in 1 ml of Freund's incomplete adjuvant (FIA). The animal was bled on weeks nine and ten. The antiserum obtained was absorbed and characterized according to the methods below and, once shown to be pure, was conjugated to fluorescein isothiocyanate (FITC) by the following method.

Conjugation of FITC to sheep anti-rabbit immunoglobulin (159): Dry FITC at a concentration of 30 µg FITC per 1 mg of antibody was added to the purified antibody, at a concentration of 10 mg/ml, containing 10% carbonate buffer, pH 9.5 and the mixture was rotated on a Matburn mixer for one hour at room temperature. The FITC, bound to protein, was separated from free dye by passage over a Sephadex G25 coarse column, dimensions - 200 x 10 mm, in PBS. The

labelling ratio of fluorochrome to protein was estimated using the formula,

$$\text{Labelling ratio} = \frac{\text{moles of fluorochrome}}{\text{moles of protein}} + \frac{xA}{Cp}$$

Where A = absorbance at λ max for FITC i.e. at 490nm
Cp = concentration of protein in ng per ml
x = a constant, 2.8 for FITC

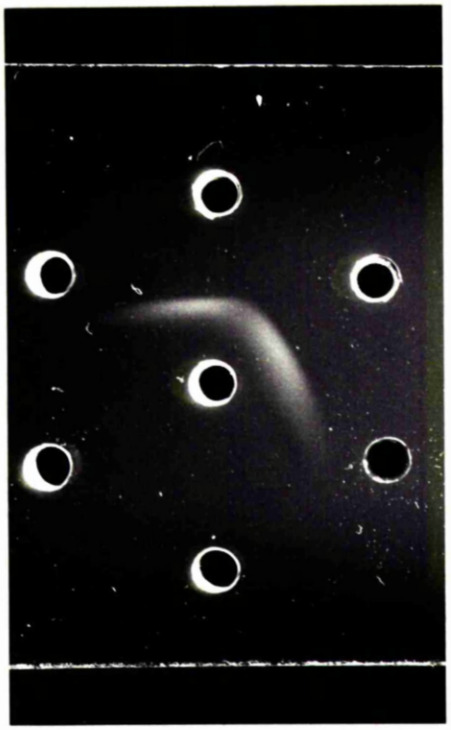
The labelling ratio of FITC to sheep anti-rabbit globulin was found to be 2.0.

Characterisation of antisera

All antisera were tested for monospecificity by double diffusion gel precipitation (160) and immunoelectrophoresis (161). Where necessary absorption techniques were employed to ensure that all antisera were monospecific.

Double diffusion gel precipitation: Microscope slides (22 x 76 mm) were coated with 3 ml of 1.5% agarose dissolved in VBS containing 0.01M EDTA and holes, of diameter 1.7mm, were cut in the agarose as shown in figure 2-1. Each antiserum for test was placed in the centre well and normal human serum or the relevant animal serum and purified antigen preparations were placed in the peripheral wells. Purified antigen preparations were obtained as indicated

IgA, IgM } Dr. D. Parrott



X 3

Figure 2-1 In this double diffusion gel precipitation test, rabbit anti-human C3 was placed in the centre well, with a purified C3 preparation in the top well and normal human serum to the right of that. A single line of precipitation was found between anti-C3 and purified C3 antigen and this shared complete identity with the single precipitation line between anti-C3 and normal human serum.

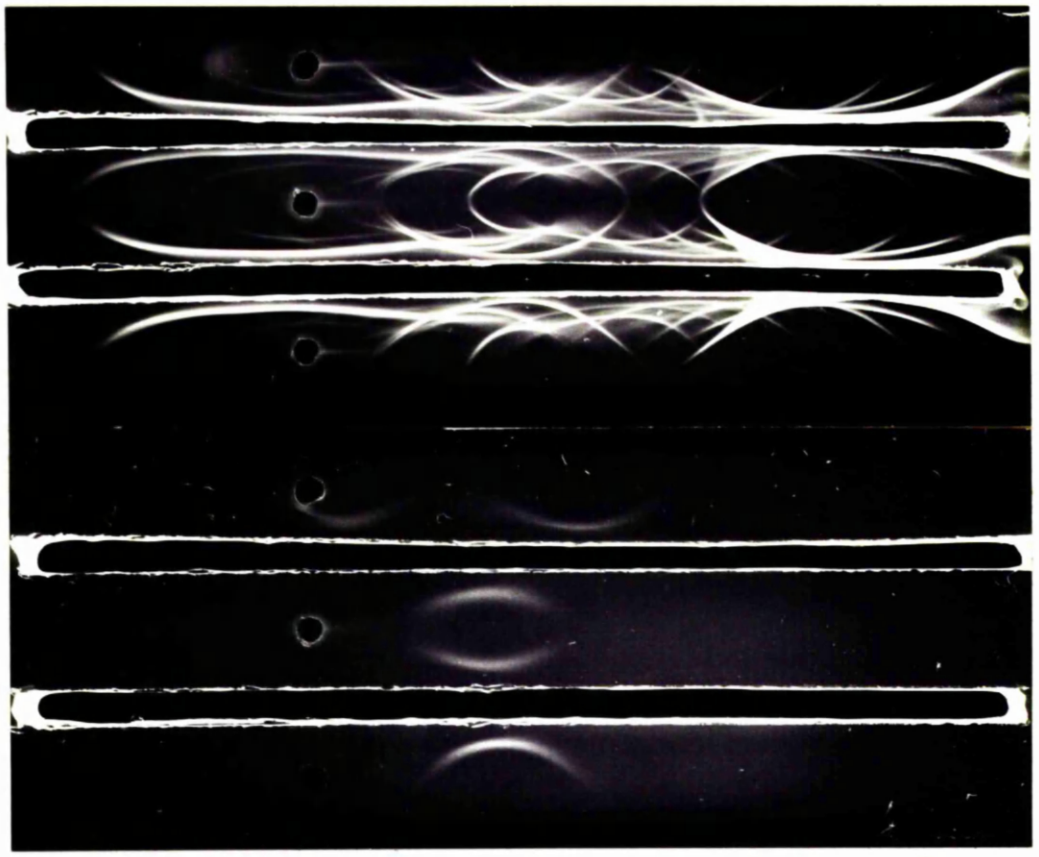
IgG, C1q, C1s, C4, Properdin, factor B, C3, C5, CĪ-INH, C3bINA, βIH	Dr. K. Whaley
Rabbit globulin, Goat globulin	Prepared as above

In the case of each antiserum used for immunofluorescence or radial immunodiffusion, only one line of precipitation was apparent between the antiserum and normal human serum and this line shared complete identity with the line between the antiserum and the pure antigen preparation except with sheep anti-rabbit globulin and rabbit anti-goat globulin where three lines were distinguished against the relevant antigen preparation and the relevant normal serum. All human antigens used were shown to be pure by double diffusion gel precipitation and immunoelectrophoresis against antibody to normal human serum by the methods shown here. C1q, C1s, C4, properdin, factor B, C3, C5, CĪ-INH, C3bINA and βIH had further been shown to be pure by sodium dodecyl sulphate polyacrylamide gel electrophoresis run under non-reducing conditions.

Immunoelectrophoresis: Microscope slides were coated with 3 ml of 1.5 per cent agarose in barbitone HCl buffer, pH 8.4.

Wells and troughs were cut using a Shandon immunoelectrophoresis cutter as shown in figure 2-2 and the agarose in these removed by suction. Normal human serum was applied to the centre well and pure antigen to the outside well. These slides were then placed in a Shandon thin layer electrophoresis tank model Q77 with barbitone-HCl buffer, pH 8.4 and with wicks made from surgical lint. The antigens were then subjected to a potential difference of 120v with a current of 20mA for one hour. After removal, antiserum was applied to the troughs and diffusion allowed to take place in a humid chamber for 48 hours at room temperature. In each case except the following only one arc was seen against normal human serum and this was in an identical position to the arc seen against the pure antigen preparation and was in an appropriate position for that antigen. With sheep anti-rabbit globulin and rabbit anti-goat globulin, three lines were visible against the relevant normal serum and these were in the appropriate positions for IgG, IgA and IgM. No pure antigens were available.

Absorption of antisera: Absorption of antisera is done to remove antibodies of unwanted specificities. It may be used in two ways. If an antiserum to a given antigen is contaminated with specificity for one or more other antigens, then the antiserum may be incubated with a preparation of



X 2

Figure 2-2 In this immunoelectrophoresis test, normal human serum was placed in the top 3 wells, zymosan treated human serum in the fourth, purified factor B antigen in the fifth and normal human serum in the sixth. Anti-normal human serum was placed in the top two troughs and anti-human factor B in the bottom two. The single arcs against purified antigen and normal human serum show the antiserum to be specific and mono-specific and the two arcs found against zymosan treated serum verify the specificity since factor B is cleaved in the presence of zymosan.

the inappropriate antigens. The contaminating antibodies will complex with these antigens and, if the antigens are in an insoluble form, the complexes may be removed by centrifugation. Alternatively, in order to prepare small quantities of very pure antibody, a pure preparation of the appropriate antigen may be prepared, insolubilized and incubated with the antiserum. All proteins except specific antibody are removed by washing and the pure antibody is eluted from the insoluble antigen. Three antisera required purification; with sheep anti-rabbit immunoglobulin both methods were employed and with anti-human properdin and anti-human factor B, only the former method was used.

Purification of Sheep anti-rabbit globulin

Glutaraldehyde polymerisation of human Cohn fraction II : 10 mg of human Cohn fraction II (supplied by the Plasma Protein Fractionation Centre, Edinburgh) was dissolved in 10 ml of PBS and dialysed against PBS at 4°C for 16 hours. To the Cohn fraction II was added 1 ml of 0.2M phosphate buffer pH 7.2 and 3 ml of 2.5% glutaraldehyde. After two hours, at room temperature, the polymerised protein was homogenised and washed several times in 10 ml of PBS by centrifuging at 1000 g at room temperature until the supernatant was free of protein as verified by the optical density reading at 280 nm.

Removal of antibodies cross reacting with human proteins:

A 10 ml aliquot of the sheep anti-rabbit antiserum was mixed with 10 mg of glutaraldehyde polymerised human cohn fraction II on a Matburn mixer for 1 hour at room temperature and then centrifuged at 1000 g for 15 minutes at room temperature thus removing antibodies cross reacting with human globulins.

Isolation of pure antibody: Using the method described for cohn fraction II, 20 mg of rabbit globulin were polymerised. Before use, the polymer was tested under eluting conditions by mixing with glycine-HCl buffer, pH 2.8, centrifuged at 1000 g and washed twice in PBS. To this polymer was added 10 ml of antiserum and the mixture was rotated on a Matburn mixer for 1 hour at room temperature. The mixture was centrifuged at 1000 g and the precipitate washed in 10 ml of PBS until the supernatant contained no protein as verified by an optical density reading at 280 nm. The final precipitate was then suspended in 5 ml of glycine-HCl buffer pH 2.8 to elute the specific antibody bound to the rabbit immunoglobulin. This was centrifuged at 1000 g, the pH of the supernatant corrected to pH 7.0 with 1.0N sodium hydroxide and dialysed against two litres of PBS for 16 hours. The antiserum was then lyophilised, redissolved to a concentration of 10 mg/ ml in PBS for conjugation to FITC as above.

Purification of rabbit anti-human properdin: The anti-properdin

antiserum was contaminated with anti-IgG. An insoluble properdin-free IgG immunoabsorbent was prepared and the antiserum absorbed.

Preparation of a sepharose 4B/ IgG immunoabsorbent (162): Twenty millilitres of a 10 mg/ ml solution of properdin free IgG (supplied by Dr. K. Whaley) were dialysed at 4°C for 16 hours against 0.1M $NaHCO_3$. 10 ml of Sepharose 4B were washed five times in distilled water, centrifuging at 1000 g for five minutes at room temperature. In a fume cupboard, 2 g of cyanogen bromide were dissolved in 20 ml of distilled water and this was mixed with the washed Sepharose 4B maintaining the pH at 11.0 with 4N NaOH until the pH was stable. This mixture was washed under vacuum with 1 litre of $NaHCO_3$ at 0°C and the "activated" Sepharose 4B was mixed with the IgG preparation and rotated on a Matburn mixer for 16 hours at 4°C. The IgG/ Sepharose 4B immunoabsorbent was washed once in $NaHCO_3$, pH 8.0, centrifuging at 1000 g, and suspended in 1.0M monoethanolamine pH 8.0 to block excess protein binding sites on the "activated " Sepharose. This was rotated for 2 hours at 4°C and washed three times in 0.1M acetate buffer in 1.0M sodium chloride pH 4.0, three times in 0.1M tris buffer in 1.0M NaCl pH 8.0 and three times in 0.01M tris in 0.15M NaCl with 0.001M EDTA pH 7.8, centrifuging, on each occasion, at 1000 g for five minutes.

Equal volumes of rabbit anti-human properdin and the IgG immunoabsorbent were mixed and rotated for one hour at room temperature. The immunoabsorbent was removed by centrifugation at 1000 g for five minutes. The anti-properdin antisera was then found to be monospecific by the criteria employed above.

Purification of rabbit anti-human factor B (163): To remove contaminating antibodies in the anti-human factor B antisera, normal human serum was depleted of factor B by addition of an equal volume of 1 molar potassium thiocyanate (KCNS) dropwise. This was incubated at 37°C for 45 minutes and the KCNS removed by dialysis against PBS at 4°C for 16 hours. This depleted serum was then diluted to a protein concentration of approximately 10 mg/ ml, using the formula that an optical density reading of 1.0 at a wavelength of 280 nm approximates to a concentration of 1 mg/ ml. The KCNS treated serum was then conjugated to Sepharose 4B as was described for IgG above.

Equal volumes of rabbit anti-human factor B and the KCNS treated serum/ Sepharose 4B immunoabsorbent were mixed and rotated for one hour at room temperature. The immunoabsorbent was removed by centrifugation at 1000 g for five minutes and the anti-factor B antiserum was shown to be pure by the criteria employed above.

Adsorption of antisera: Adsorption involves the non specific attachment of soluble substances to proteins. Conjugation to FITC causes an increase in the net negative change of some serum proteins and these may bind to tissue sections resulting in excessive background staining. These highly negatively charged proteins may be removed by one of two methods, either by adsorption with tissue powders or by ion exchange chromatography. The former method was found to be effective and convenient and was therefore used for the adsorption of all FITC conjugated antisera before these were employed in the immunofluorescence test. Pig liver powder was washed twice in PBS, centrifuging at 1000 g for five minutes and then incubated with the antiserum for one hour at room temperature on a Mathurn mixer. This mixture was centrifuged at 1000 g for five minutes and the procedure repeated with the supernatant. The supernatant, after the second adsorption, was centrifuged a second time to remove any trace of liver powder and was then aliquotted and stored.

Renal Biopsy Studies

One hundred and four renal biopsies used in this study were obtained from hospitals in the Glasgow area. So that subsequent analyses would be meaningful, only biopsies which fell into a distinct pathological category (1) were considered and biopsies which fell into categories containing fewer than ten biopsies were excluded from the study.

The categories are shown in Table 2.1.

Table 2.1: Diagnoses of Biopsies

Diagnosis	Number of Biopsies
Membranous glomerulonephritis	22
Membranoproliferative glomerulonephritis	19
Focal glomerulonephritis	28
Henoch-Schönlein nephritis	14
Systemic lupus erythematosus	10
Minimal change nephrotic syndrome	11

These biopsies were examined for the presence of the following proteins: IgG, IgA, IgM, C1q, C1s, C4, properdin, factor B, C3, C5, $C\bar{1}$-INH, C3bINA and β1H. Biopsies which fell into a distinct pathological category but which were discarded because of the small numbers had the following diagnoses; focal glomerulosclerosis - 8 biopsies, rapidly progressive glomerulonephritis - 4 biopsies, anti-glomerular basement membrane disease - 5 biopsies, acute diffuse proliferative glomerulonephritis - 5 biopsies and diabetic nephropathy - 3 biopsies.

Renal Immunofluorescence Methods

Freezing and storing of biopsy material: The renal biopsies obtained were generally about two millimetres in diameter and two to five millimetres in length. Following removal from the body, the biopsy was immersed immediately in Tissue-Tek II contained within a gelatin capsule. The biopsy was aligned horizontally within the upright capsule, frozen in liquid nitrogen and stored in a sterile bijou bottle until required. When biopsies were recut after a period of storage, at least one test was repeated, usually C3, in order to check for tissue deterioration. In the few cases where some deterioration was noted, the blocks were discarded.

Immunofluorescence test: The method used was a modification of that suggested for use in the immunofluorescence trial for the International Study of Kidney Disease in Children.

Using a Sloe cryostat, 4μ sections were cut from the embedded renal biopsies and air dried for 30 minutes. Sections were washed twice for a total of 20 minutes in PBS while shaking on a Griffin flask shaker. These sections were then fixed for 10 minutes in ether alcohol at 4°C followed by 95% alcohol for twenty minutes at 4°C and washed three times in PBS for 15 minutes. The glass slides were dried leaving the sections moist and the sections were incubated with one drop of the relevant antiserum in a

humid chamber for 30 minutes at room temperature. This was followed by three washes in PBS for 20 minutes. When the indirect test was used sections were incubated with the second layer antibody and washed as above. Sections were then mounted in tris buffered glycerol pH 8.5, and stored in the dark at 4°C.

Control sections from each kidney were tested each time:-

(a) One section was incubated with PBS rather than antisera during each incubation.

(b) Sections were incubated with PBS during the first incubation and then with the relevant second layer antiserum.

(c) Sections were incubated with normal rabbit serum or normal goat serum as appropriate and with the relevant second layer antiserum in the second incubation.

Microscopy and Photography: The microscope used was a Leitz Ortholux II with a high pressure mercury vapour lamp, HBO 200, light source, a BG38 heat filter, a 4 mm BG12 interference filter, an inbuilt barrier filter at setting 3 and a supplementary barrier filter, K510. This system is designed to allow only light below a wavelength of 490nm to reach the tissue and only light above 520 nm to reach the eye pieces since the peak of FITC absorption is at 490nm and the peak of emission is at 520nm.

The specimens were examined within 24 hours of being stained although no deterioration was noted on storage at 4°C in the dark for up to two weeks. Sections were usually viewed using x10 wide-field eyepieces and a x40 objective lens. Only where localization of staining was difficult was an oil emersion x100 objective lens used.

All sections were assessed for intensity of stain on a scoring system of zero to four; zero = negative, one = trace, two = weak, three = moderate and four = intense. The sections were also described according to pattern of staining; C = capillary loop, M = mesangial, S = segmental, F = focal and P = peripheral.

All photography was done using a Leitz orthomat camera attachment. Timed exposures were preferred to automatic exposures in order to record the intensity of staining in each section. For most of the work, the film used was High Speed Ektachrome uprated to 400 ASA, with an exposure time of two minutes but latterly Ektachrome 200 professional film uprated to 800 ASA with an exposure time of 30 seconds was used. All black and white prints were made from colour transparencies.

Details of Immunofluorescence Methods

Fixation: Fixation is a process by which the molecules within tissue are stabilised thus inhibiting the diffusion of insoluble substances and preventing autolysis and bacterial decomposition of the tissue.

Various fixing techniques were tested within the immunofluorescence system as shown in table 2.2. Twenty sections from each of nine biopsies were cut. These biopsies were chosen to represent the range from negative to strongly positive biopsies and from kidneys with severe pathological damage to kidneys with mild disease. Four sections from each biopsy were fixed by each of the five fixing methods and two of each four were then stained with FITC conjugated rabbit anti-human IgG antiserum and two with FITC conjugated rabbit anti-human C3 antiserum. IgG and C3 were chosen because they are the two proteins most commonly found in glomerulonephritis. All sections were read without knowledge of the procedure which they had undergone and scored on the basis of three criteria:-

1) intensity of staining; scored 0 to 4
2) quality of staining; scored 0 to 5
3) background staining; scored 0 to 4

Since some fixatives caused staining patterns to be hazy and diffuse, quality of staining was an index of distinctness of staining. When assessing quality of stain, only those

sections which were positive were considered.

The mean scores for the intensity and quality of stain and the background intensity for each fixation method are shown in table 2.2.

Table 2.2: Assessment of various fixation methods

	Fixative	Mean Score		
		Intensity	Quality	Background
1	No Fixative	2.4	3.9	1.9
2	Ether alcohol at 4°C (10 minutes) 95% alcohol at 4°C (20 minutes)	2.2	3.9	1.4
3	Ether alcohol (10 minutes) 95% alcohol (20 minutes) both at room temp.	1.6	3.8	1.5
4	95% alcohol (30 minutes)	1.7	3.9	1.5
5	Acetone (5 minutes)	1.6	2.8	2.1

It can be seen from table 2.1 that method 2 is second only in intensity of staining to method 1 where no fixative was used. Fixation however allows sections to be kept longer and also increases adhesion of sections to the glass slides. For these reasons and also because background staining was higher in method 1, method 2 was chosen in preference to method 1. Background staining was lower for method 2 than for any other fixing method and there was

at least as much quality of staining as for the other fixatives, therefore method 2 was chosen.

Antisera: All renal biopsies are tested routinely for the presence of IgG, IgA, IgM, C1q, C4 and C3 by the direct method using FITC conjugated antisera to each of these proteins. The direct technique was employed because of the speed and convenience necessary for a routine test, because commercial conjugated antisera are readily available and because it allows comparison with the results of other laboratories since the direct is almost universally employed in routine laboratories.

In this study deposition of C1s, properdin, factor B, C1-INH, C3bINA and βIH was studied by the indirect immunofluorescence technique. The sections were incubated first with rabbit or goat antiserum to the relevant antigen, washed and incubated with antibody to rabbit or goat globulin, as appropriate, conjugated to FITC. There are two main reasons for using the indirect method:-

a) It requires conjugation to a fluorochrome only of antisera used in the second incubation.

b) It is more sensitive.

Before use, antisera, shown to be pure by double diffusion and immunoelectrophoresis, were further tested within the immunofluorescence system.

All antisera were incubated for two hours at room

temperature after being diluted in the appropriate pure antigen, bovine serum albumin at a concentration of 5mg/ ml or PBS. This mixture was centrifuged at 1000 g for 15 minutes at room temperature to remove any precipitate which may have formed. Antisera absorbed in this way were tested by immunofluorescence against at least two positive biopsies either by the direct or indirect method as appropriate. All sections were read without knowledge of the procedure which they had undergone and scored for intensity. Staining was eliminated in all cases by prior incubation with specific antigen (figure 2-3) and was not diminished by incubation with bovine serum albumin. Therefore, within the sensitivity of the immunofluorescence test employed, all antisera were shown to be specific.

To check the specificity of the FITC staining, tissue sections from at least two positive biopsies were incubated with antisera from the same species as the FITC conjugated antisera employed in the immunofluorescence test but these antisera were not conjugated to FITC. After washing, the sections were then incubated with the appropriate FITC conjugated antiserum. Again the staining was totally removed. Incubating sections initially with antisera of other specificities caused no reduction in intensity of staining when compared with sections treated normally.

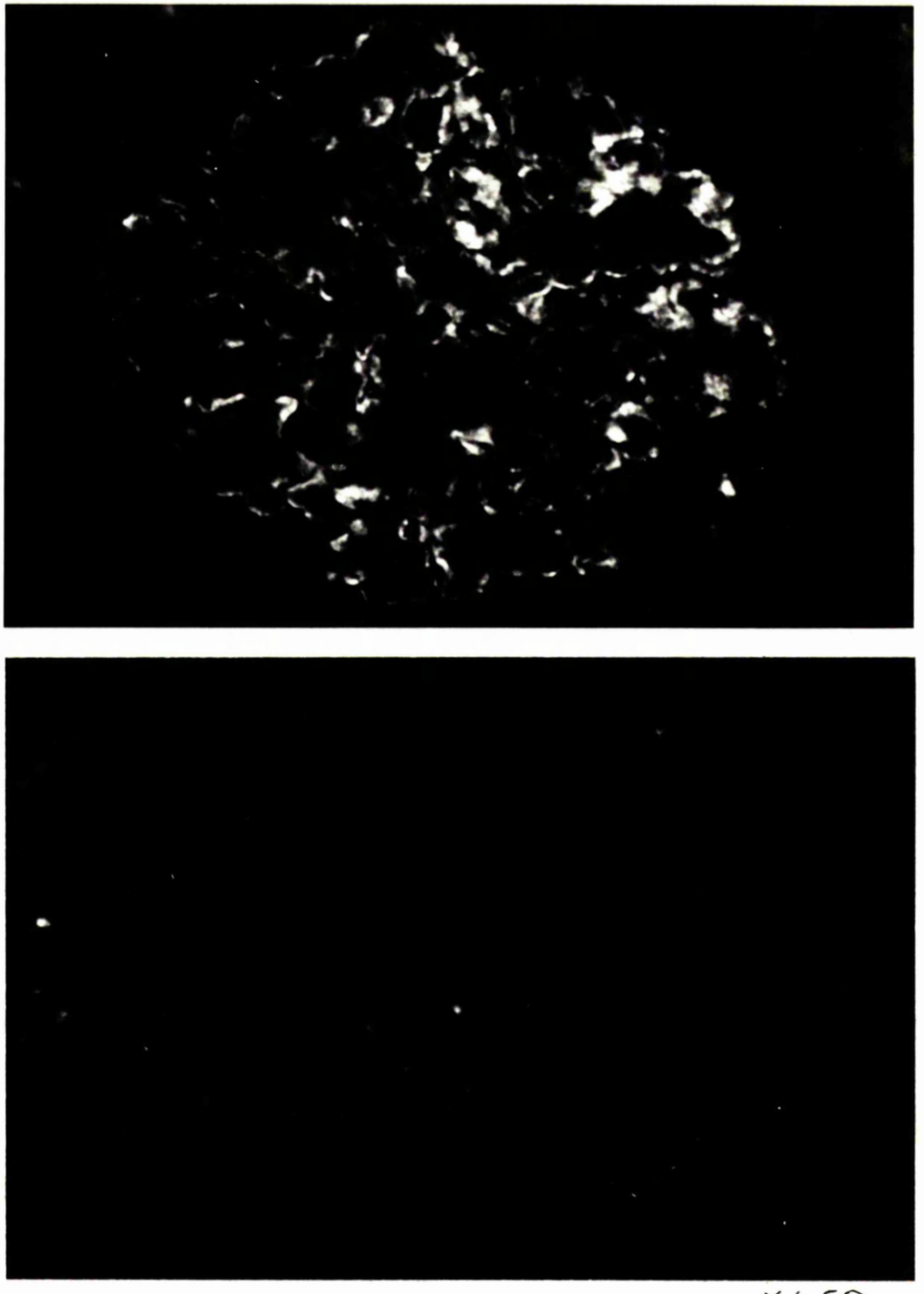


Figure 2-3 The top photograph shows the deposition of IgG from a patient with MP GN. The photograph below shows that the staining on an adjacent section has been completely blocked by prior absorption of the antiserum with purified IgG antigen.

All antisera were titrated before use to ascertain the optimal concentration. This was considered to be that concentration which gave highest intensity of staining with the lowest background staining. Doubling dilutions of each antiserum were incubated with sections from at least two positive biopsies as described above. All sections were read without knowledge of the procedure which they had undergone. In each case the optimal dilution was found at least two dilutions from the end point of staining.

All antisera were stored in small aliquots at -20°C and thawed only once.

Choice of fluorochrome: Of all the fluorochromes available FITC remains the most useful for the fluorescent antibody technique for several reasons:-

a) It has a high fluorescence efficiency, that is the ratio of light emitted to light absorbed, high.

b) It is easily conjugated to antibody and does not interfere with the antigen/ antibody union.

c) It remains attached to antibodies during long term storage at -20°C.

d) Unbound dye is easily removed because of its low molecular weight.

e) Commercial products are readily available.

Mounting: Fluorescence efficiency increases with increasing pH up to pH 8.0 (approximately). For this reason, glycerol

buffered to pH 8.5 with tris buffer was used.

Summary of Immunofluorescence Methods

1. Embed biopsies in Tissue-Tek in a gelatin capsule and snap-freeze immediately in liquid nitrogen.
2. Store at -70°C in a sealed container.
3. Cut 4μ frozen sections.
4. Air dry for 30 minutes.
5. Wash in PBS, 2 x 10 minutes.
6. Fix in ether alcohol at 4°C, 10 minutes.
7. Continue fixation in 95% alcohol at 4°C, 20 minutes.
8. Wash in PBS, 3 x 5 minutes.
9. Incubate with antisera in humid container, 30 minutes.
10. Wash in PBS, 3 x 7 minutes.
11. Repeat steps 9 and 10 for indirect test.
12. Mount in glycerol, buffered to pH 8.5
13. Examine sections and photograph.
14. Store at 4°C in the dark.

Serum Studies

Clotted blood samples were obtained, where possible, within 24 hours of renal biopsy. These were obtained from the following disease groups:-

Table 2.3: Diagnoses of patients from whom serum samples were obtained

Diagnosis	Number of Sera
Membranous glomerulonephritis	11
Membranoproliferative glomerulonephritis	17
Focal glomerulonephritis	14
Henoch-Schönlein nephritis	4
Systemic lupus erythematosus	10
Minimal change nephrotic syndrome	5

Serum samples were obtained from 50 healthy blood donors, courtesy of Dr. J. Wallace, Regional Blood Transfusion Service, Law Hospital. These were used to estimate the normal range for each complement protein studied.

The serum concentration of the following complement proteins was measured in each diseased and normal serum sample; C1q, C1s, C4, properdin, factor B, C3, C5, $C\bar{1}$-INH, C3bINA and β1H.

Radial Immunodiffusion Method

The antisera for radial immunodiffusion were those used in the immunofluorescence test apart from rabbit anti-human C1q and rabbit anti-human C3 which were supplied by Dr. K. Whaley. All antisera had been checked for mono-specificity by double diffusion gel precipitation and immunoelectrophoresis as above.

Suitable volumes of monospecific antisera, previously estimated by titration, were added to 40 ml aliquots of 1.5% agarose in VBS containing 0.01M EDTA and 3.3 ml of antibody impregnated agarose were poured into 5 cm plastic petri dishes and left to solidify at room temperature. These plates were stored in a humid chamber at 4°C until required. Wells were cut using a needle of 1.3 mm internal diameter for all plates except those for C1q, where a needle of 1.7 mm internal diameter was used. The agarose plugs were removed by suction and the wells were filled using a drawn out glass capillary tube. Each serum sample was tested in duplicate. A pool of normal human sera was aliquoted and stored at -70°C. Three wells in each radial immunodiffusion plate were filled with dilutions of this pool; undiluted (100%) 50% and 25% (dilutions were made in PBS). The serum was left to diffuse for 48 hours at room temperature in a humid atmosphere.

