ALLERGIC RESPONSE TO GLOMERULAR BASEMENT MEMBRANE IN PATIENTS WITH GLOMERULONEPHRITIS

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ABSTRACT

Glomeruli were isolated from normal human kidney cortex; glomeruli basement membrane prepared by ultrasonication of glomeruli, and soluble G.B.M. antigens finally prepared by proteolytic digestion of G.B.M. with collagenase. Specific anti-G.B.M. serum was produced in rabbits. The main antigenic components of soluble G.B.M. were immunochemically characterised as: one component of molecular weight over 200,000, the second component of molecular weight in the 20,000-100,000 and the third component containing small molecules of less than 10,000. Two high molecular components retained the antigenic properties, the last had been degraded to small non-antigenic fragments. Separate G.B.M. fractions are not uniform, but composed of multiple components of different molecular weight and size.

In assessment of humoral allergic response to G.B.M. indirect immunofluorescence was found to correlate with the direct immunofluorescent method; double diffusion was found to be insufficient for the detection of circulating anti-G.B.M. antibodies; and among passive haemagglutination methods, glutaraldehyde method of chemical linkage of G.B.M. antigens to erythrocytes was proved to be highly sensitive. Antibodies had been detected in rabbit anti-G.B.M. serum diluted more than 1:500,000. Circulating anti-G.B.M. antibodies were found in high titre in patients with linear deposits of IgG on the G.B.M. Patients

with granular deposits of IgG had low titres of anti-G.B.M. antibodies. Predominantly IgG antibodies were eluted from kidneys of a patient with Goodpasture's syndrome and were shown to fix in vitro to homogenates of various organs, but predominantly to kidneys and lung.

Evidence for cell-mediated allergic response to G.B.M. and streptococcal membrane antigens was found in patients with proliferative glomerulonephritis independently whether the immunofluorescent appearances suggested nephritis due to immune complexes or anti-G.B.M. antibodies.

CONTENTS

1. INTRODUCTION

1.1. Immunological mechanisms of glomerulonephritis (GN)

Clinical and experimental observations have established that glomerulonephritis may be the result of an allergic reaction in the glomeruli. Both humoral and cell-mediated allergic responses contribute to its pathogenesis.

Two distinct immune processes involving humoral allergic responses involved in glomerulonephritis, have been delineated (Dixon 1968). The first pathogenetic mechanism comprises the production of antibodies capable of reacting with the antigens of glomerular basement membrane (G.B.M.) - anti-G.B.M. antibodies. (Masugi 1933; Steblay 1962a; Unanue et al 1967b; Lerner et al 1970). The second immunopathologic process depends on the production of antibodies reacting with circulating, nonglomerular, endogenous or exogenous antigens with the resultant formation of circulating antigen-antibody complexes. Depending on their size and solubility immune complexes may be trapped in the glomerular capillary walls (Dixon et al 1958; Dixon et al 1961; Dixon 1968; Edington et al 1967; Germuth et al 1967; Guttman et al 1967; Lambert et al 1968; Lindberg et al 1969; Lindquist et al 1969). Either of these two processes serves to concentrate an antigen-antibody reaction in the glomeruli with consequent inflammation due to the activation of mediators such as: polymorphonuclear leucocytes, complement (Gewurz et al 1968; Henson and Cochrane 1969), kinins, vasoactive amines

(Kniker and Cochrane 1968), anaphylatoxin, blood coagulation factors and other pathways (Cochrane et al 1965; Cochrane 1969; Cochrane 1971; Carpenter 1970). Other factors, such as the phagocytic function of the mesangial cells may have important roles in determining the severity and intensity of the lesion (Carpenter 1970; Vernier 1971).

Clinically and histologically, disease depends largely on the amount of antigen-antibody interaction and the type and quantity of mediators involved. It appears that both humoral mechanisms may cause either a severe, rapidly progressive, destructive disease, or a mild, slowly developing disorder (Med. staff conference 1972), and the histological spectrum is broad, ranging from membranous GN characterised by G.B.M. thickening, to proliferative GN (Dixon 1968; Hardwicke 1968; Hardwicke 1971). In experimental animals, membranous lesion develops in those with meagre immune response secondary to prolonged exposure to a low daily level of circulating immune complexes. Severe proliferative GN develops rapidly in actively immunised animals which are given large amounts of antigen to achieve immunological balance (Dixon et al 1961; Med. staff conference 1972; Dreesman and Germuth 1972).

The clinical course, laboratory and light microscopy data are not therefore of either humoral allergic mechanism. However, these two processes have distinctive immunohistochemical characteristics, and immunofluorescent identification of the pattern of immune deposits or electronmicroscopy serves to differentiate the two major humoral pathogenic mechanisms (Rother 1969; Bariety et al 1971; Davey et al 1970). The lesions induced by anti-G.B.M. antibodies

have antibody arranged in a uniform linear pattern along the endothelial side of the G.B.M. Circulating immune complexes accumulate along the epithelial aspect of G.B.M. in irregular, lumpy or granular deposits (Med. staff conference 1972).

Another possible type of allergic response that might lead to glomerular injury is a type IV - cell mediated - response. Lymphocyte and monocyte infiltrates are often found in glomerulonephritic kidneys from either experimental (Steblay et al 1968a; Litwin et al 1971), or human GN (Benoit et al 1964; Rocklin et al 1970c). It is possible that some small lymphocytes are producing mediators such as macrophage inhibition factor (M.I.F.), chemotactic factors or other biologically important lymphokines in response to kidney antigens. It was also demonstrated by intradermal testing (Wagner and Prokop 1956) that some patients with GN exhibit in vivo cellular hypersensitivity to renal antigens. In addition, there have recently been reports of in vitro findings suggesting cellular reactivity to various renal antigens in patients with nephritis (Numo et al 1968; Rocklin et al 1970c; Mahieu et al 1971; Mallick et al 1972; Macanovic et al 1972).

Common to both humoral and cellular allergic mechanisms of glomerular damage is deposition of immune reactants in or along the glomerular basement membrane. Glomerular basement membrane is the most potent glomerular antigen in the production of nephrotoxic sera (at least twenty times more active antigenically than epithelial or endothelial cells of glomeruli (Krakower and Greenspon 1951).

The isolation, purification, immunochemical characterisation of human glomerular basement membrane and the assessment of humoral and cell-mediated allergic responses to G.B.M. in patients with glomerulonephritis were chosen as the objects of this study.

1.2. Glomerular basement membrane (G.B.M.)

Insight into the pathogenesis of most diseases affecting the glomeruli require some knowledge of the morphology, isolation and characterisation of G.B.M. Among the various types of basement membranes (B.M.), the one isolated from glomeruli has been studied most extensively and may be considered as representative of other B.M.'s which are widely distributed in tissues and which have been shown to be immunologically interrelated (Spiro 1967a). G.B.M. is a remarkable structure, the function of which in health and disease can be studied from a number of aspects. A number of diseases are closely related to G.B.M.; morphologic alterations are observed in membranous glomerulonephritis, diabetic nephropathy, lupus nephritis and others. Immune processes affecting the kidney in the development and course of many types of GN may be located to G.B.M. Glomerular basement membrane has been studied from various points of view: morphologic, embriologic, structural, chemical, functional, immunologic and others. As the immunological characterisation of human G.B.M. antigens and specially allergic responses to G.B.M. are the subject of this study, I would like to review some of the available information on chemical composition and structure of human G.B.M. A parallel will be drawn somewhere between G.B.M. and B.M. of lung capillaries because of the disorder - Goodpasture's

syndrome, where anti-G.B.M. antibodies affect both G.B.M. and basement membranes of lung capillaries.

1.21. Morphologic aspects

G.B.M. is an extracellular matrix, positioned between endothelial and epithelial cells of glomerular capillaries Balazs 1970). Histologically, it is a homogenous, amorphous structure. If examined in terms of the type of cells with which it is associated, G.B.M. beside lung B.M. of capillaries is the only basement membrane associated with both endothelial and epithelial cells. All other B.M.'s, even those from other parts of nephron (B.M. of Bowman's capsula or B.M. of renal tubules) are associated only with either epithelial or endothelial cells (Kefalides 1970). Epithelial cells contact with G.B.M. through their foot processes. The origin of G.B.M. is uncertain but some workers believe that the epithelial cells are responsible for its synthesis (Pierce et al 1967a; Pierce et al 1967b; Pierce 1970). G.B.M. turns over slowly, perhaps requiring a month for replacement (Bloodworth et al 1968; Najarian et al 1969).

One morphologic feature of G.B.M. unlike other capillary B.M.'s is that it is exposed to circulation via endothelial pores. Contrary to other B.M. associated with endothelial cells (for example B.M.'s of blood capillaries), endothelial cells of glomerular capillaries do not form a continuous layer (Von Möllendorff 1930; Bell 1946; Cessi et al 1960; Lerner et al 1967; Dixon 1968; Koffler 1969; Cochrane 1971). A third type

of cell , mesangial cells, are also in contact with G.B.M. (Farquhar et al 1962; Lerner and Dixon 1966; Michael 1967b; Linlquist et al 1969; Mauer et al 1972).

Embryologically, the glomerulus is thought to derive from differentiated mesenchymal tissue (Davies 1950; Bloodworth et al 1968; Linder 1969).

There have been reports that G.B.M. is composed entirely of collagen, but in normal G.B.M. specimens collagen fibrils with their typical 600 - 700 Å periodic structure have not been demonstrated, except in B.M. of normal rat (Latta 1961). Studies of ultrastructure of G.B.M. showed that it contains a fine, partially oriented feltwork of fibrils 20 - 40 Å in diameter, associated with an amorphous matrix (Kurtz et al 1959; Vernier 1964; Farquhar 1964). B.M.'s of alveolar capillaries have similar ultrastructural features (Karrer 1956; Weibel 1963).

In contrast to many other basement membranes, the anatomical location of G.B.M. is such that its isolation in pure form is feasible. Krakower and Greenspon (1951) described a simple procedure for obtaining large numbers of isolated glomeruli from the renal cortex of dogs. Since then, there have been a number of studies on the isolation and structure of G.B.M. of different species. However, there have not been many reports on the isolation and characterisation of human G.B.M. One purpose of this work was to develop a method for the isolation, purification of human glomeruli, and the solubilisation and characterisation of human G.B.M.

1.22. Solubility properties

Isolated G.B.M. is insoluble in water (Kefalides 1970). Variable amounts of G.B.M. can be solubilised by extraction with 8 M urea or by reduction and alkylation of disulfide bonds in 8 M urea (Kefalides and Winzler 1966; Mahieu et al 1970a; Mahieu et al 1970b). The data obtained demonstrate that following solubilisation in 8 M urea or reduction and alkylation, G.B.M. and its soluble fractions are similar in regard to aminoacid composition, carbohydrates and antigenicity (Kefalides 1970). By this procedure it is possible to solubilise over 60% of G.B.M. Kefalides (1970) demonstrated that the urea extractable fraction lacks hydroxyproline and hydroxylysine, and contains hexosamine, galactose, mannose, fucose and sialic acid. It is possible to solubilise G.B.M. with 60% trichloroacetic acid at 25°C for 6 hr (Krakower et al 1957), or with 5% trichloroacetic acid at 90°C for 45 min (Kefalides et al 1966).

Variable proportions of G.B.M. can also be solubilised by proteolytic enzymes, including trypsin (Lange et al 1964; Lange et al 1965; Mohos et al 1969; Skoza et al 1969), pronase (Spiro 1967b) and collagenase (Spiro 1967b), depending on the temperature, concentration of enzyme and length of incubation (Kefalides 1968; Kefalides 1969b). Proteolytic digestion releases almost all sialic acid in the form of glycoproteins (Kefalides 1970). Collagenase acts on the collagen component of G.B.M., hydrolyses peptide bonds and the soluble fraction obtained has also traces of collagen. Treatment of G.B.M. with collagenase does not cause metabolic alterations in antigenicity (Kefalides 1970).

The object of this study was to solubilise human G.B.M. by proteolytic digestion with collagenase in order to obtain soluble G.B.M. antigens; these were then used for the further assessment of allergic responses to G.B.M. in patients with GN.

1.23. Chemistry

Fundamental to an understanding of the role of G.B.M. in normal states and the pathogenesis of glomerulonephritis is the knowledge of its chemical structure. The intact G.B.M. possesses a complex chemical structure. Insight into the structural characteristics of G.B.M. has been obtained from studies of this substance by a number of approaches: (1) use of solubilising agents which are known to break specific chemical bonds; (2) isolation of macromolecules and characterisation of their structure by physical means; enzymatic degradation of the whole G.B.M. and its collagen component followed by structural analysis of peptides (Kefalides 1970). The modern era of direct chemical analysis of isolated G.B.M. began when Goodman et al (1955) reported the presence of collagen in canine G.B.M. based on chemical measurement of hydroxyproline. This was contrary to the then prevailing view that collagen was absent from the G.B.M., a belief based mainly on histochemical and ultrastructural studies of glomeruli. Goodman et al (1955) believed that collagen constituted almost 100% of G.B.M., a view shared by Lazarow and Spiedel (1964). Following Goodman et al (1955), a number of investigators established amino acid and carbohydrate composition of G.B.M. in various species including humans (Goodman et al 1955; Goodman and Baxter 1956; Goodman 1958;

Markowitz and Lange 1964; Dische 1965; Mahieu and Winand 1970a; Mahieu and Winand 1970b), canine (Kefalides and Winzler 1966), bovine (Spiro 1967a; Spiro 1967b), rats (Huang and Kalant 1968). From these studies it appears that there is a considerable similarity in terms of amino acid and carbohydrate composition among B.M.'s from various species.

Histochemical studies of glomeruli suggested that G.B.M. was composed of periodic acid-Schiffs (P.A.S.) positive material. However, the limits of specificity of P.A.S. are such that it means only the presence of 1,2-glycol or amino alcohol groups (Lillie 1965; Misra and Berman 1969).

It is of interest to consider the chemical composition of G.B.M. in regard to collagen. A number of studies have indicated that G.B.M. is related to collagen: on the basis of amino acid composition, especially its content of hydroxylysine and hydroxyproline and large number of glycine residues, the G.B.M. may be considered to be related to collagen (Spiro 1967c). The presence of collagen has also been demonstrated in G.B.M. by enzymatic and x-ray diffraction studies (Rothbard and Watson 1961; Misra 1964; Kefalides and Winzler 1966; Misra and Kalant 1966; Kalant et al 1966). Rothbard and Watson (1961; 1969) provided indirect evidence for the presence of collagen in the G.B.M. by localising anti-collagen antibodies in the glomeruli by immunological methods. However, from a number of other studies, it has been pointed out that marked differences exist between the composition of G.B.M. and other well characterised collagens. There is still controversy concerning the nature and quantity of collagen in the G.B.M. (Kefalides and Winzler 1966; Spiro 1967a). The difference

between G.B.M. and connective tissue collagen will be the most evident from the differences in chemical structure of these two substances. The criteria which define collagen are: glycine accounts for about 33% of the amino acid residues, the sum of proline and hydroxyproline for about 22% and hydroxylsine/hydroxyproline ratio is 0.07 (Ramachandran et al 1962). A number of studies (Kefalides and Winzler 1966; Spiro 1967a; Spiro 1967b; Kefalides 1970) have shown that the G.B.M. contains much less of glycine, that the hydroxylysine/ hydroxyproline ratio is 0.43, hydroxylysine content of G.B.M. is four times higher than that of collagen (Kefalides 1968) and that the sum of hydroxyproline plus proline is much lower than that found in connective tissue collagen. This led to the conclusion that in addition to collagen, other proteins components must be present in glomerular basement membrane.

From subsequent studies on structure and chemistry of G.B.M. (Kefalides and Winzler 1966; Kefalides 1968; Spiro 1967a; Spiro 1967b; Mahieu and Winand 1970a; Mahieu and Winand 1970b) it can be summarised that: G.B.M. is a polymer of dissimilar protein subunits rich in carbohydrate. These subunits interact by covalent and hydrogen bonds and by weak forces which involve neither of the two mentioned types of bonds. The subunits are: (1) collagen; the collagen component of G.B.M. is composed of the disacharide (glucosyl-galactose) and monosacharide (galactose), both linked glycosidically to hydroxylysine. Collagenase digestion of the native G.B.M. results in (2) a undigested residue and (3) (4) - two soluble components. Undigested residue after collagenase digestion is a large molecular glycoprotein, associated with non-collagen polypeptides (3) (4). Two soluble components (3) (4) are non-collagen glycopeptides one of molecular weight of approximately 55,000, the other over 200,000. One, of small m.w. is a disacharide

composed of an equimolar quantity of glucose and galactose linked to hydroxylysine. The second contains heteropolysacharide composed of galactose, fucose, mannose, hexosamine and sialic acid. Both components interact with each other by hydrogen bonds and with collagen by disulfide bonds.

1.24. Fractionation

There have been attempts to fractionate G.B.M. into the main components. Markowitz and Lange (1964) attempted to purify the trypsin digest of human glomeruli by column chromatography on Sephadex G-200. They achieved some separation into the elution peaks, but no effective immunologically distinctive components were obtained. Huang and Kalant (1968) separated normal rat collagenase solubilised G.B.M. into six components combining solubilisation by collagenase digestion, urea extraction and gel fractionation. Kefalides and Denduchis (1969a) and Kefalides (1970) separated dog G.B.M. into two main fractions. (1) - an insoluble undigestible residue after collagenase, which could be solubilised further by urea extraction. It contained a polysacharide unit, without hydroxylysine and hydroxyproline. (2) - a soluble portion of the collagenase digestion which after gel filtration on Sephadex G-200 separates into six peaks. Skoza and Mohos (1969) isolated glomeruli (bovine and mouse) and prepared soluble fractions by trypsin digestion. They further fractionated the soluble digests into two high (A and B), and one low molecular weight components (C). All components contained sialic acid. Pronase digestion of G.B.M. was also carried on. Pronase digest contained most of the sialic acid, but the chromatogram consisted almost entirely of the low molecular-weight

C peak, indicating that this less discriminating proteolytic enzyme completely degraded the protein portion of the G.B.M.

It will be of interest to study, in the light of the above observations, the separation pattern of soluble human G.B.M. This will be another object of the present work.

1.25. Immunochemical characterisation

Masugi (1933) described the experimental glomerulonephritis induced in rabbits by injection of duck anti-rabbit kidney serum. Since then, experimental GN induced in various animals by injection of anti-kidney serum has been studied intensively. It became well established that the kidney contains a specific antigen, the antiserum of which is capable of producing GN in experimental animals. As the kidney is a complex structure, studies of its separate components have been carried out. Additional studies (Pressman and Eisen 1950a; 1950b; Heyman et al 1950) further localised the nephritogenic antigen within the renal cortex of the glomeruli (Greenspon and Krakower 1950). Krakower and Greenspon (1951) demonstrated an easy method for the separation of glomeruli from other components of renal cortex. In a study concerned with the site of antigen within the isolated glomerulus, they established that G.B.M. was 20 times more active antigenically than the combined visceral epithelial and endothelial cells in terms of rat weight, and 50 times more active on the basis of nitrogen content (Krakower and Greenspon 1951).

As the ability to isolate G.B.M. and to extract various molecular species from it is obviously pertinent to the problem of allergic diseases affecting the kidney, many investigators have tried to isolate, purify and identify the nephrotoxic antigens located in the G.B.M., using different methods and different approaches to the problem (Thoenes and Hammer, 1967; Loewi 1967). Difficulties in isolating and characterising the G.B.M. antigens were partly due to insolubility of the native G.B.M. Attempts to define renal antigens responsible for induction of nephrotoxic antibodies have been carried on using different methods: solubilisation by chemical and biological means; or indirectly by blockade of fixation of nephrotoxic serum by previous absorption of the antiserum and subsequent application to kidney sections.

Enzymes have been used in the past in attempts to identify the nephritogenic antigens (Cruickshank and Hill 1953). A soluble material capable of neutralising the anti-kidney antibodies was isolated from proteolytic digests of the renal cortex or glomeruli (Cole et al 1951; Goodman and Baxter 1956; Goodman 1958; Shibata et al 1967). In 1951 Cole et al showed that collagenase digest of G.B.M. retained its nephritogenic capacity. These workers were able to neutralise nephrotoxic (anti-kidney) antibody by absorption and the soluble material obtained from the collagenase or trypsin digestion of homogenised kidney. This was one of the first studies that indicated that soluble G.B.M. antigens, obtained after proteolytic digestion are "nephritogenic". Unlike collagenase, digestion with pronase resulted in the loss of ability to elicit nephrotoxic antibodies (Huang and Kalant 1968). Further studies of the chemical composition and fractionation of G.B.M. were carried out simultaneously with the investigations of antigenicity of separate

components of G.B.M. These studies have indicated that G.B.M. has a complex antigenic structure, a mosaic containing both non-"nephritogenic" and "nephritogenic" antigens (Boss 1963; 1965a; 1965b).

Variable numbers of antigenic determinants have been reported in G.B.M. Cleve et al (1957) using immunoelectrophoresis (IEP) demonstrated at least twelve different antigens in crude kidney extract. Antoine and Hamburger (1966) demonstrated in human kidney 18 different tissue antigens, and in rabbit kidney about 30. These workers used anti-kidney sera prepared in rabbits and ducks and tested them in IEP against the soluble extracts of kidneys which had been used as immunising agents. But the antigens used were insufficiently defined and characterised and the absorption of the antisera was inadequate. In 1968 Huang and Kalant separated specific nephritogenic antigens from rat G.B.M. and characterised them as macromolecular glycoproteins containing 10.7 carbohydrate in the form of galactose, mannose, sialic acid, hexosamine and galactosamine. They also demonstrated that collagenase digest of rat G.B.M. retained nephritogenic activity and that further proteolytic digestion by pronase resulted in the loss of ability to elicit nephrotoxic antibody.

The method of direct and indirect immunofluorescence of kidney sections provided an opportunity for the use of enzymes on G.B.M. antigens in situ. Rothbard and Watson (1969) made the experiments with pretreatment of frozen normal rat kidney with various purified enzymes, followed by the incubation either with anti-kidney cortex serum or anti-collagen serum. The experiments were done to determine whether selective destruction or alteration of the enzyme specific

antigens would alter fixation of the antisera and so permit the identification of antigens. Therefore several proteolytic enzymes, each with substrate specificity, were tested. The experiments showed that trypsin and hyaluronidase had no effect on the subsequent fluorescence of either antibodies; papain reduced the fluorescence; pepsin and pronase acted on both collagen and glycoprotein antigens and no fluorescence was present; neuraminidase from vibrio cholerae reduced fluorescence. The last finding was interesting. Neuraminidase is an enzyme which splits sialic acid from glycoproteins. When kidney slices were treated with neuraminidase and anti-kidney serum applied thereafter, some diminuition of fixation of either antibody was observed, but no complete blockade occurred as would be expected if sialoglycoproteins were completely altered. This meant either (1) that after neuraminidase treatment some sialoglycoproteins still remain in undigested G.B.M., or (2) that sialoglycoproteins are the only nephritogenic antigens contributing to the fixation of anti-kidney sera. Treatment with collagenase completely prevented fixation of antiserum to rat collagen, but had no effect on fixation of anti-kidney sera.

Collagenase thus acts on collagen component of G.B.M. (Spiro 1967a; 1967b; Huang and Kalant 1968; Rothbard and Watson 1969) and sharply differentiates collagen antigens from nephritogenic antigens. In addition, incubating of anti-kidney cortex serum with tendon collagen does not block the activity of such serum. In conclusion, the experiments of Rothbard and Watson (1969) using direct and indirect immunofluorescence, established that antibodies to rat collagen and to rat G.B.M. are apparently directed towards different antigens, both of which are present in G.B.M.

Kefalides (1968; 1969b; 1970) using fractionation of reduced and alkylated G.B.M. was able to demonstrate two antigenic components of G.B.M. by immunodiffusion. One was a collagen-like protein having two types of carbohydrate units: a neutral hexose composed of glucosae and galactose alone and a charged unit, composed of galactose, mannose, hexosamine, fucose and sialic acid. Both components are glycoproteins, lacking collagen. These data also demonstrate that antigen specificity resides in the non-collagen peptide of G.B.M., whether linked to collagen or not.