Where antigen and antibody meet in optimal proportions, diffusion of specific antigen stops and a ring of precipitation is formed as shown in figure 2-4. The area within the ring is proportional to the concentration of antigen applied to the well. When the squares of the diameters of the 100%, 50% and 25% standards are plotted against concentration on linear graph paper, a stright line is obtained. The intercept of this line on the y - axis approximates to the square of the diameter of the well and the percentage concentration of the serum protein under test may be read from the graph against the square of the diameter of the ring size. Results were analysed using a Hewlett-Packard Desk Top Computer model 9800. The programme printed the correlation coefficient of the standard line, the intercept on the y-axis and individual concentration in terms of percentage of the normal pool and in µg/ml. Only results from plates whose standard had a correlation coefficient greater than $r = 0.997$ were accepted. Duplicates were calculated separately and repeated if they differed by more than 10% in the final concentration and samples giving readings of greater than 110% of the 100% standard were repeated after dilution in PBS.

Normal Range: The concentration of each of the proteins studied was measured in each of the 50 normal sera in terms of percentage of the standard pool.

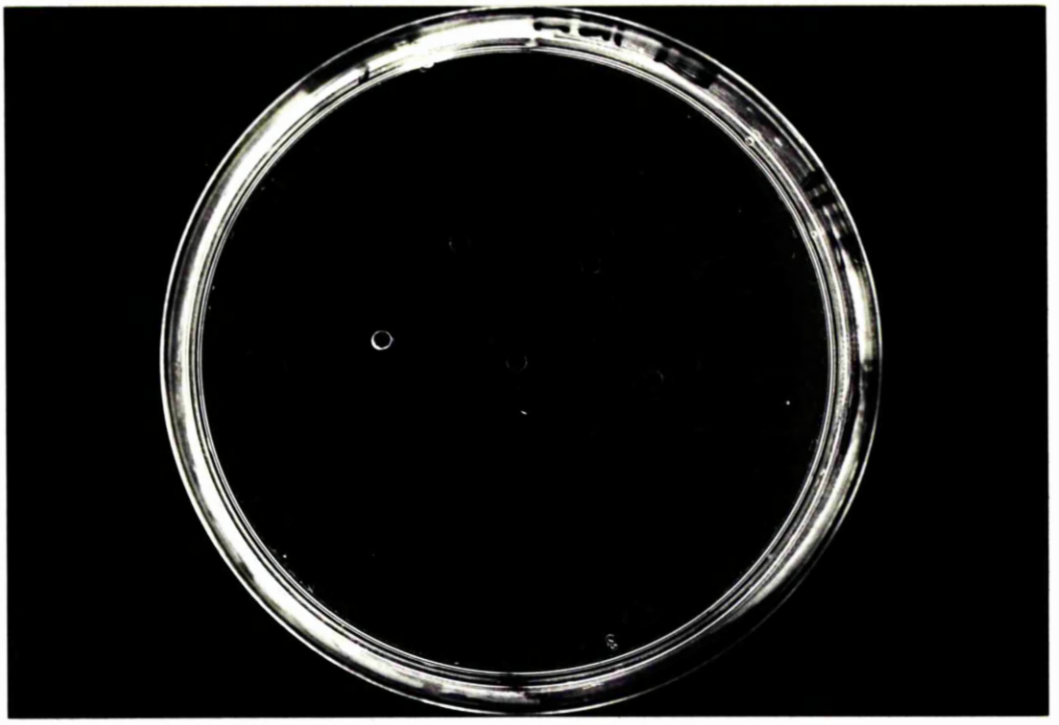


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Figure 2-4 This photograph shows a radial immunodiffusion plate in which $C\bar{1}$-INH levels were estimated. The plate was filled from the top left and each sample was tested in duplicate. The well in the centre of the plate was filled with the 50% standard with the 100% and 25% standards immediately to left and right of it respectively.

The range for each component was tested for normality using the χ^2 test for normality (165). As all ranges were found to be normally distributed the mean $\pm$ two standard deviations was calculated and used as the normal range. Serum concentrations of each component, except C3bINA, were converted to µg/ ml using reference sera supplied by Dr. P. Schur and Dr. K. Whaley and the normal ranges are shown below.

C1q	283-672 ug/ ml
C1s	24-49 "
C4	149-550 "
properdin	13-41 "
factor B	111-303 "
C3	769-1794 "
C5	84-170 "
$C\bar{1}$-INH	121-303 "
C3bINA	55-123% of normal
βlH	156-356 µg/ ml

C3 Nephritic factor assay

All membranoproliferative GN (MPGN) sera and all other sera with C3 levels below the normal range were examined for the presence of nephritic factor (NF).

One hundred microlitres of test serum were incubated with 300 µl of normal human serum, either in the presence of 0.01M EGTA with 0.01M magnesium chloride or 0.01M EDTA for 20 minutes at 37°C. As controls, a known positive and a known negative were included each time. After 20 minutes, the reaction was stopped by the addition

of 200 µl of 0.1M EDTA at 0°C. Magnesium EGTA allowed only activation of the alternative pathway while EDTA inhibited all complement activation. The residual C3 was measured by a haemolytic titration of C3 and the percentage utilisation of C3 due to alternative pathway activation was calculated for each sample.

Ten normal serum samples were each tested on four occasions in order to establish the lower limit of normal. The mean of these results was 93.0% with a standard deviation of 13.7%. The lowest result was 68% residual C3 and this was accepted as the lower limit of normal. When the GN sera were tested only 3 MPGN sera were positive and no borderline values were found; the highest of the three subnormal values was 43 per cent and the others were nine and four per cent residual C3.

Haemolytic C3 assay

The following reagents, required for this assay, were supplied by Dr. K. Whaley; rabbit anti-sheep haemolysin, human C1 and human C2.

Preparation of EAC4 cells (166): 2.5×10^{11} sheep erythrocytes were washed twice in 100 ml of PBS, centrifuged at 1000g for ten minutes, and resuspended finally in 0.01M EDTA/ GVB$^{=}$ at a concentration of 1×10^{9} cells per ml (250 ml). An equal volume of rabbit anti-sheep haemolysin at a dilution of 1/ 100

(previously titrated) in 0.01M EDTA/ $GVB^{=}$ was added and the mixture was incubated at 37°C for 30 minutes. This was centrifuged at 1000g for ten minutes and resuspended in 500 ml $DGVB^{++}$. To these cells was added 2×10^5 units of human C1 in 500 ml of $DGVB^{++}$ and these were incubated at 37°C for 15 minutes, centrifuged for 15 minutes at 1000g, resuspended in 500 ml $DGVB^{++}$ and cooled to 0°C. To 500 ml of EAC1 cells, thus prepared, was added a one in ten dilution of normal human serum in 0.01M EDTA/ $GVB^{=}$ and the mixture was incubated at 0°C for 15 minutes while shaking. The cells were then washed twice in 0.04M EDTA/ $GVB^{=}$ to dissociate to the C1 complex, centrifuged at 0°C at 1000g for 15 minutes, made up to a volume of 500 ml in 0.04M EDTA/ $GVB^{=}$ and incubated at 37°C for 30 minutes to decay C2 from the $EAC4\bar{2}$ complex. The cells were washed once in 0.04M EDTA/ GVB= and twice in $DGVB^{++}$ centrifuging at 1000g for 15 minutes. The cells were then in the EAC4 state.

Preparation of EAC14 cells (166): To 10 ml of EAC4 cells at a concentration of 1×10^8 in $DGVB^{++}$ were added 2,000 units of human C1 in 10 ml of $DGVB^{++}$ and this mixture was incubated at 37°C for 15 minutes. The EAC14 cells were centrifuged at 1000g for 5 minutes and made up to 10 ml in $DGVB^{++}$.

Oxidation of human C2 (86) and preparation of $EAC14^{oxy}$ 2 cells:

Ten microlitres of potassium iodide/ iodine oxidising agent

(KI/I_2) were diluted in 2 ml of 0.1M phosphate buffer, pH 6.0 and 50 µl of this was added to 50 µl of human C2 containing 2,000 units. This mixture was incubated for five minutes at room temperature and then made up to 10 ml in $DGVB^{++}$ and mixed with 10 ml of EAC14 cells in $DGVB^{++}$. Oxidation of C2, in this way, has been shown to increase the stability of the $C\overline{42}$ complex. The cells and oxidised C2 were incubated at 37°C for 15 minutes and the $EAC14^{oxy}2$ cells were centrifuged at 1000g for five minutes and made to a final concentration of 1×10^8 cells in $DGVB^{++}$.

Preparation of C3 depleted serum (R3) (167): To 10 ml of normal human serum was added 2.5 ml of 0.3M hydrazine hydrate and this was incubated for two hours at 37°C, adjusted to pH 7.0 and dialysed at 4°C against 1 litre of PBS.

C3 Assay: 50 µl of a 1/400 dilution of C3 nephritic assay product in 0.04M EDTA $GVB^{=}$ was incubated with 50 µl of a 1/50 dilution of the C3 depleted serum (R3) in 0.04M EDTA $GVB^{=}$ and 100 µl of $EAC14^{oxy}2$ cells at a concentration of 1×10^8 in $DGVB^{++}$ for one hour at 37°C. Two ml of PBS were added at 0°C and the tubes centrifuged at 1000g for five minutes. The optical density of the supernatant was measured at a wavelength of 414nm. As a control, to test the effect of the R3 reagent, the C3 nephritic factor assay product was replaced in the C3 haemolytic assay by 50 µl of 0.04M EDTA $GVB^{=}$

and the optical density reading of this reagent blank was subtracted from all test readings before the percentage of residual C3 was calculated. The concentration of the R3 reagent had been titrated previously to give less than 10% lysis in the above assay.

Statistical Tests

1) Mean and Standard deviation

The mean and standard deviations of sets of values were calculated using a Hewlett Packard calculator, model 9800.

2) χ^2 Test for normality (165)

This test was used to ascertain whether a set of values was normally distributed in order to establish which statistical tests were appropriate for further analysis of the groups. The basis of the test was to divide the observed values into suitable class intervals. Using the mean and standard deviation of the observed values, the expected number of values in each class interval was calculated assuming that the values were truly normally distributed. The observed and expected number of values in each class interval were then compared using the χ^2 test with the Null Hypothesis that there was no difference between the expected and the observed results. The number of degrees of freedom was calculated as the number of class intervals minus 3. χ^2 tables show the level of probability that the observed results vary significantly from the expected.

The normal range for the serum concentration of each component when analysed in this way, was found to be normally distributed. The serum levels within the disease groups, however, were often not normally distributed. This was particularly true for MPGN and SLE. For this reason serum levels were analysed using the median test and the

Spearman rank correlation test.

3) Median Test (169)

This test was used to see if the serum levels of any single complement component in any group varied significantly from normal. The parametric student t test could not be employed since many of the sets of values were not normally distributed. The null hypothesis was that the two groups were from populations having the same median. The values in the two groups to be compared were combined and the median of this combined group found. The two sets of scores were then split at the combined median and set in the table below.

	Group I	Group II
Number of scores above combined median	A	B
Number of scores below combined median	C	D

Since the combined sample size was always greater than 50 the χ^2 test corrected for continuity was used to analyse the results.

The formula is:-

$$\chi^2 = \frac{N(|AD-BC| - \frac{N}{2})^2}{(A+B)(C+D)(A+C)(B+D)}$$

χ^2 tables were used to find the level of probability that the two groups were from populations having the same median.

4) Spearman rank correlation test (169)

The Spearman rank correlation test is used to measure the level of association between two sets of paired variables which are on an ordinal scale or which are on an interval scale but are not normally distributed. The analysis was carried out using the appropriate programme in the "Statistical Pack for the Social Sciences" at the University of Newcastle computer using a terminal at the University of Glasgow.

This test was used to assess the correlations between serum levels of each component within each disease group since sets of values were often not normally distributed. Correlations between intensities of staining of each component within the glomeruli in each disease group were also analysed in this way. Tissues which were negative for any antigen were given a score of zero for that antigen and ranked accordingly.

CHAPTER 3

Membranous Glomerulonephritis

Membranous Glomerulonephritis

Introduction

The term membranous glomerulonephritis (MGN) was used to describe a pathological entity involving thickened basement membranes and associated with nephrotic syndrome (170). Differentiating between MGN and MCNS at one extreme and MPGN at the other was greatly simplified by the introduction of the methenamine silver stain (171). This showed that, rather than being uniformly thickened the basement membrane had spikes projecting outwards and round silver-negative material. By electron microscopy, the silver-negative material was found to consist of electron dense deposits. Little or no cellular proliferation is present and this has led some pathologists to prefer the term glomerulopathy.

Immunofluorescence studies have shown that the capillary loops are studded with deposits which stain for IgG. There is debate regarding the deposition of complement components. Some authors have frequently found C3 deposition (172-175) while others have seldom found it (176). The stage of the disease may be important since Zollinger found that biopsies taken early in the course of the disease were often negative for C3 while C3 was frequently present in biopsies taken at a later stage (177).

The evidence for an immune complex pathogenesis is

as follows:-

a) The deposition of IgG and C3 round capillary loops is in a position similar to that of electron dense deposits.

b) There is a close similarity in morphology between MGN and the Heymann model of GN (178) and chronic serum sickness (65) - both immune complex diseases.

c) In SLE, a known immune complex disease, one pattern of glomerular lesion is indistinguishable from that of MGN (53).

d) Various antigens have been found to be deposited in MGN biopsies in the same distribution as IgG (see table 1.1).

e) Immune complexes have occasionally been found in the sera of MGN patients (179-181, 191).

In contrast to this supportive evidence however,

a) Immune complexes generally cannot be found in serum (179-181, 191).

b) Circulating levels of complement components are generally not reduced (173, 182) although in one study two of 17 patients had reduced C3 concentrations (183).

c) The inflammation, normally associated with a type III hypersensitivity reaction, e.g. infiltration of polymorphonuclear and mononuclear leucocytes, is not evident in the glomeruli.

Despite this, the balance of evidence would appear to favour an immune complex pathogenesis. The possibility that

immune complexes may form within the capillary loop in MGN rather than being deposited from the circulation was discussed in the introduction (40). If this is the case it would account for the general absence of circulating immune complexes and also, to some extent, for the absence of reduced serum complement levels.

Materials

Immunofluorescence studies were performed on renal biopsies obtained from 22 MGN patients. All of these had proteinuria at the time of biopsy ranging from 1 g to 18.7 g/ 24 hours (mean = 7.2 g/ 24 hours). Serum creatinine levels all fell within the normal range.

Between 1 and 10 glomeruli (mean = 3.9) were examined by immunofluorescence in each biopsy and serum samples were obtained at the time of biopsy from 11 patients.

Results

Renal Biopsies

Glomerular deposition of immunoglobulins, complement and control proteins

The number of biopsies positive and the mean intensity of staining for each protein examined is shown in table 3.1 (see over).

Table 3.1 Glomerular deposition of immunoglobulins, complement components and control proteins

	Immunoglobulins			Classical Components			Alternative Pathway Components		Terminal Components		Control proteins		
	G	A	M	C1q	C1s	C4	P	B	C3	C5	C1-INH	C3bINA	fB1H
Number Positive	22/ 22	0/ 22	4/ 22	4/ 20	6/ 20	14/ 19	4/ 20	0/ 20	21/ 22	18/ 19	15/ 20	0/ 20	18/ 20
Mean Intensity	2.6		1.0	1.8	1.0	1.5	1.5		1.8	2.1	1.6		1.8

Mean intensity = the mean of the scores given for the intensity of deposition of each protein in each biopsy

Deposition of immunoglobulins

IgG was found, with a high mean intensity, in all 22 biopsies. IgA was not deposited in any and IgM was present in only four of 22 (18%) kidneys examined.

Deposition of classical pathway components

Fourteen of 19 biopsies (73%) were positive for C4 although C1 subcomponents were found in only six of 20 biopsies (30%).

Deposition of alternative pathway components

In four of twenty biopsies (20%) there was deposition of properdin. This was of low mean intensity. Factor B was not found.

Deposition of C3 and C5

Twenty one of 22 biopsies (95%) were positive for both C3 and C5. The intensity of C3 was lower than in other groups where C3 was frequently found (appendix I).

Deposition of control proteins

Deposition of C1-INH was seen in 15 of 20 (75%) and FH in 18 of 20 (90%). C3bINA was not detected in any MGN biopsies.

Correlations in intensities of staining between the proteins studied

Using the Spearman rank correlation test, the intensities of staining of each protein studied was compared with the

intensities of all others (Table 3.2). Because of the small number of biopsies positive for IgM, C1q and properdin and the absence of IgA, factor B and C3bINA, no statistical analyses were performed on the results of these. R represents the Spearman correlation coefficient and p the probability of significance.

Significant correlations are shown in red.

Table 3.2: Correlations in intensities of staining between the proteins studied

MGN		G	C1s	C4	C3	C5	C1̄-INH
C1s	R	0.02					
	p	NS					
C4	R	0.19	0.02				
	p	NS	NS				
C3	R	0.57	0.15	0.19			
	p	0.01	NS	NS			
C5	R	0.37	0.26	0.21	0.51		
	p	NS	NS	NS	0.02		
C1̄-INH	R	0.37	0.13	0.25	0.14	0.03	
	p	NS	NS	NS	NS	NS	
βIH	R	0.56	0.02	0.28	0.39	0.52	0.36
	p	0.01	NS	NS	0.05	0.02	NS

NS = not significant

The correlations of classical pathway components

The intensities of deposition of C1s and C4 did not show significant correlations with the intensities of any other protein.

The correlations of C3 and C5

The intensities of deposition of C3 correlated with those of IgG and C5.

The correlations of the control proteins

The intensities of $C\bar{1}$-INH deposition did not correlate with those of any other component, while the intensities of BlH deposition correlated with those of IgG, C3 and C5.

The concordance between deposition of the control proteins of complement and the components whose activity they regulate

$C\bar{1}$-INH

Table 3.3 The concordance between biopsies positive for C1s, C4 and $C\bar{1}$-INH

C1s/ $C\bar{1}$-INH	No. of Biopsies	C4/ $C\bar{1}$-INH	No. of Biopsies	C1s/ C4	No. of Biopsies
C1s + $C\bar{1}$-INH+	5	C4 + $C\bar{1}$-INH+	12	C1s+ C4+	6
C1s + $C\bar{1}$-INH-	1	C4 + $C\bar{1}$-INH-	2	C1s+ C4-	0
C1s - $C\bar{1}$-INH+	10	C4 - $C\bar{1}$-INH+	3	C1s- C4+	8
C1s - $C\bar{1}$-INH-	4	C4 - $C\bar{1}$-INH-	2	C1s- C4-	5
Total	20	Total	19	Total	19

Table 3.3 shows the concordance between biopsies positive for C1-$\bar{1}$NH and those positive for C1s and C4. C1s and $C\bar{1}$-INH were found together in only five biopsies while, in ten, $C\bar{1}$-INH was present without C1s. Better concordance was seen between C4 and $C\bar{1}$-INH, with 12 biopsies being positive for both. In

two, C4 was found without $C\bar{1}$-INH and in three $C\bar{1}$-INH was deposited in the absence of C4. All biopsies, with deposition of C1s, were positive for C4. In eight, however, C4 was present without C1s.

βlH

Table 3.4 The concordance between biopsies positive for C3, C4, C5, properdin and βlH

C3/ βlH	No. of biopsies	C4/ βlH	No. of biopsies	C5/ βlH	No. of biopsies	P/ βlH	No. of biopsies
C3+ βlH+	18	C4+ βlH+	12	C5+ βlH+	17	P+ βlH+	4
C3+ βlH-	1	C4+ βlH-	2	C5+ βlH-	2	P+ βlH-	0
C3- βlH+	0	C4- βlH+	5	C5- βlH+	1	P- βlH+	14
C3- βlH-	1	C4- βlH-	0	C5- βlH-	0	P- βlH-	2
Total	20	Total	19	Total	20	Total	20

Table 3.4 shows the concordance between biopsies positive for βlH and C3, C4, C5 and properdin. Of the twenty biopsies examined for deposition of C3 and βlH, both proteins were found in 18, in one C3 was found alone and in one neither was present. C4 and βlH were present together in 12 biopsies. In two, C4 was found in the absence of βlH and in five βlH was deposited without C4. Concordance was shown between biopsies positive for βlH and C5 in 17 biopsies, in two C5 was present without βlH and in one βlH without C5. βlH was found in all four biopsies positive for properdin.

Comparisons of immunofluorescence patterns

The staining pattern for each protein within each biopsy was assessed separately. In all cases, for all proteins, except IgM, staining was found in a diffuse granular capillary loop pattern. IgM, where present was deposited segmentally. Close examination of the patterns of C1s and C1-INH and C3 and βIH (Figure 3-1), on adjacent sections, showed identical patterns of distribution.

Serum samples

The concentration of each component in the serum of each of 11 patients is shown on figure 3-2.

The concentrations of classical pathway components

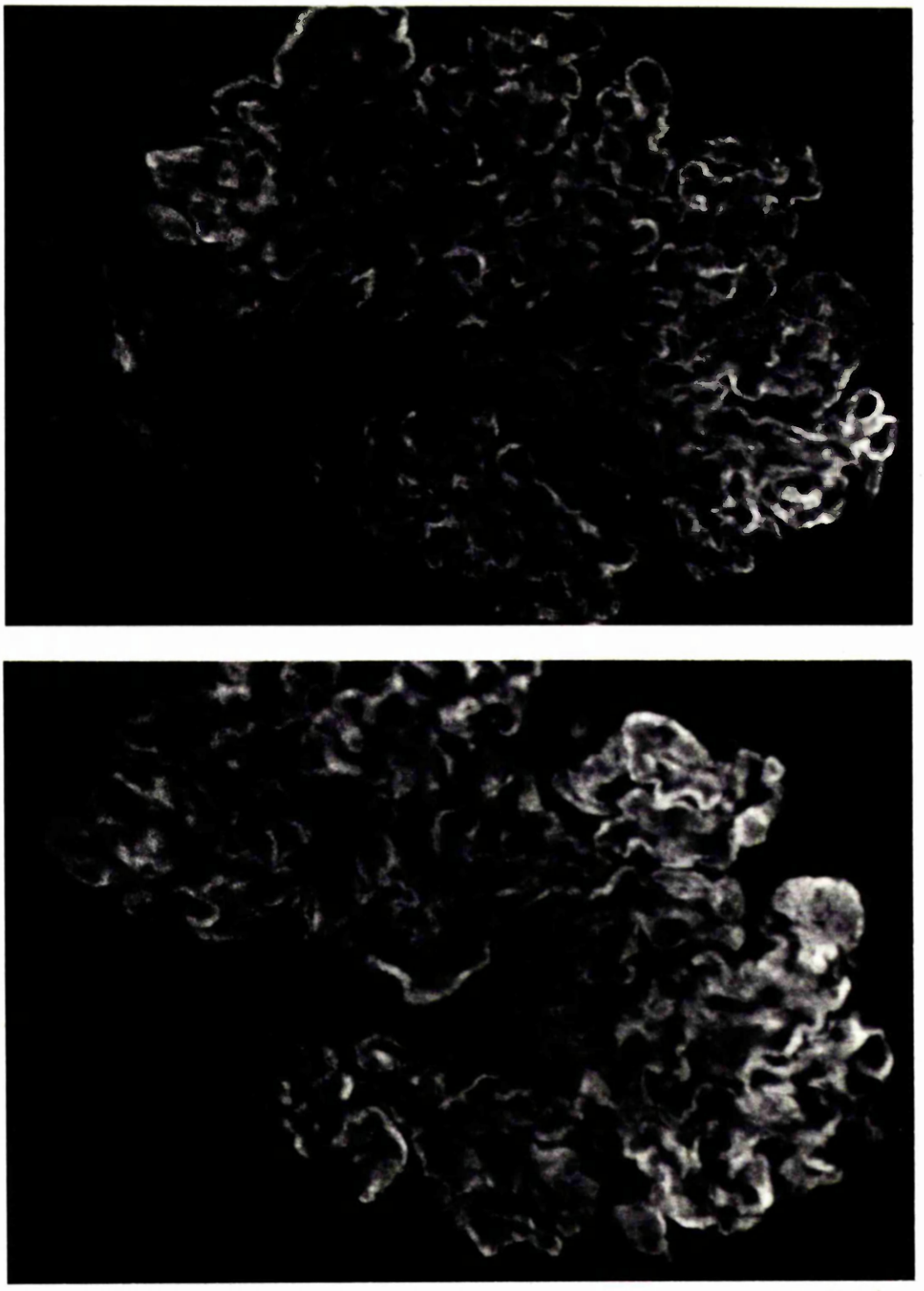
The median levels of C1q and C4 were significantly raised in MGN while C1s values did not differ significantly from normal. No subnormal concentrations were found for any of the classical pathway components studied.

The concentrations of alternative pathway components

Neither properdin nor factor B values as a group varied significantly from normal. Four factor B concentrations and one properdin level were above normal. No subnormal values were found in the MGN group.

The concentrations of C3 and C5

The median values for both C3 and C5 concentrations were significantly raised. No values were found below the normal



×650

Figure 3-1 The top photograph shows a glomerulus stained for C3 and the photograph below shows the same glomerulus on an adjacent section stained for βlH.

range and high values, particularly of C5, were common.

The concentrations of the control proteins

All MGN patients had $C\bar{1}$-INH concentrations above the normal range. The C3bINA median did not differ significantly from normal. One C3bINA concentration was below and another above the normal range. All but three β1H concentrations were raised and the median value for β1H was significantly raised.

The correlations between serum concentrations of complement components and the control proteins

The correlations between the serum concentrations of each of the components studied were calculated using the Spearman rank correlation test and the results are shown in table 3.5. R represents the Spearman rank correlation coefficient and p represents the probability of significance. As can be seen, very few significant correlations were found.

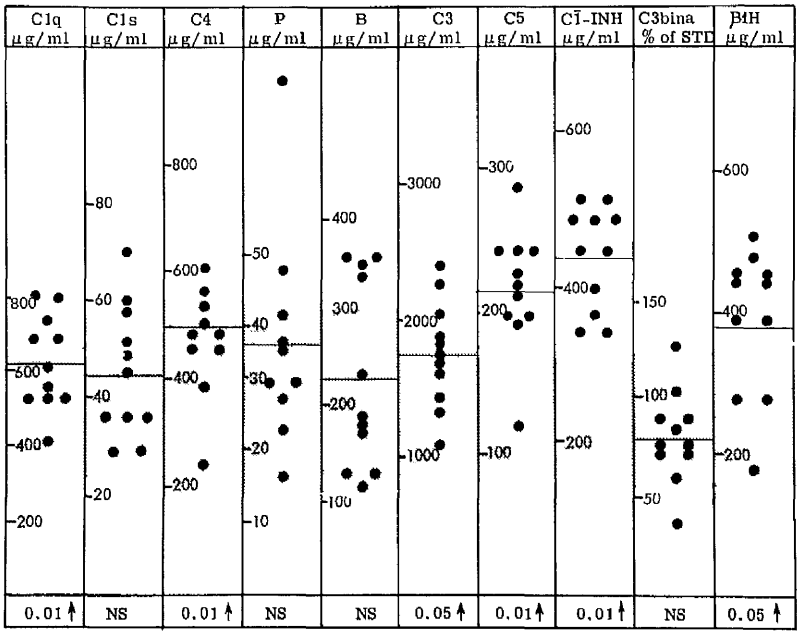


Figure 3-2 The concentrations of each protein studied in each of the 11 MGN serum samples are shown. The shaded areas represent the normal ranges and the horizontal lines represent the mean concentration for each protein. The results of the median test are shown at the bottom of each column with arrows indicating whether the concentrations are significantly higher or lower than normal.

NS = not significant.

Table 3.5 Correlations in serum concentration of the proteins studied

		C1q	C1s	C4	P	B	C3	C5	C1-INH	C3bINA
C1s	R	0.15								
	p	NS								
C4	R	0.00	0.31							
	p	NS	NS							
P	R	0.14	-0.30	0.14						
	p	NS	NS	NS						
B	R	0.35	0.02	-0.62	-0.10					
	p	NS	NS	0.05	NS					
C3	R	0.44	0.22	-0.15	0.34	0.41				
	p	NS	NS	NS	NS	NS				
C5	R	-0.16	-0.22	0.18	0.06	0.19	0.03			
	p	NS	NS	NS	NS	NS	NS			
C1-INH	R	-0.04	0.29	0.22	0.12	-0.13	0.66	0.35		
	p	NS	NS	NS	NS	NS	0.05	NS		
C3bINA	R	0.05	-0.27	-0.62	0.00	0.74	0.07	0.05	-0.26	
	p	NS	NS	0.05	NS	0.01	NS	NS	NS	
pIH	R	0.24	0.67	0.06	-0.49	0.09	0.29	0.21	0.55	-0.34
	p	NS	0.05	NS	NS	NS	NS	NS	0.05	NS

NS = not significant

The correlations of the classical pathway components

There was a weak inverse correlation between serum concentrations of C4 and factor B. No other significant correlations were found.

The correlations of the alternative pathway components

No significant correlations were found apart from those that were for C4 and factor B.

The correlations of C3 and C5

C3 and C5 concentrations did not correlate with any other complement protein.

The correlations of the control proteins

Weak correlations were found between concentrations of $C\bar{1}$-INH and C3 and β1H, between β1H and C1s and inversely between C3bINA and C4. A stronger correlation was found between concentrations of C3bINA and factor B.

The relationship between serum concentrations and intensity of glomerular deposition of complement components and control proteins

Using the Spearman rank correlation test, the intensity of deposition was compared with serum concentration at time of biopsy of each component except for factor B, C3bINA, C1q and properdin, which were not deposited or present in too few biopsies. The results are shown in table 3.6. R represents the correlation coefficient and p, the probability of significance.

Table 3.6 The correlations between serum concentrations and intensity of deposition of each complement component

	C1s	C4	C3	C5	$C\bar{1}$-INH	β1H
R	-0.61	0.28	0.51	-0.09	0.42	-0.46
p	NS	NS	NS	NS	NS	NS

NS = not significant

No significant associations were found between serum levels and intensity of deposition of any compound in MGN.

DISCUSSION

Intraglomerular evidence of complement activation

The involvement of immunoglobulins

The finding of fine granular deposition of immunoglobulins, particularly of IgG, in all biopsies is in agreement with other studies (172, 173, 177, 184). This satisfies one of the criteria of an immune complex disease. None of the biopsies in this group contained demonstrable quantities of IgA although deposition of IgA has been found occasionally in other studies (172, 173). Since IgG immune complexes activate the classical pathway, the deposition of C3 in all but one biopsy and the correlation between the intensity of deposition of IgG and C3 (Table 3.2), supports the view that the IgG found in the kidney is complexed to antigens in the form of immune complexes.

The involvement of the classical pathway

In the majority of MGN biopsies, there was evidence of classical pathway activation. C1 subcomponents were less frequently found than C4. Several factors may be responsible for this:-

a) There may be differences in the antibody potency of the anti-C1s, C1q and anti-C4 antisera. This would explain why, in another study (173) 70 percent of biopsies were positive for C1q, compared with 20 percent in this study (Table 3.1). However, the results of Berger (176) agree with the findings here.

b) It is also possible that antigenic determinants on C1s may be masked by other components; C1-INH is known to do this (115).

c) Activation of a single $C\bar{1}$ molecular complex may result in the deposition of many C4 molecules (185). It is therefore possible that, in biopsies apparently negative for C1 subcomponents, these were present in amounts sufficient to activate C4 but not to be demonstrated by immunofluorescence. This may occur particularly where complement activation is limited.

d) The binding of C1 to immunoglobulin is known to be weak at physiological ionic strength (186) and C1 may therefore dissociate from the $C\bar{1}$-IgG complex leaving IgG and C4 in the kidney.

The involvement of the alternative pathway

Properdin was deposited in four of 20 biopsies and factor B was found in none. Since MGN is normally associated with immune complex deposition and classical pathway activation, few studies have examined the glomeruli of MGN biopsies for deposition of alternative pathway components. Westberg (187) failed to find properdin in four biopsies examined, while in another study, 27% of MGN patients had glomerular deposition of properdin (187a).

In one of the properdin positive biopsies, classical pathway components were not found and therefore C3 deposition may have been the exclusive result of activation of the alternative pathway. In the other three properdin positive biopsies, classical pathway

components and properdin were deposited together. In these cases, the alternative pathway may have been activated directly by some factors in the kidney or plasma or as a result of recruitment by the classical pathway. The serum C3bINA concentration was markedly reduced in one of these three patients (36% of normal, figure 3-2). Absence of C3bINA is known to cause spontaneous activation of the alternative pathway in vitro (89) and in vivo (93). It is possible that reduced levels of C3bINA may be associated with increased alternative pathway activation. Correlations between concentrations of C3bINA and C3, factor B and properdin in other diseases suggests that such a relationship may exist (188, 196, 197). In this one patient therefore activation of the alternative pathway may have been due to low levels of C3bINA allowing amplification of activation.

It must also be considered that properdin may bind to C3b alone (189) or to the C3b/ βIH complex (190). It is therefore possible that deposition of properdin in the glomeruli does not represent activation of the alternative pathway but merely the presence of C3b. This will be discussed in more detail in Chapter 9. Presence of factor B would be a surer indication of alternative pathway activation although the absence of factor B does not exclude the possibility of alternative pathway activation as factor B decays rapidly from the C3bBb complex (109).

The terminal sequence

In four biopsies, C3 was found in the absence of either classical or alternative pathway components. There are several possible explanations for this.

a) C3 may be cleaved independently of complement activation by plasmin or other proteases.

b) C3 may be trapped in the kidney in its native form.

Neither of these is likely to be generally responsible for C3 deposition since IgG was present in all four biopsies and the correlation between IgG and C3 (Table 3.2) suggests that the presence of C3 is related to complement activation.

c) The most probable explanation is that since C3 activation is an enzymatic process (107), small quantities of $C4\bar{2}$ cause considerable cleavage of C3. Thus, in some cases undetectable quantities of C4 may be present resulting in significant C3 deposition.

d) It is also possible that C3 may remain in the kidney after the removal of C1 subcomponents and C4 or alternative pathway components.

MGN is characterised by extensive immunoglobulin deposition. C3, however, is not always found in the glomeruli (172, 176, 177) and, in this study, although present in all but one biopsy, the intensity of C3 staining was weaker than in most other disease groups (Appendix 1). Serum levels of complement

components are generally normal or raised in MGN (Figure 3-3) (182-184). These two facts suggest that complement activation in MGN is not marked.

Factors which may affect the rate of complement activation in MGN are:-

a) The number of immune complexes present. Various immune complex assays generally fail to detect circulating immune complexes in MGN (179-181, 191). The inability to demonstrate immune complexes may mean that they are not present, that they are present but in insufficient quantities for detection or that the assays used fails to detect the type of complex involved in MGN. If the assumption that MGN is an immune complex disease is correct then it is possible that small quantities of complexes are deposited within the kidney perhaps over many months. Complement activation may therefore be slight, intermittent or both.

b) The ability of immune complexes to activate complement is another factor and may involve the size of the complex (192), the antibody affinity (193) and the class and subclass of antibody (83). There is no data available from this study to assess this possibility.

c) The position of immune complexes in MGN may affect complement activation within the kidney. The electron dense deposits, assumed to be immune complexes, are deposited

in a subepithelial position and are therefore not in direct contact with the plasma. This may result in less efficient activation.

The pathogenic significance of complement activation in MGN cannot be assessed directly since experimental models of this disease are long-term models and complement depletion cannot be maintained over the required period. The absence of marked cellular proliferation and infiltration of inflammatory cells suggests that complement activation may not be performing its usual role as inflammatory mediator. It is possible that it may play a role in deposition of immune complexes although, in the acute serum sickness model, immune complex deposition was a complement independent process (58). The main morphological change in MGN is the thickening of the basement membrane. This may occur in response to the presence of immune complexes at the subepithelial aspect of the basement membrane. Complement is not known to be involved in this process.

Intraglomerular evidence of regulation of complement activation

In order to estimate the regulatory influence of $C\bar{1}$-INH, C3bINA and βIH in the glomeruli, four factors were considered:-

a) The presence of control proteins in the kidney,

b) The concordance between biopsies positive for control proteins and those proteins regulated,

c) The correlation between the intensity of deposition of the control protein and the protein which it controls,

d) The correspondence in patterns of deposition of C1s and $C\bar{1}$-INH, C3 and C3bINA and C3 and β1H.

The role of $C\bar{1}$-INH

$C\bar{1}$-INH was frequently present in MGN biopsies (Table 3.1) and where both C1s and $C\bar{1}$-INH were deposited the patterns of staining were very similar. The concordance between $C\bar{1}$-INH and C1s however was poor (Table 3.3), and there was no correlation between the intensities of deposition of these two proteins (Table 3.2). The fact that $C\bar{1}$-INH was present more frequently than C1s (Table 3.1) may have several possible explanations.

a) $C\bar{1}$-INH may mask antigenic sites on the $C\bar{1}$s molecule; it has been shown that this may occur (115).

b) The anti-C1s antisera used may have been less potent than the anti-$C\bar{1}$-INH antisera.

c) It is possible that $C\bar{1}$-INH is regulating enzymes of other humoral mediator pathways, such as the coagulation, fibrinolytic and the kinin systems (194).

The failure of $C\bar{1}$-INH to prevent further complement activation is shown by the deposition of C4 and C3. Since the complement system is primarily a defense mechanism, it is unlikely that regulation by $C\bar{1}$-INH would result in the total inhibition of complement activation. The presence of C4 therefore need not suggest a deficiency in regulation by $C\bar{1}$-INH.

Therefore the evidence from this study for the role of $C\bar{1}$-INH in modulating classical pathway activation rests on its presence in the kidney and the similarity in the distribution patterns of $C\bar{1}$-INH and C1s.

The role of C3bINA

C3bINA was not present in the glomeruli of any MGN biopsy. There is therefore no evidence that it participates in the regulation of complement activation in MGN. C3bINA acts enzymatically and is not consumed during the inactivation of C3b (195). Its absence in the kidney is therefore not unexpected in view of its action.

The role of βlH

That βlH binds to C3b in the kidney is suggested by the frequent deposition of βlH, the close concordance (Table 3.4), the good correlation between intensities of deposition of C3 and βlH (Table 3.2) and the similarities in the distribution patterns of C3 and βlH (figure 3-1). Evidence against the role of βlH as a regulator of C3b is the frequent presence of C5 (Table 3.1) and the correlation between C5 and βlH (Table 3.2). This may reflect the mutual dependence of C5 and βlH on C3b deposition.

In MGN, therefore, there is some evidence for the regulation of C1s by $C\bar{1}$-INH and particularly C3b by βlH;

but the role of C3bINA is unclear. In any disease where immune complexes are present, as is likely to be the case in MGN, the absence of marked complement activation is circumstantial evidence suggesting that effective regulation of complement activation is occurring.

Evidence of complement activation in the circulation

The Classical Pathway

In MGN, there was no reduction in the serum concentration of any of the classical pathway components and many increased concentrations were found (figure 3-2). The proteinuria present in the urine of all patients in this group has not resulted in a reduction in the serum concentrations of complement proteins. Increased levels probably resulted from increased synthesis rather than reduced catabolism since some activation was shown to have taken place.

The Alternative Pathway

There was no evidence of complement activation via the alternative pathway, as no reduced levels were found and again several concentrations were increased.

Evidence of regulation of complement activation in the circulation

There is little evidence in favour of the role of control proteins in the circulation in MGN. One patient, however, had a reduced C3bINA level and the corresponding biopsy was

one of the four to show deposition of properdin. It is possible therefore that the reduced C3bINA level allowed activation of the alternative pathway. The normal serum levels of C3 and factor B suggests that any activation which did take place was not marked. There were no reduced β1H or C$\bar{1}$-INH concentrations

Several significant correlations were present between serum concentrations of complement components and control proteins. The correlation found between C3bINA and factor B levels (Table 3.5) has been found in other studies (188, 196, 197, 198) where it was suggested that C3bINA, by exerting a modulatory influence over, and causing catabolism of C3b, prevented utilization of factor B. All other correlations were at the five per cent probability level and, since at least two correlations would be expected due to chance at this level, care must be taken in interpreting these. The correlations between concentrations of C$\bar{1}$-INH and both C3 and β1H, and C1s and β1H may have arisen due to chance or to some common factor controlling the synthetic rates of the proteins. The negative correlations between concentrations of C4 and both factor B and C3bINA are difficult to explain. Since concentrations of factor B and C3bINA show a positive correlation, it is not surprising that they should share a similar relationship with C4. The inverse relationship may be due to some factor which causes increased C4 synthesis

and inhibits synthesis of C3bINA and factor B or vice versa, or be due to a sparing of C4 in the presence of alternative pathway activation. This is unlikely in view of the limited evidence of alternative pathway activation in MGN. It is also possible that these correlations being at the five per cent probability level, arose due to chance.

The relationship between deposition and serum concentration of complement components

No significant correlations were found between the intensity of deposition and the serum concentrations of any protein studied. The absence of such correlations may have several possible explanations.

a) Serum concentrations reflect the immediate extent of complement activation while immunofluorescence may demonstrate, within the kidney, the products of previous complement activation.

b) Immune complexes, within the kidney, may retain the ability to activate complement after the removal of immune complexes from the circulation. It is also possible in MGN that immune complexes formed in situ and were therefore never present in the circulation.

c) Increased synthesis of components may have masked the hypocomplementaemic tendency induced by immune complexes.

SUMMARY

1. In this study, therefore, MGN was not found to involve marked complement activation and reasons for this were discussed.
2. Complement activation was found to be primarily via the classical pathway and correlation results suggest that IgG was the main immunoglobulin class involved.
3. The deposition of properdin in four biopsies suggests that the alternative pathway may be involved to a limited extent and several methods of activation were discussed.
4. The association between deposition of C3 and βIH, in terms of biopsies positive and distribution patterns of each was good. Although weaker, there was also evidence to suggest that C1-INH was present in response to deposition of C1s.
5. C3bINA was not present in any MGN biopsy and reasons for this were discussed.
6. Serum concentrations of control proteins show that no generalised deficiency of these was responsible for the complement activation present in MGN. The limited amount of activation in the presence of immune complexes suggests that complement activation was being regulated efficiently.

CHAPTER 4

Membranoproliferative Glomerulonephritis

Membranoproliferative Glomerulonephritis

Introduction

Membranoproliferative glomerulonephritis (MPGN) was originally included within the chronic GN group and gained separate disease status around 1965 (199, 200). There are a variety of alternative names, including mesangiocapillary GN (201), persistent hypocomplementaemic GN (199), chronic lobular GN (202) and mixed membranous and proliferative GN (203).

Histologically, diffuse changes are apparent with increase in both the number of glomerular cells and the amount of mesangial matrix. The capillary loops appear thickened and, using a periodic acid silver methenamine stain, the basement membrane appears double in places. Epithelial cell crescents may be present in some glomeruli.

Using electron microscopy, the apparent splitting of the basement membrane has been shown to be caused by the interposition of mesangial cells between the basement membrane and the endothelial cell. The results of electron microscopic studies have permitted further subdivision of this disease (204) into a subendothelial type and dense deposit disease. These have also been designated types I and II respectively (205). In the subendothelial type of MPGN, electron dense deposits may be seen primarily along the endothelial aspect of the basement

membrane and within the mesangium while, in dense deposit disease, electron dense material is deposited within the basement membranes of capillary loops and also of Bowman's capsule and tubules.

In the subendothelial type of MPGN, C3 is always present (206-209) and is usually deposited in a peripheral granular capillary pattern and may also be found within the mesangium. IgG and IgM may be deposited in association with C3 (207-209) as may properdin (208). In some biopsies, with subendothelial electron dense deposits, there is no apparent deposition of immunoglobulins. In the dense deposit variant, C3 is again always present (206, 208, 210) and is found in discrete deposits within the mesangium and may also be found in a "ribbon-like" pattern round the capillary loops. IgM and IgG may be present in trace amounts (206, 207). Davis, however, using the more sensitive indirect technique frequently found IgG and IgM (211). Factor B has been demonstrated in dense deposit disease (210) but, surprisingly, significant amounts of properdin have not been found (208, 209).

Most MPGN patients have markedly reduced serum concentrations of C3 (205, 208, 209, 212-214). Properdin and factor B levels may also be reduced (208, 209, 213). Low serum concentrations are seen particularly in patients who have circulating C3 nephritic factor (NF) (208).

The pathogenesis of MPGN is unknown. Where immune complexes are found in the circulation (180, 181) and subendothelial electron dense deposits are present along with capillary loop deposition of immunoglobulins, it is probable that immune complexes play a role in pathogenesis. Not all patients with the subendothelial type of MPGN however have evidence of immunoglobulin deposition (206, 207) suggesting that the subendothelial deposits may not always represent immune complexes. In the dense deposit type of MPGN, subendothelial deposits and capillary loop deposition of immunoglobulins are infrequently found (205, 206, 208, 210). It is therefore unlikely that immune complexes are the only factor involved in the pathogenesis of MPGN.

Since many MPGN patients are hypocomplementaemic, it has been suggested that the hypocomplementaemic state may result in the development of GN (216). Two indirect pieces of evidence argue in favour of this; 1) In patients with NF, most complement activation is independent of the kidney since C3 levels do not rise after nephrectomy (217) and 2) NF is found in some patients with partial lypodystrophy who have no evidence of renal disease (216). Both of these suggest the possibility that MPGN patients may be hypocomplementaemic before the onset of GN. This predisposition to GN may either be a direct consequence of hypocomplementaemia or be the

result of persistent infection resulting from the complement deficiency. Recent evidence from experiments in mice where a hypocomplementaemic state was maintained for up to twelve months did not result in the formation of noticeable renal abnormalities suggesting, at least in this model, that hypocomplementaemia, of itself, did not cause GN (218). Since immunoglobulins are not always present, if the disease is attributable to persistent infection, then the mechanism must involve something other than immune complex deposition.

The composition of the intra-membranous dense deposit in the dense deposit type of MPGN is not known. It is possible that C3 may be one of the components, but, since in some patients there is no deposition of C3 round the capillary loops (209) C3 is unlikely to be the sole component. Until the composition of the deposit is known, it is unlikely that the role which the deposit plays will be understood.

It is probable that no single pathogenic mechanism results in the formation of MPGN since there are at least two distinct histological appearances. Also some patients have evidence of immune complex deposition while others do not, and only some patients have detectable NF. One unifying feature is the extensive complement activation universally found and it is possible that this is involved in the pathogenesis of MPGN.

Materials

Immunofluorescence studies were performed on renal biopsies obtained from 19 MPGN patients. Two of these had no proteinuria at the time of biopsy. In the others proteinuria ranged from 0.5g to 10g per 24 hours (mean = 3.9g/ 24 hours). Serum creatinine levels ranged from 70 to 961 μmoles/ l (mean = 301 μmoles/ l) with nine elevated values.

Between one and 13 glomeruli (mean = 5.5) were examined by immunofluorescence in each biopsy. Electron microscopic data were available from ten biopsies; nine were found to be of the subendothelial type while the tenth was a dense deposit type. Serum samples were obtained from 17 patients at the time of their biopsy.

Advanced forms of the nephritis of SLE and HSN (1) may be morphologically indistinguishable from MPGN, there was however, no clinical evidence of systemic disease in any of the 19 patients in the MPGN group.

Results

Renal Biopsies

Glomerular deposition of immunoglobulins, complement and control proteins

The number of biopsies positive and the mean intensity of staining for each protein studied is shown in table 4.1.

Table 4.1 Glomerular deposition of immunoglobulins, complement and control proteins

	Immunoglobulins			Classical Components			Alternative Pathway Components		Terminal Components		Control proteins		
	G	A	M	C1q	C1s	C4	P	B	C3	C5	$C\bar{1}$-INH	C3bINA	βIH
Number Positive	9/ 19	4/ 18	11/ 19	9/ 19	9/ 15	10/ 17	12/ 16	2/ 16	19/ 19	12/ 17	3/ 15	4/ 15	16/ 16
Mean Intensity	2.1	1.5	1.7	1.4	2.0	1.7	2.0	1.5	2.4	2.6	2.1	1.0	2.5

Mean intensity = the mean of the scores given for the intensity of deposition of each protein in each biopsy.

Deposition of immunoglobulins

Immunoglobulins were deposited in 15 of 19 biopsies (79%). In five, IgG was found without IgM, in six IgM without IgG and in five IgG and IgM were deposited together. Both IgG and IgM were present in the four biopsies positive for IgA. Trace amounts of IgM were found in the biopsy from the patient with dense deposit disease. No other immunoglobulins were detected. The corresponding serum sample contained NF. Of the other NF positive patients, all three immunoglobulins were present in one and only IgM was found in the other.

Deposition of classical pathway components

At least one classical pathway component was found in 14 of 19 (74%) biopsies. Deposition of classical pathway components was not apparent in the dense deposit disease biopsy but C1q was found in one of the other NF positive biopsies and C1s and C4 were present in the other.

Deposition of alternative pathway components

Factor B was found in two biopsies in this group; neither of these was NF positive. Properdin was deposited in 12 of 16 biopsies (75%) including two NF positive biopsies but was not found in the biopsy from the patient with dense deposit disease. Properdin was deposited in both biopsies positive for factor B.

Deposition of C3 and C5

C3 was present in all biopsies with high intensity of staining (appendix 1) and C5 was found in 12 of 16 biopsies (75%).

Deposition of the control proteins

$C\bar{1}$-INH was deposited in eight of 15 (53%) biopsies. C3bINA was found in trace amounts in biopsies from four patients. The serum samples from these patients did not contain nephritic factor. β1H was present in all biopsies with comparatively high intensity of staining (appendix 1).

Correlations in intensity of staining between the proteins studied

Using the Spearman rank correlation test, the intensity of staining of each protein studied was compared with the intensity of all others. The results are shown in table 4.2. R represents the correlation coefficient and p the probability of significance. Because of the small number of biopsies positive for IgA, factor B and C3bINA, no analyses were performed on the results of these. Significant correlations are shown in red.

(see over).

Table 4.2 Correlations in intensity of staining between the proteins studied

MPGN		G	M	C1q	C1s	C4	P	C3	C5	C$\bar{1}$-INH
M	R	0.05								
	p	NS								
C1q	R	0.62	0.44							
	p	0.01	0.05							
C1s	R	0.23	0.05	0.40						
	p	NS	NS	NS						
C4	R	0.73	0.05	0.59	0.32					
	p	0.001	NS	0.01	NS					
P	R	0.19	0.13	0.16	0.39	0.18				
	p	NS	NS	NS	NS	NS				
C3	R	0.17	0.01	0.14	0.36	0.18	0.24			
	p	NS	NS	NS	NS	NS	NS			
C5	R	0.04	0.28	0.03	0.48	0.12	0.24	0.80		
	p	NS	NS	NS	0.05	NS	NS	0.001		
C$\bar{1}$-INH	R	0.52	0.44	0.86	0.43	0.79	0.21	0.13	0.08	
	p	0.05	0.05	0.001	0.05	0.001	NS	NS	NS	
BlH	R	0.17	-0.43	0.20	0.59	0.17	0.49	0.53	0.61	0.12
	p	NS	0.05	NS	0.01	NS	0.05	0.02	0.01	NS

NS = not significant

The correlations of classical pathway components

There were significant correlations between the intensities of deposition of C1q and IgG, IgM and C4. The intensities of C1s staining correlated with those of C5 and a strong correlation was found between the intensities of deposition of C4 and IgG.

The correlations of the alternative pathway

There were no significant correlations between the intensities of deposition of properdin and those of any other protein studied.

The correlations of C3 and C5

The intensities of deposition of C5 correlated with those of C3 and C4.

The correlations of the control proteins

The intensities of deposition of $C\bar{1}$-INH correlated significantly with those of IgG and IgM and all three classical pathway components. There were also significant correlations between the intensities of deposition of βlH and IgM, C1s, C3 and C5. The correlation between the intensities of staining of βlH and IgM demonstrated an inverse relationship.

The concordance between deposition of control proteins of complement and the components whose activity they regulate

$C\bar{1}$-INH

The concordance between biopsies positive for $C\bar{1}$-INH and C1s and C4 is shown in table 4.3.

Table 4.3 The concordance between biopsies positive for C1s, C4 and $C\bar{1}$-INH

C1s/ $C\bar{1}$-INH	No. of Biopsies	C4/ $C\bar{1}$-INH	No. of Biopsies	C4/ C1s	No. of Biopsies
C1s + $C\bar{1}$-INH+	6	C4+ $C\bar{1}$-INH+	8	C4+ C1s+	7
C1s + $C\bar{1}$-INH-	3	C4+ $C\bar{1}$-INH-	2	C4+ C1s-	3
C1s- $C\bar{1}$-INH+	2	C4- $C\bar{1}$-INH+	0	C4- C1s+	1
C1s- $C\bar{1}$-INH-	4	C4- $C\bar{1}$-INH-	4	C4- C1s-	3
Total	15	Total	14	Total	14

There was concordance between biopsies positive for C1s and $C\bar{1}$-INH in 10 of 15 biopsies (67%).

In two, $C\bar{1}$-INH was present without C1s and in three C1s was found without $C\bar{1}$-INH. Better concordance was found between C4 and $C\bar{1}$-INH. C4 was present more often than C1s although on one occasion C1s was found in the absence of C4.

Table 4.4 The concordance between biopsies positive for C3, C4, C5, properdin, β1H and C3bINA

C3/ C3bINA	No. of Biopsies	C4/ C3bINA	No. of Biopsies	C5/ C3bINA	No. of Biopsies	P/ C3bINA	No. of Biopsies	β1H/ C3bINA	No. of Biopsies
C3+ C3bINA+	4	C4+ C3bINA+	4	C5+ C3bINA+	2	P+ C3bINA+	3	β1H+ C3bINA+	4
C3+ C3bINA-	11	C4+ C3bINA-	5	C5+ C3bINA-	9	P+ C3bINA-	9	β1H+ C3bINA-	11
C3- C3bINA+	0	C4- C3bINA+	0	C5- C3bINA+	2	P- C3bINA+	1	β1H- C3bINA+	0
C3- C3bINA-	0	C4- C3bINA-	4	C5- C3bINA-	2	P- C3bINA-	2	β1H- C3bINA-	0
Total	15	Total	13	Total	15	Total	15	Total	15

C3bINA

In table 4.4, the concordance between C3bINA and the proteins with which it interacts is shown. C3bINA was not found in the absence of C3, C4 or βIH; C3 and βIH being present in all biopsies. In one biopsy, C3bINA was found in the absence of C5 and properdin and in another in the absence of properdin.

βIH

The concordance between biopsies positive for βIH and C3, C4, C5 and properdin is shown in table 4.5.

Table 4.5 The concordance between biopsies positive for βIH and C3, C4, C5 and properdin

C3/βIH	No. of biopsies	C4/βIH	No. of biopsies	C5/βIH	No. of biopsies	P/βIH	No. of biopsies
C3+βIH+	16	C4+βIH+	10	C5+βIH+	12	P+βIH+	12
C3+βIH-	0	C4+βIH-	0	C5+βIH-	0	P+βIH-	0
C3-βIH+	0	C4-βIH+	4	C5-βIH+	4	P-βIH+	4
C3-βIH-	0	C4-βIH-	0	C5-βIH-	0	P-βIH-	0
Total	16	Total	14	Total	16	Total	16

Since βIH was present in all biopsies in this group, no other component was found in the absence of βIH. βIH was found in the absence of C4, C5 and properdin in four cases.

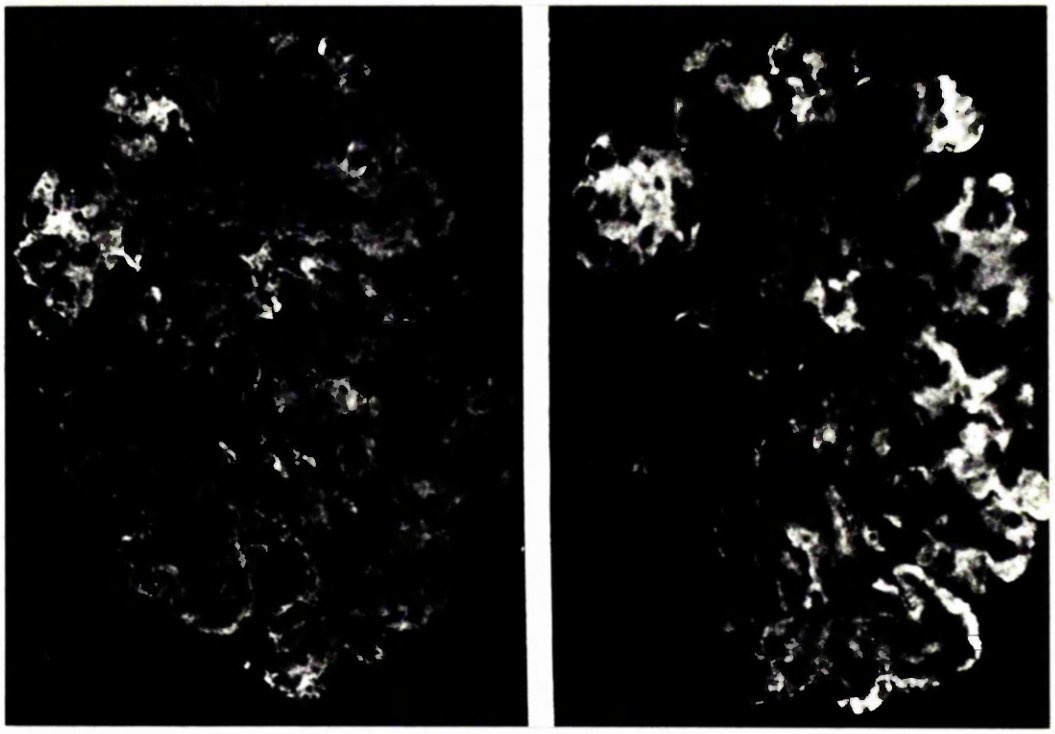
Comparison of immunofluorescence patterns

The uniformity of distribution pattern seen in MGN was

not present in MPGN. Most proteins in the majority of biopsies stained with a predominantly peripheral capillary loop pattern, while in others the predominant distribution was mesangial. When patterns of C1s and $C\bar{1}$-INH were considered, in three biopsies the staining for both was in a peripheral capillary loop distribution, in one mainly mesangial, and, in the remaining two biopsies positive for both proteins, C1s was found round peripheral capillary loops whereas $C\bar{1}$-INH was found not only round peripheral capillary loops but also in the mesangium. The patterns were therefore identical in four of six biopsies and the predominant pattern was the same in the remaining two. When adjacent sections were stained for C1s and $C\bar{1}$-INH, the areas staining for each protein were found to correspond closely (figure 4-1).

C3bINA was found in a segmental peripheral capillary loop pattern in the four biopsies where it was present (figure 4-2). In three of these, C3 was deposited in a diffuse peripheral capillary loop distribution and, in the fourth, the staining was in a diffuse capillary loop plus mesangial pattern. C3bINA was therefore found in areas where C3 was also present. Serial sections were not analysed.

The patterns of distribution were identical for C3 and β1H in 11 of the 16 biopsies positive for both. In the remaining five, β1H was found round the capillary loops and in the



×220

Figure 4-1 The glomerulus on the right was stained for C1s and the same glomerulus on an adjacent section is shown on the left, stained for $C\bar{1}$-INH.

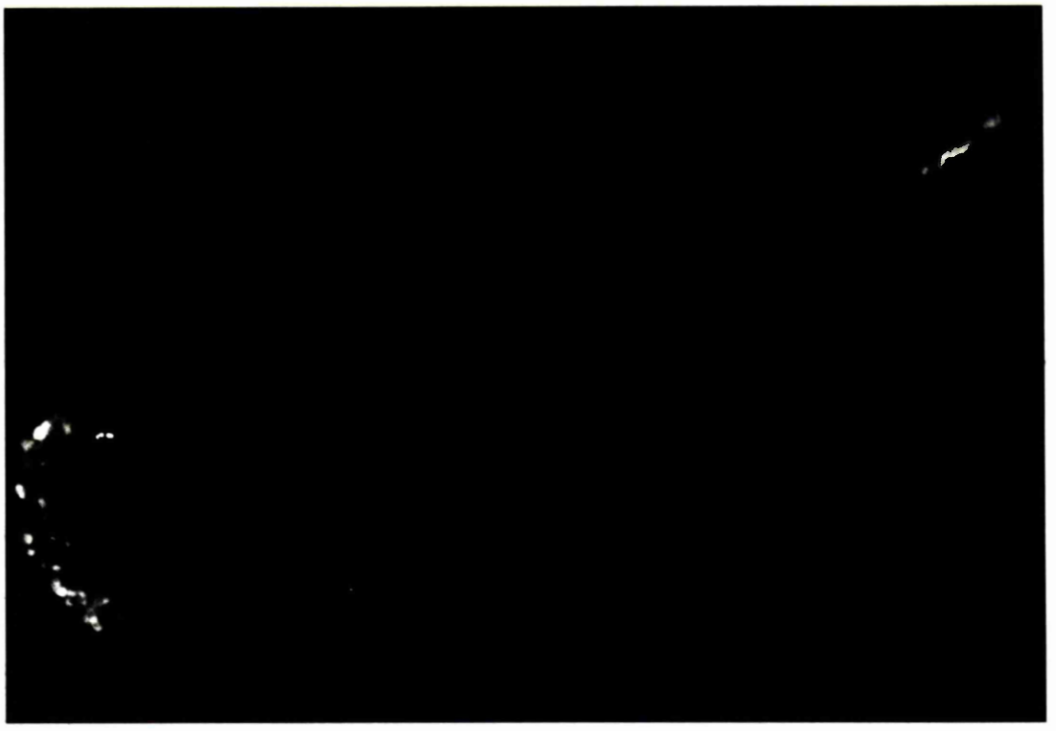


Figure 4-2 The segmental peripheral capillary loop deposition pattern of C3bINA in an MPGN biopsy.

mesangium while C3 was deposited only round capillary loops. Figure 4-3 shows the staining patterns of βlH and C3 in the same glomerulus on adjacent sections. The areas of distribution are seen to be almost identical.