The residue after collagenase digestion and its antigenicity have been studied by several groups. Huang and Kalant (1968) found that sediment after collagenase digestion of original G.B.M. alone or further solubilised by urea extraction was able to neutralise nephrotoxic antibody. This finding accords with that of Rothbard and Watson (1969) who showed, by fluorescent methods, that undigested residue still retains antigenic sites capable of fixing anti-kidney cortex serum. These experiments show that the two glycoproteins, one extracted with collagenase, and the other retained in undigested residue, have antigenic groups in common. Skoza and Mohos (1969) obtained different results. They found that the residue after trypsin digestion of G.B.M. apparently lost all of its ability to neutralise nephrotoxic antibodies. Simultaneously, they investigated the sialic acid content of the soluble G.B.M. and undigested residue, and concluded that the nephritogenic antigens were sialoglycoproteins. On gel filtration (Sephadex G-200) these workers separated trypsin soluble glycoproteins to two high molecular weight components and one low molecular weight component. Only high molecular components

retained the ability to absorb out nephrotoxic antibodies from NTS, while low molecular weight fraction behaved similarly to pronase digest of native G.B.M., i.e. was unable to neutralise NTS. In their hands, the residue after digestion did not contain sialic acid and was unable to absorb nephrotoxic antibodies from NTS.

All the studies reviewed indicate that G.B.M. is highly polymerised substance, containing at least two major antigenic components, one being glycoprotein, the other a collagen-like protein, and that the glycoprotein component can be further fractionated to at least two glycopeptides of different molecular weight. Soluble fragments obtained after chemical or proteolytic solubilisation of native G.B.M. are antigenic in experimental animals, capable of inducing nephrotoxic antibodies. The data also suggest that soluble fractions (after collagenase digestion or urea extraction-solubilisation procedures that do not alter antigenicity) share common antigenic determinants with the intact G.B.M. The removal of collagen from the G.B.M. does not affect the nephritogenic antigenicity of the G.B.M. A number of studies (Kefalides 1970) have demonstrated also that there exist immunologic cross-reaction among homologous B.M.'s derived from different tissues, and between heterologous glomerular basement membrane.

1.3. Humoral allergic response to G.B.M.

The concept that anti-kidney antibodies might cause glomerulonephritis dates back to 1900 when Lindemann demonstrated the nephrotoxic and nephritogenic properties of heterologous anti-kidney antibodies. Subsequent work on nephrotoxic serum nephritis (NTN) has provided more precise information concerning the nature and properties of heterologous nephritogenic antibodies, the location and immunochemical characteristics of the glomerular antigen and some of the mediators of inflammation activated by the antigen-antibody reaction (Masugi et al 1932; Masugi 1933; Smadel and Farr 1937; Kay 1942; Seegal and Loeb 1946; Seegal et al 1965; Bevans et al 1955; Lerner et al 1967). Glomerulonephritis was produced in various species of experimental animals, not only with heterologous, but also with homologous and autologous kidney extracts or more specified kidney antigens (Heymann et al 1950; Steblay 1962a; 1962b; 1963; Milgrom et al 1964; Lerner and Dixon 1966; Unanue 1966; Unanue and Dixon 1967a; Lerner et al 1968b; Paronetto and Koffler 1967; Gang and Kalant 1970a; Gang et al 1970b; Gang et al 1970c). The passive serum transfer of this form of GN in sheep (Lerner and Dixon 1966) and rabbits (Unanue and Dixon 1967a) to normal homologous recipients provided definite evidence that nephritogenic antibodies are producing glomerulonephritis. This was demonstrated by the following experiment of Lerner and Dixon (1966): serum globulin from donor sheep made nephritic by immunisation with G.B.M. and subsequently nephrectomised, contained specific kidney fixing antibodies and was capable of inducing an immediate but transient GN, when injected into unilaterally

nephrectomised lambs. The nephritis was characterised by immediate proteinurea, PMN infiltration into the glomeruli, and linear deposits of IgG and C3 along the recipients' glomerular capillary walls. They were able to absorb the nephritogenic antibodies by incubating NTS with sheep G.B.M. In addition, anti-G.B.M. antibodies eluted from kidneys of rabbits (Lerner and Dixon 1968a) or sheep Steblay and Rudofsky 1968b) with autoimmune GN transfer the disease to normal homologous recipients. This work, and specially the work on autoimmune GN, was a step towards implicating similar pathogenetic mechanism in human GN (Lerner et al 1967).

These extensive studies elucidated one of the two animal models of immunologically mediated immune injury - the anti-G.B.M. antibody mediated GN. As the detection of anti-G.B.M. antibodies is the main object of the present work, the other model, i.e. immune-complex nephritis will not be described in detail. There are a lot of aspects that make experimental autoimmune GN different from the other experimental allergic diseases, but here I would like just to stress one characteristic. The antigen, G.B.M. capable of inducing the disease is not anatomically sequestered and remoted from circulating antibodies (as is myelin or spermatogenetic elements in two other experimental autoimmune diseases). Here antigen is exposed via endothelial pores and circulating antibodies have easy access to their specific antigen.

1.31. Anti-G.B.M. antibodies in human glomerulonephritis

The presence of anti-human kidney antibodies has been claimed (Pfeiffer and Bruch 1952; Brod et al 1958) and denied (Vorleander 1952; Goodman 1958). In 1939 Schwenteker and Comploier postulated the following mechanism for human GN as a working hypothesis by which streptococcal infection produced kidney lesion: as a result of streptococcal infection, streptococcal products are released, damage the kidney, alter renal proteins which are released into the general circulation. The renal tissue proteins in contact with reticulo-endothelial system act as "foreign", thereby stimulating the production of antibodies.A similar mechanism was later postulated for human GN by Cavelti and Cavelti (1945). These studies induced the subsequent work on the detection of such anti-kidney antibodies. Vorleander (1952) using complement fixation technique with human kidney has demonstrated an organ specific autoantibody to kidney in acute and chronic GN. Other groups of investigators have had varying degrees of success in demonstrating circulating anti-human kidney (AHK) antibodies. Goodman (1958) failed to demonstrate AHK antibodies by either the complement fixation method or by Boydens' haemagglutination procedure. Gajdusek (1958) using the complement fixation technique demonstrated circulating autoantibodies to kidney, placenta and liver in patients with GN, lupus erythematosis and infectious hepatitis. Wagner et al (1959) found positive titres of circulating antibodies to kidney, liver and placenta in patients with preeclampsia and eclampsia. The main deficiency of the techniques used by these workers is that they used poorly characterised antigens.

Liu and McCrory (1958) obtained unusually high percentages of positive results in double diffusion method, when kidney homogenate was tested with sera of patients with GN. However, not only patients with GN, but also patients with other diseases were positive in the high percentages. As the supernatants of normal foetal kidney used was likely to have contained plasma proteins and other cellular material, it might be that anti-nuclear antibodies, C-reactive protein, rheumatoid factor, paraproteins and other non-specific factors resulted in the high proportion of presumably non-specific results. Complement fixation technique also gave a high percentage of positive results (Vorleander 1952; Schwenteker and Comploier 1939; Gajdusek 1958). Such reports of circulating AHK antibodies in patients with nephritis remain difficult to interpret as the nature of renal antigens involved was not established and no relationship of such antibodies to the pathogenesis of nephritis had been established.

The final step in demonstrating the participation of anti-G.B.M. antibodies in human GN was achieved with the isolation of such antibodies from either (or both) the serum and kidneys of nephritic patients (Lerner et al 1967). In 1967, Lerner, Glassock and Dixon established the presence of anti-G.B.M. antibodies in the circulation and in the kidneys of six patients with GN and demonstrated their specificity and nephritogenicity by the following observations (Lerner et al 1967; Dixon 1968; Lerner et al 1970): (1) the pattern of fixation of host IgG in smooth linear fashion along the glomerular capillary walls in vivo corresponds precisely with the distribution of heterologous anti-G.B.M. antibodies in experimental autoimmune version of the disease; (2) isolated from serum of nephritic patients, or eluted from their kidneys, anti-G.B.M. antibodies combined in vitro

to the frozen sections of normal human kidney in the identical linear pattern as in vivo; (3) when eluted from the kidneys, these antibodies on injection into normal squirrel monkeys were fixed together with complement in a linear pattern along the recipients' glomerular capillary walls; (4) transfer experiments showed that injection of small amounts of globulin eluted from human anti-G.B.M. type nephritic kidneys into monkeys immediately caused a severe GN; (5) in one nephrectomised patient it was possible to demonstrate circulating anti-G.B.M. antibodies prior to transplantation. After transplantation the antibodies were not detectable in the serum, but fixed promptly to the G.B.M. of the renal allograft in smooth, linear pattern. An immediate glomerulonephritis developed in transplanted kidney. This case demonstrated not only the immunological reactivity, but also "nephritogenic" properties of anti-G.B.M. antibodies. Based on the transfer of GN and on the presence of antibodies at the site of injury in the nephritic kidneys of both the patients and the recipient monkeys, Lerner et al (1967) concluded that the anti-G.B.M. antibodies are at least a contributary, if not primary, cause of glomerular injury. It is now established that anti-G.B.M. antibodies are present in all cases of Goodpasture's syndrome, and some patients with rapidly progressive glomerulonephritis, and that anti-G.B.M. antibody GN is responsible for probably less than 5 per cent of the remaining type of nephritis glomerulonephritides.

The second immunopathologic mechanism of glomerular injury - immune complex mechanism - is well established in the pathogenesis of many different forms of GN, among others in lupus nephritis (Kirshnan and Kaplan 1967; Koffler et al 1967) subacute bacterial

endocarditis (Cordeiro et al 1965), following the infection of artificial valve (Stickler et al 1968; Kaufman and McIntosh 1971), malarial nephrosis (Ward and Kibukamusoke 1969), post-streptococcal GN (Michael et al 1966), luetic nephrosis, and in many other conditions where specific antibodies combine with endogenous or exogenous antigens and form circulating soluble complexes. The frequency of GN in animals with persistent viral infections suggests that viral antigens may play an important role in human immune complex nephritis of unknown cause. This pathogenetic mechanism appears to be responsible for at least 90 per cent of human nephritis.

In connection with the incidence and pathogenetic role of anti-G.B.M. antibodies, we shall present some features of the syndrome of pulmonary haemorrhage and nephritis (Goodpasture's syndrome) mediated by specific anti-G.B.M. antibodies.

1.32. Goodpasture's syndrome

Ernest Goodpasture (1919) described the first case of diffuse pulmonary haemorrhage and proliferative glomerulonephritis. Since then, the number of such cases reported has grown to over one hundred, review and a list of references on cases reported up to 1964 are in the paper of Benoit et al (1964) and the references for newer cases in a paper of Proskey et al (1970). We shall present only some of the features of this syndrome, specially those connected with the production and role of anti-G.B.M. antibodies.

The clinical characteristics of the syndrome are: haemoptysis, anaemia, haematuria, pulmonary infiltrates, and the rapid development of renal failure (Stanton and Tange 1958; Rusby and Wilson 1965; Proskey et al 1970). The disease is generally regarded as a distinct clinical identity. Lung changes are usually seen to antedate the development of nephritis, but there are reports of renal disease preceding the pulmonary manifestations and one report of pulmonary haemorrhage developing after nephrectomy (Maddock et al 1967; Cleveland et al 1964; Bloom et al 1965; Krauss et al 1969).

The pathogenesis of the renal and pulmonary lesions has been ascribed to an allergic mechanism and the antigenic similarity of lung and kidney basement membranes had been established (Hill and Cruickshank 1953; Sophian 1970). Experimental nephritis has been induced in animals by anti-lung serum or by immunisation with human lung in adjuvant (Eisen et al 1950; Chikamutsue 1940; De Govin et al 1963; Steblay and Rudofsky 1968a). Anti-lung antibodies induced in animals have been shown to fix to the glomerular capillaries (Mellors et al 1955). Haemorrhagic pneumonitis has been induced experimentally simultaneously with glomerulonephritis following the injection of anti-lung antibody (Willoughby and Dixon 1969; 1970). Hagadorn et al (1969) reported the development of acute and chronic pulmonary and renal lesions characteristic of Goodpasture's syndrome in rats given injections of rabbit anti-rat lung serum. As fluorescent microscopy shows remarkably smooth linear deposition of IgG along the glomerular capillary basement membrane (Duncan et al 1965), anti-G.B.M. antibody appears to be a major pathogenetic factor in Goodpasture's form of glomerulonephritis (Lerner and Dixon 1966; Lerner et al 1967).

Although the aetiology of Goodpasture's syndrome remains uncertain, there is clear evidence that the production of injury depends on antibodies to lung and kidney basement membrances directed against G.B.M. and most probably lung B.M.'s may be responsible for the lesions observed in both organs. Thus Koffler et al (1969) found acid eluates of lung tissue from patients with Goodpasture's syndrome yielded an antibody with in vitro specificity to both human and lung B.M. Similarly, antibody with identical specificity was eluted from the patient's kidney. However, the initiating event responsible for anti-G.B.M. antibody production is unknown. Lerner et al (1968a) proposed the development of autosensitisation to normal G.B.M. antigens.

1.33. Source of immunising antigens

The following possibilities for the initiating events in the production of anti-G.B.M. antibodies have been offered:

(1) autosensitisation to normal G.B.M. antigens present in urine. The studies in the rat of both Lazarow and Speidel (1964) and Kurtz and Feldman (1962) suggested that G.B.M. is continuously being catabolised and replaced. In the normal rat, this turnover rate may be of such magnitude that the entire membrane is replaced every 30 - 60 days. In view of this finding the recent finding of G.B.M. antigens in normal and rabbit urine is not surprising and may reflect continuing membrane catabolism and anabolism (Hawkins 1967; Hawkins and Cochrane 1968; Lerner et al 1967; Lerner et al 1968b; Mc Phaul and Dixon 1970b).

The nephritogenicity of these antigens when injected to the animals from which they were obtained suggested that such urinary antigens might be possible immunogens in human GN. Any abnormal handling of glomerular filtrate containing the G.B.M. antigens might expose immunologically competent cells to normally secluded antigens (Poskitt 1970). In such instances, the presence of infectious agents or their products might have an adjuvant effect. The additional finding that during active GN the amount of these antigens in the urine increase provides a possible self-accelerating stimulus (Lerner et al 1967). However, this hypothesis appears to be more adaptable for anti-G.B.M. mediated GN other than GPS. The antibodies formed in contact with G.B.M. antigens might have higher affinity for G.B.M. than for lung B.M.'s, together with anatomical favours would fix to G.B.M. Besides cross-reactivity of G.B.M. and lung B.M., this hypothesis does not explain lung damage, specially for the cases where lung damage precedes glomerular lesions.

(2) the other alternative is the immunising with environmental antigens antigenically identical, or cross-reactive with G.B.M. Among these antigens, the most investigated are streptococcal membrane antigens. It has been established that membranes of the streptococci and G.B.M. share the antigenicity (Hanson 1959; Markowitz and Lange 1964; Lange and Markowitz 1965; Markowitz et al 1967; Holm 1967; 1970; Treser et al 1969; Rapaport et al 1969; Lange 1969; Markowitz and Lange 1970a). Markowitz and Lange (1970a) proposed that a common glycopeptide determinant grouping(s) present on the protoplasmatic membrane of the nephritogenic streptococcus and human G.B.M. could be responsible for the production of antibodies reacting with both

antigens and presented chemical, physical, immunological and biological data in support of this hypothesis.

(3) as in the great majority of patients with GPS viral infection of the upper respiratory tract preceded lung and kidney damage, viral aetiology for production of anti-G.B.M. antibody was postulated (Benoit et al 1964). Either normal lung basement membranes exposed to circulation as the result of viral damage, or virus altered lung antigens might elicit antibody production (Poskitt 1970). However, the apparently greater affinity of the anti-G.B.M. antibodies for the kidneys and the absence of data on cross reactivity of viral with lung or G.B.M. antigens weakens the argument for either normal or virus altered lung antigens in the antibody initiation. Except for one case (Duncan et al 1965) where virus-like particles were shown in glomeruli of patients with GPS, no other evidence was offered for the direct viral damage of affected tissues. The recent description of anti-G.B.M. nephritis following an anfluenza A2 infection supports the concept of viral aetiology for anti-G.B.M. antibodies production (Wilson and Smith 1971b; Wilson 1972).

(4) the initiating agents for the production of anti-G.B.M. antibodies might also be native G.B.M. altered by toxins, infections or trauma (Tan and Kaplan 1963). There have recently been reports of the presence of low titre of anti-G.B.M. antibodies following acute tubular or cortical necrosis (Macanovic et al 1972; Mahieu et al 1972).

(5) even if not directly related, the presence of anti-G.B.M. antibodies in anti-lymphocyte sera (ALS) may be considered together with the problem of the source of immunising antigens for the production of anti-G.B.M. antibodies. Guttman et al (1967) reported the occurrence of autologous phase of nephrotoxic serum nephritis in rats during the treatment with ALS. The nephritis was caused by anti-G.B.M. antibodies present in the ALS. Anti-G.B.M. antibodies were found in the sera prepared by immunising animals with human lymphoid cells isolated from lymphoid tissues with a reticulin stroma, such as the thymus lymph nodes, tonsils and spleen. The sera raised with blood lymphocytes or cells obtained from lymphoid cell cultures or thoracic duct fluid did not contain anti-G.B.M. antibodies. Most probably inclusion of vascular fragments from the lymphoid tissues contributed to the production of anti-G.B.M. antibodies together with anti-lymphocyte antibodies. The presence of anti-G.B.M. antibodies in ALS have been reported by a number of workers (Dielhelm 1969; Orr et al 1970; Thiel et al 1971; Katz et al 1970; Feltkamp and Balner 1972; Balner 1971; Wilson et al 1971a).

1.34. The methods for the detection of anti-G.B.M. antibodies

1.341. Double diffusion

Liu et al (1958) tested by Ouchterlony's gel-precipitation method sera of patients with glomerulonephritis against whole extract of human foetal kidney. Of the 14 sera tested, 5 were found to have precipitin lines. Those sera were from patients with glomerulonephritis; out of

11 nephrotic sera, 3 were positive. They obtained a high percentage of positive results which were later impossible to reproduce. The positive results were most probably due to non-specific precipitation reactions between sera tested and plasma proteins of normal foetal blood present in antigen used. The antigen was also used in very high concentration - 50% in saline. Lerner et al (1967) tested sera of patients with GN for their ability to precipitate soluble G.B.M. antigens in 1% agarose using a micro Ouchterlony technique. Precipitating antibodies were detected only in 2 out of 23 bilaterally nephrectomised patients and in none of the 50 patients before the nephrectomy. The two positive patients had an active GN. Markowitz and Rapaport (1970b) demonstrated the presence of anti-G.B.M. antibodies in the serum of two anephric patients using immunoprecipitation and autoradiography performed with ^{131}I labelled G.B.M. antigens.

1.342. Passive haemagglutination

Agglutination and Boyden's haemagglutination methods are considered to be extremely sensitive methods for the detection of circulating antibodies (Stavitsky and Arquilla 1953; Stavitsky 1954; Stavitsky and Ingrahm 1964; Björklund and Paulsson 1962). Lange et al (1949) using a modified collodion particle method of Cavelti (1947) reported the presence of antibodies to foetal kidney supernatant in sera of patients with nephritis. In 12 patients studied within one year after the onset of the disease, 68% sera were positive, and in 11 cases (more than one year from the onset of the disease) 78% were positive. However, in a large control group 19% positive results were recorded as well. The method employed probably contributed to the variability of the results and caused

Lange (1951) to discard collodion agglutination technique. Pfeiffer and Bruch (1952) using the same method, demonstrated anti-kidney antibodies in the blood of 72% of patients with acute glomerulonephritis and in 84% of patients with chronic glomerulonephritis.

Using Boyden's haemagglutination method and tannic acid treated sheep red blood cells (r.b.c.) on which antigen, tryptic digest of kidney was coupled, Liu et al (1958) demonstrated the anti-kidney haemagglutinating titres of 1:20 or higher in 71% of patients with acute GN and in 89% of the patients in the active phase of nephrotic syndrome. But elevated titres of "anti-kidney antibodies" were also found in patients with infectious diseases, rheumatic fever, rheumatoid arthritis and SLE.

Kramer et al (1961) using polystyrene latex suspension to which kidney antigens were absorbed as a colloid particle for agglutinating antibodies, demonstrated in 15 out of 36 patients with GN anti-human kidney antibodies. They also demonstrated that the persistence of a high titre of AHK antibodies was a poor prognostic sign. Again, the antigen used, foetal kidney homogenate, might have influenced the high percentage of positive results.

As passive haemagglutination is more sensitive method than precipitin reactions, we considered several of the passive haemagglutination (P.H.) methods for detecting anti-G.B.M. antibodies. The r.b.c. coupled with antigens and direct agglutination of such cells by specific antibody has proved to be a valuable method for detecting the minute amount of antibodies (Onkelinx 1969; Avrameas and Ternynck 1967; 1969b; Das 1970).

As the method of coupling the antigen to the r.b.c. surface may influence the amount of antigen linked, we tried several methods: tanned-cell technique, bisdiazotised benzidine, chromium chloride and glutaraldehyde.

(a) Tanned cell coupling of G.B.M. to red blood cells. A technique utilising the adsorbtion of various proteins to tannic acid (T.A.) treated r.b.c. was described by Boyden (1951): such protein-conjugated cells are agglutinated by highly diluted specific antisera (George et al 1962b). Treatment of r.b.c. with T.A. brings about a change in the properties of these cells, rendering them capable of adsorbing soluble proteins from solution in saline. Liu et al (1958) using tanned-cell technique, were able to demonstrate the presence of anti-kidney antibodies in sera of patients with GN. Feltkamp et al (1972) used passive haemagglutination of tanned-cells for detecting anti-G.B.M. antibodies in rabbit anti-human G.B.M. antisera, eluates of kidneys with anti-G.B.M. nephritis and for detecting anti G.B.M. antibodies in anti-lymphocyte sera. On tanned r.b.c. collagenase solubilised G.B.M. antigens were coated.

(b) Bisdiazotised benzidine. In 1942 Pressman et al described the method of chemical coupling protein to r.b.c. by means of stable covalences using bivalent reagents such as bisdiazotised benzidine (B.D.B.). Such"sensitised cells" were subsequently agglutinated by specific antisera. The method was modified later and used for chemical coupling of various proteins to r.b.c. (Stavitsky and Arquilla 1958; Gordon et al 1958; Ling 1961a; 1961b; Epp 1962; Butler 1963; Müller and Gramlich 1963; Cua-lim et al 1963).

(c) Chromium chloride ($CrCl_3$). One feature of the interaction between metallic cations and r.b.c. is the ability of several metals, among them $CrCl_3$, to attach proteins to r.b.c. surface (Jandl and Simmons 1957). R.b.c. so treated could thereafter be agglutinated by specific antisera. Recently Mahieu et al (1972) using the $CrCl_3$ method of Lavergne et al (1965) for linking soluble G.B.M. antigens to r.b.c. were able to demonstrate anti-G.B.M. antibodies in 7 per cent of patients with nephritis, and they were able to demonstrate the presence of antibody G.B.M. antibodies in patients with linear deposits of IgG on renal biopsies. However, low titres of circulating anti-G.B.M. antibodies were also found in patients with granular deposits and a variety of other renal disorders-including renal infarction.

(d) Glutaraldehyde. Because chemical linkage of protein to r.b.c. frequently results in haemolysis, r.b.c. were stabilised before sensitisation. Formaldehyde was most often used as stabilising agent (Csizmas 1960). Ling (1961a; 1961b) used glutaraldehyde (GA) as a stabilising agent. He was the first to observe that GA treated r.b.c. were able to fix proteins from solution even without subsequent addition of coupling agent. This observation was confirmed by Bing et al (1967). In 1969, Avrameas (1969a) and Avrameas et al (1969b) together with Onkelinx et al (1969), described a sensitive P.H. technique for the attachment of proteins to r.b.c. with GA. In efforts to link enzymes covalently to proteins, Avrameas and Ternynck (1967) found that GA was the most effective. In 1969, they noticed that low concentrations of GA should be used, otherwise insoluble material resulted (Avrameas and Ternynck 1969b). Onkelinx et al (1969) used GA for direct coupling of proteins to r.b.c. and developed standard procedure using serum albumin and specific antiserum. A standard

procedure was devised by which other proteins could be coupled and tested with specific antisera. They also noticed that the titres were comparable or higher than with classical BDB method. Free amino groups seem to be the main functional group by which GA link protein to r.b.c. (Avrameas 1969a). Various proteins have been coupled to sheep r.b.c. with GA: lypopolysacharides (Eskenazy and Petrunov 1971), bovine serum albumin and transferrin (Onkelinx et al 1969), human IgG, and human serum albumin (Avrameas 1969a).

One object of this study was to evaluate these techniques for their sensitivity and specificity in the detection of anti-G.B.M. antibodies in the sera of patients with glomerulonephritis before nephrectomy.

1.343. Direct immunofluorescence

Direct immunofluorescence of renal histology was used in this work to broadly categorise patients with nephritis into immune-complex or anti-G.B.M. glomerulonephritis. Immunofluorescent methods have contributed greatly to the understanding of the mechanisms involved in GN (Koffler and Paroneto 1965). The key for distinguishing the two mechanisms of immunologically mediated GN is immunofluorescent study of frozen kidney sections stained with fluorescein-labelled antisera specific for IgG. Immune complexes appear as granular deposits along the G.B.M. but anti-G.B.M. antibodies, because they react with the antigens on and in the G.B.M., appear as smooth linear deposits. This linear pattern of IgG deposition on G.B.M. was observed in experimental animals with

nephrotoxic nephritis sera (Baxter and Goodman 1956; Ortega and Mellors 1956). Animals immunised with heterologous or homologous G.B.M. preparations who developed nephritis, also had characteristic linear deposits of IgG on frozen kidney sections examined by fluorescent method (Steblay 1962a; 1962b; 1963; 1966b; Steblay and Rudofsky 1968a; Unanue and Dixon 1967a; Unanue et al 1967b; Lerner and Dixon 1966; Paronetto and Koffler 1967).

In humans, immunofluorescent studies have demonstrated the presence of smooth, linear, continuous, uniformly thin (approximately the width of the G.B.M.) deposits of IgG in patients with Goodpasture's syndrome (Duncan et al 1965; Scheer and Grossman 1964; Beirne et al 1968; Hayslet et al 1971). Linear deposits of antibodies have also been found on B.M.'s of alveolar septa in Goodpasture's syndrome (Sturgill and Westervelt 1965; Berger et al 1969; Lerner et al 1967; Markowitz et al 1968; Koffler et al 1969; Poskitt 1970). While the presence of linear deposits had been established before when Duncan et al (1965) presented the cases of Goodpasture's syndrome with linear deposits along the G.B.M., the pathogenetic significance of linear deposits and their identification with anti-G.B.M. antibodies became apparent following the work of Lerner et al (1967). Such linear immunofluorescent staining pattern has come to be regarded as a morphologic indicator of nephritis mediated by anti-G.B.M. antibodies, and is interpreted to signify deposition of auto-antibodies which react with G.B.M. (Unanue et al 1967b; Lerner et al 1967; Steblay and Rudofsky 1968b; Hardman et al 1967). Elution experiments and transfer experiments provided the definite proof for the pathogenetic role of anti-G.B.M. antibodies in human GN. Linear deposits of IgG in humans are typically

found in Goodpasture's syndrome and some other patients with proliferative nephritis. Linear deposits are also found in diabetic nephropathy and lupus nephritis, but these conditions are not associated with circulating anti-G.B.M. antibody, and anti-G.B.M. antibody has not been eluted from the diseased kidney (Gallo et al 1968; Churg and Grishman 1972). Only occasionally, linear deposits were observed along tubular basement membranes (Berger et al 1963; Berger and Galle 1963). Sequential renal biopsies have revealed disappearance of both the immunoglobulins and ultrastructural deposits as clinical recovery ensues.

The third component of complement (C3) is usually found together with IgG in linear immune deposits along the G.B.M. and is indicative of complement fixation by the anti-G.B.M. antibodies (Duncan et al 1965; Lerner et al 1967; Beirne et al 1968; Markowitz et al 1968; Koffler et al 1969). However, in a review of cases of Goodpasture's syndrome, Martinez and Kohler (1971) found approximately 30% of reported cases without detectable C3 deposits (Lewis et al 1970; Poskitt 1970). Gallo(1970) studied 5 cases with glomerular IgG deposits in vivo in linear pattern and showed, with elution experiments, that linear pattern of IgG without C3 deposition may not represent specific antibody to G.B.M. antigens. It has been suggested that an absence of IgG_3 subclass anti-G.B.M. molecules and relative increase of IgG_2 and IgG_4 which fix complement poorly or not at all, could explain the lack of C3 fixation Lewis et al 1970).

True linear deposits are often unrecognisable on electron microscopy as they do not form a distinct layer in the capillary wall, but are located in the G.B.M. (Churg and Grishman 1972; Gray et al 1966). The G.B.M.

showsmoderate, sometimes considerable, diffuse increase in density. Using the immunoferritin technique, Andres et al (1970) have demonstrated that the anti-G.B.M. antibody is deposited in and presumably bound to the inner, subendothelial half of G.B.M.

In contrast to anti-G.B.M. mediated GN, kidneys from patients with immune complex nephritis show granular, interrupted, lumpy depsoits of IgG and C3 along the subepithelial side of G.B.M. (Germuth and McKinnon 1957; Guttman et al 1967; Kirshnan and Kaplan 1967; Koffler et al 1967; Morel-Maroger et al 1970). On electronmicroscopy examination, immune-complexes can be visualised as subepithelial deposits along the G.B.M. (Feldman 1958).

1.344. Indirect immunofluorescence

Lerner et al (1967) described an indirect immunofluorescent assay for the detection of circulating anti-G.B.M. antibodies. Sera of 75 patients with GN were diluted 1:1 with saline, laid over normal human, chimpanzee or rat kidney, washed and stained with fluorescent anti-human IgG. They found the presence of anti-G.B.M. antibodies in sera of 6 patients. Using similar assay, Paronetto and Koffler (1967) demonstrated the presence of anti-G.B.M. antibodies in actively immunised animals given injections of G.B.M. In a large series of patients, McPhaul and Dixon (1969a) found circulating anti-G.B.M. antibodies only in patients in whom direct immunofluorescent studies showed linear deposits of immunoglobulin. Despite the sensitivity of the technique, only 40% of patients known to have this type of GN were shown to have circulating anti-G.B.M. antibodies.

In the present study, we have used the indirect immunofluorescent assay for the detection of anti-G.B.M. antibodies in some patients with GN, in an attempt to determine the correlation between passive haemagglutination method and other methods for the detection of circulating anti-G.B.M. antibodies.

1. 345. Elution of antibodies

Acid elution of homogenates of kidneys from numerous patients with anti-G.B.M. nephritis yielded anti-G.B.M. antibodies, and has made possible the characterisation of these antibodies, also the determination of their specificity and pathogenicity (Lerner et al 1967; McPhaul and Dixon 1970a).

Lerner et al (1967) studied eluted antibodies by IEP and showed that they consisted of IgG and in some cases IgG + IgA. Mc Phaul and Dixon (1970a) demonstrated by immunodiffusion studies that in eluates of kidneys from patients with Goodpasture's syndrome, IgG types 1, 2 and 3 were represented in normal proportions. In the case of Poskitt (1970) the anti-G.B.M. antibody eluted contained both kapa and lambda light chains and IgG subgroups 1, 2 and 4, but was deficient in IgG_3. In this patient, neither C_{1q} nor C3 were present on G.B.M., but the eluted antibody fixed complement in vitro with purified G.B.M.

Human IgG subgroups differ in the structure, concentration in serum, synthetic and catabolic rates and in certain biologic properties (Natvig et al 1967; Fahey and McKelvey 1965; Morell et al 1969). The IgG subgroups differ in the ability to fix complement: IgG_1 and IgG_3 fix complement well,

IgG_2 fixes complement poorly, and IgG_4 foes not fix complement (Ishizaka et al 1967; Müller-Eberhard and Calcott 1966).

The pathogenicity of eluted anti-G.B.M. antibodies was established by transfer experiments. From kidneys observed to have linear fixation of host IgG by direct immunofluorescence, anti-G.B.M. antibodies have been eluted and transferred passively to normal heterologous recipients, causing an immediate glomerulonephritis (Lerner et al 1967; Koffler et al 1969; Lerner and Dixon 1968a; Poskitt 1970). It seems that antibodies eluted from kidneys with Goodpasture's syndrome tend to fix more frequently to non-glomerular antigenic sites than antibodies eluted from anti-G.B.M. mediated nephritis other than Goodpasture's syndrome. Eluted antibodies fix strongly to sections of normal kidney in vitro (Lerner et al 1967). A discrete linear, localisation of eluted globulin among G.B.M., Bowman's capsule and tubular basement membranes of kidney sections was found (Mc Phaul and Dixon 1970a). By contrast to anti-G.B.M. antibodies, eluted antibodies from patients with lupus nephritis contained IgG antibodies with no affinity to G.B.M. but directed against nuclear antigens (Krishnan et al 1967; Koffler et al 1967; Gallo 1970).

1.4. Cell mediated allergic response to G.B.M.

1.41. Historical review

The pathogenetic role of humoral immune mechanisms in the pathogenesis of experimental and human glomerulonephritis is well established. The technical basis for in vitro demonstration of humoral immunity was early established, the methods being developed and refined (Waksman 1971).

The consequence of this is that the knowledge of allergic states has for a long time been based predominantly on the humoral type (Søborg and Bendixen 1967a). But, to explain a hypersensitivity state, it is necessary to make a total estimation of both humoral and cellular allergy. In addition, humoral reactions to renal antigens do not explain the progressive course of some human glomerulonephritides over months or years, apparently independent of the initial immune injury. To explain the pathogenesis of progressive glomerulonephritis, theories of autoimmunity have been advanced and have gained some experimental support (Bendixen 1968). According to them, cellular as well as humoral allergic mechanisms may be considered instrumental in the development and course of progressive renal damage (Bendixen 1968). Infiltrates of lymphocytes and monocytes throughout the renal cortex in some cases of glomerulonephritis also support the suggestion that cell-mediated allergic response may be pathogenetically significant in GN.

The existence of type IV allergic reactions (the terms delayed hypersensitivity, cell-mediated allergic response and cell-mediated immunity will be used interchangeably) has been known since the time of Jenner and Koch, but evaluation of its importance in health and disease has only recently become possible. The focus of this review will be in its assessment of the cell-mediated allergic reactions, especially in relation to renal antigens.

Analogously to tuberculin hypersensitivity, skin tests with kidney antigens have been carried out with inconclusive results. Such methods may be hazardous, and there is a risk of producing an undesirable effect

in transplantation studies, as the prospective recipient may be sensitised to the donor and may (in theory) provoke the formation of anti-G.B.M. antibodies.

An in vitro technique for evaluation of cellular hypersensitivity enables the investigation of specifically sensitised cells and recording their reactivity. Attempts to understand the mechanism of cell-mediated immunity by tissue culture methods were pioneered by Rich and Lewis (1932). They examined migration of leucocytes and spleen cells from tuberculin sensitive guinea pigs in the presence of tuberculin in tissue culture (T.C.) medium. The technique has been developed and used in similar experiments with macrophages, spleen cells, peritoneal exudate cells, lymph node cells and peripheral leucocytes (Darlington and Scherago 1960; Svejcar and Johanovsky 1961a; 1961b; George and Vaughan 1962a; Carpenter 1963; David et al 1964a). Besides tuberculin, other antigens have been tested: brucella bacteria (Heilman 1963), histoplasmin (Johnson and Scherago 1960), streptococci (Morn 1936), tumour antigens (Field and Caspary 1970b), thyroglobulin (Field et al 1970a).

The technique described by George and Vaughan (1962a) and later modified by David et al (1964a; 1964b) provided a simple, reproducable and quantitative in vitro system for evaluation of cellular hypersensitivity state in vivo. In this method, white cells are transferred to a capillary tube which is placed in a chamber filled with tissue culture medium. The cells migrate from the tube into the medium. The cells can be cultured in the medium alone, or in the medium plus the antigen. The migration area is then measured by planimetry and the migration index (M.I.) is calculated on the basis of measures of the cell migration area in the culture with antigen divided with the area of migration in the absence of antigen.

The most important *in vitro* system studied by this technique was the migration of peritoneal macrophages from hypersensitive guinea pigs in the presence of specific antigen. In this system, the lymphocyte possesses the immunologic information, the macrophage serving as an indicator cell that migrates (Bloom and Glade 1971). Antigen induced migration inhibition or production of migration inhibitory factor (MIF) has also been studied in other animals and found to correlate well with skin hypersensitivity (George and Vaughan 1962a; Johnson and Scherago 1960; Carpenter 1963; David et al 1964a; Thor et al 1968; Weir and Suckling 1971).

Thor et al (1968) and Rocklin and David (1970b) demonstrated that mediators produced by the incubation of sensitised human peripheral leucocytes with the specific antigen, could be used to inhibit the migration of guinea pig macrophages. The test is a useful *in vitro* correlate of delayed hypersensitivity (Lochskin 1969), is positive in patients with agammaglobulinemia who possess type IV sensitivity, and negative in patients with DiGeorge syndrome who do not exhibit delayed hypersensitivity (Bloom and Glade 1971). Inhibition of macrophage migration has been found to correlate well with skin hypersensitivity in humans, although the performance of each test requires up to 7 days. In this study, the test will be referred to as an indirect MIF assay. In 1967, Søborg and Bendixen (1967a) described an *in vitro* test based on the inhibition of migration of human peripheral blood leucocytes in the presence of specific antigen. This test is termed the direct MIF assay and was first used in patients with skin hypersensitivity to brucella antigens. Buffy coat cells from brucella-positive patients were allowed to migrate from capillary tubes in the presence and absence of killed organisms; antigen

specific inhibition of the migration of buffy coat cells was found to correlate well with skin hypersensitivity, but not with antibody (Søborg 1967b). This system was found to be simple, reliable and reproducible (Field et al 1971a; 1971b) and was applied to detect hypersensitivity to other antigens (Bendixen 1967; Søborg and Halberg 1968a; Lundgren et al 1968; Clausen and Søborg 1969; Nerup et al 1969; Field et al 1970a; Field and Caspary 1970b; Field and Caspary 1971a; Field and Caspary 1971b). The direct MIF assay has already been employed to investigate microbial infections, autoimmune states, connective tissue diseases (Bendixen and Søborg 1969; Williams et al 1970; Maini et al 1970; Nerup et al 1969), transplantation immunity (Smith et al 1969; Falk et al 1969), clinical allergic states (Brostoff and Roit 1969), sarcoidosis (Caspary et al 1971), chronic hepatitis (Vergani et al 1972), Grave's disease (Field et al 1970a), colon antigens in ulcerative colitis and tumour antigens (Bendixen and Søborg 1970). When applied to the tuberculin system, the test was successful in many instances (Mookerje et al 1969; Rosenberg and David 1970; Federlini et al 1971). However, several groups have reported their inability to successfully use this technique to demonstrate delayed hypersensitivity to soluble tuperculoproteins antigens (PPD) (Kaltreider et al 1969; Lockshin 1969). But, with minor modifications of the technique, Rosenberg and David (1970) and Federlini et al (1971) demonstrated that the test correlated excellently with the Mantoux reaction. It was found that the direct assay responds particularly well to semisoluble and particulate antigens, derived from human tissues and microbial preparations (Federlini et al 1971).

Besides the difficulties some groups (Kaltreider et al 1969; Lockshin 1969) have experienced in correlating the results *in vitro* with direct cutaneous testing other difficulties have been reported; because neutrophils are required for migration inhibition (Søborg 1969; Clausen 1970) and as some classes of immunoglobulins, especially IgG bind firmly to neutrophils (Ishizaka et al 1967) there is a possibility that antibodies, specially cytophilic antibodies (Amos et al 1967; Heisse et al 1968) might influence the results (Bloom and Glade 1971). Various experiments indicate that lymphocytes from an organism with cellular hypersensitivity have a marked, specific affinity for the corresponding antigen, but this observation does not preclude the existence of cytophilic antibodies (Søborg and Bendixen 1967a). But leucocyte migration inhibition has been clearly demonstrated in guinea pigs lacking cytophilic antibody (Bloom and Glade 1971). There is also a possibility that inhibition of migration may be produced by causes other than delayed hypersensitivity, for example antigen-antibody complexes (Bloom and Bennet 1966; Spitler and Lowrence 1969b; Spitler et al 1969a) or aggregated gamma-globulin (Christian 1960).

In addition, it has been shown that, in contrast to type I allergy and reactions involving antibody which are hapten specific, the migration inhibition tests can be shown to be carrier specific (David et al 1964b; David and Schlossman 1968; Schlossman et al 1966; Marcus 1970).

The available evidence therefore indicates that the measurement of changes in migration activity of human peripheral leucocytes in the presence of specific antigen provides an *in vitro* assessment of cellular hypersensitivity (Falk et al 1969). The direct assay was therefore employed

in the present study for the assessment of cellular allergic response to G.B.M. antigens in patients with glomerulonephritis. First, the participating cells and mediators will be considered.

1.42. Participating cells and mediators

At cellular level, both direct and indirect assay require participation of thymus derived lymphocytes, cell populations depleted of lymphocytes show no inhibition of migration with specific antigen (Søborg 1969; Cohn 1968; Federlini et al 1971; Pick 1972). The cells responsible for the production of factor(s) that inhibit migration of macrophages (in indirect assay) or mononuclear cells and PMN (in direct assay) are the small lymphocytes (Bloom and Glade 1971). As few as 1% of sensitised lymphocytes were sufficient to inhibit the migration of the population of normal, non-sensitised cells. There is disagreement in regard to the cells which are inhibited in their migration in the direct assay: some think that the target cells are mostly mononuclear (Rosenberg and David 1970; 1971) while others believe that the inhibited cells are granulocytes (Federlini et al 1971; Egeberg et al 1972).

The change in the migration which occurs after antigenic confrontation is in the whole cell population, not only in those cells which have the ability to recognise antigen specifically. This observation has led to the hypothesis that immunologically committed cells release an active substance which inhibits the migration of surrounding cells (Falk et al 1969). A number of biological activities are associated with factors released by stimulated lymphocytes. These include: migration

inhibitory factor (MIF), chemotactic factor which affect macrophage, lymphotoxin and growth inhibitory factors, blastogenic factors and factors affecting lymphocytes, interferon, antibodies and skin-reactive factors (Bloom and Glade 1971). Rosenberg and David (1971) demonstrated that supernatants of purified human lymphocytes incubated with specific antigen contain soluble mediators which can cause inhibition of leucocyte migration of non sensitised leucocytes. Others have demonstrated that an active and non-dialysable migration inhibitory substance may be obtained from sensitised human buffy coat cells, incubated with PPD, whether or not PMN are present (Thor et al 1968; Rocklin 1969).

1.43. Cell-mediated immunity in renal diseases

In vivo attempts to study cell-mediated immunity in patients with glomerulonephritis by intradermal testing with renal tissues as antigen have been inconclusive (Wagner and Prokop 1956) and this approach abandoned because of potential hazard of sensitisation. Attempts to transfer renal disease to normal animals by means of whole leucocytes (Pfeiffer et al 1962) or lymph node cells (Heymann et al 1962; Hess et al 1962; Litwin et al 1971) and by means of parabiosis experiments have been successful. However, the interpretation of these experiments is difficult as there is a possibility that the transferred cells included cells capable of antibody production (Glassock et al 1969).

The evaluation of cellular hypersensitivity directed towards antigenic components of renal parenchyme in patients with GN has been limited by the lack of suitable in vitro methods. The development of the indirect MIF assay (Thor et al 1968; Rocklin and David 1970b) and the direct MIF

assay (Søborg and Bendixen 1967a) initiated a number of studies in vitro cellular allergic response to kidney antigens in patients. Cellular hypersensitivity to human foetal kidney homogenate was investigated by Bendixen (1968) by the direct assay. He found antigen induced inhibition of cell migration in patients with glomerulonephritis. Rocklin et al (1970c) using the indirect MIF assay obtained positive results in patients where immunofluorescent studies suggested anti-G.B.M. antibody mediated GN. In these early studies the patients had not been categorised according to modern histological criteria. Recently Mallick et al (1972), using the direct assay and foetal kidney homogenate supernatant as an antigen, demonstrated the inhibition of cell migration in patients with the "minimal change" steroid sensitive nephrotic syndrome, in most patients with diffuse or focal glomerular lesions, and in approximately half of a group of patients with epimembranous or membrano-proliferative changes. Recently Mahieu et al (1971; 1972) using the direct assay and purified G.B.M. an antigen, were able to demonstrate the presence of cell-mediated immunity in patients with severe proliferative GN or nephropathies with major vascular lesions.

The direct assay was used for studies of cellular hypersensitivity to renal antigens in several groups of patients after renal transplantation. These studies showed evidence of cellular hypersensitivity to kidney antigens during rejection episodes and also following the infarction of the graft (Falk et al 1969; Weeke et al 1970; Ellis et al 1971; House et al 1972).

As the exact nature of the mechanisms involved in progressive human GN is still undefined, there is the additional possibility that altered cellular reactivity to streptococcal membrane antigens may play a

pathogenetic role. This hypothesis initiated the investigations by Zabriskie et al (1970) who, using the direct assay, demonstrated that lymphocytes of patients with progressive GN were sensitised to particulate streptococcal membrane antigens. Patients with unrelated renal disorders failed to respond to the streptococcal antigens. The antigens used were from type 12 and type 5 streptococci. The degree of inhibition in both types was similar and significant, although type 5 is not nephritogenic. Dardenne et al (1972) found sensitivity to streptococcal membrane antigens in 46% of cases of chronic proliferative GN, while non-proliferative glomerular lesions and control cases did not show hypersensitivity. The above results are not unexpected in view of the known cross-reactivity between group A streptococcal membranes and human G.B.M. (Markowitz and Lange 1964; Holm 1967). We also decided to test the allergic response to streptococcal antigens in parallel with the assessment of cellular hypersensitivity to G.B.M. antigens, in the same group of patients, and to correlate the results obtained with the histological and immuno-fluorescent finding.

2. MATERIAL AND METHODS

2.1. G.B.M. Isolation and characterisation

Pooled kidneys from patients who died with no history of renal disease were used. Kidneys were frozen and stored at - 20°C until used.

2.11. Preparation of glomeruli

The isolation procedure for preparation of glomeruli was based on the method of Krakower and Greenspon (1951) and modifications by Spiro (1967a) and Westberg and Michael (1970). All equipment was sterile and all material was handled with sterile gloves. During the entire procedure care was taken to keep the tissue on ice as much as possible. The capsule was stripped and the kidneys were sliced approximately 5 mm in thickness. The medulla was dissected away from the cortex and discarded. The weight of the kidney and of the cortex were recorded. The cortex was cut into very small pieces with scissors.

The disruption of the renal cortex and separation of glomeruli was achieved with the use of sieves. Stainless steel sieves (Fig. 1) of

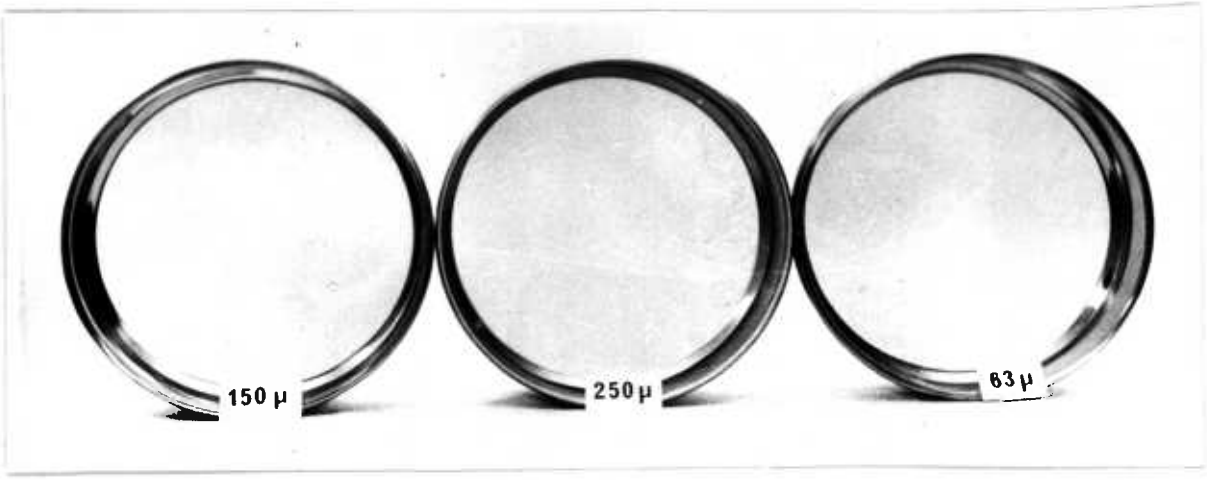


Figure 1

three different pore sizes were placed one on top of the other, the Buchner funnel of slightly greater diameter was used as the support and was placed on the receiving pan (10 litre volume) which was kept on ice (Fig. 2). Portions (20 g) of the cortex were transferred to the top sieve (150 microns pore size). The tissue was forced through the sieve with a moderate pressure using the bottom surface of a small beaker, and repeated washings with cold 0.85% sodium chloride solution, 0.5 - 1 litre for one kidney. The pressing was continued for about 30 minutes per application. Only the portion of the cortex readily sieved was collected and the material remaining on the top surface of the sieve was discarded. A clean sieve was used for each application of the cortical material.