Serum Samples

The serum concentration of each component in each of the 17 samples is shown in figure 4-4.

The concentrations of classical pathway components

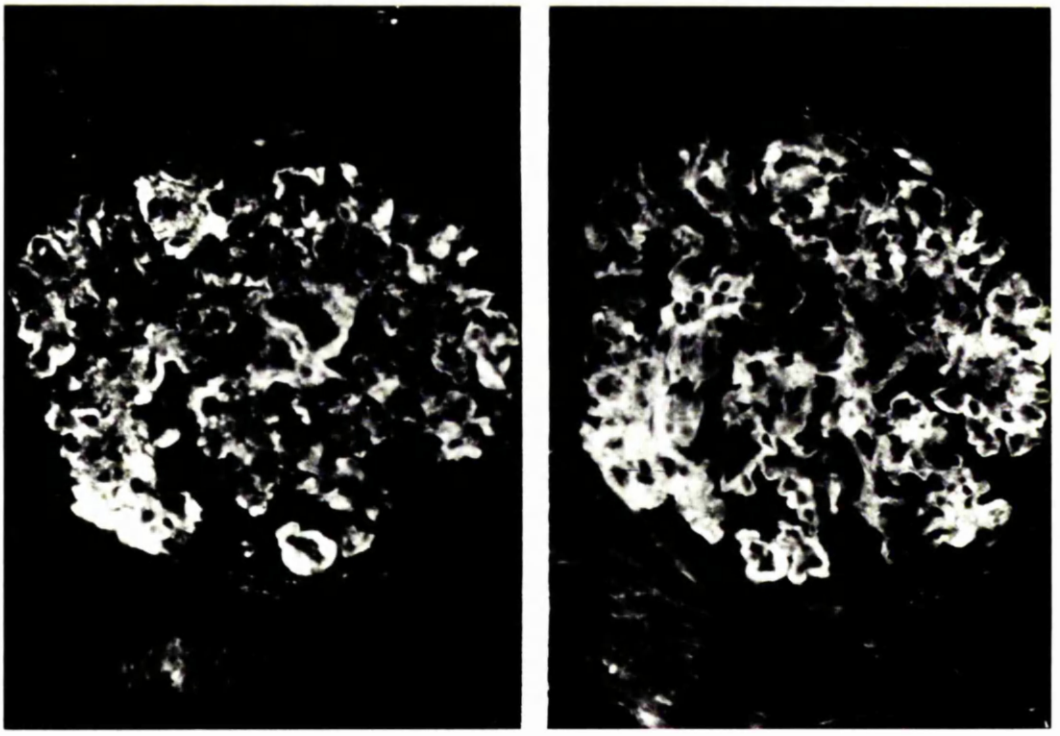
There was no significant difference between levels of classical pathway components and normal serum levels using the median test. One patient with NF, had subnormal concentrations of C1q, C1s and C4 while two NF negative patients had reduced concentrations of either C1q or C4. Raised levels of each component were frequently found.

The concentrations of alternative pathway components

Properdin levels did not vary significantly from normal and no reduced levels were found. Factor B levels as a group were reduced ($p > 0.05$) and one nephritic factor positive sample had a reduced factor B concentration.

The concentrations of C3 and C5

C5 concentrations were significantly lower than normal with six values below the normal range and a single raised value. All three NF positive patients had reduced C3 levels.



X 220

Figure 4-3 The glomerulus on the right was stained for C3 and the same glomerulus on an adjacent section is shown on the left stained for βlH.

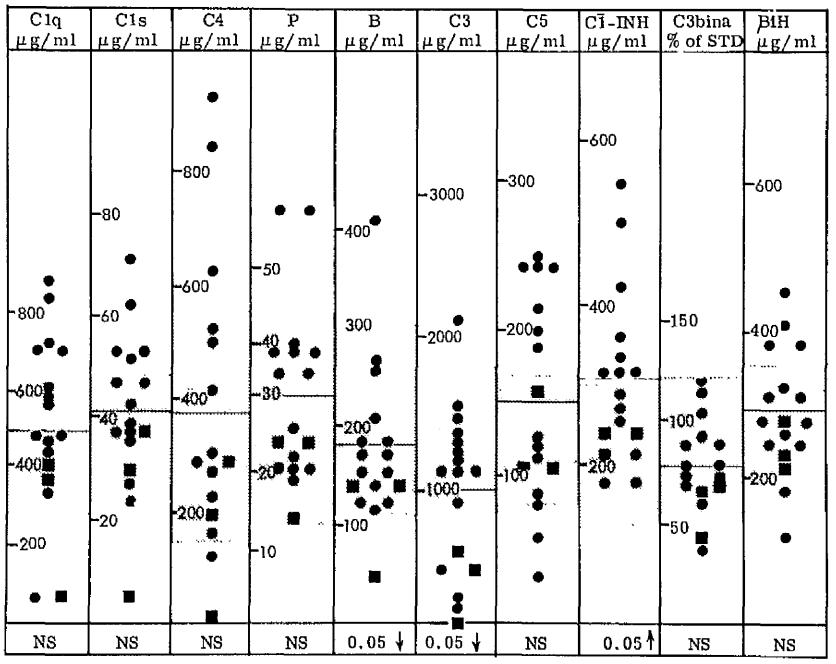


Figure 4-4 The concentration of each protein studied in each of the 17 serum samples from MPGN patients is shown. The shaded areas represent the normal ranges and the horizontal lines represent the mean concentrations for each protein. The values from the NF positive patients are shown as squares. The results of the median test are shown at the bottom of each column with arrows indicating whether the values are significantly higher or lower than normal. NS = not significant.

C5 levels did not vary significantly from normal although two were below the normal range.

The concentrations of the control proteins

The median $C\bar{1}$-INH value was significantly raised and no subnormal values were found. C3bINA and β1H levels as a group did not vary significantly from normal although there were two low C3bINA concentrations, including one from a NF positive patient, and one low β1H level.

The correlations between serum concentrations of complement components and control proteins

The correlations between the serum concentration of each of the components studied were calculated using the Spearman rank correlation test and the results are shown in table 4.6. R = the correlation coefficient and p = the probability of significance. Significant correlations are shown in red.

(Table 4.6 overleaf).

Table 4.6 **The correlations between the serum concentrations of the proteins studied**

		C1q	C1s	C4	P	B	C3	C5	C1̄-INH	C3bINA
C1s	R	0.38								
	p	NS								
C4	R	0.74	0.14							
	p	0.001	NS							
P	R	0.57	0.19	0.28						
	p	0.01	NS	NS						
B	R	0.68	0.75	0.43	0.49					
	p	0.01	0.001	0.05	0.05					
C3	R	0.40	0.43	0.53	0.59	0.59				
	p	NS	0.05	0.05	0.01	0.01				
C5	R	0.38	0.40	0.39	0.41	0.50	0.71			
	p	NS	NS	NS	NS	0.05	0.001			
C1̄-INH	R	0.35	0.41	0.15	0.26	0.58	0.19	0.10		
	p	NS	NS	NS	NS	0.01	NS	NS		
C3bINA	R	0.26	0.13	-0.57	0.35	0.29	0,79	0.45	0.18	
	p	NS	NS	0.05	NS	NS	0.001	0.05	NS	
pH	R	0.20	0.47	-0.13	0.53	0.49	0.43	0.37	0.48	0.39
	p	NS	0.05	NS	0.05	0.05	0.05	NS	0.05	NS

NS = not significant

The correlations of classical pathway components

The serum concentrations of C1s correlated with the concentrations of C3 and factor B. C1q levels correlated with those of C4, properdin and factor B and C4 values correlated with factor B and C3. concentrations.

The correlations of alternative pathway components

Apart from the correlations between the alternative

pathway components and the classical pathway components noted above, properdin concentrations correlated with those of factor B and C3 and factor B levels correlated with C3 and C5 concentrations.

The correlations of C3 and C5

The concentrations of C3 correlated with those of C1s, C4, properdin, factor B and C5. C5 levels also correlated with those of factor B.

The correlations of the control proteins

There was a significant correlation between concentrations of $C\bar{1}$-INH and both factor B and β1H. C3bINA levels correlated with levels of C4, C3 and C5 and β1H concentrations correlated with C1s, properdin, factor B, C3 and $C\bar{1}$-INH concentrations.

The relationship between serum concentrations and intensities of glomerular deposition of complement components and control proteins

Using the Spearman rank correlation test, the intensity of deposition was compared with the serum concentration of each component except for factor B and C3bINA which were present in too few biopsies. The results are shown in table 4.7. R represents the correlation coefficient and p represents the probability of significance.

Table 4.7: The correlations between serum levels and the intensities of deposition of each complement component

	C1q	C1s	C4	P	C3	C5	C1-INH	BIH
R	-0.22	0.58	-0.32	0.11	-0.56	-0.31	0.34	-0.13
p	NS	<0.05	NS	NS	<0.05	NS	NS	NS

NS = not significant

There was a significant correlation between the serum concentrationsand the intensities of deposition of C1s and an inverse correlation between serum levels and intensities of deposition of C3.

DISCUSSION

Intraglomerular evidence of complement activation

The involvement of immunoglobulins

The deposition of immunoglobulins in this study, compares well with the results in other reports (206-208). IgG and IgM were found in equal numbers of biopsies but not necessarily together (table 4.1). Only one biopsy of the ten examined by electron microscopy was of the dense deposit variant and, in this only trace amounts of IgM were found. This is consistent with the findings of others (206, 210).

The involvement of the classical pathway

Classical pathway activation was evident in 14 of 19 (74%) biopsies (table 4.1) including those from two patients with

circulating NF. Immunoglobulins were present in 13 of these. In these patients, activation of the classical pathway may have been due to immune complexes. This is substantiated by the significant correlations between the intensities of deposition of both C1q and C4 with IgG and C1q with IgM (table 4.2). It is also possible that the classical pathway may have been activated by the rather unique immune complex formed by NF and the alternative pathway C3 convertase (219).

In one biopsy, significant quantities of C1s were deposited in the absence of immunoglobulins and there was no evidence of circulating NF at the time of biopsy. There are various possible explanations to account for this a) activation of C1s by plasmin may occur (220), b) there may be a non-specific trapping of molecules caused by increased capillary permeability or finally c) it is possible that NF stabilised C3 convertase caused the activation and deposition of C1s prior to the removal of the biopsy at which time NF was no longer present in the circulation. NF may appear and disappear in the circulation particularly in the subendothelial type of MPGN (205).

The fact that neither the intensity of deposition of immunoglobulins nor classical pathway components correlated with the intensity of deposition of C3 (table 4.2) may suggest that, while classical pathway activation is probably dependent on the deposition of immunoglobulins, this activation does not control

the extent of activation of C3. Other explanations will be discussed with reference to the alternative pathway.

The involvement of the alternative pathway

The deposition of properdin in 12 of 16 biopsies (75%) including two biopsies from patients with circulating NF suggests that activation of the alternative pathway is frequently present in MPGN. The possibility of the presence of properdin in the absence of activation of the alternative pathway will be considered fully in chapter 9. Immunoglobulins and classical pathway components were present in ten of these 12 biopsies and the alternative pathway activation may have been secondary to classical pathway activation in these cases. Direct activation of the alternative pathway may occur particularly where NF is present in the circulation, as was the case in two patients. In the other two biopsies, there was no evidence of immunoglobulin deposition although C1s was present in one biopsy. Factor B was present in both of these biopsies. It is therefore likely that activation was independent of the classical pathway. Analysis of the corresponding serum samples failed to detect circulating NF. The mechanism is therefore not known. It is possible that NF was present in the serum prior to the investigation and caused deposition of C3b in the glomeruli and the C3b thus deposited would result in further activation. C3b formed as a result of classical pathway activation in the circulation or by

some other means could similarly deposit in the kidneys.

The absence of properdin deposition in the biopsy of the patient with the dense deposit variant of MPGN is a finding consistent with the results of others (208, 209). The marked deposition of C3 and low serum concentrations of C3 suggested that intense activation of the complement system was taking place. NF was present in the circulation of this patient and is reported to be frequently found in this disease (208). Activation is therefore likely to be by the NF stabilized, alternative pathway C3 convertase. The mechanism of activation in the glomeruli in dense deposit disease remains unclear; perhaps the question could be answered if the composition of the intramembranous deposit were known.

Like the classical pathway components, the intensities of deposition of properdin failed to correlate with those of C3. This failure of either mechanism of activation to correlate with the intensity of deposition of C3 may have resulted from the fact that both pathways were involved to varying extents in different biopsies. It is also possible that C3, perhaps as C3d, remained in the kidney after components of either pathway of activation had been removed. The use of antisera specific for C3b and C3d, if available, would have been able to clarify this.

The terminal sequence

MPGN is characterized by intense complement activation as demonstrated by reduced serum concentrations of C3 (figure 4-3 and references 205, 209, 212-214), and deposition of C3 in all biopsies (table 4.1 and references 206-210). The intraglomerular deposition of C3 in kidneys where immunoglobulins are deposited and subendothelial electron dense deposits are found is most likely due, at least in part, to activation initiated by immune complexes. Circulating immune complexes have occasionally been reported (180, 181). The subendothelial position of the immune complexes may allow more efficient activation in MPGN compared to MGN. It is also possible that the type of complex which deposits in a subendothelial rather than a subepithelial position is more efficient at activating the classical pathway.

Diffuse deposition of C3 and other components where significant quantities of immunoglobulins are not present is more difficult to explain. Several possible mechanisms may exist:

a) The state of hypocomplementaemia may predispose a patient to infection. If bacterial endotoxins, for example, were to reach the kidney, then activation of the the alternative pathway might result.

b) It is conceivable that the, as yet undefined, substance

within the basement membrane in dense deposit disease may be capable of causing local amplification of C3 turnover. No evidence exists for this.

c) Complement activation may occur in the circulation and products of that activation may deposit in the kidney. If C3b is deposited, in this way, then activation could take place in situ, particularly if the regulation of C3b by βIH was inhibited by the presence of NF.

d) Basement membranes from glomerulonephritic patients have been shown to have an abnormally low sialic acid content (214). Deficiency of sialic acid on a membrane allows uncontrolled activation of C3 to occur (97). Activation of the alternative pathway may therefore be due to biochemical changes in the basement membrane.

The intensity of complement activation in MPGN suggests that this activation is involved in the pathogenesis of this disease. Both types of MPGN are associated with inflammatory changes and it is likely the complement activation is effective in contributing to this. The role of complement in the subendothelial type of MPGN, where there is evidence of immune complexes, is possibly secondary to an immune complex pathogenesis. In dense deposit disease and also in the subendothelial type with no evidence of immune complex deposition, particularly where NF is present in the circulation, complement activation or the

effects of hypocomplementaemia may be the primary factor in the disease. Evidence from the hypocomplementaemic model (218) discussed in the introduction to this chapter suggests that other factors, such as infection, may also be involved.

Intraglomerular evidence of regulation of complement activation

The role of control proteins in the kidneys of patients with MPGN was assessed using the four criteria outlined in chapter 3.

The role of $C\bar{1}$-INH

The presence of $C\bar{1}$-INH in approximately half of the MPGN biopsies (table 4.1), the significant correlations between the intensities of deposition of C1s and $C\bar{1}$-INH (table 4.2) and the similarities in distribution patterns of C1s and $C\bar{1}$-INH (figure 4-1) suggest that $C\bar{1}$-INH is involved in regulation of classical pathway complement activation. Concordance results showed a better relationship between $C\bar{1}$-INH and C4 than between $C\bar{1}$-INH and C1s (table 4.3). This was also found in the MGN biopsies.

The role of C3bINA

C3bINA was deposited in four of the 15 biopsies examined and, since C3 was found in all MPGN biopsies, C3bINA was not found in the absence of C3. Although the patterns of distribution of C3bINA were segmental compared with the diffuse staining

pattern of C3, the areas positive for C3bINA were also positive for C3. The absence of C5 in two and factor B in four biopsies positive for C3bINA may suggest effective regulation of C3b by C3bINA in these tissues. Factor B, however, was present in only two MPGN biopsies.

The assessment of the role of C3bINA is therefore based on its presence in only four of 15 biopsies, all of which contained C3, and the compatibility of the distribution patterns. Although far from conclusive, there is more evidence for the regulation of C3b by C3bINA in MPGN than in other groups studied with the exception of HSN.

The role of βlH

βlH was present in all biopsies, as was C3 (Table 4.1). The concordance between C3 and βlH was therefore complete (Table 4.3). The intensities of deposition of βlH correlated significantly with those of C3 (Table 4.2) and the distribution patterns were almost identical (figure 4-3). The absence of properdin in four and C5 in four biopsies (three were negative for both) may suggest that effective regulation either by βlH or some other factor, perhaps C3bINA was taking place. However, βlH deposition correlated both with that of properdin and C5 and this may reflect mutual dependence of βlH, properdin and C5 on C3b.

βlH was deposited in three patients with circulating NF.

Since NF prevents the displacement of Bb from $\overline{C3bBbP}$ by βlH, the deposition of βlH in patients with NF appears anomalous. βlH deposition has been reported in the glomeruli of all patients with the subendothelial type and the dense deposit type of MPGN in one study (262). No information on the presence of NF was published but it is likely that it was present in the serum of at least some of these patients. There are several possible explanations for its presence.

1) βlH may compete with NF to bind to C3b formed either via the classical or alternative pathways. The classical pathway may be activated by the immune complex formed by the binding of NF to $\overline{C3bBbP}$ (219).

2) Intraglomerular complement activation may be independent of NF.

3) βlH may be binding to some other protein. The good correlation between, and similarity in distribution of C3 and βlH deposition in the total group would argue against this.

Therefore the evidence for the regulation of C3 by βlH in MPGN is based on its presence and good concordance between biopsies positive for C3 and βlH, the correlation between the intensities of depositions of these two proteins and the similarity in their distribution patterns. It seems likely that βlH plays a role in regulation even where NF is present.

Evidence of complement activation in the circulation

The classical pathway

Serum levels of classical pathway components have been found to be reduced in the sera of some patients, in most studies (206-208, 213). In this study, low values of classical pathway components were found in three patients (figure 4-4) one of whom was NF positive. In each of these cases, classical pathway components and immunoglobulins were deposited in the corresponding biopsy. Serum concentrations of C1s and C4 correlated with C3 levels suggesting that classical pathway activation in the circulation may affect the C3 concentration. It may also occur due to a common synthetic stimulus. There was good correlation also between concentrations of all classical pathway components and alternative pathway components, particularly factor B. This may either be due to recruitment of the alternative pathway by the classical pathway or again some common synthetic stimulus. A further possibility linking the classical and alternative pathways is the activation of the classical pathway by NF stabilised alternative pathway C3 convertase (219) where NF is present.

The alternative pathway

While only one MPGN sample had a subnormal factor B concentration, the levels as a whole were significantly reduced (figure 4-4). These findings are in agreement with others (208,

209, 213). There were, however, no reduced properdin levels and the group did not vary from normal. This is at variance with the results of others (208). Levels of properdin and factor B in this study correlated with those of C3 and at a higher level of significance than did C1s and C4 concentrations (table 4.2). These correlations suggest that the activation of the alternative pathway is responsible for significant C3 cleavage. A common synthetic stimulus may also account for the relationship between C3 and both properdin and factor B.

The Terminal Sequence

In agreement with others (205, 208, 209, 212-213), C3 levels were significantly lower than normal with six values below the normal range, including all three patients with NF. The relationships between C3 and the components of the classical and alternative pathways have been discussed above and suggest that activation of both pathways may be responsible for C3 cleavage.

Evidence of regulation of complement activation in the circulation

The role of $C\bar{1}$-INH

$C\bar{1}$-INH concentrations as a group were significantly raised (figure 4-3). The absence of correlations between levels of $C\bar{1}$-INH and classical pathway components are at variance with the correlations seen in the intensity of deposition of these components. This may suggest that:

1) Regulation is not occurring in the circulation. This is unlikely since levels of classical pathway components are only occasionally reduced.

2) $C\bar{1}$-INH may be used in other pathways.

3) Increased $C\bar{1}$-INH synthesis may more than compensate for utilization. This is most likely since $C\bar{1}$-INH levels as a group were significantly raised.

The role of C3bINA

C3bINA levels did not differ significantly from normal although two subnormal values were found. In neither of the corresponding biopsies was C3bINA deposited. The strong correlation between C3 and C3bINA and the weaker one between C4 and C3bINA suggest that the levels of C3bINA influence C3 activation in the circulation. Such correlations have been recorded by others (222) in a NF negative group. In that group, C3bINA concentrations also correlated with factor B and properdin levels. C3bINA and factor B concentrations correlate in normal serum (198) and this significant correlation may be expected since C3bINA, by controlling C3b, has a sparing effect on factor B. The absence of correlations between serum concentrations of C3bINA and factor B and properdin in this study may be due to the NF positive sera since others (222) have shown that correlations do not exist between C3bINA and C3, properdin and factor B levels in NF positive sera.

The role of βIH

βIH levels, as a group, did not vary significantly from normal and only one subnormal value was found. The concentrations of βIH correlated significantly with those of C3, properdin and factor B (table 4.2), suggesting that βIH concentrations may influence the extent of alternative pathway activation. The absence of a significant correlation between concentrations of βIH and C3bINA in MPGN has been previously reported (222).

The relationship between deposition and serum concentrations of complement components

C3 was the only component whose intensity of deposition correlated inversely with the serum concentrations. It is unlikely that the serum concentration was directly affected by the quantity of C3 deposited in the kidney since, particularly in NF positive patients, extensive activation is known to take place in the circulation, as demonstrated by degradation products of C3 (223) and the ability of NF positive sera to cleave C3 in normal serum (92). Where immune complexes are present in the circulation it is also probable that classical pathway activation will take place there. The extent of complement deposition is more likely to reflect the situation in the serum than cause it.

The positive correlation between intensities of deposition and serum concentrations of C1s is more difficult to explain. It may arise due to chance or may reflect some mechanism

for increasing synthesis in response to utilization. Since all components do not show such a correlation this latter mechanism would require to be peculiar to C1s.

Reasons for the absence of correlations for other components have been suggested in chapter 3.

SUMMARY

1. Complement activation in MPGN was intense and occurred both via the classical and the alternative pathways. No difference was found in the relative importance of each pathway. The mechanisms of activation were discussed.

2. There was reasonable evidence for the role of $C\bar{1}$-INH in the regulation of C1s, based on a reasonable concordance between biopsies positive for $C\bar{1}$-INH and C1s and a significant correlation between intensities of deposition of $C\bar{1}$-INH and C1s. There was also a close similarity in the distribution patterns of these two proteins. Although no correlation was found between serum concentrations of $C\bar{1}$-INH and C1s, absence of reduced $C\bar{1}$-INH levels suggests that deficiency of $C\bar{1}$-INH was not responsible for classical pathway activation.

3. The role of C3bINA in regulation of C3b was not so well substantiated. Evidence for the regulation was based on its presence in four of 15 biopsies and similarities in distribution patterns as well as a significant correlation between serum levels of C3 and C3bINA.

4. Good evidence was found for the role of βlH in regulation of C3b with a good concordance between biopsies positive for C3 and βlH, significant correlation between intensities of deposition of C3 and βlH and close similarities in the distribution pattern of these two proteins. The serum concentrations of C3 and βlH also correlated significantly.

5. Based on the information from this study, complement activation was more intense in MPGN than in the other disease groups studied. There was also more evidence of regulation by each of the three control proteins considered in MPGN than in the other groups.

CHAPTER 5

Focal Glomerulonephritis

Focal Glomerulonephritis

Introduction

In early clinical classifications, the term focal glomerulonephritis (FGN) was ascribed to any disease involving haematuria without hypertension or gross proteinuria (1). The disease, although generally healing quickly, was recurrent and exacerbation often followed an infection of the upper respiratory tract. When pathological evidence became available, it was found that a clinical presentation of haematuria was generally associated with focal and segmental glomerular changes. Biopsies from patients who have other clinical presentations have since been shown to demonstrate a focal glomerular lesion. FGN may also be found in a variety of systemic diseases such as SLE (53), Henoch-Schönlein nephritis (1) and anti-glomerular basement membrane disease (37a).

The focal and segmental lesions usually involve proliferation of mesangial cells and increase in matrix and may be found in association with segmental capillary necrosis. Epithelial cell crescents may be also present. By electron microscopy, electron dense deposits have been identified in the mesangium and at the subendothelial side of the basement membrane. These have been shown to be most prevalent in cases where immunofluorescence staining for immunoglobulins is strongest (224).

Unlike the focal segmental pattern seen by light microscopy,

immunofluorescence studies often show each glomerulus to have diffuse deposition of immunoglobulins and complement components. Deposition is generally mesangial and the predominant immunoglobulin class detected is IgA alone or IgA in association with IgG (224-227). This is particularly true when FGN is present in association with recurrent haematuria. One group has found deposition of IgG and/ or IgM more commonly than IgA (228). C3 is almost always present where immunoglobulins are found (224, 228, 229) and deposition of C1q, factor B and properdin (224, 227, 230) has been recorded although some workers have failed to find C1q (228, 229). In some biopsies there is no evidence of immunoglobulin deposition.

Speculation has arisen as to the mechanism of this disease and several explanations have been suggested:-

(1) IgA may be part of an immune complex within the kidney. It has been suggested that IgA in an altered form may be the antigenic component of the complex. No anti-IgA activity can be detected in the circulation (231) and IgA is the sole immunoglobulin detected in a minority of biopsies. This explanation is therefore unlikely. The possibility of an IgA anti-IgA complex cannot be excluded.

(2) Recurrence of the disease often follows an upper respiratory tract infection. It is therefore possible that secretory IgA may form a complex with the antigens of

respiratory tract viruses. Although secretory piece has been detected in the glomeruli in one study (232), other studies have failed to confirm this observation (229, 231) suggesting that the antibody is not secretory IgA.

(3) IgA eluted from the kidney of a single patient with IgA nephritis was shown to bind weakly to mesangial cells of normal kidney using an indirect immunofluorescence test (231). It is therefore possible that FGN, with mesangial IgA deposition, is an autoantibody disease. This hypothesis rests on the evidence from only one kidney. The mechanism of this disease therefore remains to be elucidated.

MATERIALS

Immunofluorescence studies were performed on renal biopsies obtained from 28 FGN patients none of whom had clinical evidence of systemic disease. Proteinuria was present in 14 of these patients ranging from 0.5g to 9.5g per 24 hours (mean = 3.5g/24 hours) and three patients had serum creatinine levels above the normal range.

Between one and 16 glomeruli (mean = 5.2) were examined by immunofluorescence and serum samples taken on the day of biopsy were available from 14 patients.

RESULTS

Renal Biopsies

Glomerular deposition of immunoglobulins, complement and control proteins

The number of biopsies positive and the mean intensity of staining for each protein studied is shown in Table 5.1.

Table 5.1 Glomerular deposition of immunoglobulins, complement and control proteins

(See over for Table)

Deposition of Immunoglobulins

IgA was the predominant immunoglobulin, being found in 19 of 27 biopsies (70%). It was present as the sole immunoglobulin in ten biopsies. Of the eight biopsies where IgA was not deposited, IgM was found alone in one, IgG in one and, in six biopsies, no immunoglobulins were present. The mean intensity of deposition of IgA in FGN was higher than in the other disease groups studied (appendix 1).

Deposition of classical pathway components

C1q and C1s were present only in seven of 28 (29%) and six of 24 (25%) biopsies respectively and the intensity of staining was weak. C4 was found in 19 of 28 biopsies (70%). At least one classical pathway component was present in 23 of 28 biopsies (82%).

Table 5.1 Glomerular deposition of immunoglobulins, complement and control proteins

	Immunoglobulins			Classical Components			Alternative Components		Terminal Components		Control proteins		
	G	A	M	C1q	C1s	C4	P	B	C3	C5	$C\bar{1}$-INH	C3bINA	βIH
Number positive	9/ 28	19/ 27	7/ 28	7/ 28	6/ 24	19/ 28	12/ 24	0/ 24	25/ 28	13/ 22	6/ 24	1/ 23	18/ 24
Mean intensity	1.7	2.3	1.1	1.0	1.5	1.5	1.6		1.8	1.5	1.3	1.0	2.2

mean intensity = the mean of the scores given for intensity of deposition of each protein in each biopsy

Deposition of alternative pathway components

Factor B was not deposited in any of the biopsies in this group and properdin was found in 12 of 24 (50%).

Deposition of C3 and C5

C3 was found in all but three biopsies. Two of these had no deposition of immunoglobulins and in the third trace amounts of IgM were present. C5 was present in 13 of 22 biopsies (59%), all of which showed C3 deposition.

Deposition of control proteins

$C\bar{1}$-INH was found in the same number of biopsies, 6 of 24 (25%) as C1s although they were not always present together. C3bINA was deposited in only one and βlH was found in 18 of 24 (75%) tissues examined.

Correlations in intensity of staining between proteins studied

Using the Spearman rank correlation test, the intensity of staining of each protein studied was compared with the intensities of all others. The results are shown in table 5.2. R represents the correlation coefficient and p the probability of significance. Because of the absence of factor B and the small number of biopsies positive for C3bINA no analyses were performed on the results of these. Significant correlations are shown in red.

Table 5.2 Correlations in intensity of staining between the proteins studied

		G	A	M	C1q	C1s	C4	P	C3	C5	C1̄-INH
A	R	0.26									
	p	NS									
M	R	0.07	0.18								
	p	NS	NS								
C1q	R	0.28	0.08	0.10							
	p	NS	NS	NS							
C1s	R	0.10	0.34	0.31	0.09						
	p	NS	0.05	NS	NS						
C4	R	0.27	0.75	0.23	0.09	0.28					
	p	NS	0.001	NS	NS	NS					
P	R	0.05	0.20	0.09	0.11	0.05	0.27				
	p	NS	NS	NS	NS	NS	NS				
C3	R	0.54	0.40	0.09	0.01	0.04	0.48	0.55			
	p	0.001	0.05	NS	NS	NS	0.01	0.01			
C5	R	0.06	0.41	0.09	0.04	0.45	0.49	0.01	0.19		
	p	NS	0.05	NS	NS	0.02	0.01	NS	NS		
C1̄-INH	R	0.30	0.42	0.10	0.35	0.42	0.51	0.23	0.32	0.34	
	p	NS	0.02	NS	0.05	0.02	0.01	NS	NS	NS	
BIH	R	0.40	0.47	0.09	0.08	0.31	0.56	0.57	0.64	0.22	0.44
	p	0.05	0.02	NS	NS	NS	0.01	0.01	0.001	NS	0.02

NS = not significant

The correlations of classical pathway components

The intensities of deposition of both C1s and C4 correlated with those of IgA and C5 and the intensities of C4 also correlated with those of C3.