Figure 2

The middle sieve (pore size 250 microns) retained a few bigger tissue fragments; the material retained on the third sieve (pore size 63 microns) was continually washed with cold 0.85% sodium chloride solution until nothing but glomeruli free of cells and tubular fragments could be seen when the sample was examined by the phase contrast microscopy. The material which emerged into the receiving pan was poured once again through the 63 micron sieve in order to collect more glomeruli, and the continued washings of the retained material repeated. The material retained on 63 micron pore size sieve was then transferred by suspension in 0.85% sodium chloride solution to the 250 ml centrifuge tubes and centrifuged at 1500 rpm for 10 minutes. The supernatant was removed by suction and the sediment examined under the phase contrast microscope. The sediment was washed four times in distilled water with centrifugations at 1500 rpm for 10 minutes and lyophilised.

2.12. Preparation of G.B.M.

The final glomerular sediment was suspended in ice-cold 1 M sodium chloride (approximately 1:20 v/v). Sonic disruption was performed on portions of 40 ml in 50-ml tall type beaker immersed in an ice bath. MSE Ultrasonic Disintegrator Model 150 W was used with a 0.5-in. stainless steel probe. The sonicator was set at no. 6 power setting and used in 2 minute bursts with 2 minute cooling time being allowed between each of the sonic bursts. The extent of glomerular disruption was followed under the phase microscope, and a total sonication time of 10 minutes was required for complete fragmentation of glomeruli.

The ultrasonically treated suspension was then passed through a sieve (63 microns pore size) to remove insufficiently disrupted glomeruli. The filtrate was transferred to 50-ml centrifuge tubes and centrifuged for 10 minutes at 3000 rpm. The sediment was then washed five times with ice-cold 1 M NaCl, followed by five washings with ice-cold distilled water. The sediment was then lyophilised. DNA analysis was performed on a sample of G.B.M. by the dephenylamine reaction (Burton 1956) and RNA determined by orcinol reaction for ribose (Brown 1946) in hydrolised membrane (Volkin et al 1954).

In order to examine the effect of prolonged sonic disruption on solubilisation of G.B.M., the total sonication time was prolonged for 20 minutes. The supernatant of this suspension was collected and tested for the presence of soluble G.B.M. antigens.

2.13. Solubilisation of G.B.M.

G.B.M. was solubilised by proteolytic enzyme (collagenase, Sigma Chemical Company, St. Louis, U.S.A.) according to the method of Spiro (1967a), except that incubation with the enzyme was prolonged to five instead of three days. The digestion was performed in 0.1 M Tris acetate buffer, Ph 7.4 in the presence of 0.005 M calcium acetate at 37°C. The basement membrane was suspended in tris buffer (25/mg/ml). The collagenase was added initially to equal 0.7% of the basement membrane. at 24, 48, 72 and 96 hours, further additions of the enzyme, equal to 0.35%, 0.10%, 0.10% and 0.10% of the weight of G.B.M. respectively, were added. A crystal of thymol was added at the beginning of the incubation to prevent the bacterial growth. During the total period of incubation (120 hours) the mixture was constantly stirred. At the end of incubation, the material was centrifuged at 2000 rpm for 15 minutes. The supernatant

containing solube G.B.M. was removed and heated at 60°C for 30 minutes. The concentration of soluble G.B.M. in the supernatant was determined by the folin phenol reagent (Lowry et al 1951).

2.14. Immunochemical characterisation of G.B.M.

2.141. Rabbit anti-human G.B.M. serum

Male, New Zealand white rabbits, weighing 2 - 2.5 kg were used. Antisera were prepared in the rabbits against the following antigens: (1) intact human G.B.M. (4 rabbits immunised) and (2) a soluble fraction obtained after collagenase digestion (2 rabbits immunised). 10 ml of blood (for control purposes) was withdrawn from each rabbit prior to immunisation. The antigen in saline (2 mg/ml) was emulsified with complete Freund's adjuvant (DIFCO) in the ratio 1:2 v/v. Approximately 1 - 1.5 ml of emulsion was given at each injection. The first injection was given in the footpads. After three weeks a second injection was given intramuscularly, and three subsequent injections were given intramuscularly at two-weekly intervals. The animals were bled one week after the last injection. Blood was collected by cardiac puncture, allowed to clot at room temperature for three hours and serum separated. The sera were pooled and stored at - 20°C. After sacrifice, frozen rabbit kidneys were stained with fluoresceinated anti-rabbit IgG.

Antisera were absorbed with lyophilised normal human plasma (5ml/5mg), incubated for three hours at 37°C and allowed to stand overnight at 4°C, centrifuged at 1800 x G for 30 minutes. Sodium azide was added to prevent bacterial growth. One batch of antiserum was absorbed by passing through

the column of CNBr activated Sepharose 4B coupled to normal human plasma.

The specificity of the antiserum was investigated by indirect immunofluorescence method. Frozen sections of normal human kidney were incubated with the rabbit anti-human G.B.M. antiserum, washed and stained with fluoresceinated anti-rabbit IgG. Controls included normal kidney sections without previous incubation with anti-G.B.M. serum and pre-incubation of antiserum with human G.B.M.

2. 142. Double diffusion

Double diffusion in gel method of Ouchterlony (1953; 1958) was used. 1.5% agarose (B.D.H.) in saline was melted, cooled to about 60°C, sodium azide to the concentration of 0.02% was added, and plates poored immediately. Glass plates 2" x 2" were used, and 3.4 ml of agarose was added. The antigen, soluble G.B.M. in concentration of 2 mg/ml in saline was used. The antiserum, rabbit anti-human G.B.M. serum was used undiluted. After adding the antigen and the antiserum into the separate wells in the gel, the plates were kept at room temperature in a humid chamber for three days.

2.143. Immunoelectrophoresis

Immunoelectrophoresis was done according to the method of Schediegger (1955) - an adaption of the method of Williams and Grabar (1955). Soluble G.B.M. was used in a concentration of 2 mg/ml in saline, and the rabbit anti-human G.B.M. serum was used undiluted. The buffer was Na barbital/ barbitone 0.075 M, pH 8.6, diluted 1:2 for use in the electrophoresis tank

and in the preparation of agarose. Agarose (B.D.H.) solution was prepared by dissolving 1.5 gm of agarose in diluted buffer to give 1.5% solution. The agarose/buffer mixture was boiled in a water bath till the solution was clear. 2.5 ml of hot agarose was poured on a standard 3" x 1" slide. A 2 mm diameter hole was cut using a steel cutter. The slides were run from negative to positive pole. The voltage was adjusted to give constant readings at 6 v/cm, the slide was run at the constant voltage for 45 minutes, then a trough in gel, 2 mm wide, was cut, and the antiserum added. The plates were incubated for 1 - 3 days at room temperature in a humid chamber.

2.144. Antigen-antibody cross-electrophoresis

Antigen-antibody cross-electrophoresis technique of Laurell (1965) was used. The first dimension run was performed by the same method as described for IEP. After the first dimension run, gel containing fractionated protein was cut with a razor blade, 0.5" wide, 2" in length, and placed on a side of a 2" x 2" glass slide.

A modification of the technique was used for the second dimension run (modification used by André de Bats, St. Helier Hospital, Surrey, U.K.) as follows: 3.4 ml of agarose was added to three tubes containing 0.12, 0.10, and 0.08 ml of antiserum. The hot mixture was poured on to three glass 2" x 2" slides. The agarose was allowed to set. After setting, a slip 0.5" wide and 2" in length was cut in agarose, and placed on the slide next to the slip containing the antigen. Next to the antigen, the slip containing the highest concentration of antiserum was placed, then two

others, in decreasing order of concentration (see fig 7). The plate was run at 3 v/cm for 16 hours and read under indirect light or stained.

After immunodiffusion, IEP and cross-electrophoresis, the slides were washed extensively in saline and distilled water for at least 24 hours and dried under the filter paper in a 37°C oven. The dried slides were stained with a 1% amido black solution in 7% acetic acid for 5 minutes. The slides were destained in methanol/acetic acid (9:1) until the background was clear, and photographed.

2.145. Fractionation of G.B.M. antigens

Columns of Sephadex G-150 were used for the fractionation of the collagenase digested human G.B.M. A standard column (K 26/100, Pharmacia, Uppsala, Sweden) 2.6 x 100 cm was packed to a height of 75 cm with Sephadex G-150. The samples, 5 ml of 20 mg/ml concentration of G.B.M. in the buffer, were applied to the column. The columns were equilibrated with 0.1 M tris/acetate buffer + 0.6 M NaCl + 0.02% NaN3, pH 7.4. Elution was achieved with the same buffer at the rate of 16 ml/hr, and fractions of 2.65 ml were collected and monitored on the LKB Uvicord II at 280 mμ and 254 mμ for 40 hours. The approximate molecular weight of the fractions was determined by chromatographing blue dextran, ^{125}I labelled bovine serum albumin (BSA) on the same column. Aliquots of the fractions were analysed in gel diffusion (Ouchterlony) and in cross-electrophoresis against rabbit anti-human G.B.M. serum. The fractions in each elution peak were marked and pooled.

For further fractionation of the material emerging with the void volume from Sephadex G-150 column, a column (1.5 x 90 cm) of Sepharose 6B was used. The first peak, which was excluded from Sephadex G-150, was further fractionated on Sepharose 6B in the same buffer. Aliquots of the fractions were analysed in gel diffusion (Ouchterlony) and cross-electrophoresis with rabbit anti-human G.B.M. serum. The flow rate of Sepharose column was adjusted to 16 ml/hr, the sample 10 mg of protein from peak A, from Sephadex G-150 column in 1 ml of buffer.

2.2. Patients

A group of patients from the Renal Unit, Hammersmith Hospital, were chosen for the study. The diagnosis was confirmed by light microscopic and immunofluorescent examination of 14 renal biopsies. The histology and immunofluorescent examination of renal biopsies was routinely undertaken by Dr. D. Evans, Senior Lecturer in Pathology, Royal Postgraduate Medical School, Hammersmith Hospital. For light microscopic examination, specimens were fixed in formalin, embedded in parafin, cut at 2 - 3 μ in thickness, stained with haematoxylin and eosin, Martius scarlet blue and periodic acid/silver methamine. The immunofluorescent method used was the standard method (Coons and Kaplan 1950). Monospecific fluorescinated antisera to human IgG, C3, IgA and IgM were obtained from the Wellcome Laboratories.

Some sera were obtained from patients at other London hospitals and l'Hopital Tenon, Paris.

2.3. Detection of circulating anti-G.B.M. antibodies

2.31. Indirect immunofluorescence

Indirect immunofluorescent examination of frozen normal human kidney sections for IgG fixation in vitro from serum of patients with glomerulonephritis, was made by slight modification of the technique of McPhaul and Dixon (1969a). Normal human kidney sections were processed as for the direct immunofluorescence (cut in the cryostat, fixed in dry acetone, washed in phosphate buffered saline - PBS) and sera of patients with linear deposits of IgG and control subjects (granular deposits, normal human sera) were incubated with the kidney slices for 60 minutes, washed extensively in PBS, incubated with FITC anti-human IgG, washed and mounted.

2.32. Double diffusion

Gel prepared of 1.5% Agarose in saline and 0.02% NaN_3 was used. The sera of patients with linear deposits of IgG in vivo on direct immunofluorescent examination of renal biopsies were used undiluted. The antigen used was collagenase solubilised G.B.M. at concentrations of 1, 2, 3, 4 and 5 mg/ml in saline.

2.33. Passive haemagglutination tests

The passive haemagglutination (P.H.) tests were carried out in Microtiter plates (FLOW LABORATORIES) and the other equipment was from standard micro-haemagglutination kit, Flow Laboratories.

Group "O" Rh-negative red blood cells were used. The blood was collected in acid citrate dextrose solution (20 ml/4 ml) and well meixed. R.b.c. were washed three times with saline. The sera tested were from patients with various renal and non-renal diseases, normal persons, and rabbit anti-human G.B.M. serum. Sera were decomplemented by heating at 56°C for 30 minutes. They were subsequently absorbed with human washed red blood cells (3 vol. of serum per 1 vol. of r.b.c.) by contact for 2 hours at room temperature.

G.B.M. antigens, soluble, collagenase digest, was used in concentration of 2 mg/ml in saline. This concentration was found to be optimal after testing different concentrations of antigen.

The tanned red cell agglutination technique of Boyden (1951) modified by Marder (1971) was used. Cells were suspended in 200 ml of phosphate buffered saline to give 2% suspension. 200 ml of tannic acid solution (1:40,000 in saline was mixed with the cell suspension, incubated at room temperature for 15 minutes, washed three times in citrate buffer (pH 7.2). To the tanned red blood cells suspension, 200 ml of citrate buffer containing 40 mg of solube G.B.M. was added, incubated at room temperature for 1 hour, washed in citrate buffer three times, and at the end, coated cells were used as 2% suspension in citrate buffer for antibody titration.

Chemical coupling of G.B.M. antigens to r.b.c. by means of bisdiazotised benzidine (BDB) was performed according to the technique of Stavitsky and Arquilla (1955). 0.23 of benzidine was dissolved in 45 ml of 0.2 N HCl. The mixture was cooled in an ice bath. The whole procedure

was carried out in a cold room (+4°C). 0.175 g of $NaNO_2$ was dissolved in 5 ml of distilled water, cooled, and added to the benzidine solution slowly, with constant stirring. After 30 minutes 2 ml of the reagent was pipetted to a small vial. The vials were kept at -180°C (stored in liquid nitrogen). To 3 ml of solution of G.B.M. (2 mg/ml) 0.1 ml of 50% suspension of well packed washed r.b.c. was added. The content of a vial of BDB was thawed, diluted 1:30 in PBS, and one ml of the diluted BDB was added to the antigen and cells suspension, incubated at room temperature for 15 minutes. The mixture was then centrifuged at 4°C, the supernatant removed and cells washed three times in PBS. The coated cells were finally used as 2% suspension for antibody titration.

Coupling of G.B.M. antigens to r.b.c. was also performed by chemical linkage with chromium chloride using the technique of Lavergne et al (1965). The 0.1 ml of thrice washed packed erythrocytes were added to 0.5 ml of G.B.M. solution (2 mg/ml in saline), incubated for 5 minutes at room temperature, and gently stirred. Then 0.3 ml of 0.05% $CrCl_3$ solution in saline was added and suspension was incubated at room temperature for 15 minutes. After incubation the reaction mixture was treated in the same way as above, i.e. after washing used as 2% r.b.c. suspension for antibody titration in micro-haemagglutination system.

The technique described by Avrameas et al (1969a; 1969c) was specially modified for coupling of G.B.M. antigens to r.b.c. with glutaraldehyde (GA). GA was obtained as 26% aqueous solution from Tubb Laboratorities, and was used without further purification. For use in the actual test, it was diluted to 2.5% solution with saline. R.b.c. used were the same as in other P.H. procedures, i.e. human, group "O" Rh-negative, washed three times in saline. The sera tested were sera from patients with various kidney diseases, sera from normal subjects and rabbit anti-human

G.B.M. serum. Sera were previously decomplemented (30 minutes at 56°C) and absorbed with r.b.c. (3:1 v/v). To 1.25 ml of saline containing G.B.M. antigens (2 mg/ml conc.) 0.05 ml of washed, well packed erythrocytes was added. The suspension was incubated at room temperature for 5 minutes. While gently stirred, 0.25 ml of 2.5% GA solution was added. The stirring was continued for one hour at room temperature and centrifuged. The packed, sensitised cells were washed two times in saline. The following procedure was the same for all four P.H. techniques used:

The third washing of cells was performed in a stabilising serum. We used 0.5% rabbit serum in saline (Atkin 1909; Fulthorpe 1957; Gill 1964). Finally the cells were suspended in 5 ml of 0.5% rabbit serum saline to give 1% cell suspension (in tanned-cell procedure, $CrCl_3$ and BDB the 2% cell suspension was used). For the haemagglutination test, serial dilutions of serum were placed in the wells of a microtitre plate. Dilutions of serum were made by mixing equal parts of serum and 0.5% rabbit serum saline (0.05 ml/0.05 ml) in the wells of microtitre plates. To 0.05 ml of serum (and various dilutions of it), 0.025 of 1% suspension of sensitised cells was added. The plate was sealed and gently agitated for 5 minutes. After incubation period of three hours at room temperature, the agglutination was registered. Usually the plates were also read after 12 hours. A smooth matt of the cells at the bottom of the wells was designated as positive (+) and a "button" of cells in the centre of the wells as negative (-). The end point of the antibody titre was taken to be the highest dilution of serum causing complete agglutination of the cells, and the titre of the serum was expressed as reciprocal of the initial dilution of the serum.

Controls consisted of: (1) sensitised cells and normal serum; (2) cells sensitised with an heterologous protein (B.S.A.) or lyophylised normal human plasma and serum; (3) sensitised cells and saline; (4) non sensitised cells and serum; (5) positive sera absorbed with G.B.M. and tested again with sensitised cells.

2.34. Elution of antibodies

The method used in elution procedure was a modification of previously described methods (Lerner et al 1967; Krishnan 1967; Koffler et al 1967; Kitagawa et al 1967; McPhaul and Dixon 1970a).

Kidneys were obtained at autopsy from a patient with Goodpasture's syndrome. Detailed clinical and other data concerning the patient are presented elsewhere (Peters and Evans 1972). Prior to elution, a piece of the tissue was prepared for fluorescent studies by snap freezing in a liquid nitrogen bath, cut in the cryostat, fixed and stained with fluorescein conjugated goat anti-human IgG and C_3.

The cortex of both kidneys was dissected from the medulla, minced with scissors, mixed with three parts of PBS (pH 7.2) and homogenised in a Waring Blender at maximum speed for 30 minutes. The homogenate was spun at 2000 g in a refrigerating centrifuge for 30 minutes at 4°C. Washing in PBS and spinning as above were repeated until the supernatant was colourless. The washed sediment was incubated with 10 vol. of 0.02 M citrate buffer, pH 3.2 for 2.5 hours at 37°C with constant stirring. After centrifuging for 30 minutes at 2000 g in a refrigerating centrifuge (+ 4°C), the supernatant was immediately brought to pH 7.0 with 0.5 M NaOH, and then dialysed against PBS solution, pH 7.2 for 42 hours at +4°C with

several changes of the buffer. The dialysate was concentrated by ultrafiltration to a protein concentration of 10 mg/ml for indirect immunofluorescence and to 10 - 30 mg/ml for IEP and immunodiffusion studies.

The indirect immunofluorescence method of McPhaul and Dixon (1969a) was used for the detection of anti-G.B.M. antibodies in the eluate. Normal human kidney section was snap frozen, cut in the cryostat, fixed and overlaid with eluate for 45 minutes, washed twice with PBS for 8 minutes. Sections were incubated for 30 minutes with fluorescent goat anti-human IgG and then washed in two changes of PBS. Control sections of the same normal human kidney were stained directly with fluorescent goat anti-human IgG serum.

The eluted antibody was studied in IEP using rabbit anti-whole human serum and anti-IgG; immunodiffusion studies were performed also on eluate with rabbit anti-whole human serum, anti-IgG, anti IgG light chains, anti-IgA and anti-IgM serum, all sera prepared in rabbits. IEP and immunodiffusion were repeated using eluate previously absorbed on insoluble human G.B.M. (0.5 ml: 10 mg, v/v). Eluate was absorbed with insoluble G.B.M. and B.S.A. separately. 10 mg of G.B.M. was added to 0.5 ml of eluate, incubated at 37°C for three hours, allowed to stand overnight at 4°C, centrifuged at 1800 g for 30 minutes. The absorption with B.S.A. served as a control of the specificity.

The eluate was labelled using the iodine monochloride technique (McFarlane 1958) with ^{131}I, then exposed to weighed homogenates of various organs including kidney, lung, liver, spleen, brain, skeletal muscle and cartilage at +4°C for 24 hours. The homogenates were thoroughly washed until there

was no free radioactivity in the supernatant, and bound radioactivity was then counted.

2.4. Cell-mediated allergic response in GN

2.41. Peripheral blood leucocyte migration-inhibition test to G.B.M. antigens

The method used was a modification of previously described methods (Søborg 1971; Rosenberg and David 1971).

Material and equipment: Hanks' Balanced Salt Solution, TC Medium 199, Normal Horse Serum were from WELLCOME REAGENTS, Beckenham Kent. Heparin: mucous, loooI.U./ml, PAINES AND BURNE LTD., Greenford. 50 ml plastic syringes (GILLETE.SCIMITAR). Migration chambers (STERILIN - S25). Silicone high vacum grease (EDWARDS HIGH VACUM LTD, Manor Royal, Crawley, Sussex). Coverslips, 20 mm x 20 mm. plastic test tubes. 30 ml plastic test cubes. Capillary tubes, external diameter 1.5 mm, internal diameter 1.1 mm (BENJAMIN HAEMATOCRIT TUBES, Daventry, Northants, England). Glass pipettes. Seal ease (CLAY ADAMS, Division of Becton and Comp., N.J.) Projection microscope (WILD). Planimeter (GALLENKAMP).

Method: 40 ml of blood was drawn from the cubital vein into a 50 ml plastic syringe containing 1 ml of heparin (1000 units). The syringe was inverted several times. The blood was then distributed into 4 sterile 10 ml plastic tubes, left to stand upright for about 90 minutes at 37°C by which time the red cells had settled at the bottom, leaving a white cell-rich plasma layer above. The red cell poor supernatant plasma was

aspirated with a glass Pasteur pipette so that only a few mm of supernatant was left above the sedimented erythrocytes. The plasma supernatant was transferred to one plastic 30 ml test tube and centrifuged at 700 rpm at room temperature for 5 minutes. The plasma was aspirated and discarded, the cells suspended in 15 ml of Hanks (medium) previously warmed in 37°C water bath. Resuspension was performed by repeated pipetting with 10 ml pipette. The cells were recentrifuged and suspended in Hanks medium three times. After aspirating the Hanks (medium) following the third washing, the remaining white cells paellet was resuspended in TC medium 199 containing 10% horse serum. The final cell concentration was adjusted to about 20,000 per mm^3. The cell pellet was thoroughly resuspended and sucked up into capillary tubes. The tubes were sealed with seal-ease, placed in a 10 ml test tube containing a wad of rubber at the bottom and centrifuged at 1500 rpm for 10 minutes at 20°C. After centrifugation, each capillary tube was cut 1 mm below the cell-fluid interface, and mounted on silicone dabs in the migrating chamber. One capillary tube was placed in each chamber. The migration chambers were filled with TC medium 199 containing 10% horse serum, or with the same medium containing different concentration of antigen. Antigens used were G.B.M. antigens in concentration of 0.5mg, 1 mg and 2 mg. In preliminary experiments carried out to determine the effect of high antigen concentration on cell migration in normal subjects, antigen was used in concentration of 2, 4 and 8 mg/ml of TC medium. The first and second peak chromatographic fractions obtained after gel filtration of normal, collagenase solubilised G.B.M. on Sephadex G-150 were also tested. These antigens were used in concentrations of 0.5 and 1.0 mg/ml.

Routinely 6 chambers were filled with the medium alone and at least two with each of the three antigen concentrations. The chambers were filled until a slight convex surface of the fluid was obtained and a sterile coverslip was applied. Care was taken to avoid leaving any air bubbles. The chambers were incubated on a flat surface for 24 hours in a 37°C incubator. The migration area was then projected and traced onto paper and the area of migration was measured with a planimeter. Migration indices were calculated according to the formula:

$$\text{migration index (MI)} = \frac{\text{area of migration in presence of antigen}}{\text{area of migration in absence of antigen}}$$

The resulting product was multiplied with 100.

Peripheral leucocytes of 10 patients that were found to be sensitive to G.B.M. antigens, were subsequently incubated again, separately with G.B.M. antigens and the first and second chromatographic fraction of gel filtration of normal human G.B.M. on Sephadex G-150.

2.42. Peripheral blood leucocyte migration-inhibition test to streptococcal membrane antigens

At the same time, from the same patients, whenever a sufficient number of cells was available, white cells were incubated with two different streptococcal membrane antigens. Streptococcal membrane antigens of type 5 and type 12 were used in concentrations of 0.05 mg/ml in TC 199 plus 10% horse serum. The antigens were given to us by Dr. J. Zabriskie from Rockerfeller University, New York. Their preparation was described in the paper by Zabriskie and Freimer (1966).

3. RESULTS

3.1. G.B.M.: Isolation and characterisation

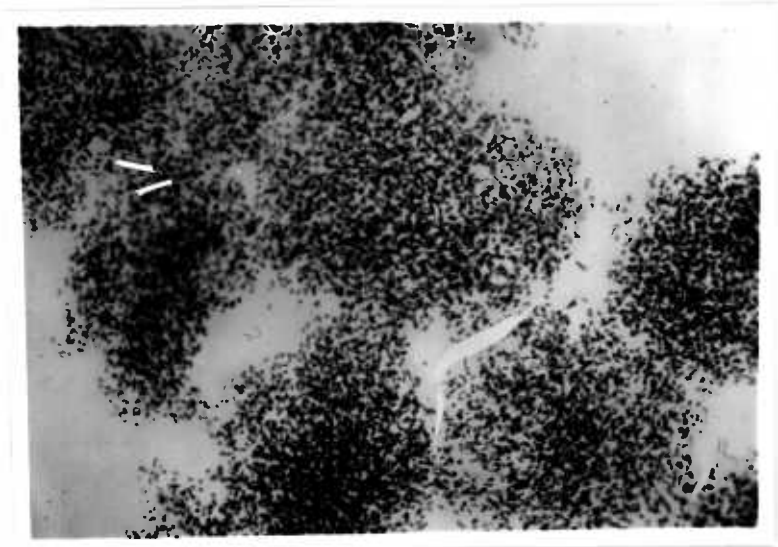
3.11. Preparation of glomeruli

More than 200 normal human kidneys were used for preparation of glomeruli and isolation of G.B.M. Average weight of the kidney cortex per one kidney was 65.2 gm. Each glomerular preparation was examined under the phase microscope and was found to be free of tubular fragments or other tissues (Fig. 3). When glomeruli were lyophilised and weighed, approximately 200 mg of glomerular preparation was obtained per one kidney cortex. As a significant number of glomeruli passed through the 61 pore size sieve during extensive washings, this material was filtered once more through the same sieve.

3.12. Preparation of G.B.M.

G.B.M. obtained after ultrasonication and extensive washings of sonicated material, examined under phase contrast microscope was free of cellular material and undisrupted glomeruli (Fig.4). Under light microscope this material appeared as amorphous structure. The average yield of lyophilised G.B.M. was 58 mg dry weight per one adult size kidney.

Figure 3. Isolated glomeruli (H.E. x 200)



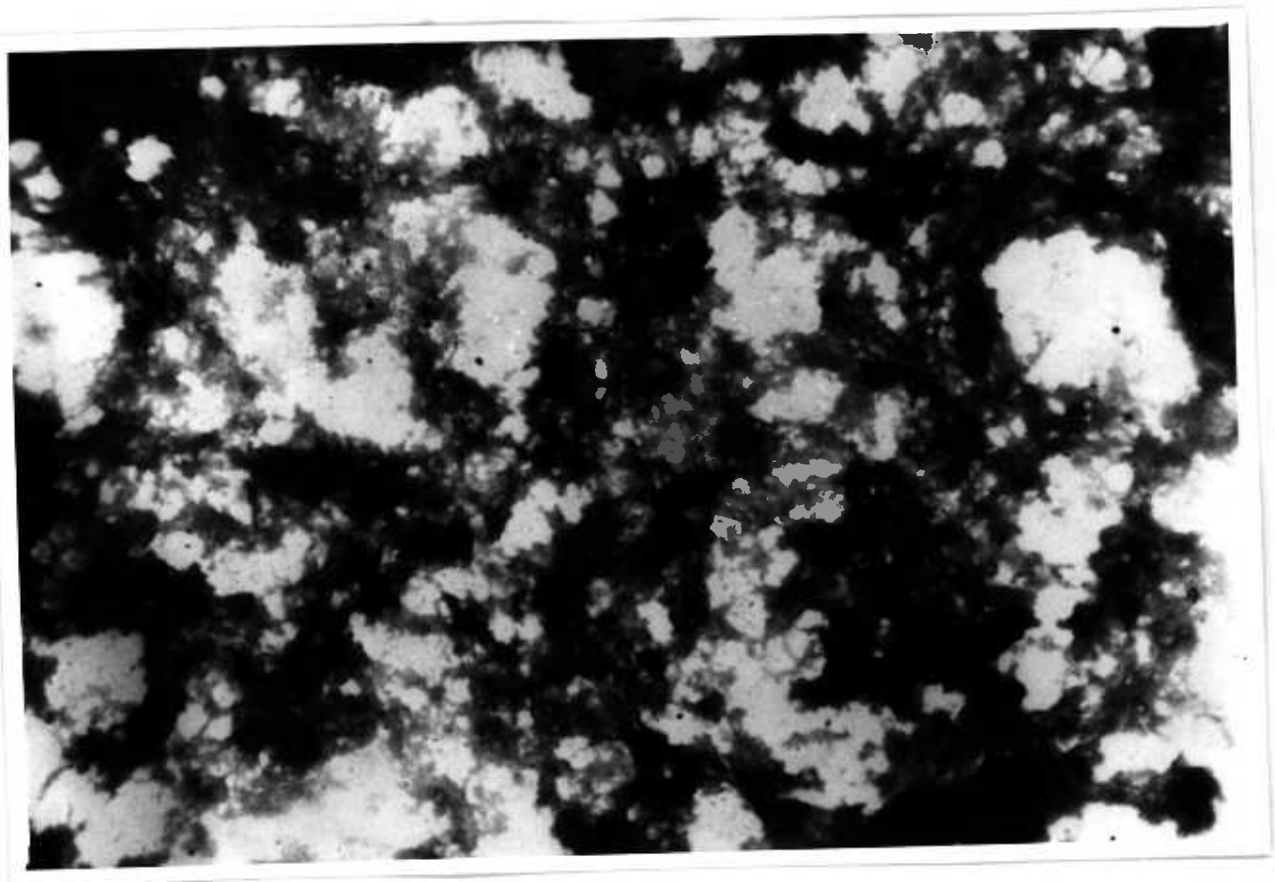


Figure 4. G.B.M. obtained after sonication of glomeruli (Periodic acid Schiff x 400)

Beside morphologic examination, chemical analyses for DNA and RNA were performed to determine the purity of G.B.M. These analyses suggested that G.B.M. preparation was free from cellular and subcellular contaminants since less than 0.1% of DNA and RNA was found.

3.13. Solubilisation of G.B.M.

For the solubilisation of G.B.M., digestion with collagenase was used. The digestion period was prolonged to five days with daily addition of small amount of enzyme. This resulted in the highest yields of solubilised G.B.M. On average, 35 mg of solubilised G.B.M. was obtained after digestion of 100 mg of the original G.B.M. (35%).

3.14. Immunochemical characterisation of G.B.M.

3.141. Rabbit anti-human G.B.M.

All rabbits immunised with undigested or soluble G.B.M. produced strong antiserum. The production of the antiserum was followed by sequential testing of serum samples from immunised animals in gel (Ouchterlony) against soluble G.B.M. in concentration of 2 mg/ml of saline. Following the immunisation schedule, strong lines of precipitation were observed in gel between serum of immunised rabbits and soluble G.B.M. No detectable differences were observed in lines of precipitation when soluble G.B.M. was tested against the antiserum produced either by immunisation with insoluble or soluble G.B.M.

Unabsorbed anti-G.B.M. produced a line of precipitation in gel, when tested against normal human plasma. Therefore the antiserum was regularly absorbed with lyophilised normal human plasma, either by incubation or by passing through the column of CnBr activated Sepharose 4B coupled to normal plasma. After absorption, no lines were detected in an Ouchterlony, when the serum was tested against normal human plasma or serum. No differences were noticed between the batch of serum absorbed by incubation with plasma, or passed through the column of activated Sepharose 4B. However, as absorption by simple incubation of serum and plasma is simpler and does not require subsequent concentration, this procedure was regularly used.

The specificity of the antiserum was established as follows: (1) incubation of anti-G.B.M. serum with insoluble G.B.M. or collagenase G.B.M. completely blocked the precipitation of the serum to G.B.M. when tested in double diffusion; BSA at the same concentration was ineffective in absorbing out the antibody activity; (2) the specificity was also tested in indirect immunofluorescence. Bright linear fluorescence of G.B.M., tubular basement membranes and B.M.'s of blood vessels was observed. As controls, normal human kidney sections stained with fluorescent anti-rabbit IgG, or sections incubated with the antiserum previously absorbed with native G.B.M., showed no fluorescence in indirect immunofluorescent assay.

3.142. Double diffusion method

Two lines of precipitation were formed in the gel (Ouchterlony test) when soluble G.B.M. and rabbit anti-human G.B.M. serum reacted. Two identical precipitating lines (Fig 5) were obtained always in each of the G.B.M. preparations and with all antisera produced in different rabbits, and at various times during two years study. Antiserum produced by immunisation with original G.B.M. and antiserum produced by immunisation with collagenase solubilised G.B.M. gave identical lines of precipitation when tested with soluble G.B.M., suggesting that collagenase solubilisation did not alter the antigenicity of G.B.M.

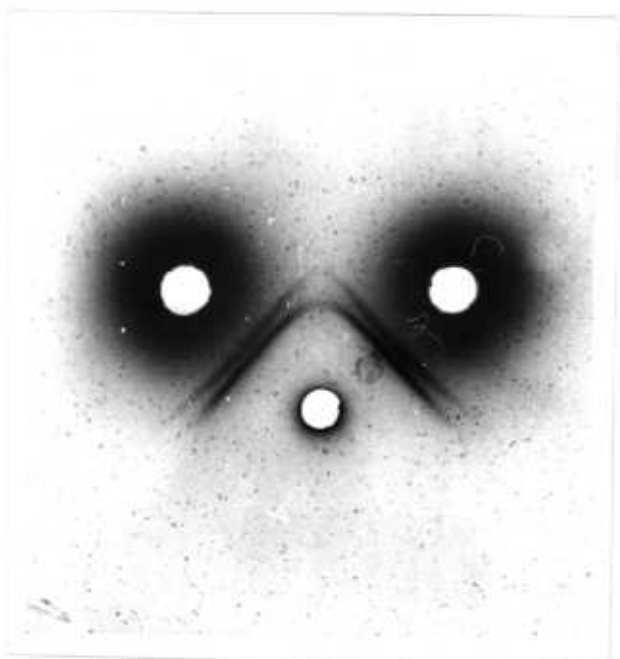


Figure 5. Double diffusion pattern of normal human G.B.M. and rabbit anti-human G.B.M. serum

3.143. Immunoelectrophoresis

The immunoelectrophoretic pattern of human G.B.M. appears in Fig. 6. Soluble human G.B.M. was used in the same concentration as for immunodiffusion studies. It is evident that G.B.M. was separated into at least 5 main electrophoretic fractions.

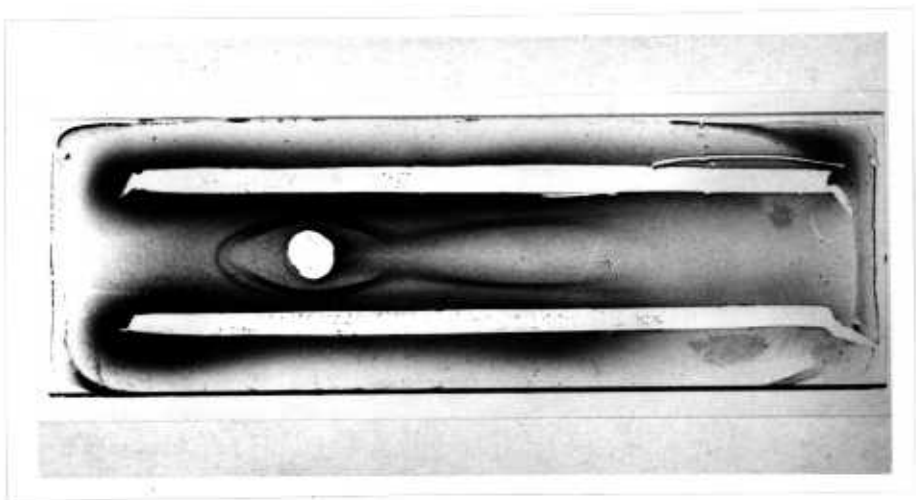


Figure 6. Immunoelectrophroesis of soluble G.B.M. The trough contains rabbit anti-human G.B.M. serum. The well contained soluble human G.B.M.

3.144. Antigen-antibody cross electrophoresis

The original technique of Laurell (1965) was specially modified, as for the second dimension electrophoretic run, three slips of decreasing concentrations of antiserum were put next to the slip containing the antigen. By this modification, the chances of obtaining optimal antigen-antibody concentrations were increased. This technique revealed in soluble human G.B.M. preparation a greater number of components of different mobility and charge than the two previous techniques of double diffusion and IEP. A typical antigen-antibody cross electrophoretic pattern obtained with normal human G.B.M. and rabbit antiserum is shown in

Figure 7.

Figure 7. Antigen-antibody cross electrophoretic pattern of human G.B.M. and rabbit anti-human G.B.M. serum

1 = 0.12 ml

2 = 0.10 ml

3 = 0.08 ml

1.145. Fractionation of G.B.M. antigens

Solubilised G.B.M. obtained by collagenase digestion of human G.B.M. was fractionated by gel filtration on Sephadex G-150 and Sepharose 6B columns. The fractionation of soluble G.B.M. material on the basis of molecular size on Sephadex G-150 is shown on diagram (Fig. 8). The soluble G.B.M. material was subsequently separated into three main fractions. The first fraction moved with blue dextran, it is of high molecular weight in the range of 2,000000 - 1000000 (Andrews 1964). The second fraction overlapped the albumin peak, its molecular weight must have been in the 2000° - 10000° range. The last fraction corresponded to small molecules of molecular weight of less than 10000. It seems that decreasing molecular weight of chromatographic patterns is a result

of collagenase digestion of original G.B.M.

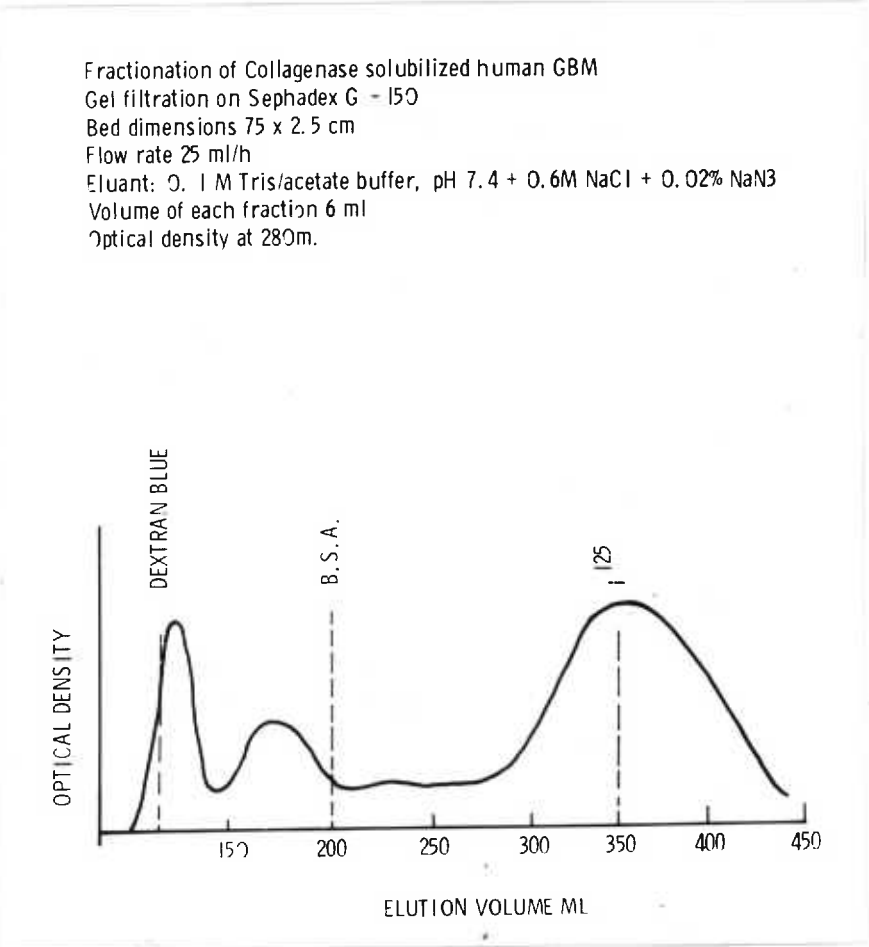


Figure 8

To study the antigenicity present in chromatographic fractions, double diffusion (Fig 9 and Fig 10) and antigen-antibody cross electrophoresis (Fig 11) of the samples within each peak were performed, using rabbit anti-human G.B.M. serum. The studies done showed that: (1) it is possible to separate two main antigenic components of human G.B.M.; (2) one antigen is of high molecular weight and present in the first chromatographic fraction after gel filtration on Sephadex G-150, the second is of smaller molecular weight and present in the second fraction after chromatography of G.B.M. on Sephadex G-150; (3) the last fraction has no antigenicity (fraction 3), most probably represents small polypeptides or aminoacids; (4) separate fractions are not uniform, but are composed of multiple components.

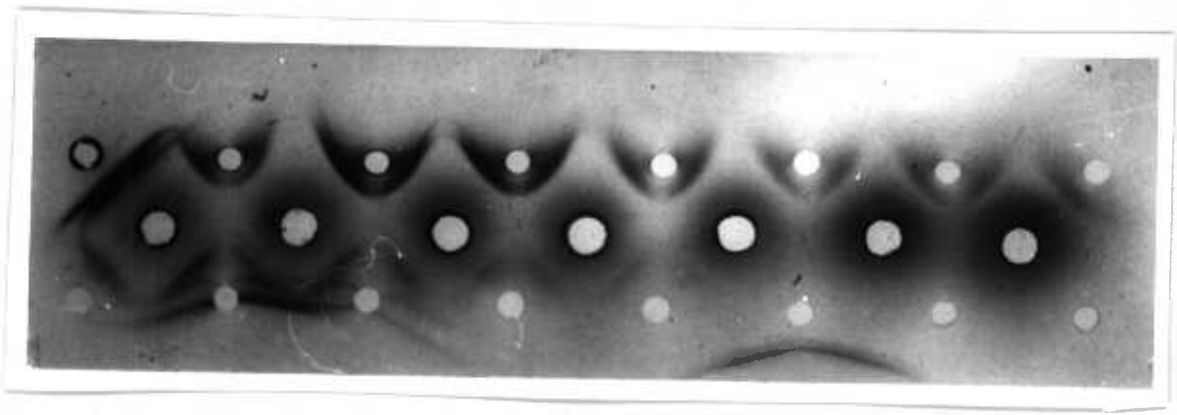


Figure 9. Double diffusion analysis of fractions obtained after chromatography of soluble G.B.M. on Sephadex G-150. Upper row contains soluble G.B.M. in the first well, then samples within first peak. Central wells contain rabbit anti-human G.B.M. serum. Lower wells: samples from the second peak and at the end, samples from the last peak

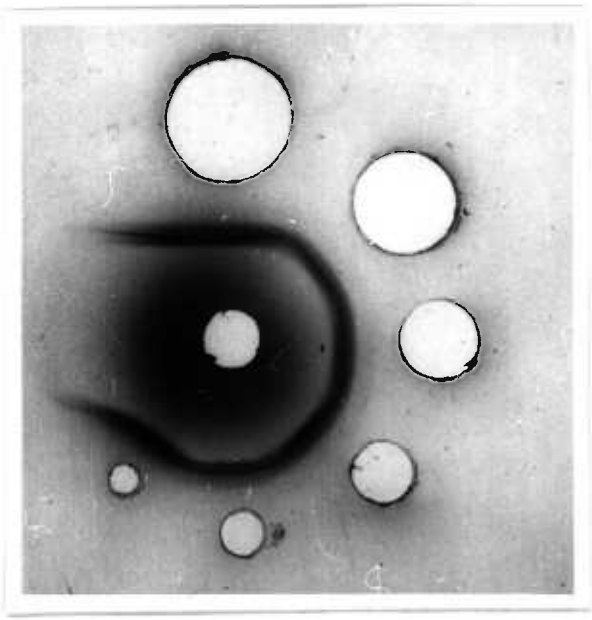


Figure 10. Double diffusion analysis: peak 1 after chromatography of soluble G.B.M. on Sephadex G-150 and rabbit anti-human G.B.M. serum

Figure 11. Antigen-antibody cross electrophoresis: soluble G.B.M., first fraction (pk I) and third fraction (marked as pk 2) after chromatography of G.B.M. on Sephadex G-150

As double diffusion (Fig. 10) and cross electrophoresis (Fig. 11) have shown that the first peak after chromatography on Sephadex G-150 was not uniform fraction, this peak was pooled, concentrated and further fractionated on Sepharose 6B. The result of fractionation on Sepharose 6B column is presented in Fig. 12. The first peak from Sephadex G-150 column was able to further fractionate in four different fractions. However, when each of these fractions were tested in immunodiffusion (Fig. 13) or antigen-antibody cross electrophoresis (Fig. 14), it became apparent that they were composed also of multiple components.