The correlations of the alternative pathway components

The intensities of deposition of properdin correlated only with those of C3.

The correlations of C3 and C5

The intensities of C3 deposition correlated with those of IgG, IgA, C4 and properdin while the intensities of C5 deposition correlated with those of IgA, C1s and C4.

The correlations of the control proteins

The intensities of $C\bar{1}$-INH deposition correlated with those of IgA, C1q, C1s, C4 and β1H and the intensities of β1H deposition correlated also with those of IgG, IgA, C4, properdin and C3.

The concordance between deposition of control proteins of complement and the components whose activity they regulate

$C\bar{1}$-INH

The concordance between biopsies positive for $C\bar{1}$-INH and C1s and C4 is shown in table 5.3

Table 5.3 The concordance between biopsies positive for C1s, C4 and $C\bar{1}$-INH

C1s/ $C\bar{1}$-INH	No. of biopsies	C4/ $C\bar{1}$-INH	No. of biopsies	C1s/ C4	No. of biopsies
C1s+ $C\bar{1}$-INH+	3	C4+ $C\bar{1}$-INH+	5	C1s+ C4+	5
C1s+ $C\bar{1}$-INH-	3	C4+ $C\bar{1}$-INH-	12	C1s+ C4-	1
C1s- $C\bar{1}$-INH+	3	C4- $C\bar{1}$-INH+	1	C1s- C4+	12
C1s- $C\bar{1}$-INH-	15	C4- $C\bar{1}$-INH-	6	C1s- C4-	6
Total	24	Total	24	Total	24

Of the nine biopsies positive for $C\bar{1}$-INH and/ or C1s, only three were positive for both. C4 was frequently deposited and of the six biopsies positive for $C\bar{1}$-INH, five had deposition of C4 and five of the six C1s positive biopsies were positive also for C4.

C3bINA

In the single biopsy positive for C3bINA, C3, C4, C5, properdin and βIH were also present.

βIH

The concordance between biopsies positive for βIH and C3, C4, C5 and properdin is shown in table 5.4.

Table 5.4 The concordance between biopsies positive for BlH and C3, C4, C5 and properdin

C3/BlH	No. of biopsies	C4/BlH	No. of biopsies	C5/BlH	No. of biopsies	P/BlH	No. of biopsies
C3+ BlH+	18	C4+ BlH+	15	C5+ BlH+	10	P+ BlH+	11
C3+ BlH-	4	C4+ BlH-	2	C5+ BlH-	3	P+ BlH-	1
C3- BlH+	0	C4- BlH+	3	C5- BlH+	6	P- BlH+	7
C3- BlH-	2	C4- BlH-	4	C5- BlH-	3	P- BlH-	5
Total	24	Total	24	Total	22	Total	24

βIH was present in 18 of 22 (82%) biopsies positive for C3 and was not found in the absence of C3. In two biopsies positive for C4, βIH was absent but was positive in three tissues where C4 was not found. C3 was found in each of the three biopsies where C5 was present in the absence of βIH and βIH was present in all but one biopsy where properdin was deposited.

A comparison of immunofluorescence patterns

Only three biopsies were positive for both C1s and $C\bar{1}$-INH and, in all three, the pattern of staining was described as mainly mesangial with some capillary loop staining for both C1s and $C\bar{1}$-INH. Serial sections stained for C1s and $C\bar{1}$-INH, showed the close similarities in the distribution of C1s and $C\bar{1}$-INH (figure 5-1). C3bNA was found within the mesangium of only one biopsy in this group, although of more extensive distribution, C3 staining was also mesangial.

The patterns of distribution of both C3 and βIH were described as mainly mesangial with some capillary loop staining in 16 of 18 biopsies where both were positive. Of the remaining two biopsies, C3 was present only round capillary loops while βIH was present in the mesangium and round capillary loops. Serial sections stained for C3 and βIH confirmed that these proteins were present in the same areas within the glomeruli (figure 5-2).

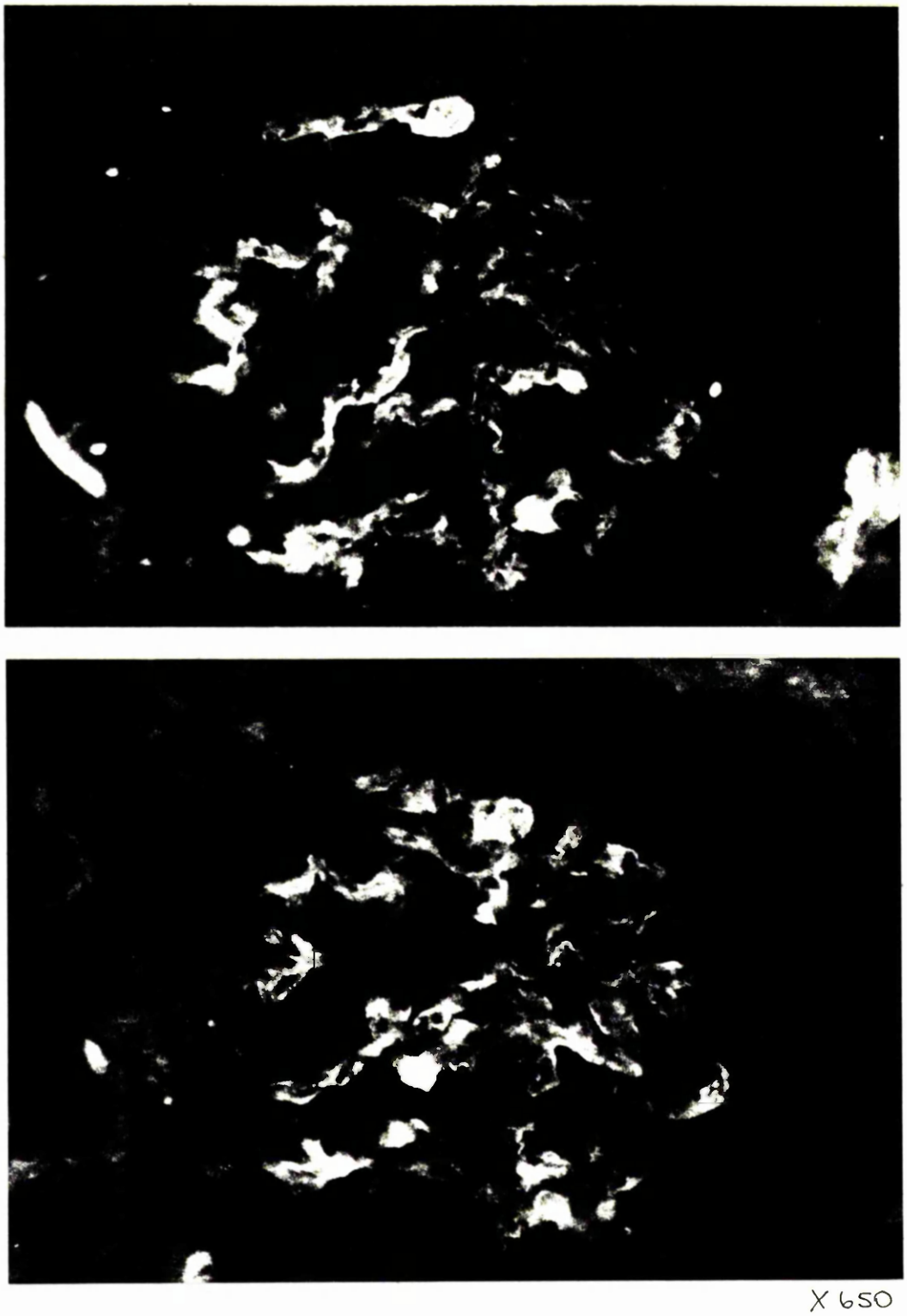


Figure 5-1 The top photograph shows the glomerulus stained for C1s and the photograph below shows the same glomerulus on an adjacent section stained for $C\bar{1}$-INH.

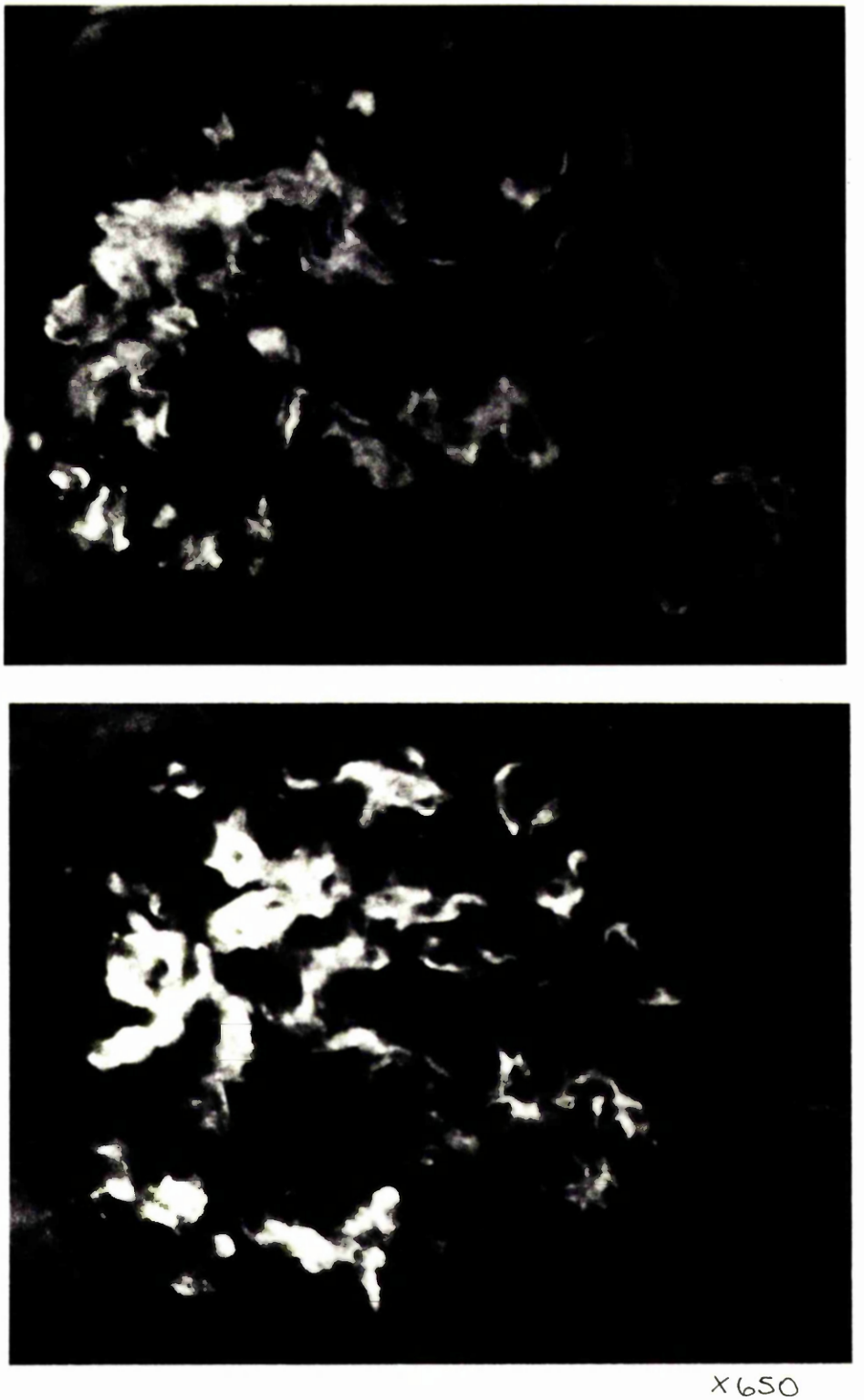


Figure 5-2 The top photograph shows the glomerulus stained for C3 and the photograph below shows the same glomerulus on an adjacent section stained for β1H.

Serum Samples

The serum concentrations of each component in each of the 14 serum samples is shown in figure 5-3.

The concentrations of classical pathway components

There were no subnormal levels of any classical pathway components and the levels as a group did not vary significantly from normal. Several raised levels were found for each component.

The concentrations of alternative pathway components

One serum sample had a marginally reduced concentration of factor B. All properdin levels were normal or raised and the median levels of both components did not differ significantly from normal.

The concentrations of C3 and C5

One patient had subnormal levels of C3 and C5. All other values were normal or raised and C3 levels, as a group, were significantly higher than normal.

The concentrations of the control proteins

$C\bar{1}$-INH levels were significantly raised. C3bINA levels were all within the normal range and β1H levels, as a group, were significantly above normal.

The correlations between serum concentrations of complement components and control proteins

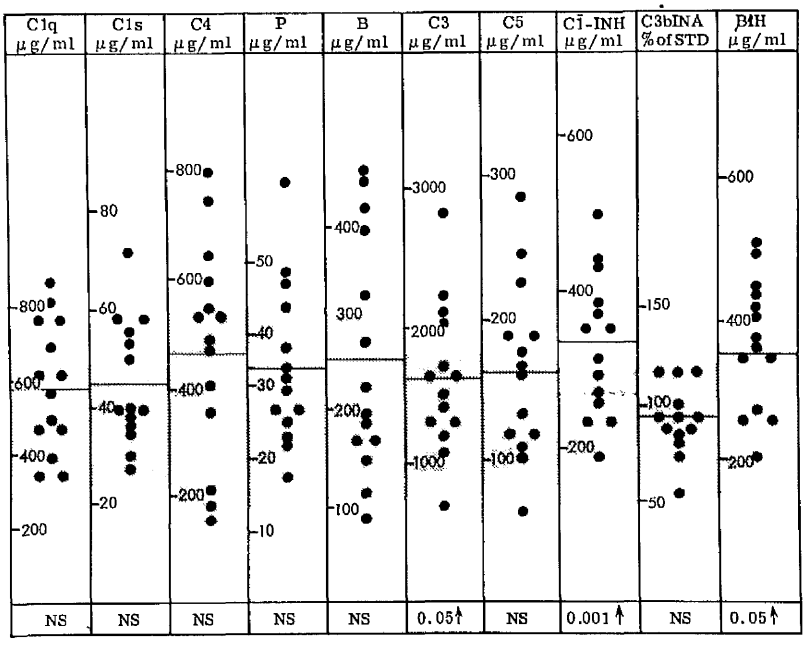


Figure 5-3 The concentration of each protein studied in each of the 14 serum samples in FGN is shown. The shaded areas represent the normal ranges and the horizontal lines represent the mean concentrations of each protein. The results of the median test are shown at the bottom of each column with arrows indicating whether the values are significantly higher or lower than normal.

NS = not significant.

The correlations between the serum concentrations of each of the components studied were calculated using the Spearman rank correlation test and the results are shown in table 5.5. R represents the correlation coefficient and p represents the probability of significance. Significant correlations are shown in red.

Table 5.5 Correlations in serum concentrations of the proteins studied

		C1q	C1s	C4	P	B	C3	C5	$C\bar{1}$-INH	C3bINA
C1s	R	0.59								
	p	0.05								
C4	R	0.12	0.44							
	p	NS	NS							
P	R	0.10	0.27	0.17						
	p	NS	NS	NS						
B	R	0.43	0.28	0.33	0.08					
	p	NS	NS	NS	NS					
C3	R	0.40	0.16	0.48	0.11	0.64				
	p	NS	NS	0.05	NS	0.01				
C5	R	-0.19	-0.16	0.44	0.11	0.15	0.62			
	p	NS	NS	NS	NS	NS	0.01			
$C\bar{1}$-INH	R	0.42	0.77	0.48	0.09	0.33	0.48	0.32		
	p	NS	0.001	0.05	NS	NS	0.05	NS		
C3bINA	R	-0.10	-0.06	0.38	0.44	0.35	0.36	0.18	-0.21	
	p	NS	NS	NS	NS	NS	NS	NS	NS	
βIH	R	0.49	0.77	0.52	0.06	0.46	0.31	0.26	0.83	-0.15
	p	0.05	0.001	0.05	NS	0.05	NS	NS	0.001	NS

NS = not significant

The correlations of classical pathway components

Only two significant correlations were found in this group. These were between C1q and C1s concentrations and between C4 and C3 levels.

The correlations of alternative pathway components

The concentrations of properdin did not correlate with those of any other component while factor B levels correlated with those of C3.

The correlations of C3 and C5

Apart from the correlations with the concentrations of C4 and factor B noted above, C3 levels also correlated with those of C5.

The correlations of the control proteins

$C\bar{1}$-INH levels correlated with levels of C1s, C4, C3 and β1H. β1H concentrations also correlated with those of C1q, C1s, C4 and factor B. C3bINA concentrations did not correlate with those of any other component.

The relationship between serum concentrations and the intensities of glomerular deposition of complement components and control proteins

Using the Spearman rank correlation test, the intensities of deposition were compared with the serum concentration of each component except for factor B and C3bINA which were

either absent or present in too few biopsies to allow analysis. The results are shown in table 5.6. R represents the correlation coefficient and p the probability of significance.

Table 5.6 The correlations between serum levels and the intensities of deposition of each complement component

	C1q	C1s	C4	P	C3	C5	$C\bar{1}$-INH	β1H
R	0.39	-0.27	-0.22	-0.04	0.12	-0.07	-0.21	0.51
p	NS	NS	NS	NS	NS	NS	NS	NS

NS = not significant

No significant correlations were found.

DISCUSSION

Intraglomerular evidence of complement activation

The involvement of immunoglobulins

Interest over recent years in the deposition of IgA in the glomeruli has resulted in a number of studies reporting and discussing mesangial deposition of IgA (225, 220, 231, 232, 233). Fewer reports of immunofluorescence studies of PGN have been published (224, 227, 230). The exact extent of the mesangial IgA pattern in PGN is hard to gauge since in many studies mesangial IgA deposition was the main selection criterion. Siscons found mesangial IgA in less than 50 per cent of focal GN biopsies (228). Other studies have found more; in one study, 11 of 24 were positive (225), in another five of six (230) and, in a third small study, four of four biopsies were found to show mesangial deposition of IgA (227). Wide variation therefore exists. In this study mesangial deposition of IgA was present in 19 of 27 biopsies (table 5.1). Variation also exists in the type and extent of accompanying immunoglobulins. IgG was most often found in association with IgA (225, 220, 230, 232). In one study, IgG was seldom found although IgM was frequently present (224). The presence of IgA, as the sole immunoglobulin, in ten of 19 biopsies positive for IgA in this study is higher than was found elsewhere, with the exception of one small series (227). Numerically, therefore, in this study, IgA was the predominant immunoglobulin detected.

The large variation in FGN results from various studies may represent real differences, perhaps environmental or genetic, in different geographical areas or may represent a discrepancy in diagnostic criteria from one centre to another.

The involvement of the classical pathway

C1q has been found deposited in the glomeruli by some workers (224) but not by others (228, 229). In the biopsies examined here (table 5.1), C1 subcomponents were seldom found but the presence of C4 in 19 of 28 biopsies suggests that classical pathway activation was taking place in a majority of these biopsies. Where there was deposition of IgG or IgM activation of the classical pathway is likely to be initiated by immune complexes involving IgG or IgM although neither intensities of deposition of IgG nor IgM correlated with those of classical pathway components. The deposition of classical pathway components where IgA was the only immunoglobulin present and the significant correlation between intensities of deposition of IgA and C1s and C4 (table 5.2) suggests a relationship between IgA and the classical pathway. IgA immune complexes are not thought to activate the classical pathway (83). One of the following explanations may be responsible for this association between IgA and C1s and C4:-

(1) amounts of IgG or IgM proportional to, but on occasions, too small to be detected by immunofluorescence were present and caused activation of the classical pathway.

(2) IgA may be the antigen involved in an IgG/ IgA immune complex. If this complex were in antigen excess masking of IgG might occur. The absence of anti-IgA activity in the circulation (231) makes this unlikely.

(3) Activation of the classical pathway may be taking place in the circulation and products of the activation deposited in the kidney. This could account for the presence of C1 subcomponents and C4 but not for the correlation with intensities of IgA deposition.

The involvement of the alternative pathway

Evidence also exists in the glomeruli for activation of the alternative pathway with 12 of 24 biopsies positive for properdin. The relevance of properdin deposition as an indicator of alternative pathway activation will be discussed in chapter 9. In other studies, four of eight biopsies (230) and four of four biopsies (227) were found to have glomerular deposition of properdin. Activation of the alternative pathway may be secondary to classical pathway activation, may be initiated directly by immune complexes containing IgA or some other unknown factor may be involved. There were no correlations between the intensities of deposition of properdin and either classical pathway components or IgA to support either possibility.

The terminal sequence

There is therefore evidence in FGN of activation of both

the classical and the alternative pathways. The significant correlations between intensities of C3 and those of C4 and properdin suggest that both pathways contribute to C3 catabolism. In two FGN biopsies, deposition of C3 was found in the absence of either classical or alternative pathway components. In both cases C3 was deposited in only trace amounts. The most likely explanation for this therefore is that classical and/ or alternative pathway components were present but in insufficient quantities for detection by immunofluorescence. Explanations, previously discussed for other types of GN must also be considered.

Intraglomerular evidence of regulation of complement activation

As before, the four criteria outlined in chapter 3 were used to assess the role of control proteins in FGN.

The role of $C\bar{1}$-INH

Evidence for the role of $C\bar{1}$-INH in FGN was inconclusive. The presence of $C\bar{1}$-INH in some biopsies (table 5.1), the significant correlation between intensities of deposition of C1s and $C\bar{1}$-INH (table 5.2) and the close similarities in distribution patterns of C1s and $C\bar{1}$-INH, where both were found (figure 5-1), argue in favour of a regulatory role for $C\bar{1}$-INH. The poor concordance between biopsies positive for C1s and $C\bar{1}$-INH (table 5.3) and the presence of $C\bar{1}$-INH in the absence of C1s suggests that the deposition of $C\bar{1}$-INH

need not mean that regulation of C1s by $\overline{C1}$-INH is taking place. Some reasons for a poor concordance have been discussed previously in chapter 3.

The role of C3bINA

C3bINA was present in one of 23 biopsies (table 5.1) and the distribution patterns of C3 and C3bINA were compatible. There is no other evidence from this study to support the role of C3bINA in the regulation of C3b in FGN.

The role of βIH

The frequent presence of βIH in FGN biopsies (table 5.1) the good concordance between biopsies positive for C3 and βIH, (table 5.3), the significant correlation between intensities of deposition of C3 and βIH (table 5.2) and the close similarities in distribution pattern (figure 5-2) all suggest that βIH is involved in the regulation of complement activation within the kidney.

Evidence of complement activation in the circulation

Marginally reduced levels of complement components were found in only two patients; one had low levels of factor B and another, low C3 and C5. The absence of generalised reductions of complement components in FGN is in agreement with others (227, 228) where normal levels of C3 and B were found. In the patients recorded here, however, some raised

values were found for each component except C3bINA. In the papers discussed (227, 228) normal ranges were not included and therefore direct comparison cannot be made. Raised levels of complement components have been discussed previously in chapter 3.

There is therefore no evidence from serum concentrations of substantial utilization of complement components in FGN. The correlation analysis, however, suggests that the relationships noted in deposition between C3 and the classical and alternative pathways also exists in the serum with significant correlation between levels of C3 and both C4 and factor B (table 5.5). The significant correlations between C1q and C1s and between C3 and C5 might have been anticipated considering their close relationship in the complement sequence. It also must be considered that synthetic factors may be responsible for correlations in concentrations of components.

Evidence of regulation of complement activation in the circulation

The role of $C\bar{1}$-INH

$C\bar{1}$-INH levels were not reduced in any FGN patients. It is therefore unlikely that a deficiency of $C\bar{1}$-INH resulted in classical pathway activation. The significant correlation between concentrations of $C\bar{1}$-INH and C1s suggests that $C\bar{1}$-INH was regulating classical pathway complement activation in the circulation.

The role of C3bINA

The failure of C3bINA concentrations to correlate with those of any other component may suggest that C3bINA is not controlling C3b. Significant correlations between serum concentrations of C3bINA and both C3 and factor B have been found elsewhere in normal serum and in other types of disease (188, 196, 197, 198) and this has been demonstrated in this study for other types of GN (tables 3.5 and 4.6). The failure to correlate may be due to the small sample size. The fact that the concentrations of complement components are not reduced suggests that regulation of complement activation was taking place normally in PGN.

The role of βlH

The correlations of βlH levels with those of Clq, Cls, C4, $C\bar{1}$-INH and factor B suggests a relationship between βlH and the classical and alternative pathways as was noted in the deposition studies of PGN. It was suggested that this may have resulted from the close relationship of βlH with C3 found in the kidney. In the serum, however, βlH did not correlate with C3. It is possible that the significant correlations between βlH concentrations and those of other components are due to common synthetic stimuli. Alternatively they may be due to an interdependence of all the components on the extent of C3 catabolism and the absence of a correlation

between levels of C3 and BIH may be the result of some other factor such as increased synthesis of C3.

The relationship between deposition and serum concentrations of complement components

As with all other groups considered, except MPGN, no significant correlations were found (table 5.6). Possible reasons for this have been previously discussed in chapter 3.

SUMMARY

1. Therefore evidence from this study suggests that activation of both the classical and the alternative pathways takes place in FGN and the mechanisms of activation of each have been discussed.
2. The correlations between the intensities of deposition of both C4 and properdin and those of C3 suggest that both pathways are involved in activation of C3. This is substantiated in the circulation by the significant correlations found between serum levels of both C4 and factor B and those of C3.
3. Evidence for the regulatory role of $C\bar{1}$-INH was based on its presence in the glomeruli, on the significant correlations between C1s and $C\bar{1}$-INH both in terms of intensity of staining and also serum concentrations and the similarities in the patterns of deposition of $C\bar{1}$-INH and C1s.
4. There is little evidence from this study for the role of C3bINA since it was found in only one biopsy and serum concentrations of C3bINA did not correlate with those of C3 or factor B.
5. As has been found in other groups, good evidence was found for the role of β1H in regulation; there was good concordance between biopsies positive for both C3 and β1H, strong correlations between intensities of deposition of C3 and β1H and close similarities in their distribution patterns although no correlation was found between serum concentrations of C3 and β1H. Reasons for this were discussed.

CHAPTER 6

Henoch-Schönlein Nephritis

Henoch-Schönlein Nephritis

Introduction

Henoch-Schönlein nephritis (HSN) is a systemic disease characterized by arthralgia, purpuric rash and abdominal pain (1). Haematuria is also a common finding and where the kidneys are involved, which is in between 12-64% of patients in various studies (1), the prognosis is poorer.

Where there is renal involvement, the histological pattern is most often a focal glomerulonephritis although severity may range from a minimal lesion to a diffuse crescentic GN and has been subdivided into five morphological types for diagnosis (1). By electron microscopy, electron dense deposits are apparent in the mesangium and also at the subendothelial aspect of the basement membrane (1). IgA, IgG and C3 deposition has been found, by immunofluorescence, mainly within the mesangium and also to varying extents round capillary loops (1, 234, 235). As in FGN, the pattern of immunofluorescence staining is diffuse in contrast to the focal histological picture most commonly found. IgA has been found to be the predominant immunoglobulin in two studies (227, 235).

Immune complexes have been detected in the circulation (236) and some of those were shown to contain IgA antibodies. Other workers failed to find circulating immune complexes (180). Serum concentrations of C3 have been reported as being normal

(237).

The pathogenesis of HSN is unclear. The close similarities often found between HSN and FGN suggest that similar pathogenetic mechanisms may be operative. Various mechanisms were suggested for the pathogenesis of FGN in chapter 5. The presence in the glomeruli of immunoglobulins and complement components, subendothelial and mesangial electron dense deposits and the presence of circulating immune complexes would suggest that immune complexes are involved.

MATERIALS

Immunofluorescence studies were performed on 14 renal biopsies obtained from 13 patients with HSN, 12 of whom were under 15 years of age. Proteinuria was present in five patients ranging from 1 to 13.6 g/ 24 hours (mean = 4.0 g/ 24 hours). Serum creatinine levels ranged from 48 to 430 μmoles/l with three values above the normal range.

Between one and 16 glomeruli (mean = 5.8) were examined by immunofluorescence and serum samples taken on the day of biopsy were available from four patients.

RESULTS

Renal Biopsies

Glomerular deposition of immunoglobulins, complement and control proteins

The number of biopsies positive and the mean intensity of staining for each protein studied is shown in table 6.1.

Table 6.1 Glomerular deposition of immunoglobulins complement and control proteins

	Immunoglobulins			Classical Components			Alternative Components		Terminal Components		Control proteins		
	G	A	M	Clq	Cls	C4	P	B	C3	C5	C1-INH	C3bINA	βIH
Number positive	9/ 14	12/ 14	4/ 14	2/ 14	4/ 14	9/ 13	11/ 14	0/ 14	13/ 14	9/ 12	8/ 14	4/ 13	13/ 14
Mean intensity	1.8	2.2	1.0	1.5	1.3	1.7	1.7		1.5	1.3	1.4	1.0	1.8

mean intensity = the mean of the scores given for the intensity of deposition of each protein in each biopsy

Deposition of immunoglobulins

As in FGN, IgA was again the predominant immunoglobulin, being present in 12 of 14 (86%) biopsies with a high mean intensity of staining (appendix 1). IgG was found in 9 of 14 (64%) and IgM in four of 14 (28%) biopsies. Neither IgG nor IgM was found in the absence of IgA. Immunoglobulins were not detected in two biopsies.

Deposition of classical pathway components

C1q and C1s were found in two of 14 (14%) and four of 14 (28%) biopsies respectively although classical pathway activation, as evidenced by C4 deposition was found in nine of 13 (69%) biopsies. Neither C1q nor C1s was found in the absence of C4.

Deposition of alternative pathway components

Properdin was present in 11 of 14 (79%) HSN biopsies but factor B was never found.

Deposition of C3 and C5

C3 was found in all but one HSN biopsy although the intensity of staining was relatively low compared with other disease groups studied (appendix 1). C5 was deposited in nine of 12 (75%) biopsies.

Deposition of control proteins

$C\bar{1}$-INH was present in eight of 14 (57%) biopsies which is twice as many as were positive for C1s. Of the regulators of

C3b, C3bINA was found in four of 13 (31%) which represents the highest percentage of biopsies positive in any group studied (appendix I) and β1H was found in 13 of 14 (93%) biopsies.

Correlations in intensities of staining between proteins studied

Using the Spearman rank correlation test, the intensities of staining of each protein studied was compared with the intensities of all others. The results are shown in table 6.2. R represents the correlation coefficient and p represents the probability of significance. Because of the absence of factor B and the small number of biopsies positive for IgM, C1q, C1s and C3bINA, no analyses were performed on the results of these. Significant correlations are shown in red.