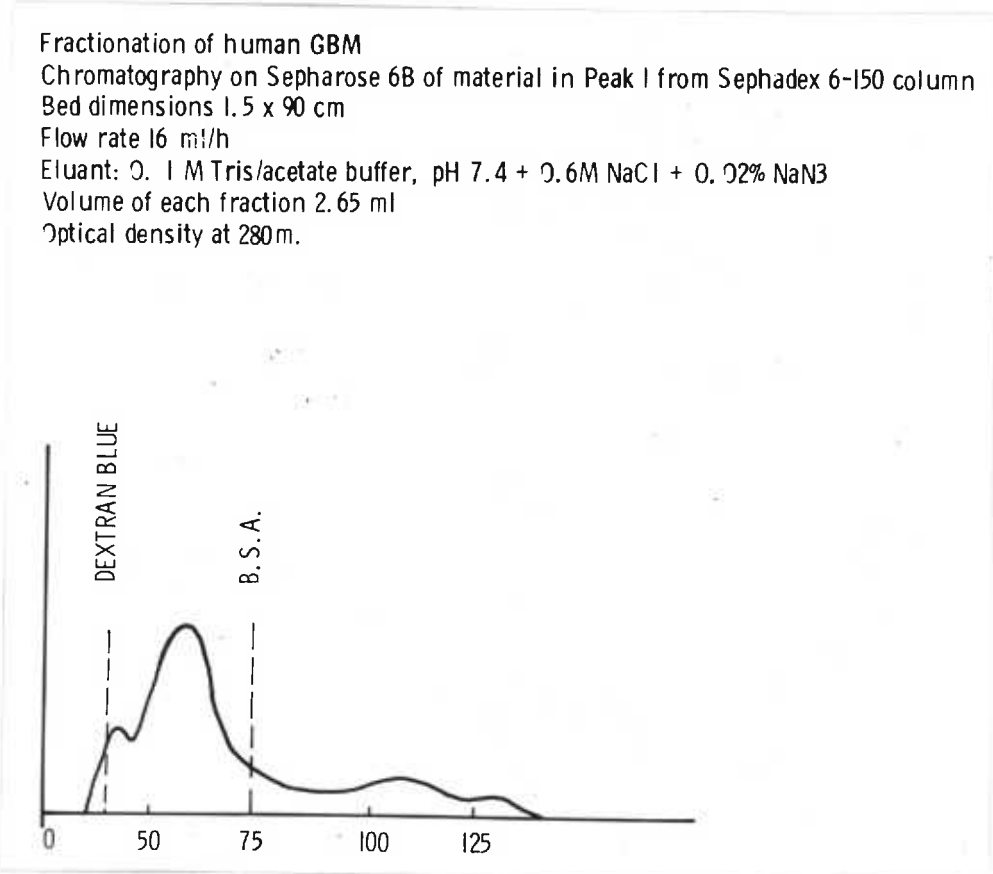


Figure 12

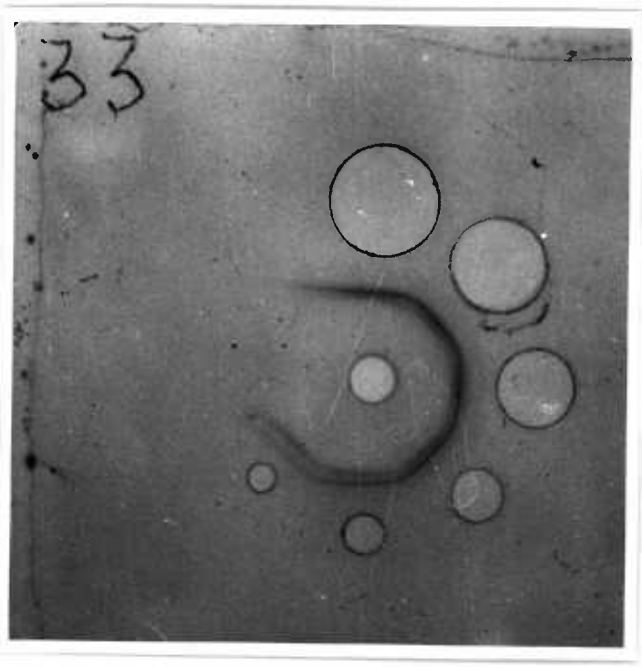


Figure 13. Double diffusion analysis: Peak I after chromatography on Sepharose 6B of the first peak from Sephadex G-150 column, and rabbit anti-G.B.M. serum

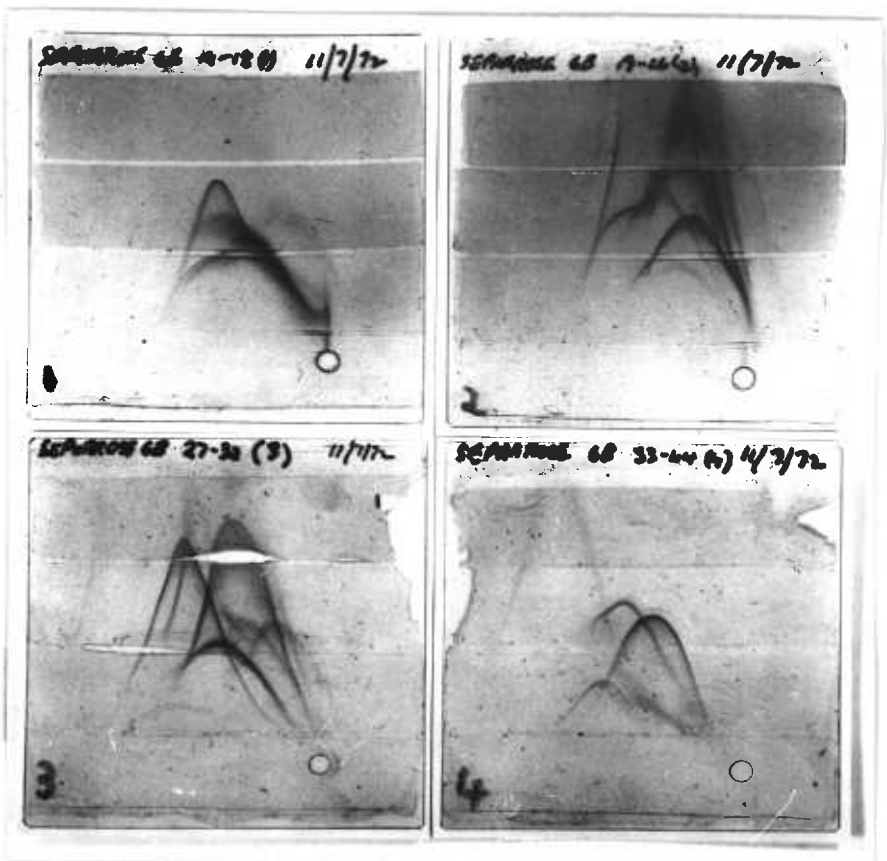


Figure 14. Antigen-antibody cross electrophoretic pattern of samples within each of the peaks from chromatography on Sepharose 6B of the first fraction separated by chromatography on Sephadex G-150

3.2. Patients

In regard to clinical, functional and other laboratory findings, and in the majority of patients renal biopsy findings, patients studied were classified as: Minimal change (MC) glomerulonephritis; Proliferative glomerulonephritis (PGN); Membranous glomerulonephritis (MGN); Chronic pyelonephritis (CP), Acute tubular necrosis (ATN), Polycystic kidneys (PK). According to the findings of immunofluorescent examination of renal biopsies, patients were categorised as having linear or granular immune deposits. Patients with Goodpasture's syndrome were grouped separately, light microscopy of their renal biopsies showed proliferative changes, on direct immunofluorescent examination linear deposits of IgG and C_3 were found. Fig. 15 demonstrates the characteristic linear pattern of IgG deposits in a patient with Goodpasture's syndrome.

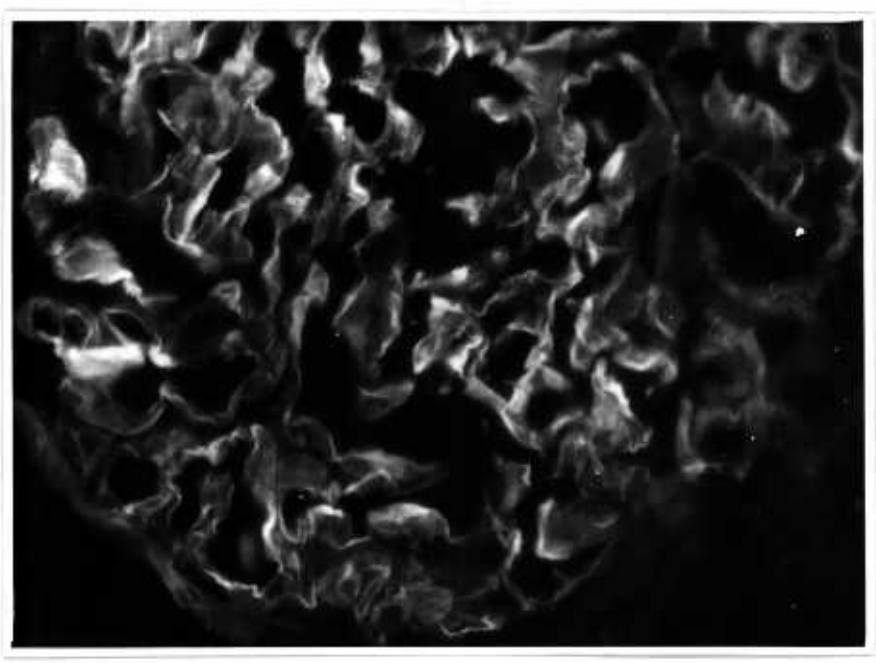


Fig. 15. Immunofluorescent stain for IgG showing typical smooth, linear deposition of antibody along the glomerular basement membrane (x 455)

3.3. Anti-G.B.M. antibodies

3.31. Indirect immunofluorescence

No instance of IgG fixation to G.B.M. of frozen normal human kidney sections were encountered among normal controls, patients with immune-complex nephritis and other non-renal diseases (98 sera tested). Among 10 patients known to have anti-G.B.M. antibody mediated GN, sera from all of them fixed unequivocally to G.B.M. of normal human kidney section also in a linear fashion (Fig. 16).

If positive sera were previously absorbed with normal human G.B.M., no fixation was possible to detect; lyophilised normal human plasma, bovine serum albumin or liver powder were ineffective in absorbing out the antibody activity.

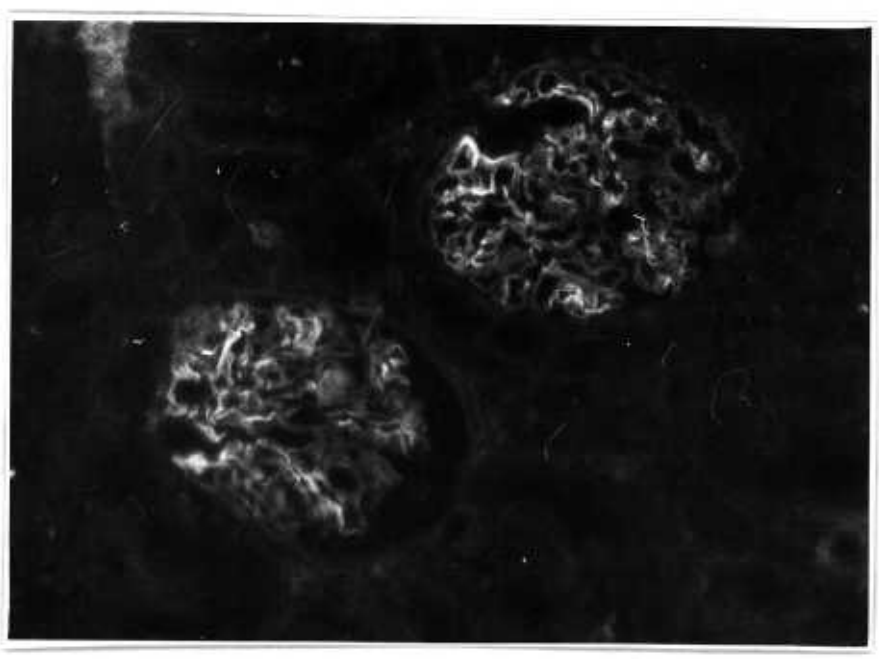


Figure 16. Demonstration of typical pattern of linear fixation of IgG anti-G.B.M. antibody to glomeruli of normal human kidney sections.

3.32. Double diffusion

Precipitating antibodies against collagenase solubilised extract of normal human G.B.M. were found in none of the sera tested. The sera tested were from 11 patients who had linear deposits of IgG on direct immunofluorescent examination of their renal biopsies. None of the patients studied was nephrectomised. Fig. 17 illustrates the negative findings of four of the sera tested.

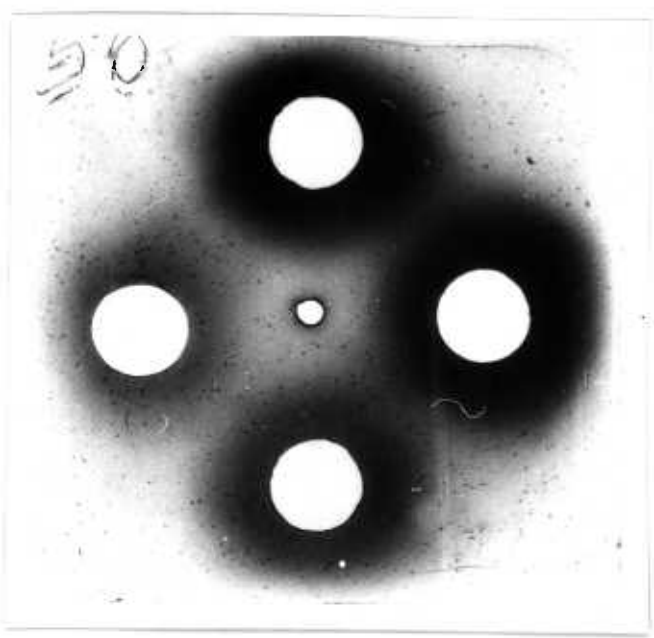


Figure 17. Double diffusion gel analysis of sera of patients with linear staining for IgG deposits in vivo (peripheral wells) and soluble G.B.M. antigens (central well)

3.33. Passive haemagglutination

Fresh r.b.c. were treated with tannic acid and coated with soluble G.B.M. antigens. Factors influencing the sensitivity and specificity such as: soluble G.B.M. antigen concentration, effect of temperature, length of incubation, and others were varied and modified in order to obtain optimal conditions. Maximal haemagglutination titre of tanned blood cells sensitised with soluble G.B.M. antigens and tested with rabbit anti-human G.B.M. serum was 1: 64. None of the sera of patients with linear deposits of IgG on the renal biopsies, or other patients' sera tested, gave positive agglutination with TA sensitised r.b.c.

G.B.M. antigens were coupled to r.b.c. with bisdiazotised benzidine and subsequently tested with rabbit anti-G.B.M. serum. Under optimal conditions, it was never possible to obtain the high titre for circulating anti-G.B.M. antibodies. The highest titre for anti-G.B.M. antibodies present in rabbit anti-G.B.M. serum was 1:128. Sera from patients with linear deposits of IgG never gave agglutination reaction when tested with bisdiazotised r.b.c.

Chemical linkage of G.B.M. antigens to r.b.c. using chromium chloride was attempted, but the maximal titre of circulating anti-G.B.M. in rabbit anti-G.B.M. serum, under optimal conditions, was never more than 1:128. Sera of patients with linear deposits of IgG on renal biopsies, gave no agglutination when tested with $CrCl_3$ sensitised cells for the presence of circulating anti-G.B.M. antibodies.

G.B.M. was coupled to r.b.c. with glutaraldehyde and sensitised cells tested with rabbit anti-human G.B.M. serum. Optimal conditions were selected in order to increase agglutinating titre without losing the specificity.

The effect of GA concentration: 2.5 mg of G.B.M. were coupled at constant reaction volume (1.25 ml) to 3.3% red blood cells suspension, using varying GA quantities. When other variables were kept constant and only amount of GA varied, it was found that at high concentrations of GA non-specific agglutination occurred in the controls. Therefore 0.25 ml of 2.5% solution of GA in saline was found to be the optimal value.

Batches of cells were simultaneously coated with solutions of G.B.M. containing between 0.5 and 8 mg/ml. It was evident that G.B.M. concentrations in excess of 2 mg/ml did not increase the sensitivity, at very high concentrations of more than 4 mg/ml, usually haemolysis occurred. There was also the evidence that the sensitivity was considerably diminished if the G.B.M. concentration was below 2 mg/ml. Therefore 2 mg per ml G.B.M. solution in saline was found to be the optimal concentration.

Cells were sensitised by standard method and concentration, except that the only variable was the length of incubation. The incubation time was varied from 15 to 80 minutes. Samples of sensitised cells were taken at regular intervals. The cells were washed, suspended in diluent and used in haemagglutination titration. Longer periods of coating the cells of more than 60 minutes did not increase the sensitivity. The coupling reaction was virtually complete after 1 hour. When incubated for shorter periods, haemagglutination titres were lower.

Preincubation of the antigen alone with GA before coupling with r.b.c. resulted in a decrease of the agglutination titre. Therefore erythrocytes and antigen were always preincubated for 5 minutes and after that GA was added. Erythrocytes treated for 15 minutes with GA alone, before the addition of G.B.M. were able to fix the antigen and most often no diminuition in the agglutination titre was observed.

It was found optimal to use 3.3% cell suspension in the coating procedure. If higher concentrations of cells were used (6% and 8%), lower titre of antibodies detected was obtained.

The experiments were performed with different concentrations of stabilising serum. Optimal stabilising of coated cells was achieved with 0.5% rabbit serum in saline. By increasing the concentration of stabilising serum to 1% or more, agglutination of the cells could be prevented. The same occurred if stabilising serum was added to early, i.e. after the first washing of the cells treated with GA, or if before coating, the cells were washed in stabilising serum.

G.B.M. coated cells were usually used on the same day as they had been prepared, lower haemagglutination titre was obtained when stored cells were used. The coating of the cells was always carried out at room temperature. Saline was always used as diluent for the cells, GA and G.B.M.

All the investigations of different variables were done in order to find the optimal conditions which will give the greatest sensitivity when rabbit anti-human G.B.M. serum was tested with G.B.M. coated cells. Under the optimal conditions, the highest dilution of rabbit anti-human G.B.M. serum causing complete agglutination of the coated cells was 1:524,288. The end point of agglutination was clear and reading was easy. When the reaction was positive, the erythrocytes coated with G.B.M. were seen as a homogenous matt of the cells on the bottom of the wells of plastic agglutination trays; when the reaction was negative, the cells could be seen as a button in the centre of the wells.

The specificity of the test was established as follows: (1) sensitised cells incubated with normal human serum gave no agglutination. Only occasionally, at the beginning of the experiments, in a few instances, non-specific agglutination in a titre of 1:2 was obtained. Later on, with more standardised procedure, no agglutination was observed with normal human serum; (2) cells sensitised with an heterologous protein (BSA) or lyophilised normal human plasma, when tested with rabbit anti-human G.B.M. serum gave no agglutination; (3) sensitised cells gave no agglutination when incubated with saline; (4) cells treated with GA, but without subsequent addition of G.B.M., gave no agglutination when incubated with rabbit anti-human G.B.M. serum; (5) agglutination of GA sensitised cells with rabbit anti-G.B.M. serum was inhibited or the titre significantly decreased if the antiserum was previously absorbed with G.B.M. (Fig. 18). (6) agglutination of GA sensitised cells with rabbit anti-human G.B.M. serum was not inhibited if the antiserum was previously incubated with non-specific protein (BSA).

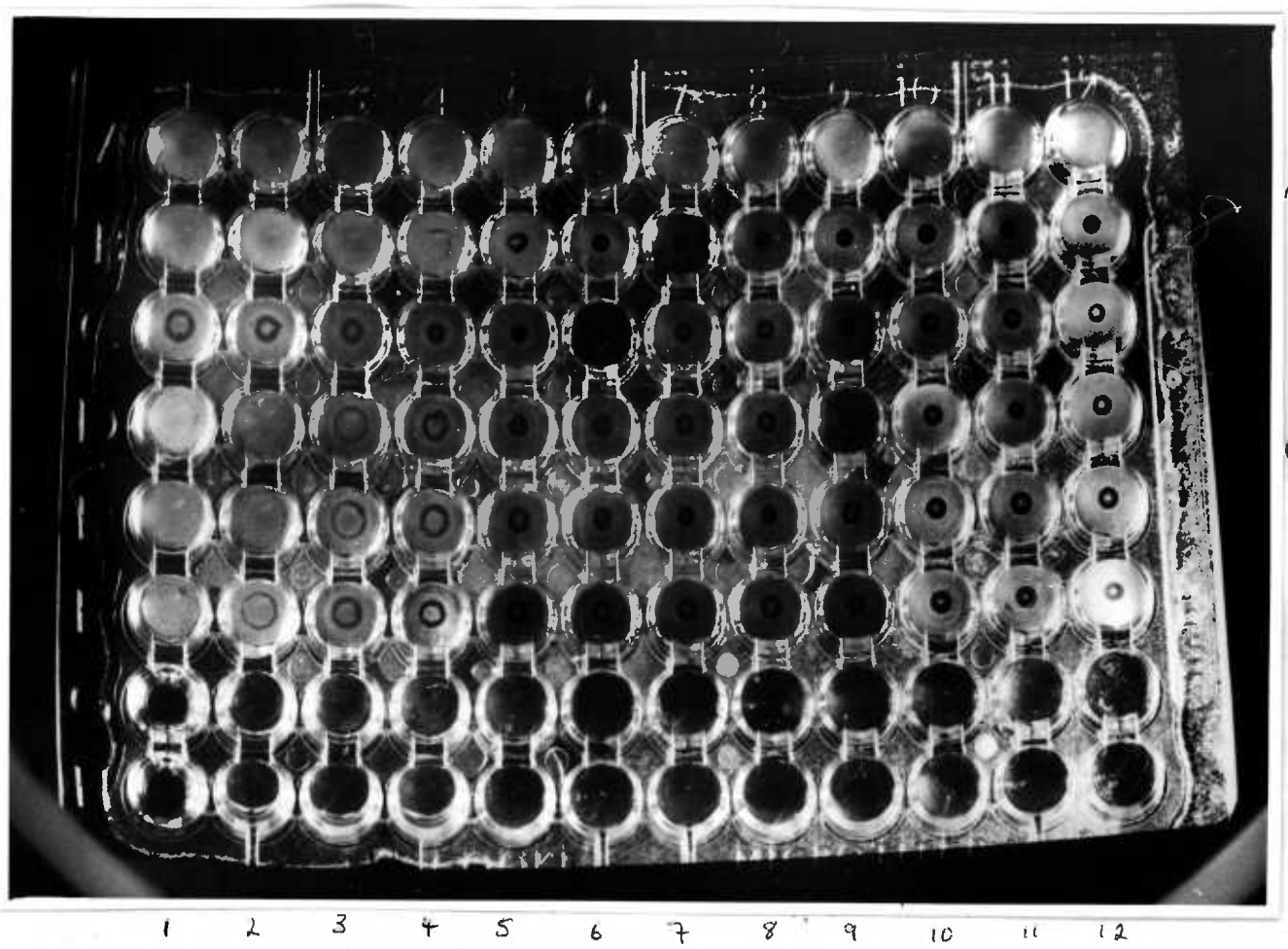


Figure 18. Haemagglutination pattern of human erythrocytes coated with human G.B.M. tested with serial (1:1) dilutions of the rabbit anti-G.B.M. serum (agglutination from A1 - B4). In horizontal rows C, D, G, H, rabbit anti-G.B.M. serum previously absorbed with different concentrations of human G.B.M.

When optimal conditions that gave the best sensitivity of the test were found, sera of patients with glomerulonephritis and other renal and non-renal diseases and sera of normal controls were tested for the presence of anti-G.B.M. antibodies. In all, 111 sera were tested. The characteristic pattern of agglutination in 8 patients tested is shown in Fig 19. The results of testing sera of patients with various renal diseases and other diseases and normal controls is shown in Fig. 20. All control subjects (except three tested at the beginning of the experiment) gave no agglutination when tested with GA sensitised erythrocytes. The maximal titres of agglutination in sera of patients with linear deposits of IgG was 1:64. The specificity of the test was established by: (1) agglutination of sensitised cells with positive serum was inhibited if the serum was previously absorbed with G.B.M.; (2) agglutination of sensitised cells with positive serum was not inhibited when the serum was previously

absorbed with BSA or lyophilised normal human plasma; (3) cells sensitised with BSA or human plasma gave no agglutination when tested with positive sera; (4) sensitised cells have no agglutination when tested with saline; (5) cells treated with GA but with no addition of G.B.M. gave no agglutination when tested with positive serum.