Table 6.2: Correlations in intensities of staining between the proteins studied

		G	A	C4	P	C3	C5	$\overline{C1}$-INH
A	R	0.76						
	p	<0.001						
C4	R	0.38	0.45					
	p	NS	NS					
P	R	0.49	0.33	0.91				
	p	<0.05	NS	<0.001				
C3	R	0.18	0.21	0.12				
	p	NS	NS	NS				
C5	R	0.62	0.47	0.40	0.49	0.37		
	p	<0.02	NS	NS	0.05	NS		
C1-INH	R	-0.20	0.05	0.41	0.30	0.14	-0.03	
	p	NS	NS	NS	NS	NS	NS	
B1H	R	0.32	0.26	0.38	0.37	0.20	-0.01	0.39
	p	NS	NS	NS	NS	NS	NS	NS

The correlations of classical pathway components

Of the classical pathway components, only C4 was present in enough biopsies to allow meaningful analysis and the intensities of C4 deposition correlated well with those of properdin only.

The correlations of alternative pathway components

As well as the correlation with C4, the intensities of properdin deposition correlated with those of IgG and C5.

The correlations of C3 and C5

Intensities of C3 deposition did not correlate with those of any other component while the intensities of C5 deposition correlated with IgG and properdin.

The correlations of control proteins

Neither the intensities of deposition of $C\bar{1}$-INH nor BlH correlated with the intensities of any other component. Analysis on C3bINA was not carried out.

The concordance between deposition of control proteins of complement and the components whose activity they regulate

$C\bar{1}$-INH

The concordance between biopsies positive for $C\bar{1}$-INH, C1s and C4 is shown in table 6.3.

Table 6.3 The concordance between biopsies positive for C1s, C4 and $C\bar{1}$-INH

C1s/ $C\bar{1}$-INH	No. of biopsies	C4/ $C\bar{1}$-INH	No. of biopsies	C1s/ C4	No. of biopsies
C1s+ $C\bar{1}$-INH+	4	C4+ $C\bar{1}$-INH+	7	C1s+ C4+	4
C1s+ $C\bar{1}$-INH-	0	C4+ $C\bar{1}$-INH-	2	C1s+ C4-	0
C1s- $C\bar{1}$-INH+	4	C4- $C\bar{1}$-INH+	1	C1s- C4+	5
C1s- $C\bar{1}$-INH-	6	C4- $C\bar{1}$-INH-	3	C1s- C4-	4
Total	14	Total	13	Total	13

$C\bar{1}$-INH was present in all C1s positive biopsies and also in four biopsies where C1s was not found. In three of the C1s negative biopsies, C4 was deposited along with $C\bar{1}$-INH and in two biopsies C4 was present without $C\bar{1}$-INH. C4 was always found where C1s was present and also in five biopsies with no evidence of C1s deposition.

C3bINA

The concordance between biopsies positive for C3bINA and C3, C4, C5, properdin and β1H is shown in table 6.4. (overleaf).

Table 6.4 The concordance between biopsies positive for C3bINA, C3, C4, C5, P and βIH

C3/ C3bINA		C4/ C3bINA		C5/ C3bINA		P/ C3bINA		βIH/ C3bINA	
C3+ C3bINA+	4	C4+ C3bINA+	3	C5+ C3bINA+	4	P+ C3bINA+	4	βIH+ C3bINA+	4
C3+ C3bINA-	8	C4+ C3bINA-	6	C5+ C3bINA-	5	P+ C3bINA-	6	βIH+ C3bINA-	8
C3- C3bINA+	0	C4- C3bINA+	1	C5- C3bINA+	0	P- C3bINA+	0	βIH- C3bINA+	0
C3- C3bINA-	1	C4- C3bINA-	3	C5- C3bINA-	3	P- C3bINA-	3	βIH- C3bINA-	1
Total	13	Total	13	Total	12	Total	13	Total	13

C3, C5, properdin and βIH were found in each of the four biopsies positive for C3bINA and C4 was present in three.

βIH

The concordance between biopsies positive for βIH and C3, C4, C5 and properdin is shown in table 6.5.

Table 6.5 The concordance between biopsies positive for βIH, C3, C4, C5 and P

C3/βIH		C4/βIH		C5/βIH		P/βIH	
C3+βIH+	12	C4+βIH+	9	C5+βIH+	9	P+βIH+	10
C3+βIH-	0	C4+βIH-	0	C5+βIH-	0	P+βIH-	0
C3-βIH+	0	C4-βIH+	3	C5-βIH+	2	P-βIH+	2
C3-βIH-	1	C4-βIH-	1	C5-βIH-	1	P-βIH-	1
Total	13	Total	13	Total	12	Total	13

There was very good concordance between deposition of C3 and βIH with 12 of 13 biopsies being positive for both and the remaining one negative for both. C4 was negative in three biopsies positive for βIH and C5 and properdin were negative in two positive for βIH. All other components were negative in the biopsy where βIH was not found.

Comparison of immunofluorescence patterns

Of the four biopsies positive for both C1s and C1-INH, three had identical patterns of staining and in the fourth both

C1s and $C\bar{1}$-INH were predominantly capillary loop while $C\bar{1}$-INH was also present within the mesangium. Serial sections stained for C1s and $C\bar{1}$-INH showed very close similarities in distribution pattern (Figure 6-1).

When C3bINA staining was considered, C3bINA was found to be deposited in a segmental capillary loop pattern while C3, in each case, was found round capillary loops and also within the mesangium.

C3 and β1H were found to have the same pattern of staining in nine of the 12 biopsies where both were positive. In the remaining three, C3 was found mainly round capillary loops while β1H was present round capillary loops and also in the mesangium. Serial sections stained with antisera to C3 and β1H showed close similarities in distribution pattern (Figure 6-2).

Serum samples

The serum concentration of each component within each sample is shown in figure 6-3.

The concentrations of classical pathway components

No subnormal values were found for any classical pathway component. In one patient, C1q and C1s levels were raised.

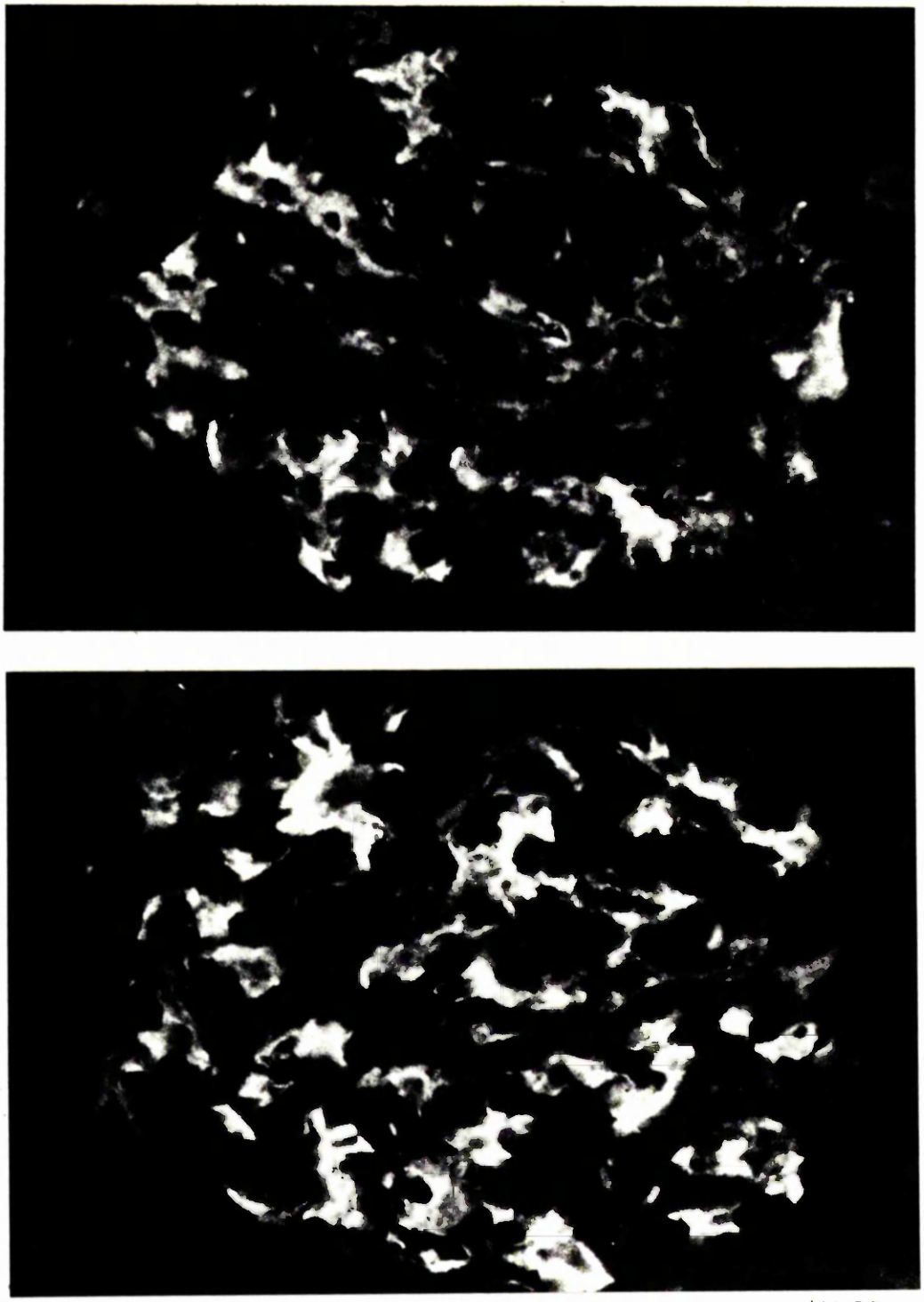


Figure 6-1 The photograph on top shows the deposition pattern of C1s and the photograph below shows the same glomerulus on an adjacent section stained for $C\bar{1}$-INH.

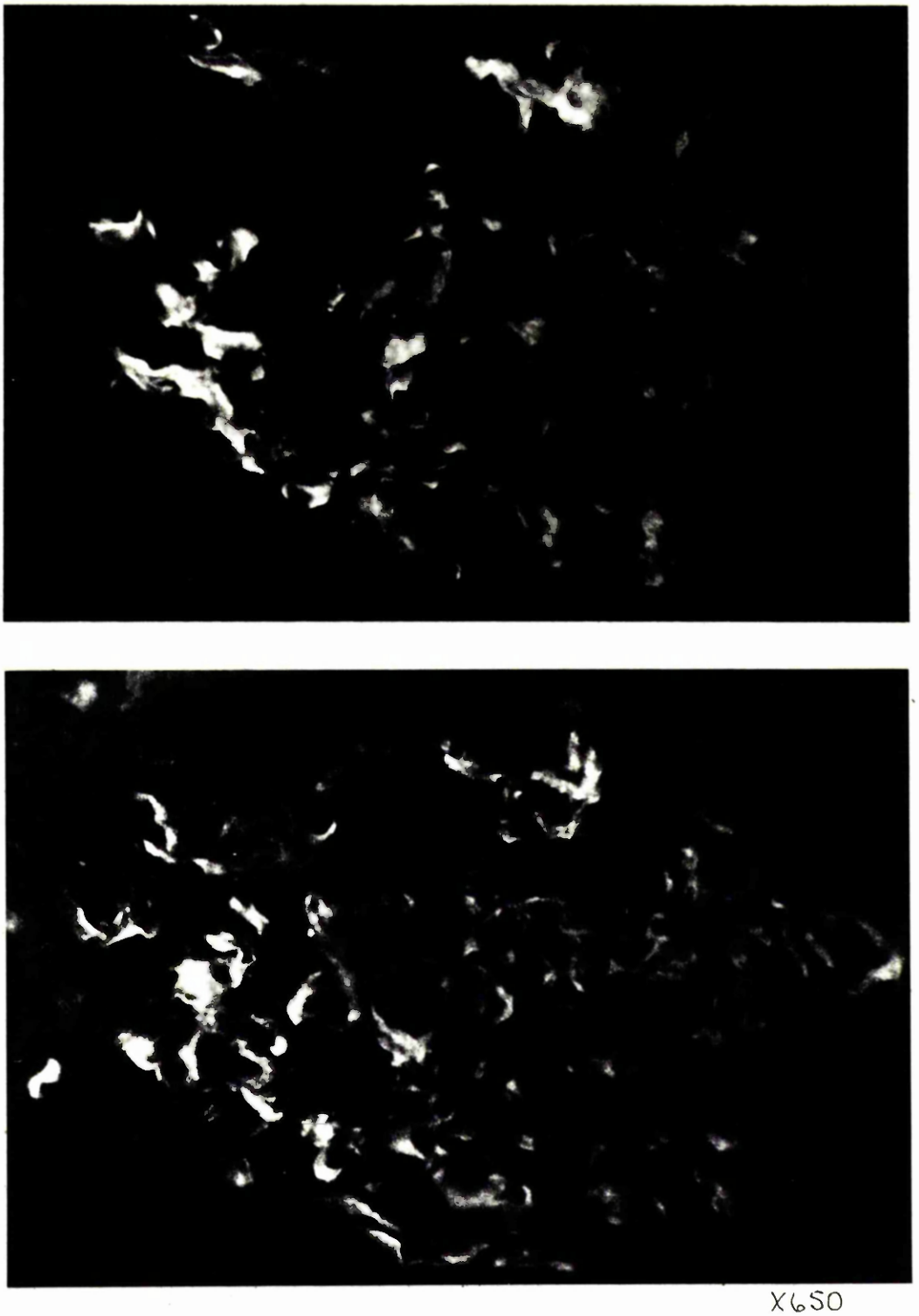


Figure 6-2 The photograph on the top shows the staining pattern of C3 and the photograph below shows the same glomerulus on an adjacent section stained for β1H.

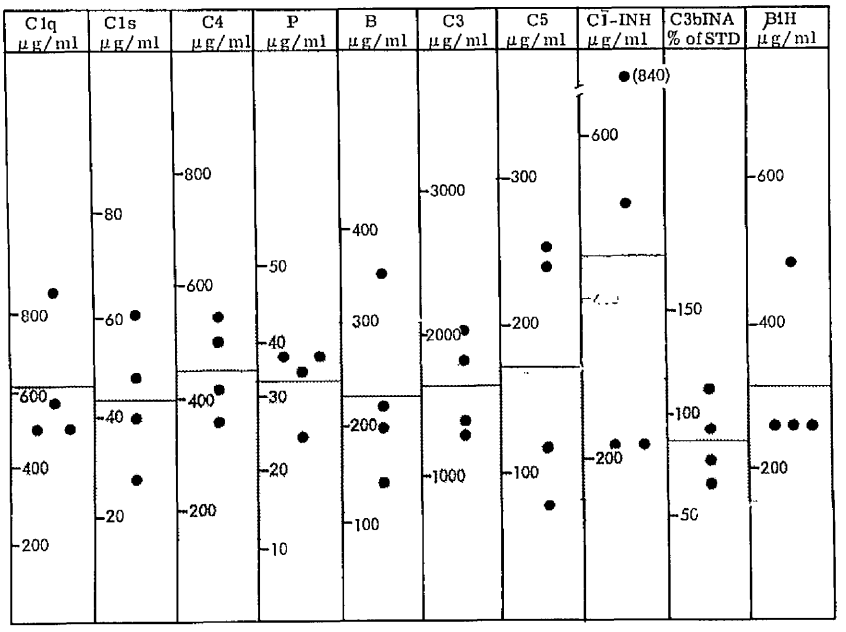


Figure 6-3 The serum concentration of each component in each HSN sample is shown here. The shaded areas represent the normal ranges of each component and the horizontal lines represent the mean of the sample concentrations for each component.

The concentrations of alternative pathway components

All properdin values were within the normal range. One serum had a raised factor B concentration while other samples had normal values.

The concentrations of C3 and C5

No subnormal C3 and C5 serum concentrations were found. One patient had raised levels of both C3 and C5 while another two patients had a raised concentration of either C3 or C5. Other C3 and C5 concentrations were normal.

The concentrations of control proteins

Subnormal serum concentrations of the three control proteins of complement activation were not found. One patient had raised levels of both $C\bar{1}$-INH and β1H and another had a raised concentration of only $C\bar{1}$-INH. All C3bINA levels were within the normal range.

Correlations between the serum concentrations of complement and control proteins could not be examined because of the small number of samples available for study. Similarly correlations between serum concentration and the intensity of glomerular deposition of components could not be studied.

DISCUSSION

Intraglomerular evidence of complement activation

The involvement of immunoglobulins

In agreement with Berger's study (235), IgA was the predominent immunoglobulin in HSN and the intensity of staining was also relatively high (appendix I). Immunoglobulins were absent from two biopsies. Urizar (238) found that fibrinogen was often deposited in the absence of immunoglobulins in very early stages of the disease and suggested that coagulation may therefore be a pathogenic mechanism in HSN. The two immunoglobulin negative biopsies in this study however showed a diffuse proliferative GN and fibrinogen was present in trace amounts in one and was absent from the other. The lack of immunoglobulin staining is therefore not associated with early stages of the disease and some other factors must be involved.

The involvement of the classical pathway

There was evidence of classical pathway activation in 71% of HSN biopsies. In eight of the ten biopsies where classical pathway components were present, IgG and/ or IgM were also found. It is likely that the pathogenesis of HSN involves deposition of immune complexes and that activation of the classical pathway is due to the presence of immune complexes containing antibody of the IgG or IgM classes.

In two biopsies, C4 was present where IgA was the only immunoglobulin apparent. Since IgA immune complexes are not known to activate the classical pathway (83), it is possible that IgG or IgM was present in these tissues in sufficient amounts to cause complement activation but in insufficient amounts to be visualised. Alternatively, activation of the classical pathway may have occurred by immune complexes or by some other means in the circulation and the products deposited in the kidneys.

The involvement of the alternative pathway

Although factor B was not present in any biopsy, activation of the alternative pathway was suggested by the frequent presence of properdin (table 6.1). The good correlation between intensities of deposition of IgG and properdin may imply that activation of the alternative pathway is secondary to classical pathway activation initiated by immune complexes containing IgG. The good correlation between the intensities of deposition of C4 and properdin supports this view. Alternatively the alternative pathway may be activated directly as the result of IgA containing immune complexes or by some immunoglobulin independent mechanism. The possibility that properdin may deposit in the absence of alternative pathway activation has been suggested previously and will be discussed more fully in chapter 9.

The terminal sequence

Activation of both the classical and the alternative pathways is found in HSN. However the intensities of deposition of C3 did not correlate with those of any other component and consequently the contribution of each pathway to C3 activation could not be assessed. If the intensities of C5 may be taken as an index of the extent of activation, then the significant correlation between intensities of staining of C5 and properdin suggests that the alternative pathway is mainly responsible for the activation of the terminal sequence.

Intraglomerular evidence of regulation of complement activation

The four criteria outlined in chapter 3 were used to assess the role of the control proteins in regulation of complement activation in HSN.

The role of $C\bar{1}$-INH

The role of $C\bar{1}$-INH in controlling classical pathway complement activation in HSN is therefore based on its presence in 8 of 14 biopsies (table 6.1), the limited concordance between $C\bar{1}$-INH and C1s and particularly C4 (table 6.3) and the similarities in distribution patterns (Figure 6-1). C1s was present in too few biopsies to allow correlation analysis to be carried out.

The role of C3bINA

C3bINA was found in a higher percentage of HSN biopsies

than in any other group (table 6.1) although it was only present in trace amounts in each of 4 biopsies. The concordance between biopsies positive for C3bINA and βlH being present in all C3bINA positive biopsies (table 6.4).

βlH being present in all C3bINA positive biopsies (table 6.4).
It may be suggested that the relatively frequent presence of C3bINA accounts for the lack of intense complement activation in HSN, based on weak glomerular deposition of C3 (table 6.1) and normal serum C3 concentrations (Figure 6-3). However, the two other groups in which C3bINA was found, MPGN and SLE, are associated with excessive complement activation (tables 4.1 and 7.1 and figures 4-3 and 7-3).

Alternatively, it is possible that C3bINA is only found where considerable complement activation is occurring, since only in this situation would one expect significant amounts of C3b to be present. One factor common to MPGN and SLE is the presence of electron dense deposits in a subendothelial position. In the four HSN biopsies with deposition of C3bINA, the staining pattern of C3 and IgA was predominantly capillary loop with two biopsies, also showing mesangial staining for C3 and IgA. Although electron microscopy was not performed on these biopsies electron dense deposits in HSN are generally found within the mesangium and in a subendothelial position (1). Therefore the subendothelial position of the complexes in these

three diseases may allow easier access of complement components and C3bINA to the complexes and the transitory C3bINA may therefore be visualised.

Despite the close similarities between PGN and HSN seen in this study and described by others (1, 227), C3bINA was found in only one of 23 PGN biopsies. The difference could be due to the fact that there is relatively more capillary loop deposition of immunoglobulins and complement components in HSN compared with PGN.

The role of βIH

As with other groups studied, βIH was frequently present (table 6.1). The concordance between biopsies positive for C3 and βIH was strong and the patterns of staining were very similar. No significant correlation was found, however, between the intensities of deposition of βIH and any other component. The evidence for the role of βIH in controlling complement activation in HSN is therefore based on the fact that three of the four criteria employed were satisfied.

Evidence of complement activation in the circulation

Too few serum samples were available from the HSN group for detailed study. No reduced values of any component was found which agrees with the work of others (238). There is therefore no evidence, from the few results available, of a deficiency of any of the regulatory proteins which would result in uncontrolled complement activation.

SUMMARY

1. There is evidence of activation of both the classical and the alternative pathways of complement in HSN and the possible mechanisms of activation were discussed.

2. The relative contribution of each pathway could not be assessed since the intensities of staining of components of neither pathway correlated with the intensities of deposition of C3 and no analysis of the serum concentrations was done. The correlation found between the intensities of staining of C5 and properdin however may suggest a greater involvement of the alternative pathway.

3. Evidence for the role of $C\bar{1}$-INH in regulating classical pathway activation was based on its presence in the kidney, the reasonable concordance between biopsies positive for $C\bar{1}$-INH and C1s and the similarities in distribution patterns.

4. Although, by no means conclusive, there was more evidence for the regulation of C3b by C3bINA in HSN than in any other group studied and reasons for this were discussed. The regulatory role of C3bINA was based on its presence in four biopsies which also contained properdin and C3 and the compatibility of the distribution patterns of C3 and C3bINA.

5. There was good evidence for the role of βIH based on its presence, good concordance between biopsies positive for C3 and βIH and the similarities in distribution patterns of these two proteins.

6. Since the serum concentrations of the three control proteins were either normal or raised, activation of the complement system in HSN was not due to a serum deficiency of these proteins.

CHAPTER 7

Systemic Lupus Erythematosus

Systemic Lupus Erythematosus

Introduction

In a large number of published series of SLE patients, the frequency of renal involvement was found to range from 37 to 87 percent of cases (1) and was a frequent cause of death.

There is a variety of possible histological patterns in this disease with evidence of progression from mild to more severe forms in some patients (239). The single diagnostic characteristic is the appearance in some glomeruli of haematoxyphil bodies. Some biopsies show a focal proliferative pattern while in others, the proliferation may be diffuse and accompanied by thickening of capillary walls. Crescents may also be present. In the absence of proliferation, capillary walls may be focally or diffusely thickened, giving the appearance of MGN.

Variations are similarly found at an ultrastructural level with electron dense deposits in one or more of the subendothelial intramembranous or subepithelial positions. These deposits may have a characteristic "finger-print" appearance.

Immunofluorescence, where little or no urinary abnormalities exist and no histological lesion is found is reported as showing linear capillary loop deposition of IgG (53, 246). Where the histological pattern is that of a focal GN, IgG and C3 and often also IgA and IgM are found mainly in the mesangium (246). In the diffuse proliferative forms, the staining is most often in both

the mesangium and capillary loops and, in the type resembling MGN, staining is in a fine granular capillary loop pattern. All three main immunoglobulin classes are often found together particularly in diseases involving mesangial deposition (240-241). DNA antigens may be demonstrated with immunoglobulin and C3 in the glomeruli (242) and antibodies with specificity for native DNA may be eluted from the kidneys of SLE patients (41).

Circulating immune complexes have frequently been found using a number of different assays (179, 180, 242a, 242b) and although antibodies to native DNA are generally considered diagnostic for SLE (243), antibodies to denatured DNA, RNA, ribosomes, polynucleotides and nuclear proteins are also found in the serum (53).

Deposition of both classical and alternative pathway complement components have been recorded (241) and reduced serum concentrations of C3, C4, factor B are common (245). Increased catabolic rates of C3 have also been noted. There is therefore evidence of both classical and alternative pathway activation in SLE.

Evidence based on the presence of electron dense deposits and antigens with specific antibody in the glomeruli and immune complexes in the circulation strongly suggests that the pathogenesis of SLE involves deposition of immune complexes. The significance of type II hypersensitivity was discussed in chapter 1 and may contribute to the pathogenesis particularly in biopsies where deposits are in a subepithelial position.

MATERIALS

Immunofluorescence studies were performed on renal biopsies obtained from ten patients. Proteinuria was found in five patients ranging from 2.1 to 10.6 g/ 24 hours (mean = 4.4 g/ 24 hours) and the serum creatinine level was raised in one patient. All patients, at some time prior to biopsy, had had circulating antibody to DNA as measured by the Farr technique (243). At the time of biopsy, nine patients had positive DNA binding capacities ranging from 42 to 99 percent (mean = 81%).

Between 1 and 10 glomeruli (mean = 5.6) were available for immunofluorescence examination and serum samples taken on the day of biopsy were available from all ten patients.

RESULTS

Renal Biopsies

Glomerular deposition of immunoglobulins, complement and control proteins

The number of biopsies positive and the mean intensity of staining for each protein studied is shown in table 7.1. (see overleaf).

Table 7.1 Glomerular deposition of immunoglobulins, complement components and control proteins

	Immunoglobulins			Classical Components			Alternative Components		Terminal Components		Control proteins		
	G	A	M	C1q	C1s	C4	P	B	C3	C5	$C\bar{1}$-INH	C3bINA	βlH
Number of biopsies positive	10/ 10	8/ 10	9/ 10	10/ 10	9/ 9	10/ 10	7/ 9	0/ 9	10/ 10	8/ 8	7/ 9	2/ 9	9/ 9
Mean intensity	2.3	1.6	1.7	2.3	2.0	1.7	1.4		2.6	2.0	2.3	1.5	2.7

Mean intensity = the mean of the scores given for the intensity of deposition of each protein in each biopsy.

Deposition of immunoglobulins

IgG was deposited in all biopsies with IgA and IgM present in eight out of ten (80%) and nine of ten biopsies (90%)

Deposition of classical pathway components

Deposition of classical pathway components

All three classical pathway components were present in each of the biopsies examined.

Deposition of alternative pathway components

Properdin was present in seven of nine biopsies (78%) studied while factor B was never detected.

Deposition of C3 and C5

C3 and C5 were each present in all biopsies studied.

Deposition of control proteins

$C\bar{1}$-INH was deposited in seven of nine SLE biopsies (78%) while C3bINA was only found in two (22%). β1H was present in each of nine biopsies examined.

Correlations in intensity of staining between the proteins studied

Using the Spearman rank correlation test, the intensities of staining of each protein studied was compared with the intensities of all others. R represents the correlation coefficient and p the probability of significance. The results of this are shown on table 7.2. Because factor B was not present and C3bINA was only found in two biopsies, no analyses

were performed on the results of these. Significant correlations are shown in red.

Table 7.2: Correlations in intensity of staining between the proteins studied

		G	A	M	C1q	C1s	C4	P	C3	C5	C1̄-INH
A	R	0.85									
	p	0.001									
M	R	0.78	0.82								
	p	0.01	0.001								
C1q	R	0.57	0.34	0.50							
	p	0.05	NS	NS							
C1s	R	0.62	0.46	0.53	0.10						
	p	0.05	NS	NS	NS						
C4	R	0.44	0.26	0.13	0.29	0.41					
	p	NS	NS	NS	NS	NS					
P	R	0.46	0.65	0.71	0.36	0.28	0.08				
	p	NS	0.05	0.02	NS	NS	NS				
C3	R	0.43	0.24	0.35	0.80	0.38	0.15	0.41			
	p	NS	NS	NS	0.01	NS	NS	NS			
C5	R	0.19	0.28	0.32	0.09	0.42	0.09	0.55	0.43		
	p	NS	NS	NS	NS	NS	NS	NS	NS		
C1̄-INH	R	0.46	0.51	0.41	0.24	0.28	0.27	0.90	0.51	0.55	
	p	NS	NS	NS	NS	NS	NS	0.001	NS	NS	
βIH	R	0.51	0.61	0.73	0.29	0.41	0.25	0.83	0.50	0.76	0.85
	p	NS	0.05	0.02	NS	NS	NS	0.001	NS	0.01	0.001

NS = not significant

The correlations of classical pathway components

The intensities of staining of C1q and C1s correlated weakly with those of IgG and C1q intensities also correlated with those of C3.

No other significant correlations were present.

The correlations of the alternative pathway

Factor B was not present in SLE and the intensities of properdin deposition correlated only with those of IgA and IgM.

The correlations of C3 and C5

The intensities of C3 deposition correlated only with those of C1q while the intensities of C5 deposition failed to correlate with those of any other component.

The correlations of the control proteins

The intensities of deposition of $C\bar{1}$-INH correlated well with those of both properdin and β1H. The intensities of β1H deposition also correlated with those of IgA, IgM, properdin and C5.

The concordance between deposition of the control proteins of complement and the components whose activity they regulate

$C\bar{1}$-INH

The concordance between biopsies positive for $C\bar{1}$-INH and those positive for C1s and C4 is shown in table 7.3.

(see over).

Table 7.3: The concordance between biopsies positive for C1s, C4 and $C\bar{1}$-INH

C1s/ $C\bar{1}$-INH	No. of biopsies	C4/ $C\bar{1}$-INH	No. of biopsies	C4/ C1s	No. of biopsies
C1s+ $C\bar{1}$-INH+	7	C4+ $C\bar{1}$-INH+	7	C4+ C1s+	9
C1s+ $C\bar{1}$-INH-	2	C4+ $C\bar{1}$-INH-	2	C4+ C1s-	0
C1s- $C\bar{1}$-INH+	0	C4- $C\bar{1}$-INH+	0	C4- C1s+	0
C1s- $C\bar{1}$-INH-	0	C4- $C\bar{1}$-INH-	0	C4- C1s-	0
Total	9	Total	9	Total	9

C1s and C4 were present in all nine biopsies examined and since $C\bar{1}$-INH was present in seven of these the concordance was good.

C3bINA

The two biopsies positive for C3bINA also showed deposition of C3, C4, C5, properdin and β1H.

β1H

The concordance between biopsies positive for β1H and C3, C4, C5 and properdin is shown in table 7.4.

Table 7.4: The concordance between biopsies positive for β1H and C3, C4, C5 and properdin

C3/ β1H	No. of biopsies	C4/ β1H	No. of biopsies	C5/ β1H	No. of biopsies	P/ β1H	No. of biopsies
C3+ β1H+	9	C4+ β1H+	9	C5+ β1H+	8	P+ β1H+	7
C3+ β1H-	0	C4+ β1H-	0	C5+ β1H-	0	P+ β1H-	0
C3- β1H+	0	C4- β1H+	0	C5- β1H+	0	P- β1H+	2
C3- β1H-	0	C4- β1H-	0	C5- β1H-	0	P- β1H-	0
Total	9	Total	9	Total	8	Total	9

Since all biopsies studied were positive for βIH, C3, C4 and C5, complete concordance existed between each of these and βIH was present in each of the seven biopsies positive for properdin.

Comparison of immunofluorescence patterns

In eight biopsies in the SLE group, most proteins studied were found both in the mesangium and round the capillary loops. In one, the pattern was mainly mesangial and, in another, most proteins were found almost exclusively round the capillary loops.

When the patterns of C1s and $\overline{C1}$-INH were considered, it was found that C1s and $\overline{C1}$-INH were both found round capillary loops and within the mesangium in four and, in the remaining three, $\overline{C1}$-INH was present again round capillary loops and within the mesangium, while C1s was deposited mainly round capillary loops. Staining of adjacent sections with antisera to C1s and $\overline{C1}$-INH demonstrated a very close similarity in the distribution patterns (figure 7-1).

In one of the two biopsies positive for C3bINA, C3 and C3bINA were both found in a mesangial and capillary loop distribution although C3bINA staining was patchy while C3 staining was diffuse. In the other biopsy, C3 was again found in a mesangial and capillary loop distribution but C3bINA was deposited focally and segmentally round capillary loops.

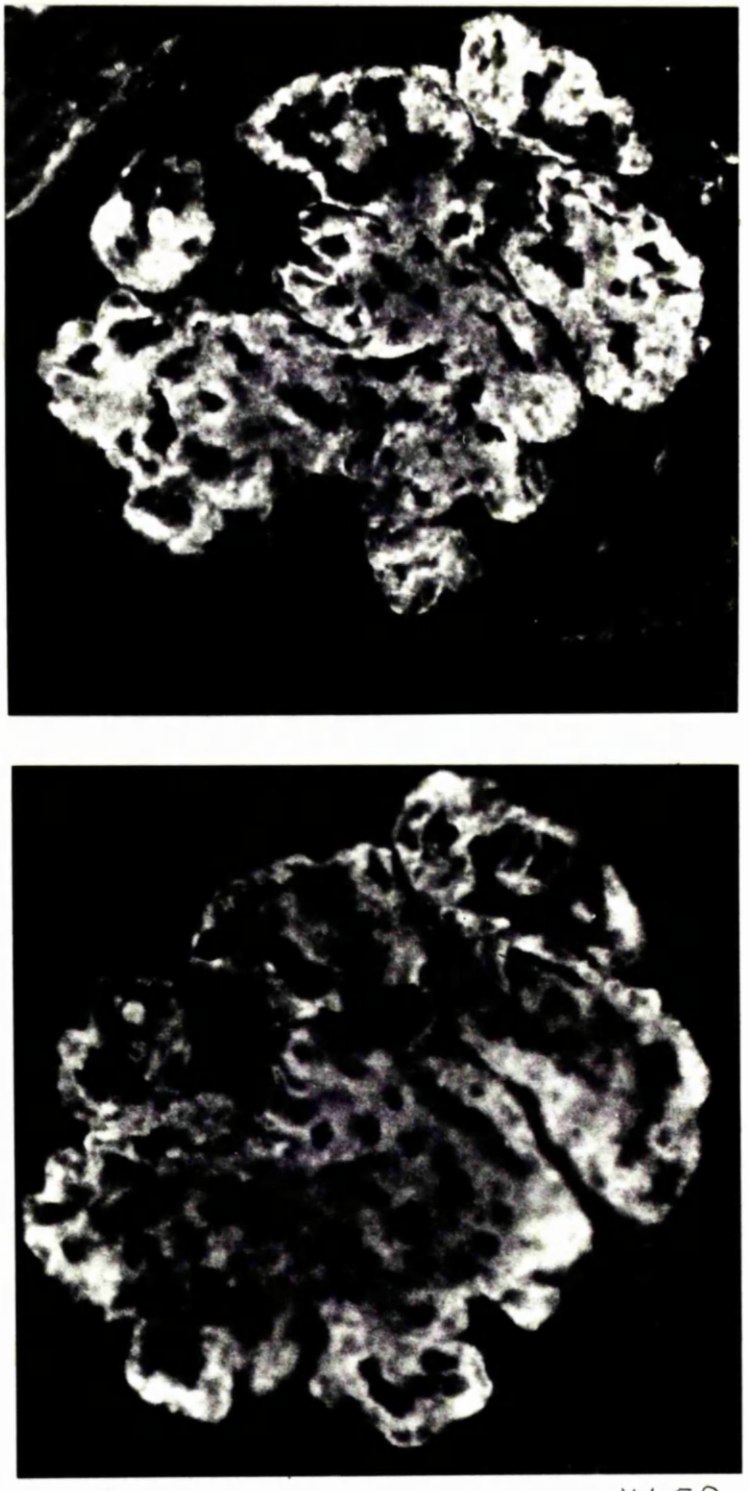


Figure 7-1 The top photograph shows the staining pattern for C1s while the photograph below shows the same glomerulus on an adjacent section stained for $C\bar{1}$-INH.

The patterns of deposition for C3 and βIH were identical in eight out of nine biopsies. In the ninth C3 was found both in the mesangium and round capillary loops while βIH showed only a capillary loop staining pattern. The distribution on serial sections was again very similar (figure 7-2).

Serum Samples

The serum concentration of each component in each of the ten serum samples is shown in figure 7-3.

The concentrations of classical pathway components

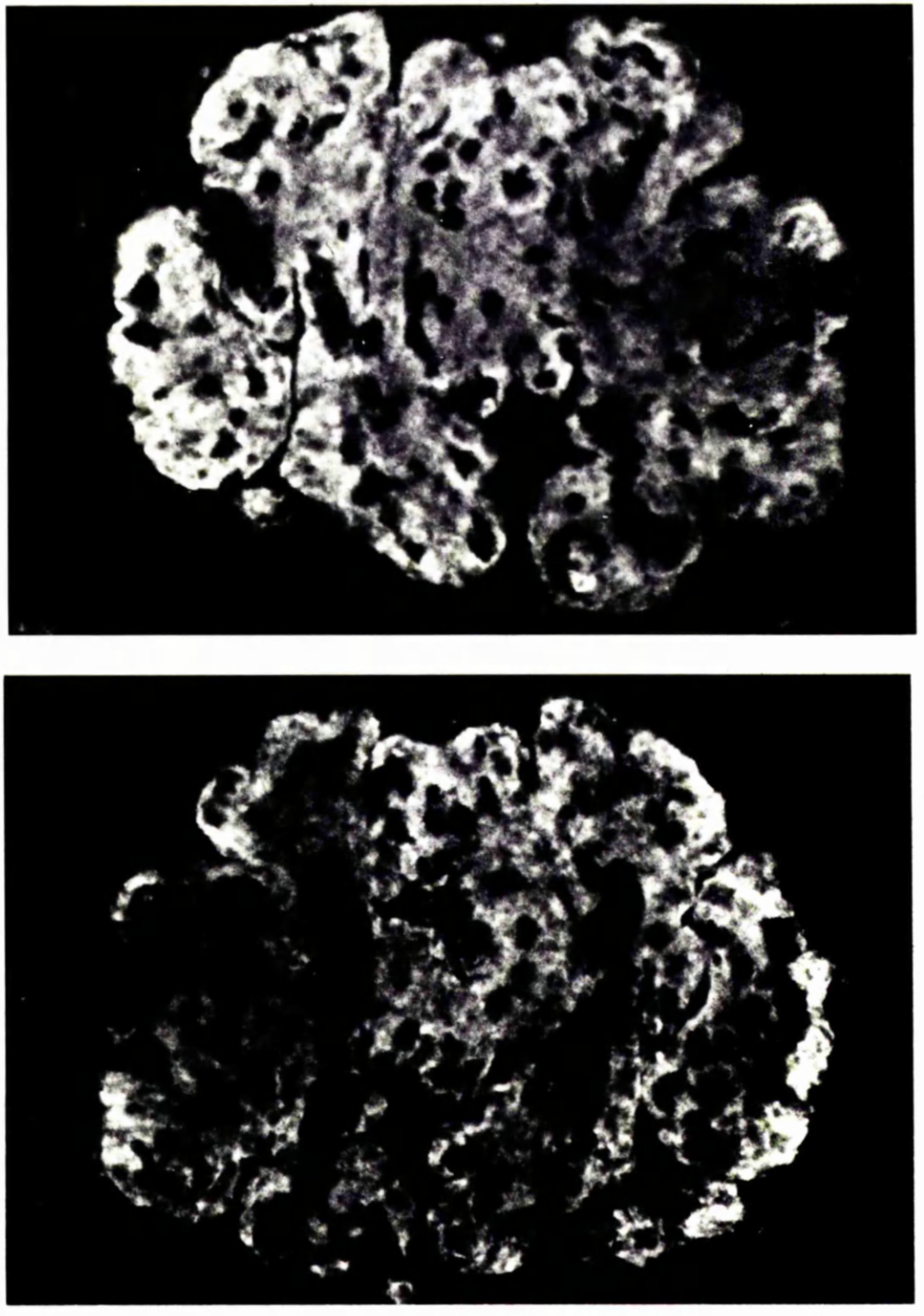
Reduced levels of complement components were found in seven of the ten samples studied and the median values of C1q, C1s and C4 were significantly lower than normal.

The concentrations of alternative pathway components

Values of factor B and properdin as a group did not vary significantly from normal although one patient had reduced levels of factor B and properdin and factor B levels were subnormal in a further two patients.

The concentrations of C3 and C5

Eight of ten C3 concentrations were reduced and the group was significantly lower than normal. This marked reduction in C3 levels was not noted for C5 where only one sample had a subnormal value and the levels in two samples were raised.



X450

Figure 7-2 The photograph on top shows the staining pattern for C3 while the photograph below shows the same glomerulus on an adjacent section stained for βlH.

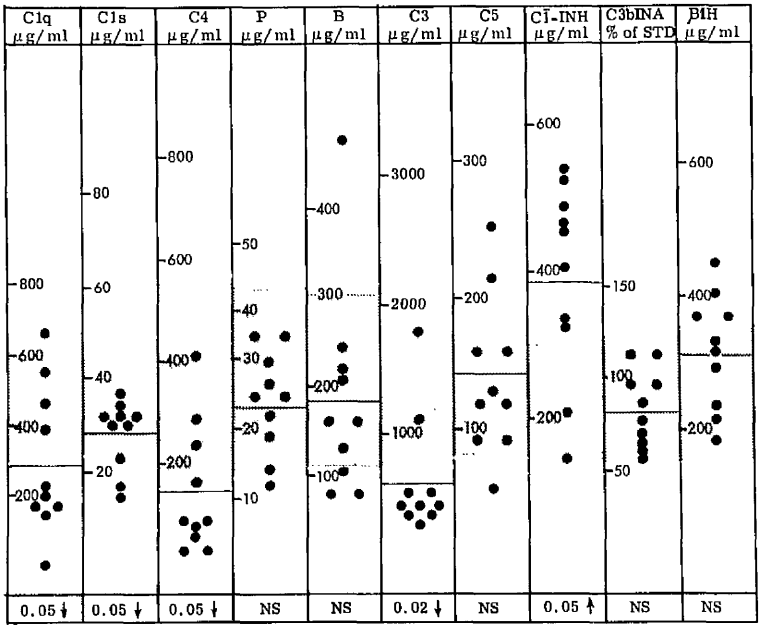


Figure 7-3 The concentration of each protein studied in each of the ten SLE serum samples is shown. The shaded areas represent the normal ranges and the horizontal lines represent the mean concentration for each protein. The results of the median test are shown at the bottom of each column with arrows indicating whether the values are significantly higher or lower than normal.
NS = not significant.

The concentrations of control proteins

$C\bar{1}$-INH levels were significantly increased with only two values remaining within the normal range. No reduced C3bINA or BlH levels were noted and the values for the group did not vary significantly from normal. Two raised C3bINA and four raised BlH concentrations were found.

The correlations between serum concentrations of complement components and the control proteins

The correlations between serum concentrations of each of the components studied were calculated using the Spearman rank correlation test and the results are shown in table 7.5. R represents the correlation coefficient and p the probability of significance. Significant correlations are shown in red.

Table 7.5: Correlations between serum concentrations of the proteins studied

		C1q	C1s	C4	P	B	C3	C5	$C\bar{1}$-INH	C3bINA
C1s	R	0.50								
	p	NS								
C4	R	0.65	0.85							
	p	0.02	0.001							
P	R	-0.53	0.26	0.03						
	p	NS	NS	NS						
B	R	0.15	0.38	0.42	-0.14					
	p	NS	NS	NS	NS					
C3	R	0.41	0.72	0.74	-0.10	0.80				
	p	NS	0.02	0.01	NS	0.01				
C5	R	0.34	0.27	0.53	-0.37	0.78	0.69			
	p	NS	NS	NS	NS	0.01	0.01			
$C\bar{1}$-INH	R	-0.15	0.42	0.33	0.82	0.13	0.10	-0.07		
	p	NS	NS	NS	0.001	NS	NS	NS		
C3bINA	R	0.09	-0.03	0.10	-0.13	0.37	0.29	0.64	-0.10	
	p	NS	NS	NS	NS	NS	NS	0.05	NS	
BlH	R	-0.24	-0.05	0.15	0.16	0.69	0.41	0.62	0.33	0.49
	p	NS	NS	NS	NS	0.01	NS	0.05	NS	NS

The correlations of classical pathway components

The concentrations of C4 correlated with those of C1q and C1s and levels of both C1s and C4 correlated with C3 concentrations.

The correlations of alternative pathway components

Factor B levels correlate with levels of C3 and C5.

The correlations of C3 and C5

Levels of C3 correlated with C1s, C4, factor B and C5 concentrations. C5 also correlated with C1s.

The correlations of control proteins

$C\bar{1}$-INH levels correlated only with properdin, C3bINA correlated with C5 and β1H with factor D and C5.

The relationship between serum concentrations and intensities of glomerular deposition of complement components and control proteins

Using the Spearman rank correlation test, the intensities of deposition were compared with the serum concentrations of each component except for factor B and C3bINA which were not deposited or present in too few biopsies. The results are shown in table 7.6. R represents the correlation coefficient and p the probability of significance.

Table 7.6: The correlations between serum levels and intensities of deposition of each complement component

	C1q	C1s	C4	P	C3	C5	$C\bar{1}$-INH	β1H
R	-0.05	-0.36	-0.32	0.27	-0.28	0.01	-0.14	0.29
p	NS	NS	NS	NS	NS	NS	NS	NS

NS = not significant

No significant correlations were found.

DISCUSSION

Intraglomerular evidence of complement activation

The involvement of immunoglobulins

In agreement with other studies of immunoglobulin deposition in SLE, IgG was always present (53, 240, 241). IgA and IgM were also frequently found in the biopsies included here (table 7.1), although in other studies IgM and particularly IgA deposition was seldom seen (240, 241). Koffler (246) found IgM and IgA frequently deposited except where the staining had a linear pattern.

The involvement of the classical pathway

All three classical pathway components were present in each of the biopsies (table 7.1) with relatively high intensity of staining particularly for C1q and C1s (appendix 1) suggesting that marked activation of the classical pathway had taken place. Since significant correlations existed between IgG and C1q and C1s (table 7.2) it is likely that immune complexes containing IgG were normally responsible for the activation of the classical pathway.

The involvement of the alternative pathway

The presence of properdin in seven of nine SLE biopsies suggests that the alternative pathway was also activated. The alternative pathway may be activated directly, independent of the

classical pathway. Evidence for this is the weakly significant correlation between the intensity of deposition of IgA and properdin and the lack of correlations between the intensities of deposition of properdin and classical pathway components. As a means of directly activating the alternative pathway in SLE, a "nephritic-factor like" substance has been described. This was found, however, to be dependent on calcium ions and therefore activated the classical rather than the alternative pathway. (247).

In favour of a recruitment of the alternative pathway, secondary to classical pathway activation, is the correlation between intensities of deposition of IgM and properdin. Direct and indirect activation of the alternative pathway need not be mutually exclusive.

The absence of factor B need not necessarily argue against alternative pathway activation since deposition of factor B has been found very rarely even where marked alternative pathway activation is known to occur. In one study, factor B was found in six of eight renal biopsies from patients with SLE (247a). A discussion of the general absence of factor B in this study with reference to the above communication is included in chapter 9.

The terminal sequence

The presence of C3 and C5 in all biopsies is consistent with the findings of others (240, 241). C3 activation is likely to result from the activation of both the classical and the alternative pathway but since the intensities of C3 deposition correlated only

with those of C1q, it is perhaps most likely that the classical pathway is the main pathway of complement activation.

Because of the diversity of types of renal damage in SLE, is is not possible to estimate the significance of complement activation in a general way. All the biopsies in this study had evidence of an inflammatory process most often represented by cellular proliferation and it is likely that complement activation would be involved in this. The role of complement in the type of SLE resembling MGN, with no evidence of inflammation is unclear. Several possible mechanisms were suggested for idiopathic MGN and these may apply here.

Intraglomerular evidence of regulation of complement activation

The role of complement proteins in SLE biopsies was assessed using the four criteria outlined in chapter 3.

The role of $C\bar{1}$-INH

The frequent presence of $C\bar{1}$-INH in the glomeruli of SLE patients (table 7.1) the good concordance between biopsies positive for C1s and $C\bar{1}$-INH (table 7.3) and the similarities in distribution patterns (figure 7.1) suggest that $C\bar{1}$-INH is present in the kidney in response to the presence of C1s. Against a regulatory role for $C\bar{1}$-INH is the absence of a significant correlation between the intensities of deposition of $C\bar{1}$-INH and C1s. This may be due to the fact that classical pathway activation is intense in SLE. $C\bar{1}$-INH may be present in the kidney in response to a need for regulation

but may be inadequate for the control of activation. The reduced serum concentrations of classical pathway components (figure 6.3) would support this view.

The role of C3bINA

C3bINA was present in two renal biopsies and was present in areas also positive for C3. No other evidence exists from the deposition of C3bINA in SLE to suggest that C3bINA is controlling C3b.

The role of βIH

βIH was present in all biopsies. The concordance between C3 and βIH was therefore, by necessity, perfect. The distribution patterns suggest that βIH was binding to C3b. The correlation results, however, present conflicting evidence for the role of βIH. The intensities of deposition of βIH did not correlate with those of C3 although they did relate to the intensities of C5. The significant correlation between deposition of C5 and βIH may reflect a mutual dependence of βIH and C5 on C3b. This point has been discussed previously.

The failure of the intensities of deposition of $\overline{C1}$-INH and βIH to correlate with C1s and C3 respectively may have several possible explanations.

1) The sample size may be too small.
2) Activation may be too great for effective regulation. This is supported by the observation that the serum concentration of

C4 are often reduced in the presence of high levels of C1-INH.

3) The absence of correlations may be caused by the inherent insensitivity of the immunofluorescence technique at high intensities. The intensity of staining for each protein was scored on a scale from zero to four. It is unlikely that these scores represent a linear relationship between the score and the quantity of antigen in the tissue. It is more probable that, with increasing scores, the difference in quantity of antigen represented between two scores also increases due to steric hindrance and perhaps other factors. The intensities of staining of C3 and βIH were particularly high in SLE.

4) It is also possible that, in SLE, C1-INH and βIH intensities do not correlate with those of C1s and C3 respectively because they are deposited independently. This may be true of C1-INH which may bind to several other enzymes (240). Based on the distribution patterns and the good concordance between biopsies positive for the control protein and the protein regulated this would seem improbable.

Evidence of complement activation in the circulation

The Classical Pathway

Serum concentrations of classical pathway components have been measured in several studies (245, 247) and low values of these components have frequently been found. In this study 70% (7 of ten) patients had evidence of intense classical pathway

activation as demonstrated by subnormal levels of at least one classical pathway component. The C3 concentration for each of the patients with subnormal classical pathway components was also low and levels of C3 correlated significantly with C1s and C4 suggesting that classical pathway activation is responsible for a significant increase in C3 catabolism.

The alternative pathway

Concentrations of alternative pathway components were reduced in three of ten samples. These results are similar to those of others (245, 247) and concentrations of factor B correlated with those of C3 and C5. This may either represent a direct activation of the alternative pathway or a recruitment of the alternative pathway secondary to classical pathway activation. The concentrations of alternative pathway components did not correlate with classical pathway levels. The alternative pathway however is an amplification system (249) which, once triggered, may be independent of the generation of C3b by the classical pathway C3 convertase. Serum factors capable of activating the alternative pathway are not present in SLE (247). Recruitment of the alternative pathway via the classical pathway is therefore the most probable means of activation. Some direct activation by IgA immune complexes cannot be excluded since IgA was frequently found in renal biopsies (table 7.1). Therefore as was found in the deposition studies, there is evidence in SLE for activation of both the classical and the alternative pathways.

Evidence of regulation of complement activation in the circulation

The role of $C\bar{1}$-INH

Elevated levels of $C\bar{1}$-INH, lack of correlation between levels of $C\bar{1}$-INH and C1s and reduced C1s serum concentrations may suggest that $C\bar{1}$-INH plays no role in the modulation of the classical pathway in SLE. It is possible either that the small sample size in the group reported here may account for the lack of correlation or a relationship between C1s and $C\bar{1}$-INH may have been masked by an increase in $C\bar{1}$-INH synthesis.

The strong correlation between serum levels of properdin and $C\bar{1}$-INH, like the correlation between the intensities of their deposition in the kidney is difficult to explain. $C\bar{1}$-INH is not known to play any role in the control of alternative pathway activation. It is possible that activation of the alternative pathway, secondary to classical pathway activation, may have resulted in this relationship. If this were true, however, a correlation would also have been expected between the serum levels of $C\bar{1}$-INH and classical pathway components. This was not found (table 7.5). It is possible that some other, as yet unknown, interaction may exist between $C\bar{1}$-INH and the alternative pathway.

The role of C3bINA

C3bINA levels all fell within the normal range. Only C5 levels correlated with those of C3bINA and since low levels of C3bINA could result in increased C3 turnover and consequent

C5 catabolism, this correlation supports the regulatory role of C3bINA. The small sample size may again account for lack of correlation between C3 and C3bINA.

The role of βIH

βIH levels were either raised or normal and did not differ significantly from normal. Correlation existed between concentrations of βIH and factor B and C5 again supporting the role of βIH as a regulator of complement activation in the circulation.

The relationship between deposition and serum concentration of complement components

No correlations existed between the intensities of deposition and the serum levels of any of the components (table 7.6). Several reasons have already been suggested for this. Another reason exclusive SLE and HSN is the fact that they are systemic diseases with a variable amount of renal involvement. Since the complement system may be activated in other tissues, the serum levels may reflect activation in sites other than in the kidney.

SUMMARY

1. Evidence from this study suggests that intense activation of both the classical and the alternative pathways occurred in SLE and the mechanisms of activation were discussed.

2. Correlation analyses of the intensities of deposition of components suggests that the classical pathway is the main pathway of activation in the glomeruli.

3. Concordance results and the study of distribution patterns suggests that $C\bar{1}$-INH and β1H were present in response to deposition of C1s and C3 respectively and were therefore likely to be exerting a regulatory effect on these proteins. The correlations between the intensities of deposition of these proteins, however, did not substantiate this and reasons for this were discussed.

4. Some evidence existed in two biopsies for the role of C3bINA as a regulator of C3b since the patterns of deposition of C3b and C3bINA were compatible.

5. There was no deficiency in the serum concentrations of $C\bar{1}$-INH, C3bINA or β1H to account for the intense complement activation seen in SLE.

CHAPTER 8

Minimal Change Nephrotic Syndrome

Minimal Change Nephrotic Syndrome

Introduction

Originally called lipoid nephrosis, minimal change nephrotic syndrome (MCNS) is a disease, primarily of childhood, associated with nephrotic syndrome and minimal or no histological abnormalities. By electron microscopy, the most noticeable change is the fusion of epithelial cell foot processes (1). Most studies have found that immunoglobulins and C3 are not deposited in the glomeruli of MCNS patients (13, 173) although occasionally trace amounts have been noted (16). IgE was found in four of five patients in one study (13) but this finding has not been confirmed by others (12, 16, 17). There is therefore little evidence that MCNS involves the deposition of immune complexes.

Some evidence exists, however, to suggest that MCNS involves immunological mechanisms. Although serum complement levels are normal, Ngu (251) found raised immunoconglutinin levels in MCNS patients. Lymphocyte changes have also been noted. Lymphocytes from MCNS patients appear to have an enhanced ability to kill renal target cells (76). Also the formation of both EA (181) and EAC (252) rosettes has been shown to be inhibited by prior incubation of the cells with serum from MCNS patients suggesting the presence of circulating immune complexes. The possibility that immune complexes, although apparently not deposited, may be involved in the pathogenesis of MCNS has been

suggested (252). One proposed mechanism is that complexes cause the release of lymphokines from lymphocytes which results in damage to the glomerular basement membrane and loss of protein into the urine.

The association between MCNS and type 1 hypersensitivity (13, 14, 15) may suggest that the increased permeability of the capillary loop may in some cases be due to the release of vasoactive amines from sensitised mast cells.

Because of the failure to detect immunoglobulins and complement proteins in the glomeruli of MCNS patients, this group could, to some extent, be treated as a control group for the study of deposition of complement components and more particularly regulatory proteins.

MATERIALS

Immunofluorescence studies were performed on renal biopsies obtained from eleven patients. Proteinuria was present in only five patients at the time of biopsy and ranged from 2.1 to 20 g per 24 hours (mean = 11.6g/ 24 hours). However, the remaining six patients had had at least one episode of proteinuria prior to the time of biopsy. The serum creatinine level of one patient was raised with a value of 300 μmoles/ l, the levels in the other patients ranged from 25-132 μmoles/ l (mean = 70 μmoles/ l). Six of the 11 patients in this group were children under the age of 15.

RESULTS

Renal Biopsies

Glomerular deposition of immunoglobulins, complement and control proteins

The number of biopsies positive for each of the proteins studied and the mean intensity of staining for each protein is shown in table 8.1 (see over).

Table 8.1: Glomerular deposition of immunoglobulins, complement and control proteins

	Immunoglobulins			Classical Components			Alternative Components		Terminal Component		Control proteins		
	G	A	M	Clq	C1s	C4	P	B	C3	C5	$C\bar{1}$-INH	C3bINA	βIH
Number positive	2/ 11	2/ 11	3/ 11	2/ 11	1/ 11	2/ 10	1/ 11	0/ 11	3/ 11	1/ 10	4/ 11	0/ 11	2/ 11
Mean intensity	1.0	1.0	1.3	1.5	1.0	2.0	1.0		1.7	1.0	2.0		2.0

mean intensity = the mean of the scores given for intensity of deposition of each protein in each biopsy

Deposition of Immunoglobulins

Three of 11 (27%) biopsies were positive for IgM. In one of these, the staining was in a focal and segmental capillary loop pattern. In the other two, the staining was also patchy capillary loop but not restricted to a single area of the glomerular tuft. The latter two biopsies also had trace amounts of staining for IgA and IgG. A photograph of the patchy capillary loop staining pattern is shown in figure 8-1.

Deposition of classical pathway components

The two biopsies with trace amounts of all three immunoglobulins were positive for C1q and C4 andlwas also positive for C1s. Classical pathway components were only present where immunoglobulins were deposited.

Deposition of alternative pathway components

Properdin was present in trace amounts in one of the immunoglobulin positive biopsies. Factor B was not found.

Deposition of C3 and C5

C3 was present in three of the 11 biopsies. Two of these were the biopsies where immunoglobulins were found in a patchy capillary loop pattern. In the third, trace amounts of C3 were present in the absence of immunoglobulins.

Deposition of control proteins

C1-INH was present in four of 11 biopsies.

X 650

Figure 8-1 The pattern of deposition of IgM in a biopsy from a patient with MCNS.

C3bINA was not present in any of the biopsies and BIH was found in the two biopsies positive for all three immunoglobulins.

The correlations in intensities of staining between the various components studied were not examined because too few biopsies were positive for any of the proteins.

The concordance between deposition of control proteins of complement and the components whose activity they regulate

$C\bar{1}$-INH

The concordance between biopsies positive for $C\bar{1}$-INH, C1s and C4 is shown in table 8.2.

Table 8.2: The concordance between biopsies positive for $C\bar{1}$-INH, C1s and C4.

C1s/ $C\bar{1}$-INH	No. of biopsies	C4/ $C\bar{1}$-INH	No. of biopsies	C1s/ C4	No. of biopsies
C1s + $C\bar{1}$-INH+	1	C4+ $C\bar{1}$-INH+	2	C1s+ C4+	1
C1s + $C\bar{1}$-INH-	0	C4+ $C\bar{1}$-INH-	0	C1s+ C4-	0
C1s- $C\bar{1}$-INH+	3	C4- $C\bar{1}$-INH+	2	C1s- C4+	1
C1s- $C\bar{1}$-INH-	7	C4- $C\bar{1}$-INH-	6	C1s- C4-	8
Total	11	Total	10	Total	10

In one of the $C\bar{1}$-INH positive biopsies, C1s and C4 were present while in another only C4 was found. In the remaining two biopsies, $C\bar{1}$-INH was found in the absence of any other complement protein. C1s and C4 were not found in the absence of $C\bar{1}$-INH.

C3bINA

C3bINA was not present in any MCNS biopsy.

βlH

The concordance between biopsies positive for βlH and C3, C4, C5 and properdin is shown in table 8.3.

Table 8.3: The concordance between biopsies positive for βlH, C3, C4, C5, and properdin

C3/βlH	No. of biopsies	C4/βlH	No. of biopsies	C5/βlH	No. of biopsies	P/βlH	No. of biopsie
C3+ βlH+	2	C4+ βlH+	2	C5+ βlH+	1	P+ βlH+	1
C3+ βlH-	1	C4+ βlH-	0	C5+ βlH-	0	P+ βlH-	0
C3- βlH+	0	C4- βlH+	0	C5- βlH+	0	P- βlH+	1
C3- βlH-	8	C4- βlH-	8	C5- βlH-	9	P- βlH-	9
Total	11	Total	10	Total	10	Total	11

C3 and C4 were both present in the two biopsies where βlH was found. C5 was deposited in one and properdin in the other. C3 was found where βlH staining was absent in one biopsy. βlH was not found in the absence of C3, C4 or C5.

Comparison of immunofluorescence patterns

In the single biopsy positive for C1s and C1-INH both proteins were found to be deposited round capillary loops and within the mesangium. C3bINA was not found and in the two biopsies where both C3 and βlH were present, the patterns of staining were again the same. Because of the small number of biopsies positive and

the low intensity of staining, it was not possible to obtain photographs of serial sections stained for C1s and $C\bar{1}$-INH and C3 and βlH.

Serum samples

The serum concentration of each component within each sample is shown in figure 8-2. Because of the small number of sera available for study no statistical analyses were carried out.