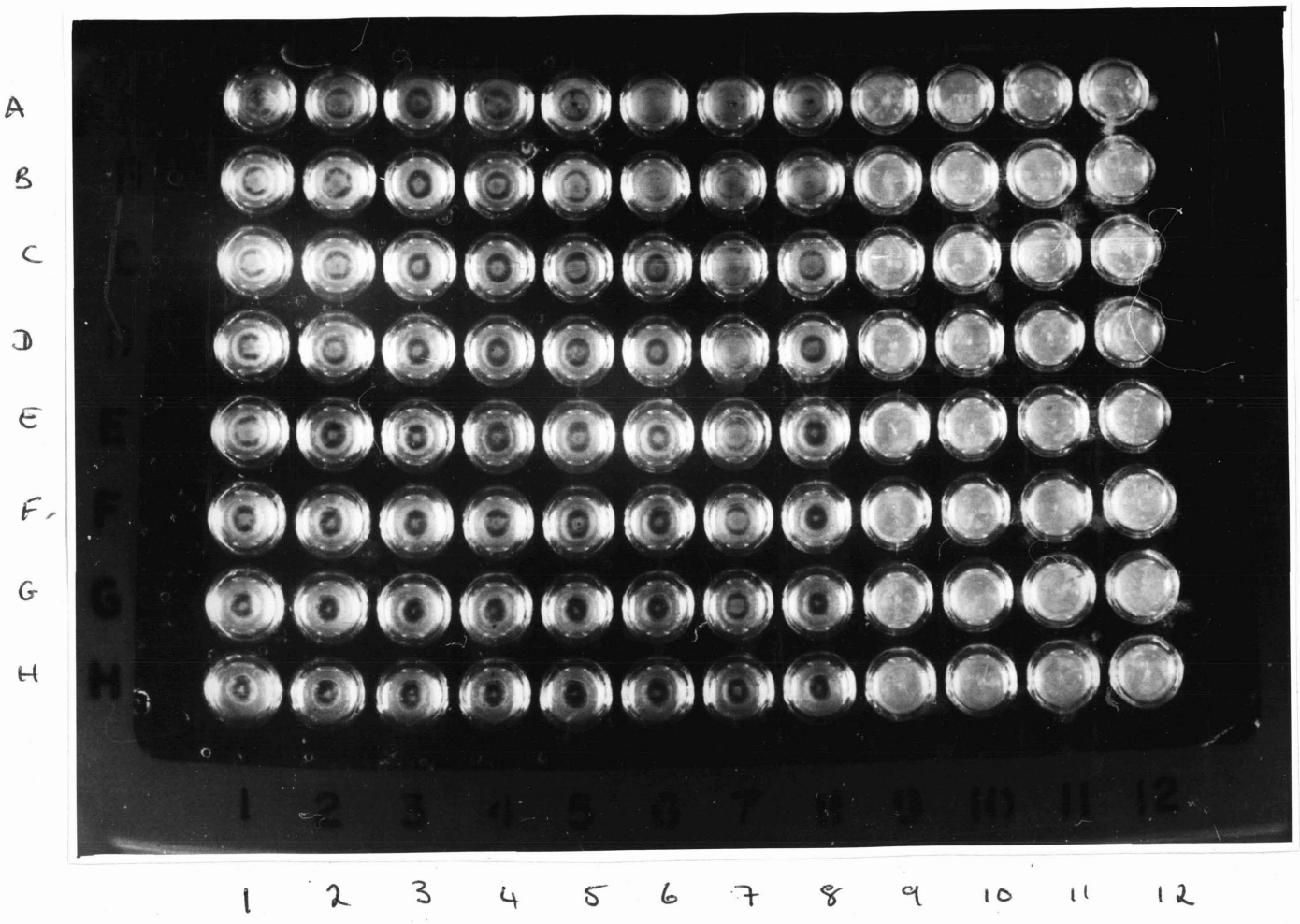


Figure 19 . Haemagglutination patterns of human r.b.c. coated with G.B.M. antigens and sera of patients with glomerulonephritis or normal controls. Dilutions of sera in vertical order (A-H). The titre of the serum No. 7 is 1:16, the others 1:2 or negative.

The correlation of humoral allergic response to G.B.M. as measured by the titre of circulating anti-G.B.M. antibodies and light microscopy findings is given in Fig. 20. All control subjects had negative passive haemagglutination test for the presence of circulating anti-G.B.M. antibodies. Patients with proliferative glomerulonephritis and some patients with acute tubular necrosis had raised titre of circulating anti-G.B.M. antibodies. All patients with Goodpasture's syndrome had the maximal titres of 1:32-1:64. As in every group of patients there were cases with elevated titre of circulating anti-G.B.M. antibodies, it seems that kidney damage, whatever the cause, within 2-3 weeks of onset, may cause elevated titres of anti-G.B.M. antibodies. But more information concerning the nature of these anti-G.B.M. antibodies is obtainable when they are correlated with the immunofluorescent findings.

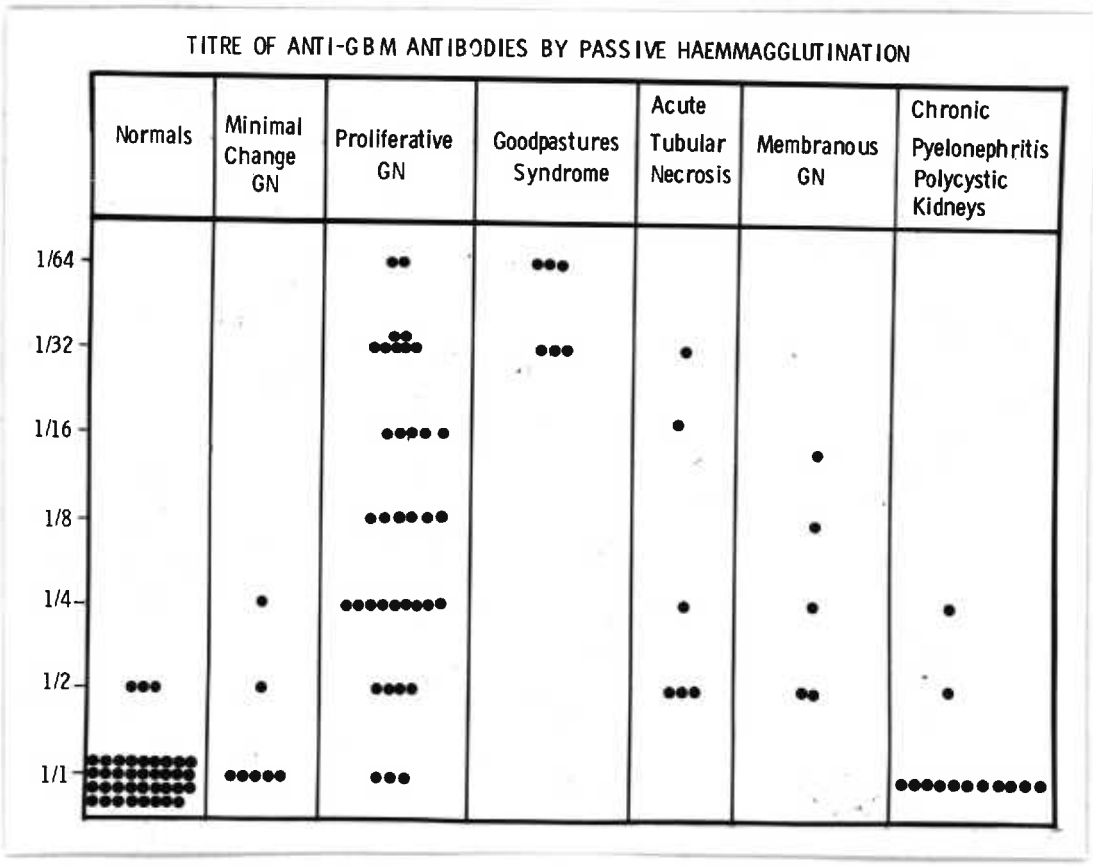


Figure 20

Fig. 21 illustrates the correlation between the titre of circulating anti-G.B.M. antibodies established by passive haemagglutination method and the findings of direct immunofluorescent examination of renal biopsies within a group of patients. It is apparent that the highest titres are present in patients with linear immune deposits, i.e. patients having anti-G.B.M. antibody mediated glomerulonephritis. In the majority of patients with granular immune deposits lower but nevertheless raised titres were observed.

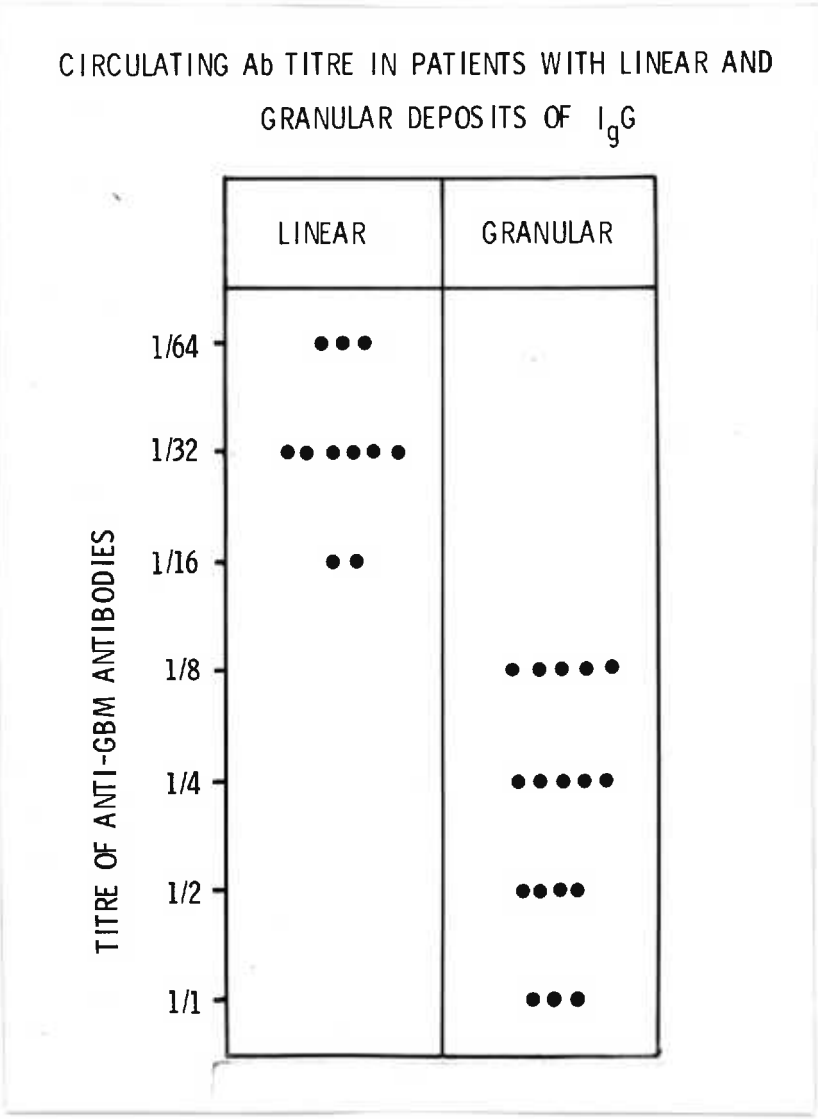


Figure 21

The correlation of data obtained from passive haemagglutination test for the presence of circulating anti-G.B.M. antibodies and indirect immunofluorescent assay for the same antibodies, is presented in Fig. 22. It is evident that sera containing high titre of circulating anti-G.B.M. antibodies fixed in vitro to normal human kidney sections (in a linear fluorescent pattern). Sera with low titre of circulating anti-G.B.M. antibodies established by PH method, using glutaraldehyde sensitised r.b.c., did not fix in vitro to normal human kidney sections.

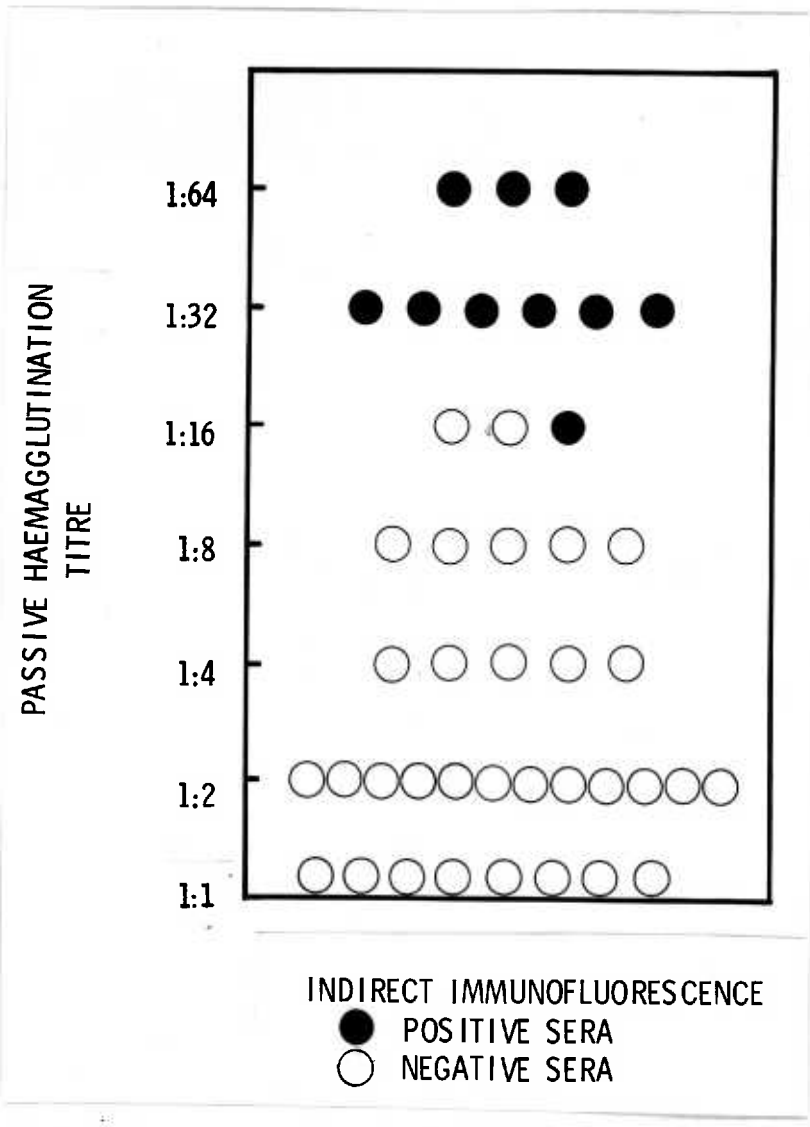


Figure 22

3.34. Studies on eluted anti-G.B.M. antibodies

Antibodies were eluted from the kidneys of a patient with Goodpasture's syndrome. The diagnosis was established by clinical, laboratory and autopsy findings. The deposition of IgG and C3 along the glomerular capillary walls could be shown with certainty to be uniformly linear. Both kidneys were eluted. Eluted antibodies fixed strongly to the section of normal human kidney in vitro, and indirect immunofluorescent staining after incubation with eluate, demonstrated linear staining pattern of normal kidney basement membranes treated with goat anti-human IgG. Complete inhibition of the immunofluorescent reaction of eluate and G.B.M. was obtained by incubating the eluate with the sonicated G.B.M.

Immunoelectrophoresis of the eluate with the anti-whole human serum, anti-IgG, anti-IgM and anti-IgA demonstrated that eluate contained primarily IgG, although trace amounts of other immunoglobulins were also present. In double diffusion (Ouchterlony), eluate produced a single line reacting with anti-whole human serum, anti-IgG and anti-Ig light chains, demonstrating that the IgG was the main antibody class present (Fig.23).

Figure 23. Double diffusion analysis of eluted antibodies against anti-human sera: anti-whole human serum, anti-IgG and anti-Ig light chains. No precipitation developed against the wells containing anti-IgA and anti-IgM

When eluted antibodies were labelled with ^{131}I and exposed to homogenates of various organs, the highest binding was obtained to the kidneys and lung (Table 1)

	kidney	lung	liver	spleen	brain	skeletal m.	cartilage
% labelled IgG bound /G	79	74	43	39	39	28	7

Table 1. Absorption by normal human tissues of immunoglobulin eluted from kidneys of patients with Goodpasture's syndrome

3.4. Peripheral leucocyte migration-inhibition test

The in vitro test used for the assessment of cellular allergic response to G.B.M. antigens and streptococcal membrane antigens was peripheral blood leucocyte migration-inhibition test. An example of the inhibition of migration of peripheral leucocytes in the presence of G.B.M. antigens is illustrated in Fig. 24. Several aspects of the technique were altered before deriving the method as described. Separation of the white cells was achieved by simple sedimentation of the blood for up to 90 minutes at 37°C. With spontaneous sedimentation, the risk of inducing non-specific changes in the cellular reaction capacity by means of polyvinylpyrrholidone or dextran was eliminated. A sufficient number of white cells (lymphocytes, monocytes, polymorphonuclear leucocytes) was obtained in the plasma after sedimentation of erythrocytes. Erythrocyte contamination of the leucocyte suspension was reduced to a minimum. Red cell contamination of the leucocyte suspension was usually less than 1:1, and no particular advantage in higher purity (which could be achieved by different centrifugation or by flash-lysis with tris-buffered NH_4Cl) was obvious in the sensitivity of the test. Indeed, red-cell-free leucocyte migration had less distinct image on projection and made it harder to read the result.

Of importance in obtaining good results are: the very clean migrating chambers and cover slips; clearly cut capillaries which were not allowed to dry after cutting; exclusion of air bubbles by careful manipulation; a strict adjustment of tissue culture medium pH between 7.2-7.4; sterile conditions during all the procedure. Non-siliconised capillaries and other equipment was used with no particular disadvantage. The heparin used should contain benzyl alcohol and not phenol as preservative. The amount of heparin should not vary much, higher concentrations from the indicated could be toxic for the cells, lower concentrations can result in clumping of the cells during washing procedure. The experimental procedure was carried out as quickly as possible in order to obtain as high viability of the leucocytes as possible. The time from taking the blood until the cells were in the culture medium filled migrating chambers never exceeded 150 minutes. Samples were always tested in duplicate, sometimes in triplicate for each concentration of antigen. Six capillary tubes containing cells were always incubated in migrating chamber containing TC medium plus horse serum alone, without antigen, the mean migration of the cells incubated without antigen was calculated from the migration of 6 separate tubes. The incubation of cells was carried out in an atmosphere with 5% CO_2 and water saturated at 37°C.

After 24 hours of incubation, the migrating area was composed of a dense inner area surrounded by lighter halo. In our experience, when inhibition of migration occurred, both areas were smaller than in the control chambers, without antigen. This is nicely illustrated in Fig. 24.

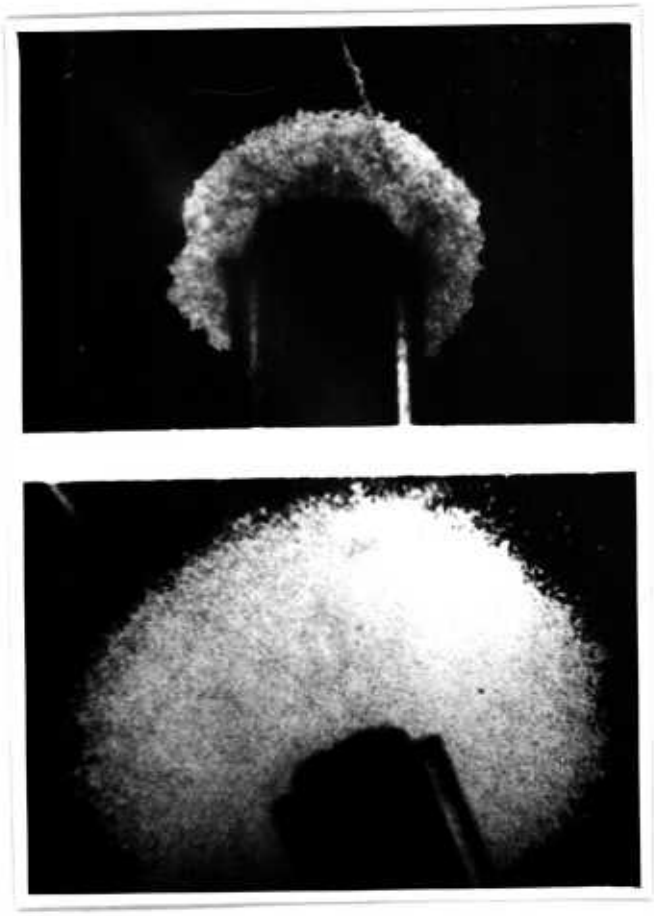


Figure 24. Appearance of inhibited migration in the presence of G.B.M. (up) and normal leucocyte migration in the absence of antigen (below)

3.41. Cellular hypersensitivity to G.B.M. antigens

The peripheral leucocyte migration inhibition test was performed using soluble collagenase digest of normal human G.B.M. A high concentration of antigen (1 mg/ml) was found to be the optimal antigen concentration. Concentrations of 2, 4 and 8 mg/ml of G.B.M. were found to be toxic and inhibit migration of cells from normal subjects. The dose of 1 mg/ml was found to be the optimal antigen concentration. The leucocyte migration inhibition test was performed on 79 subjects. The results are presented in Fig. 25. The white cell migration of normal persons appears to be unaffected by the presence of G.B.M. antigens. The migration index calculated as mean of M indices of individual normal subjects was 92.72, S.D. 6.49, and the dotted line in Fig. 25 represents 2 standard deviations from the mean.

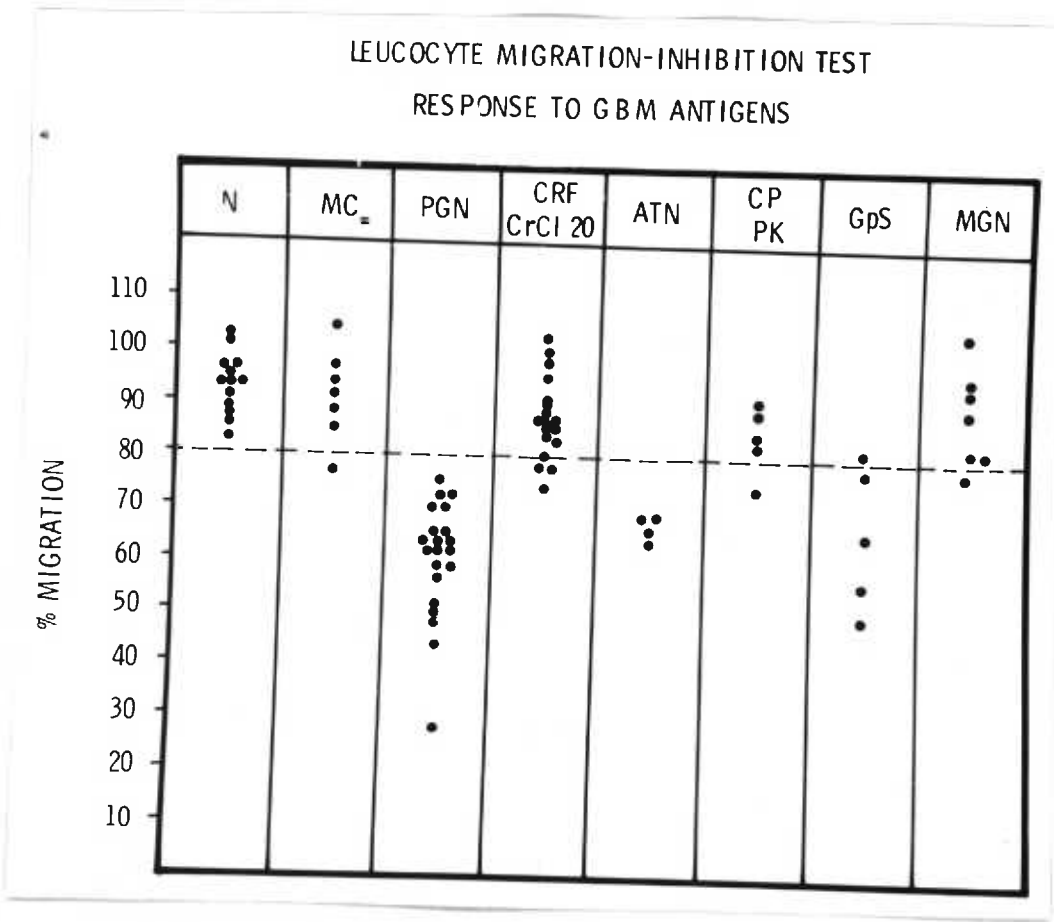


Figure 25

The findings of the test in 21 patients with proliferative glomerulonephritis are shown in the third column of Fig. 25. All values in the group are below the range of normal (mean of MI in normal group minus 2 S.D.); the mean of MI in the group is 53.7, S.D. 17.8. When compared with normal, this group is significantly different ($p < 0.001$).

The white cell migration of patients with chronic renal insufficiency did not show significant difference from the group of normal patients, even if the mean of migration is slightly lower (mean 86.16, S.D. 7.7). The mean white cell migration of patients with chronic renal insufficiency is significantly different from the group with proliferative glomerulonephritis ($p < 0.001$).

In the group of patients with "minimal change" (MC) glomerulonephritis and membranous glomerulonephritis (MGN) only one patient in each group had MI slightly below the normal range, and the groups were not significantly different from normal.

Patients with acute tubular necrosis (test performed 3 weeks from the insult) had M Indices below the normal range, and were significantly different from the normal ($p < 0.001$).