Serum concentrations of classical pathway components

No subnormal values of any classical pathway components were found. Two patients had raised levels of all three components and a third had a supranormal C4 concentration.

Serum concentrations of alternative pathway components

Three MCNS patients had raised properdin concentrations. One of these also had a raised factor B level while, in a second, the factor B concentration was subnormal. Other values were within the normal range.

Serum concentrations of C3 and C5

Four MCNS patients had raised concentrations of both C3 and C5 and in the fifth the C3 level was raised.

The serum concentrations of the control proteins

All $C\bar{1}$-INH concentrations, one of the five C3bINA concentrations and four of five βlH concentrations were above the normal range in MCNS. One patient had a subnormal C3bINA level.

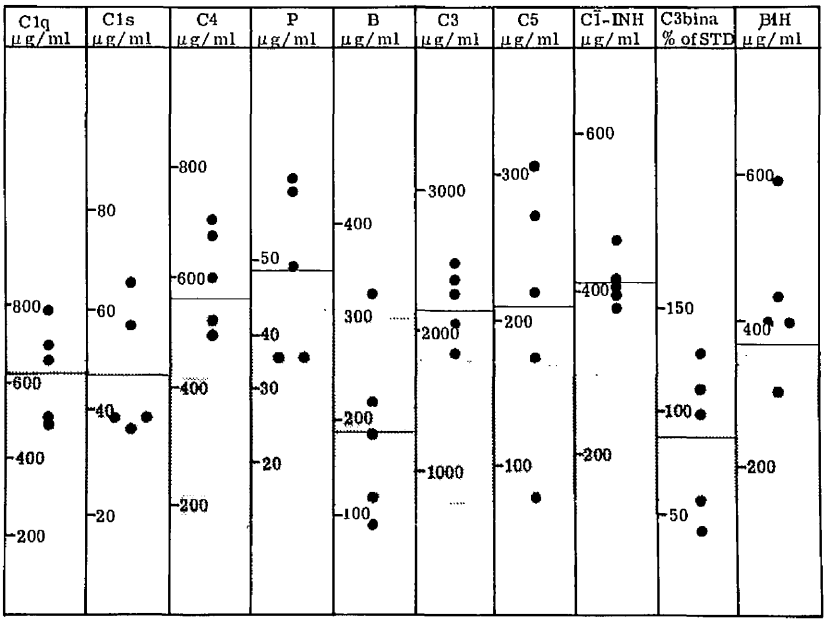


Figure 8-2 The serum concentration of each component in each MCNS sample is shown here. The shaded areas represent the normal ranges and the horizontal lines represent the mean of the sample concentrations for each component.

DISCUSSION

Intraglomerular evidence of complement activation

The involvement of immunoglobulins

Three biopsies showed deposition of immunoglobulins in MCNS. One had trace amounts of only IgM in a focal, segmental capillary loop pattern. This pattern is more often associated with focal glomerulosclerosis (FGS) than MCNS (1). Differentiation between these two diseases is complicated by the focal nature of FGS (253). Twenty glomeruli were available for histological analysis in this IgM positive biopsy and all glomeruli were either normal or showed minimal mesangial increase with no segmental lesions. Since the biopsies in this study were categorised according to their histological appearance, this biopsy was therefore considered in the MCNS group. It is possible that, had a different sample of glomeruli been available for light microscopic examination, the diagnosis would have been FGS. Whether MCNS and FGS are two distinct diseases or whether one is a variant of the other is a subject of debate (254).

In the other two biopsies, IgG, IgA and IgM were all found in a trace or weak patchy capillary loop pattern. As can be seen from figure 8-1, the staining pattern is unlike that seen in other types of GN. It is possible, therefore, that the staining does not represent deposition of immune complexes. One possible explanation is that the staining is caused by the

passage of proteins through the capillary loops. MCNS is associated with selective proteinuria with excretion mainly of albumin. In a situation where albumin passes through the loops into the urine, it may be possible that larger protein molecules are trapped within the loops and demonstrated by immunofluorescence.

In each of the biopsies with evidence of deposition of immunoglobulins, the intensities of staining were either trace or, in the case of IgM in one biopsy, weak. The significance of staining of such low intensity must be considered dubious.

In the remaining eight MCNS biopsies, there was no glomerular immunoglobulin deposition evident. These findings are consistent with the results of others (18, 173).

The involvement of the classical pathway

The fact that the two biopsies positive for all three immunoglobulins studied also showed deposition of classical pathway components and all other nine biopsies were negative for classical pathway proteins, may suggest, either that the immunoglobulin staining does, in fact, represent deposition of immune complexes or at least that the immunoglobulins in the loops are capable of activating the classical pathway. It is also possible that these proteins were trapped in the capillary loops as was discussed for immunoglobulins.

In the remaining nine MCNS biopsies, there was no evidence of deposition of classical pathway components.

The involvement of the alternative pathway

Only one biopsy showed trace deposition of properdin and none was positive for factor B. The properdin positive biopsy was also positive for the three immunoglobulins studied and the three classical pathway components. Staining of such weak intensity however is of doubtful significance. If the staining does represent specific deposition rather than non specific trapping then it may mean direct activation of the alternative pathway, activation secondary to the classical pathway or trapping of properdin independently of complement activation. In the other ten biopsies, alternative pathway proteins were not found.

The terminal sequence

C3 was found in the two biopsies with deposition of immunoglobulins and classical pathway components and in trace amounts in one biopsy where no immunoglobulins were found. The close concordance between deposition of immunoglobulins, classical pathway components and C3 in this group suggests that the staining represents specific deposition and may be circumstantial evidence for the intraglomerular activation of complement by immune complexes.

If the staining of all these components merely represented trapping then it may be expected that proteinuria would be greater in the two biopsies with deposition. In fact, at the time

of biopsy, these two patients had less than 0.5g of protein in the urine per 24 hours. In order to assess the possibility of trapping, it may have been useful to examine the biopsies for the presence of proteins of similar size but which are not known to be involved in the immune response.

In most biopsies, there is neither deposition of immunoglobulins or complement components and MCNS is therefore a useful control group for studying the role of regulators of complement activation in GN.

Intraglomerular evidence of regulation of complement activation

No correlation studies could be done because so few biopsies were positive for any of the proteins considered. Assessment, therefore, of the role of regulators was based on their presence in the kidney, the concordance between biopsies positive for the control protein and the component regulated and a comparison of the distribution patterns of the two proteins involved.

The role of $C\bar{1}$-INH

$C\bar{1}$-INH was found more frequently in MCNS than any other protein studied. It was present in two biopsies positive for classical pathway components. The distribution pattern of $C\bar{1}$-INH in these two biopsies was the same as that of the classical pathway components. $C\bar{1}$-INH however, was found in two biopsies without evidence of deposition of any other complement protein. Only six other biopsies in the rest of the disease groups showed

$C\bar{1}$-INH in the absence of any classical pathway components and in each of these six, C3 was present. There are three possible explanations for $C\bar{1}$-INH being found in the absence of all other components: a) the insensitivity of the immunofluorescence test results in a failure to demonstrate other classical pathway components where they are present. b) $C\bar{1}$-INH deposition may be in response to some other mechanism. While fibrinogen was present in the two biopsies where C4 and C3 were found it was absent where $C\bar{1}$-INH was found alone. For this reason it is perhaps improbable that $C\bar{1}$-INH is present as a regulator of one of the components of the coagulation or fibrinolytic systems. c) $C\bar{1}$-INH may be trapped on passage through the capillary loop. Absence of other proteins would make this unlikely. In these two biopsies, therefore, the role of $C\bar{1}$-INH is unknown. This may place in doubt the significance of $C\bar{1}$-INH as a control protein of complement activation where only concordance results support the regulatory role of $C\bar{1}$-INH.

The role of C3bINA

C3bINA was not deposited in MCNS and there is therefore no evidence for a regulatory role for C3bINA in this disease.

The role of β1H

β1H was present in the two biopsies where C3 staining was weak but was not found in the biopsy with only trace C3 deposition.

In the two biopsies positive for both C3 and βIH, the staining patterns were compatible with each other. No βIH deposition was found in the eight biopsies where C3 was absent. Evidence for a regulatory role for βIH is therefore based on the presence of βIH in two biopsies, the good concordance between biopsies positive for C3 and βIH and the similarities in distribution patterns of C3 and βIH.

Evidence for complement activation and regulation in the circulation

MCNS is a disease associated with marked proteinuria and hypoalbuminaemia. The loss of protein in the urine, however, has not resulted in a generalised reduction in serum complement concentrations. In fact, as in other GN groups, the levels are often raised above normal (figures 3-2, 4-3, 5-3, 6-3, 7-3 and 8-2). Two subnormal values were found in two separate samples and these were factor B and C3bINA levels. It is possible that these proteins were lost in the urine of these patients both of whom had high levels of proteinuria (6g and 20g per 24 hours). With molecular weights of around 90,000 for factor B and C3bINA respectively (255, 256), these are relatively small molecules and may be excreted in preference to other complement components. Other reasons for low levels would be either reduced synthesis or increased catabolism. The low C3bINA concentration does not appear to have resulted in an uncontrolled activation since C3 and factor B concentrations were normal.

SUMMARY

1. Therefore in most MCNS patients there was no evidence of activation of either the classical or the alternative pathways or subsequent catabolism of C3.
2. Four biopsies showed deposition of immunoglobulins and/ or complement components and the significance of this was discussed.
3. The absence of deposition of the control proteins of C3b where C3 was also absent helps to substantiate the relevance of the concordance between biopsies positive for C3 and both β1H and C3bINA found in other disease groups. This is important because of the high percentage of biopsies positive for C3 in the other types of GN studied.
4. The presence of $C\bar{1}$-INH in the absence of any other complement proteins suggests that care is needed in interpreting the significance of concordance between biopsies positive for C1s and $C\bar{1}$-INH in the other disease groups.

CHAPTER 9

DISCUSSION

FINAL DISCUSSION

Complement activation may occur by one of two pathways; the classical and the alternative pathways. The purpose of this thesis was to study the pathways of activation in human GN. Since activation of complement is controlled by several plasma proteins, the role of these proteins in the modulation of complement activation was also examined. Six major types of GN were considered and renal biopsies and serum samples from patients with these diseases were studied.

Of the disease groups studied, MGN and SLE are generally considered to involve classical pathway activation (173, 176). The activation in MGN is slight with generally no evidence of reduced levels of complement components (182-184) and weak intensities of deposition of complement proteins in the glomeruli. In some biopsies C3 is absent (172, 176, 177) while, in SLE, serum concentrations of C3 and classical pathway components are frequently reduced (213, 245) and C3 and classical pathway components are almost always present in the glomeruli (137, 240, 244). Both classical and alternative pathway activation, are thought to be involved in MPGN (205-210) although particularly where NF is present, the activation of the alternative pathway may be predominant (205). The intense activation of complement in a disease where there is often little evidence of an immune complex pathogenesis makes MPGN particularly interesting for study.

Little work has been done on complement activation in FGN and HSN. The fact that IgA is the predominant immunoglobulin in both (224-227, 235) suggests that the alternative pathway may be involved in complement activation since IgA immune complexes do not activate the classical pathway (83). The presence of other immunoglobulin classes may result in classical pathway activation. MCNS was chosen to represent a control group because there is generally no deposition of immunoglobulins or complement components and serum levels of these are not reduced (18, 173). There are however several disadvantages; the mechanism of MCNS is not known but the presence of raised immunoconglutinin levels (251) and some abnormalities of lymphocyte function (76, 181, 252) suggest that immunological processes are involved, also high levels of proteinuria may result in non specific trapping of proteins within the capillary loop. In the absence of normal renal biopsy material, MCNS was accepted as a control group.

Activation of complement is known to occur in the circulation of patients with certain types of GN, for example MPGN and SLE. This is demonstrated by reduced serum complement concentrations (figure 4-3 and 7-3 and references (205, 208, 209, 212-214, 245-248) and by factors in the serum capable of causing activation of either the classical or the alternative pathway of

complement in normal serum (208, 247). In other disease groups such as MGN, FGN and HSN, there is little evidence that activation occurs within the circulation (see figures 3-2, 5-3, 6-3).

It is more difficult to establish whether activation occurs within the glomerulus or whether components localise by one of the following alternative methods:- 1) trapping of components which are in the native state. This may be expected to occur where capillary permeability is increased. In MCNS, however, where high levels of proteinuria suggest that capillary permeability is increased, complement components are generally not present in the kidneys (18, 173 and table 8.1). 2) trapping of complement components which have been activated in the circulation. It is possible that C3b formed in the circulation may bind to C3b receptors in the glomeruli in this way (70), or 3) complement components may attach to the immune complexes in the circulation and be deposited as part of the complex.

The Spearman rank correlation analysis failed to show significant correlations between intensities of glomerular deposition of complement components and the serum levels of these components within each disease group except for an inverse correlation for C3 and a positive correlation for C1s in MPGN. Reasons for the absence of correlations have already been discussed and some suggestions were made to explain the

somewhat anomalous correlation of C1s in chapter 4. Despite the general absence of useful information from the correlation analyses, it has been shown that diseases which have intense complement activation in the circulation, as demonstrated by low median C3 levels, for example, SLE and MPGN also show high intensity of deposition of C3 while less deposition of complement components is found where serum levels are normal, for example in MGN, HSN and FGN. This may suggest that deposition in the glomeruli is secondary to activation in the circulation. It is also true however that the same factors which cause activation in the circulation in SLE, that is, immune complexes, are present in the glomeruli. In MPGN, the means of activation is less well defined. Where complexes are involved, the mechanism may be same as for SLE. Since NF may bind to the alternative pathway C3 convertase, it is possible that NF may also deposit in the kidney with resulting activation. It is also possible, though less likely, that low serum levels result from intraglomerular activation of complement. The persistently reduced serum complement levels after nephrectomy in MPGN argues against this (217). No direct evidence exists to verify that activation takes place within the kidney in human GN but several studies suggest that it may occur; 1) activation of the alternative pathway may take place, in vitro, on kidney sections

from rabbits with acute serum sickness (257). 2) Surfaces with a low sialic acid content are known to allow amplification of alternative pathway activation, and basement membrane material from patients with GN has been shown to be low in sialic acid (214). The potential for activation may therefore reside in the basement membrane itself, and 3) if, as has been suggested for MGN, immune complexes may form at the subepithelial aspect of the basement membrane rather than being deposited in a preformed state, then activation must occur within the glomerulus.

The role of complement in the circulation may be different from that in the kidney. In the plasma, complement may bind to immune complexes via C1q and the activation will result in the opsonisation of the complex, aiding the ingestion of the complex by phagocytic cells, bearing C4b or C3b receptors. Similarly, the alternative pathway may be activated directly by gram-negative bacteria and the binding of C3b to these will again aid ingestion. Lysis of these micro-organisms may also result by the actions of the C5b-9 lytic complex. In the kidney, the activation of complement may result in extensive damage. However, the complement system is essentially a protective mechanism and an important function may be to cause the solubilization of intraglomerular immune complexes.

It has been shown that antigen is removed more slowly

from the kidneys of rabbits with deposited immune complexes if the animals are depleted of complement (257). If the removal of antigen involved the complement dependent solubilisation of immune complexes then this would require that activation, particularly of the alternative pathway, occurred within the glomerulus (145).

The activation of the classical pathway

Activation of the classical pathway was a feature of all the disease groups studied with the exception of MCNS. It was demonstrated by deposition of classical pathway components within the kidney in all groups although reductions in serum concentrations of these components were only found in SLE and MPGN. Normal serum concentrations of complement components in MGN (182-184), FGN (227, 228) and HSN (238) have been found by others and reduced classical pathway components are a general feature of SLE (245), and particularly the subendothelial type of MPGN (205). The correlations which existed between intensities of deposition of classical pathway components and IgG and IgM in MPGN and SLE offered evidence that immune complexes were involved in activation in these two disease groups and may suggest that activation is occurring within the glomeruli. The presence of correlations between the intensities of deposition of classical pathway components and C3 in FGN and SLE may suggest that classical pathway activation

contributes to C3 catabolism in those disease groups. Absence of a relationship between intensities of staining of classical pathway components and C3 need not exclude the classical pathway as an important factor in complement activation. Where no correlations were present between intensities of deposition of C3 and either classical or alternative pathway components as was the case in MPGN, it is possible that both pathways contributed to varying extents in different patients. Absence of correlations may also be due to different rates of removal of various complement proteins from the kidney in human GN although no information is available to assess this possibility. Care was necessary when analysing the correlation results to consider that with correlation values with probabilities of 0.05, 5% of the correlations would be due to chance. Since large numbers of correlation analyses were performed it is likely that some of the significant correlations occurred due to chance.

The activation of the alternative pathway

Components of the alternative pathway were demonstrated in the glomeruli of at least 50% of the biopsies from each disease group except MCNS and MGN. In agreement with one large study (187a) however, factor B was rarely present. There are several possible explanations for this; 1) the C3b, factor B complex, $\overline{C3bBb}$ is unstable, with Bb decaying rapidly from the complex (109). Also factor B may be displaced from the convertase

by βIH (127) which was deposited in a high percentage of biopsies. The absence of factor B in the presence of intense deposition of βIH, C3 and also often properdin may suggest that βIH is extremely effective in displacing factor B from C3b in the glomerulus. For these reasons absence of factor B is not incompatible with alternative pathway activation. 2) the antiserum may have been too weak to detect factor B. The antiserum used for immunofluorescence was also used for the radial immunodiffusion studies at a dilution comparable with other antisera and in the immunofluorescence test a higher concentration of antiserum was employed than was used for the other non-fluoresceinated antisera. 3) It is possible that the method of fixation used in this study may have destroyed the B antigenicity or factor B may be soluble in the fixative. In support of this is the fact that Wyatt (247a) found factor B deposition in six of eight biopsies from SLE patients using unfixed frozen tissue sections although factor B was only present in one of 18 biopsies from MPGN patients. Other workers (187a) however have failed to find factor B in the glomeruli of SLE patients using unfixed tissue sections. Also the method of fixation used in this study was able to demonstrate factor B in the cytoplasm of cultured peripheral blood monocytes (258).

The intraglomerular evidence of activation of the alternative

pathway was therefore almost entirely based on the presence of properdin within the glomeruli. Properdin may bind to C3b in the absence of factor B (189) and consequently in the absence of activation of the alternative pathway. It is therefore possible that deposition of properdin is not a good index of alternative pathway activation. The presence of both properdin and βIH in the glomeruli of patients with GN suggests that C3b is present and it is probable that factor B would bind to C3b to form the alternative pathway C3 convertase and subsequently decay or be displaced by βIH. That activation of the alternative pathway occurs in GN is further suggested by the occasional low concentrations of components in the sera of patients with MPGN, FGN, SLE and MCNS and by the fact that factor B levels in the serum of MPGN patients were significantly lower than normal. The correlations in the intensities of staining between properdin and C3 in FGN and between serum concentrations of C3 and at least one alternative pathway component in MPGN, FGN and SLE suggest that the alternative pathway contributes significantly to C3 catabolism.

The role of the alternative pathway in solubilising immune complexes is unclear. If it were a universal protective mechanism then all biopsies would be expected to show evidence of activation. Also if the activation of the alternative pathway was beneficial, one would expect that damage would be less severe where

activation occurred. This is not the case, since there was evidence of activation particularly in MPGN and SLE, both associated with severe pathological damage. It is impossible to judge, however, whether these kidneys would be more badly damaged in the absence of alternative pathway activation. In some biopsies, at least, therefore, damage may be caused and repair may be made by the activation of the alternative pathway.

On the basis of the results from this study, MGN involves essentially classical pathway activation although there was deposition of properdin in four biopsies. The significance of properdin deposition has been discussed and this deposition may indicate that alternative pathway activation may play a limited role in MGN. In MPGN and HSN, both pathways of complement activation are involved, although activation is more intense in MPGN, and approximately equal numbers of biopsies were positive for classical and alternative pathway components. Reduced serum concentrations of components of both pathways were found in MPGN although only the median level of factor B was reduced. Since there was no correlation between the intensities of deposition of C3 and either classical or alternative pathway components it is not possible to say which pathway is primarily responsible for C3 catabolism in MPGN and HSN. In FGN both pathways are again involved and the significant

correlation between intensities of deposition of C3 and both C4 and properdin suggest that both contribute to a significant extent in C3 activation. Although there is evidence of activation of both pathways in SLE, more biopsies were positive for classical pathway components and there was more evidence of classical pathway activation in the circulation. Also there was a significant correlation between intensities of deposition of C3 and C1q but not alternative pathway components. For these reasons, this study shows that the classical pathway is predominant in SLE.

The modulation of complement activation

$C\bar{1}$-INH

$C\bar{1}$-INH controls the activation of the classical pathway by stoichiometrically inhibiting C1s; thus preventing it from cleaving C4 and C2 to form the classical pathway C3 convertase, $C\overline{42}$ (110). This regulation is known to be relatively inefficient and amounts of $C\bar{1}$-INH, markedly in excess of physiological levels are necessary to inhibit activation of complement in the presence of immune complexes (259). The role of $C\bar{1}$-INH may therefore be to prevent spontaneous activation of the classical pathway. This suggestion is substantiated by the fact that diseases may result from a deficiency of $C\bar{1}$-INH. The most common of these is hereditary angioedema (111) but SLE with GN has also been reported (260). The data reported in this

study suggest that $C\bar{1}$-INH is involved in regulation of the classical pathway. This is based on the presence of $C\bar{1}$-INH in the kidneys of patients with each type of GN, the correlation between the intensities of deposition of C1s and $C\bar{1}$-INH in MPGN and FGN and the similarities in distribution patterns in all biopsies where both proteins were deposited. There was no evidence that classical pathway activation was the result of a deficiency of $C\bar{1}$-INH since serum concentrations were either normal or raised in all samples studied. There are however several factors which do not support a regulatory role for $C\bar{1}$-INH in GN. Firstly, $C\bar{1}$-INH is not effective in halting complement activation. This is demonstrated by the presence of C4 and C3 with C1s and $C\bar{1}$-INH in the kidneys. The inefficiency of $C\bar{1}$-INH was discussed at the beginning of this section and the presence of $C\bar{1}$-INH may represent only limited regulation. Secondly, the concordance between biopsies positive for C1s and $C\bar{1}$-INH was generally poor with $C\bar{1}$-INH present in the absence of C1s. Possible reasons for this have been outlined in Chapter 3.

The presence of C1s and $C\bar{1}$-INH in the kidney may appear somewhat anomalous since the binding of $C\bar{1}$-INH to C1s and $C\bar{1}$r on aggregated IgG is known to cause the dissociation of a C1r C1s ($C\bar{1}$-INH_2) complex from the $C\bar{1}$ complex (261). It is therefore possible that where C1s and $C\bar{1}$-INH are found together in the kidney, they are not attached to immune complexes via

C1q but are lying free and this may account for the fact that C1s is often absent from kidneys positive for IgG, C4 and C3.

Control proteins of C3b

C3bINA and βIH are both control proteins of C3b but act in different ways. C3bINA is an enzyme which causes the degradation of C3b to form an inactive molecule which is further catabolised by trypsin or like enzymes to form C3c and C3d (121). C3bINA cannot bind to C3b which is bound to factor B. βIH acts to prevent the amplification of the alternative pathway by displacing factor B from C3b (127) and thus aiding the enzymatic degradation by C3bINA and by binding to C3b to prevent the C3 convertase from reforming. Thus βIH inhibits stoichiometrically.

C3bINA

There is little evidence from this study to suggest that regulation of C3b by C3bINA was occurring, since only seven biopsies showed deposition of C3bINA. Absence of C3bINA is not however, incompatible with regulation since the mechanism of action is enzymatic and C3bINA would not be expected to remain in position after inactivating C3b. Two other studies have failed to demonstrate deposition of C3bINA in various types of GN (247a, 262). In the circulation, regulation by C3bINA would not result in reduced serum concentrations of C3bINA since this enzyme is not consumed during its inactivation

of C3b (195). Low C3bINA levels may however result in amplification of the alternative pathway. The existence of a positive correlation between serum concentrations of C3bINA and C3 in MPGN may argue that, in MPGN, C3bINA is involved in the regulation of activation.

βIH

That βIH was involved in regulation of C3b in GN was well substantiated by its frequent presence in the kidney, good concordance between biopsies positive for C3 and βIH in all groups, good correlation between intensities of deposition of C3 and βIH in MGN, MPGN and FGN and close similarities in the distribution patterns of these two proteins in all biopsies where both were found together. There was also no evidence of a generalised deficiency of βIH in the serum. There is therefore close association between C3 and βIH in the kidney in each of the disease groups studied and since βIH acts by binding to C3b to form the relatively stable complex C3bβIH, the close relationship suggests that βIH is functioning to control C3b in GN. This appears to be the case even where renal biopsies were obtained from patients with circulating nephritic factor and reasons for this were discussed in Chapter 4. βIH was similarly found to be frequently present in kidneys from GN patients by others (247, 262).

Despite the close relationship between C3 and βIH in the

kidney, it is still possible that the presence of βIH does not represent effective regulation of the activities of C3b by βIH. Two explanations for the presence of βIH in the absence of regulation may be suggested. It is known that sialic acid deficient surfaces offer a "protected" site for C3b, preventing effective regulation by βIH and basement membranes isolated from glomerulonephritic kidneys have been shown to contain only half the sialic acid content of normal basement membranes (214). The mechanism for this loss of sialic acids is not known but it is possible that either micro-organisms or cells, such as neutrophils or macrophages which accumulate in the kidney because of the inflammatory process may contain neuraminidase which removes the sialic acid. These membranes may therefore, "protect" C3b allowing amplification of the alternative pathway. Studies using neuraminidase treated sheep erythrocytes, have shown that in this "protected" situation about 10 per cent of C3b may bind βIH (98) and therefore, in the kidney, the presence of βIH may indicate that it is controlling only a small percentage of the C3b molecules; the remainder retaining their activity. Although this possibility could not be positively excluded, the similar intensities of C3 and βIH in all groups studied may suggest that, at most, only a small proportion of C3b molecules are "protected" in this way.

The second possibility is that the anti-βIH antiserum may

have been contaminated with antibodies to C4-binding protein (C4-bp). Fujita et al. (117) showed that C4-bp binds to C4b and catalyses the degradation of C4b by C3bINA. These authors also showed that anti-β1H antisera often contained anti-C4-bp specificity. Thus some or all of the β1H staining could have been due to the presence of C4-bp in the kidney. Although again we cannot exclude this possibility, the β1H antigen used in the preparation gave a single line on sodium dodecyl sulphate polyacrylamide gel electrophoresis and did not bind significantly to EAC4, potentiate the inactivation of C4b by C3bINA or destabilise $C\overline{42}$. The antiserum obtained by immunisation of rabbits with the antigen was shown to be pure by double diffusion and immunoelectrophoresis and the staining obtained was completely blocked by prior absorption of the antiserum with the pure antigen preparation. Further evidence to support this comes from the fact that the only biopsy in which C4 was deposited in the absence of C3, did not stain for β1H.

Therefore, in this study, the methods and extent of complement activation in human GN have been assessed and the role of the control proteins examined. While the fact that complement activation in GN is extensive, has been established, no attempt has been made to define the effects of this activation.

It is probable that the complement system developed to rid the body of infection and the importance of the system is

demonstrated by the fact that patients with deficiencies of classical pathway components have a higher incidence of immune complex disease than normal (263, 264) and also that patients with primary deficiency of C3 (265, 266) or deficiency of C3 resulting from a genetic deficiency of C3bINA (93) suffer from persistent bacterial infections. The complement system is essentially a beneficial process and it is probable that complement is involved in the prevention of the development of GN by its role in aiding the removal of antigens. Whether intraglomerular complement activation is beneficial to the kidney is less likely since it causes cell lysis, attraction of inflammatory cells and increased capillary permeability, all of which will result in damage to the kidney. It has recently been shown that activation causes the solubilization and removal of immune complexes from the capillary loops of rabbits with acute serum sickness. Whether, on balance, intraglomerular activation does good or harm therefore remains a matter for conjecture.

It is possible that artificial alternation of the complement system, perhaps by increasing the concentrations of control proteins may become a therapeutic tool in the management of GN. This may be most useful in diseases not involving the presence of an ongoing infection since the dangers of reducing the effects of complement during an infection would be likely to outweigh the advantages to the kidney. Such diseases may be those involving endogenous antigens, particularly anti-glomerular basement membrane disease, but also immune complex diseases involving self antigens.

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Glomerular deposition of immunoglobulins, complement and control proteins

Disease		Immunoglobulins			Classical pathway Components			Alternative pathway Components		Terminal Components		Control proteins		
		G	A	M	C1q	C1s	C4	P	B	C3	C5	$C\bar{1}$-INH	C3bINA	β1H
Membranous glomerulo-nephritis	Number of biopsies positive	22/ 22	0/ 22	4/ 22	4/ 20	6/ 20	14/ 19	4/ 20	0/ 20	21/ 22	18/ 19	15/ 20	0/ 20	18/ 20
	Mean intensity	2.6		1.0	1.8	1.0	1.5	1.5		1.8	2.1	1.6		1.8
Membrano-proliferative glomerulo-nephritis	Number of biopsies positive	9/ 19	4/ 18	11/ 19	9/ 19	9/ 15	10/ 17	12/ 16	2/ 16	19/ 19	12/ 16	8/ 15	4/ 15	16/ 16
	Mean intensity	2.1	1.5	1.7	1.4	2.0	1.7	2.0	1.5	2.4	2.6	2.1	1.0	2.5
Focal glomerulo-nephritis	Number of biopsies positive	9/ 28	20/ 27	7/ 28	7/ 28	6/ 24	19/ 28	12/ 24	0/ 24	25/ 28	13/ 22	6/ 24	1/ 23	18/ 24
	Mean intensity	1.7	2.3	1.1	1.0	1.5	1.5	1.6		1.8	1.5	1.3	1.0	2.2
Henoch Schonlein nephritis	Number of biopsies positive	9/ 14	12/ 14	4/ 14	2/ 14	4/ 14	9/ 13	11/ 14	0/ 13	13/ 14	9/ 12	8/ 14	4/ 13	13/ 14
	Mean intensity	1.8	2.2	1.0	1.5	1.3	1.7	1.7		1.5	1.8	1.4	1.0	1.8
Systemic lupus erythema-tosus	Number of biopsies positive	10/ 10	8/ 10	9/ 10	10/ 10	9/ 9	10/ 10	7/ 9	0/ 9	10/ 10	8/ 8	7/ 9	2/ 9	9/ 9
	Mean intensity	2.3	1.6	1.7	2.3	2.0	1.7	1.4		2.6	2.0	2.3	1.5	2.7
Minimal change nephrotic syndrome	Number of biopsies positive	2/ 11	2/ 11	3/ 11	2/ 11	1/ 11	2/ 10	1/ 10	0/ 11	3/ 11	1/ 10	4/ 11	0/ 11	2/ 11
	Mean intensity	1.0	1.0	1.7	1.5	1.0	2.0	1.0		1.7	1.0	2.0		2.0