Patients in the group of Goodpasture's syndrome did not show uniform results. This might be due to the fact that some patients in this group had creatinine clearance below 20 ml/min. Some patients in the group had the disease diagnosed several years ago, and no signs of disease activity when the test was performed.

When 14 patients with proliferative glomerulonephritis (Fig.26) were divided into those with linear and those with granular deposits by immunofluorescence, no significant difference was present between the two groups.

Experiments were performed which demonstrated that incubation of cells from normal subjects in plasma of positive individuals for 30-60 minutes at 37°C did not cause inhibition of migration of these cells when subsequently incubated in the presence of G.B.M. antigens. Thus, the specific reactivity to G.B.M. antigens was not transferred to cells by plasma.

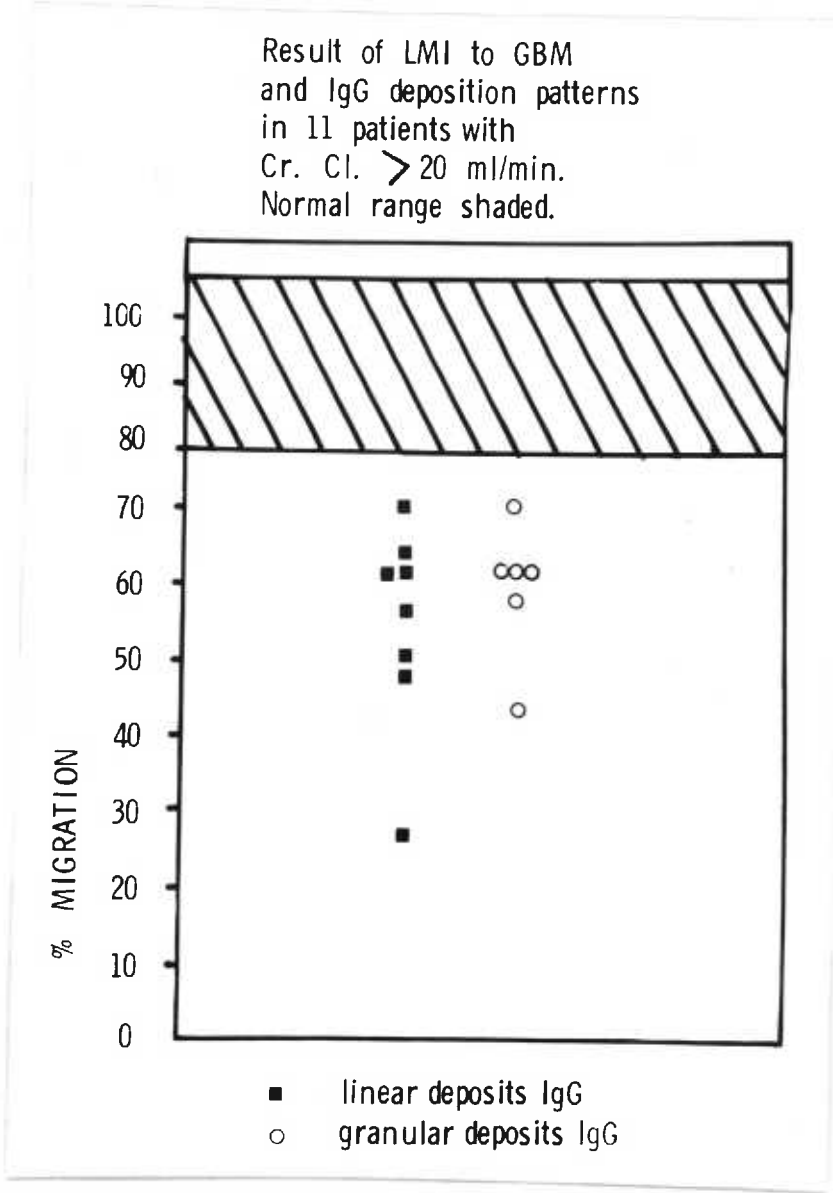


Figure 26

Peripheral leucocytes of ten patients that were found to be sensitive to G.B.M. antigens were subsequently incubated with the first and second chromatographic fraction obtained after chromatography of collagenase solubilised G.B.M. on Sephadex G-150. Both fractions were found to inhibit cell migration, the effect of the first fraction being more prominent. However this and the previous experiment were preliminary studies, and more detailed investigation is needed.

An attempt was made to show the correlation of agglutination titre for specific anti-G.B.M. antibodies and migration-index in the group of patients investigated, but it appeared that there was no correlation between these parameters.

3.42. Cellular hypersensitivity to streptococcal antigens

When sufficient number of cells were balanced, hypersensitivity to streptococcal membrane antigens of type 5 and 12 was investigated. Streptococcal membrane antigens were from group A streptococci. Fig. 27 illustrates the results of leucocyte migration inhibition test against streptococcal membrane antigens. The overall picture was similar to that obtained with G.B.M. antigens, although the migration indices were slightly higher than the ones obtained when G.B.M. antigens were used. Beside other factors, this might be due to the dose of the antigen used. White cells from patients with proliferative GN showed significant inhibition of migration in the presence of particulate streptococcal membrane antigens. Leucocytes from patients with unrelated renal disorders and normal controls exhibited minimal reactivity to streptococcal membrane antigens.

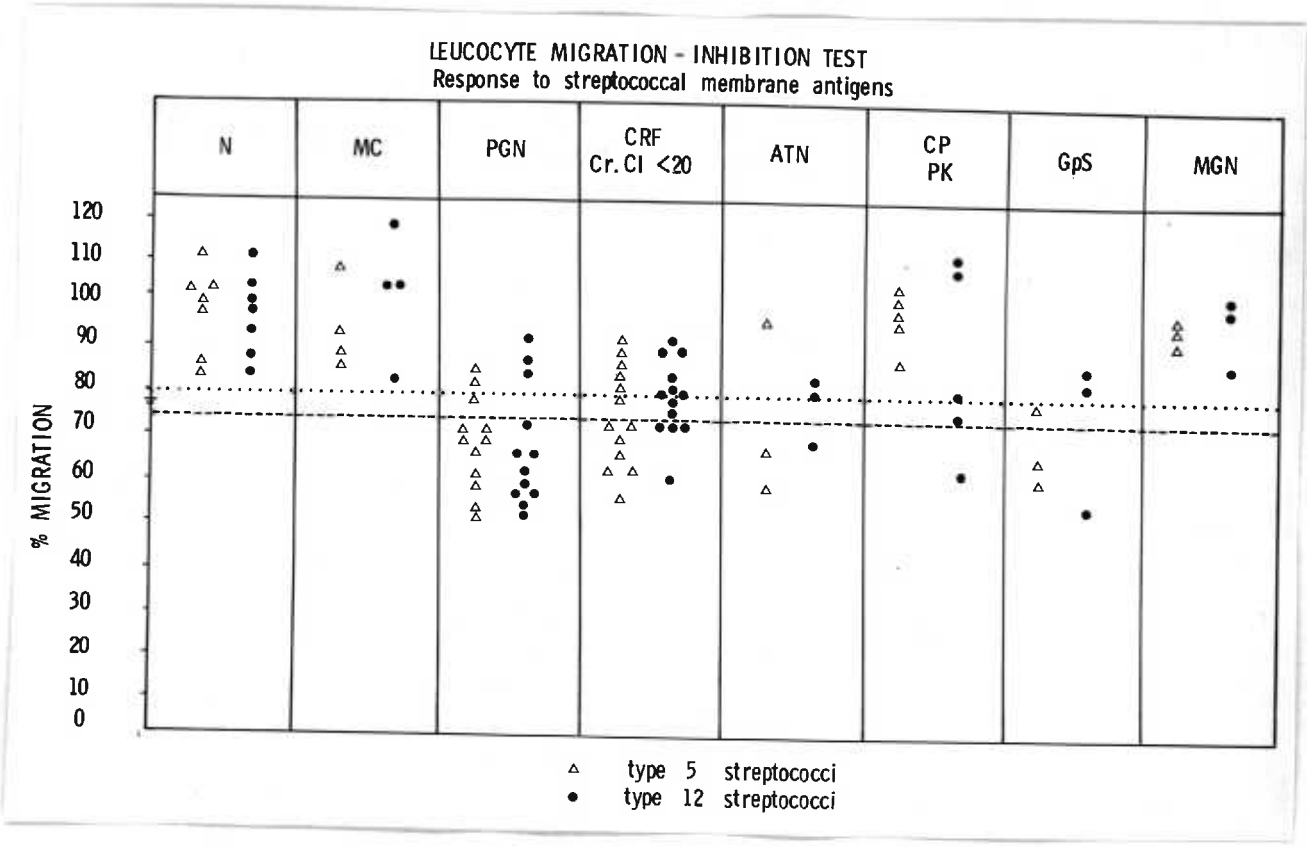


Figure 27

4. DISCUSSION

4.1. Glomerular basement membrane

4.11. Isolation of human G.B.M.

The present work indicates that it is possible to obtain high purified G.B.M. from normal human renal cortex. The first step in G.B.M. preparation is isolation of renal glomeruli. The anatomical localisation of glomeruli is such that purely physical methods, use of sieves of different pore size, are sufficient for their isolation. The sieves were used for both the tissue disruption and separation of glomeruli from other components of kidney cortex. There are reports on isolation of glomeruli by the use of sieves in various animal species and humans (Krakower and Greenspon 1951; Spiro

1967a; Huant and Kalant 1968; Steblay and Rudofsky 1968a; Westberg and Michael 1970; Mahieu and Winand 1970a; Nagi and Kirkwood 1972). The method used in this study was the slightly modified method from Spiro (1967a) and based on the original method of Krakower and Greenspon (1951). The modifications included the use of sieves of slightly different pore size of 150, 240 and 63 microns instead of 141, 251 and 61 microns. Repeated washing with ice cold saline is necessary to remove blood and other free cellular elements as much as possible and to wash out plasma proteins. Based on morphological criteria, the end product was highly purified glomerular suspension, free of parietal capsules and tubular fragments. The G.B.M. obtained from such glomerular preparation represent only B.M.'s from the capillary tufts.

In order to prepare G.B.M., isolated glomeruli are ultrasonically disrupted. Sonication period was in general longer than described in previous studies. Sometimes it is necessary to prolong sonication up to 10 minutes for complete disruption of the glomeruli. Filtration through 63 microns pore size sieve after sonication is also necessary to obtain G.B.M. preparation free of any cellular elements by morphologic criteria. By chemical criteria of purity, the negligible amounts of RNA and DNA present in the membrane preparations, indicate that it is free from significant cytoplasmatic and nuclear elements. In conclusion, the present study indicates that a highly purified preparation of G.B.M. is obtained from renal cortex of normal human kidneys and that such a preparation is used in further studies of solubilisation of G.B.M.

4.12. Solubilisation of human G.B.M.

Soluble G.B.M. is obtained from native G.B.M. by collagenase digestion. Solubilisation of G.B.M. is undertaken to prepare soluble antigens which could be used in subsequent tests of humoral and cell-mediated allergic response to such antigens in patients with glomerulonephritis. The method of solubilisation is that of Spiro (1967a), modified in two respects. Firstly, the length of incubation of enzyme and substrate is 120 hours instead of 72 hours. It is shown that longer incubation results in higher yield of soluble material. Secondly, it is found imperative to heat the enzyme/substrate mixture immediately after digestion for 30 minutes at 60°C to inactivate the remaining enzyme. In addition, greater amounts of enzyme are added, the total amount being 1.35% instead of 1.15%. The yield of soluble G.B.M. obtained is about 35% of the original G.B.M. before the solubilisation. The yield is comparable to the other studies where collagenase was used, but it is very low if compared to the weight of the original material: kidney cortex or isolated glomeruli.

Collagenase was chosen for solubilisation of G.B.M. because the previous studies have shown that this enzyme does not alter the antigenicity of G.B.M. (Huant and Kalant 1968; Kefalides 1970). It is also an enzyme with high specificity, unlike trypsin or pepsin. The digestion with these two enzymes resulted in a smaller degree of solubilised membrane (Skoza and Mohos 1969). Collagenase sharply differentiates collagen antigens and could bring almost complete solubilisation of the peptide and carbochydrate portions (Skoza and Mohos 1969). The G.B.M. is disrupted by collagenase action, which is known to break peptide bonds (Huant and Kalant 1968) and degrade the crystalline region of collagen into dialysable tripeptides (Huant and Kalant 1968).

It has been the problem of solubilisation that made difficult, for a long time, the fractionation and immunochemical characterisation of G.B.M. G.B.M. is insoluble in water, some methods of solubilisation render soluble antigens in the solvents inconvenient for immunological tests (Allan and Crumpton 1971). Common to all solubilisation procedures is the problem whether soluble fragments represent substantial material and the major antigenic components, or whether they are degraded to non-characteristic fragments of the original material. On the basis of several previous reports, it has been established that the method of solubilisation with collagenase render soluble G.B.M. antigens that retain the major nephritogenic antigens. Some results of the present study represent additional support to the theory that collagenase digestion does not alter the antigenicity of the G.B.M.

A batch of native normal human G.B.M. was exposed to prolonged ultrasonication of 20 minutes. After centrifuging, the supernatant contained soluble G.B.M. material which, upon testing with rabbit anti-human G.B.M. antiserum, gave the identical precipitation lines as material solubilised by collagenase. The yield of solubilised material obtained by this procedure is very low, the experiment was performed to verify that entirely mechanical extraction is efficient in elaborating soluble G.B.M. material. Similarly, Naruse and Shibata (1972) found that the supernatant after 15 minutes of sonication of glomeruli produced strong nephrotoxic antigen, capable of induction of potent nephrotoxic serum if injected into rabbits.

Preliminary experiments with sodium desoxycholate and trypsin digestion of native G.B.M. showed that even if the material was digested, it was degraded to an extent that resulted in loss of antigenicity. This might have resulted from the use of high concentrations of the enzyme, as other workers have reported the extraction of soluble G.B.M. antigens by trypsin digestion (Hawkins 1968b).

4.13. Fractionation and immunochemical characterisation

The antigenicity of G.B.M. and fractions of G.B.M. was investigated with the anti-G.B.M. serum produced in rabbits. On direct examination of the kidneys of rabbits immunised with human G.B.M., smooth linear deposits of IgG were present following the outline of glomerular capillary loops, with no fluorescence of cellular or cytoplasmatic elements. The immunofluorescent staining was also investigated after direct application of antiserum to frozen sections of normal human kidney. The incubation with rabbit anti-human G.B.M. serum was followed (after washings) by the fluorescein conjugated anti-rabbit IgG. Again characteristic linear smooth deposits on glomerular basement membranes were found. However, not only G.B.M., but also tubular B.M. and vascular basement membranes of small vessels were brightly fluorescent. This difference in fluorescence is most probably due to greater amounts of antibody reaching tubular basement membranes in vitro. In vivo, all antibodies were fixed to the free antigenic sites on G.B.M., and little antibody reached tubular basement membranes, perhaps because the covering cells prevented fixation of antibodies to tubular basement membranes. In vitro, these factors do not operate.

The above experiments also show the antigenic similarities between glomerular basement membranes in different species. Even if immunised against human G.B.M., rabbits showed bright, linear, extensive fluorescence on their G.B.M. in vivo. It is apparent that antibodies produced in response to human G.B.M. are not able to discriminate between human and rabbit G.B.M. This is further support for the observation of antigenic cross-reactivity among mammalian glomerular basement membranes. Lerner et al (1967) found that antibodies eluted from human kidney with anti-G.B.M. antibody mediated GN stained the glomerular and tubular basement membranes of kidney sections of mouse, rat, guinea pigs, rabbit, pig, and lamb.

The first steps in studying the antigenicity of G.B.M. in the present work are double diffusion analyses. Two lines of precipitation are always obtained in gel when soluble G.B.M. antigens were tested either with antiserum prepared by immunisation with native or soluble G.B.M. Both antisera produced identical lines. These experiments indicate that: (1) collagenase solubilised G.B.M. is antigenic in rabbits and (2) collagenase digestion does not cause detectable alteration in the antigenicity of G.B.M. G.B.M. and specific antiserum gave two lines in double diffusion, confirming earlier reports (Milgrom et al 1964; McPhaul and Dixon 1969; Shulman and Wypych 1969) that soluble human G.B.M. possesses two main antigenic components, represented by two separate fractions which give two separate lines of precipitation. Although two precipitin lines were usually seen in double diffusion between soluble G.B.M. present in urine and sheep anti-human G.B.M. serum (Pierce and Nakane 1967a; McPhaul and Dixon 1969a; Pierce 1970), three or more lines were noted in some instances. The heterogenous physicochemical composition of human G.B.M. was anticipated by electrofocusing experiments where one antigen localised over a very broad isoelectroc zone, from pH 1.7 to 3.5 (McPhaul and Dixon 1969a).

The complexity of antigenic structure and heterogeneity of human G.B.M. is evident from immunoelectrophoretic and particularly antigen-antibody cross electrophoretic studies. Not only two, but regularly many more precipitin lines were seen. This multiplicity of antigenic structure induced the experiments on fractionation of G.B.M. But, before the comments on fractionation experiments, it would be of importance to consider the relationship between collagenase digested soluble G.B.M. antigens and the antigenicity of non digested residue.

Huang and Kalant (1968) compared the antigenicity of collagenase soluble rat G.B.M. and undigested residue which was further solubilised by urea extraction. Cross reactions in both directions between components solubilised by collagenase and by urea extraction indicated that they share at least one common antigenic determinant. The cross reaction between the isolated fractions was further demonstrated by absorption experiments. Collagenase solubilised G.B.M. was able to absorb out completely the antibodies against urea solubilised G.B.M. from anti-G.B.M. serum. On the basis of chemical composition, it is not possible to determine whether the glycoproteins solubilised by collagenase digestion are identical to that solubilised by urea extraction; however, immunologic evidence obtained in double diffusion and absorption experiments strongly suggest that the two glycoproteins are similar, or at least contain common antigenic groups.

Our fractionation experiments show that upon gel filtration on Sephadex G-150, soluble collagenase digestion produced digest of normal human G.B.M. is separated into three main fractions. These fractions are of different molecular weight, the fraction A being of more than 200,000, fraction B about 70,000 and the last fraction of very small molecular weight, eluted around the peak of ^{131}I. All chromatographic fractions isolated

were investigated after gel filtration on Sephadex G-150 immunologically for antigenicity. Tests were done using immunodiffusion, IEP and cross electrophoresis methods. Results of these studies demonstrate that:

(1) it is possible to separate two main antigenic components of G.B.M., i.e. the components that give two lines of precipitation when soluble G.B.M. is tested in double diffusion against rabbit anti-human G.B.M. serum. These two fractions differ in regard to molecular weight and could be correlated to the fractions that Kefalides (1970) obtained upon gel filtration on Sephadex G-200 of soluble canine G.B.M. The fractions isolated were non-collagen polypeptides, one of molecular weight of over 200,000 and the other of m.w. about 55,000, both linked to collagen by disulfide bonds. As both fractions reacted with anti-G.B.M. serum, both might be nephritogenic antigens. Skoza and Mohos (1969) solubilised bovine and mouse G.B.M. by enzymatic digestion using trypsin. After gel filtration on Sephadex G-200, soluble G.B.M. antigens were separated into two high and one low molecular components. All fractions contained sialic acid. They studied nephritogenicity of the fractions in vivo, for anti-kidney serum neutralising capacity (which was after absorption injected into animals). They found that high molecular weight fragments possessed the antibody neutralising capacity, while low molecular weight fragment was completely inactive in ability to absorb the nephrotoxic antibodies from NTS. Huang and Kalant (1968) fractionated collagenase solubilised rat G.B.M. on Sephadex G-75, and G-200, into six fractions. Chemical analyses of the fractions indicated that they were glycoproteins in nature, and that they differ quantitatively, not qualitatively, in chemical composition.

(2) The last fraction after chromatography of soluble G.B.M. had no antigenicity when tested with rabbit anti-G.B.M. serum in immunodiffusion, IEP or antigen-antibody cross EP. Low molecular weight fragments after chromatography on Sephadex G-200 of bovine or mouse trypsin digested G.B.M. had no neutralising capacity for anti-kidney serum (Skoza and Mohos 1969). Similarly, it was not possible to obtain any lines of precipitation when low molecular fragments after gel filtration of soluble rat G.B.M. were tested against specific antiserum (Huang and Kalant 1968). It seems that, on the basis of our study and others cited, proteolytic digestion of glycoproteins constituting the G.B.M. results in a progressive protein breakdown, with the loss of antigenic capacity in low molecular weight fractions. This finding can be interpreted also as indirect proof that the nephritogenic antibodies are directed towards the high molecular antigenic components of G.B.M. When native G.B.M. was digested with pronase (Spiro 1967a; Skoza and Mohos 1969), similar conclusions were reached. Pronase completely degrades the protein portion of the antigen with the destruction of the molecular integrity necessary to elicit nephrotoxic antibody. After digestion with pronase, a single fraction was obtained if soluble material was further fractionated on the Sephadex column. This single peak consisted entirely of low molecular fragment with similar characteristics to the third fraction we obtained upon gel filtration of collagenase solubilised human G.B.M. on Sephadex G-150 column. The last peak obtained after gel filtration of collagenase solubilised G.B.M. on Sephadex G-150 gave no lines in gel when tested with anti-G.B.M. serum, presumably because of its small molecular size and could well be the smallest in a range of degradation products caused by progressive protein breakdown during the proteolytic digestion.

(3) double diffusion and antigen-antibody cross electrophoresis show the presence of multiple antigens in each of the separate G.B.M. fractions. If we exclude a number of conditions which might give double lines from a homogenous antigen (Ouchterlony 1962), multiplicity of lines in gel diffusion between each separate G.B.M. fraction and specific anti-G.B.M. antiserum might suggest heterogeneity.

The multiplicity of lines observed in double diffusion and cross electrophoresis when separate fractions were tested with the anti-G.B.M. serum may be the result of the progressive breakdown of native G.B.M. resulting in a wide range of components, different in regard to molecular weight, size and charge. Proteolytic digestion resulted, not in a few sharply differentiated uniform fractions within each of the elution peaks on gel filtration, but in a large number of fragments, polydispersed from large fragments of more than 2 million molecular weight to polypeptides. It is most probable that within each peak after chromatography on Sephadex, there exist a range of constituents different in molecular weight. These differences may be sufficient to produce several lines when tested in gel with specific antiserum. This is nicely illustrated with further fractionation of pooled peak I from Sephadex G-150 on Sepharose 6B. The peak I is fractionated further into 4 fractions, each of them producing multiple lines when tested in double diffusion method with specific anti-G.B.M. antiserum.

However, as there are no substantial differences in antigenicity between separate fractions of collagenase soluble extract of normal human G.B.M., soluble G.B.M. obtained after collagenase digestion of native G.B.M. may be regarded as well characterised, specific, nephritogenic component of normal human G.B.M. This preparation was used in subsequent tests of

humoral and cellular allergic response to G.B.M. in patients with glomerulonephritis. The preparation contained no collagen and non-specific reactions to collagena will be excluded. The use of this antigen is much more specific than the use of foetal kidney homogenate or renal kidney cortex, as many authors have used in assessment of immunity to kidney antigens in GN.

4.2. Humoral allergic response to G.B.M.

Using passive haemagglutination technique and glutaraldehyde as a coupling agent, we demonstrated the presence of circulating anti-G.B.M. antibodies in patients with the immunofluorescent evidence for anti-G.B.M. mediated GN. All sera with the high titre of circulating anti-G.B.M. antibodies fixed in vitro to frozen normal human kidney sections as proved by indirect immunofluorescent method. In addition to the high titre of circulating anti-G.B.M. antibodies in patients with linear deposits of IgG, some patients with immune-complex GN and renal necrosis exhibited low, but significantly raised, titre of circulating anti-G.B.M. antibodies. These results will be discussed in the light of previous reports on the pathogenetic role and incidence of anti-G.B.M. antibodies in patients with glomerulonephritis.

Observations by Lerner et al (1967) demonstrated that some cases of human GN are mediated by anti-G.B.M. antibodies. This specific autoimmune disease process appears to be more frequent among the patients with a rapidly progressive GN such as Goodpasture's syndrome and subacute GN. McPhaul and Dixon (1969a) and Lerner et al (1967) as well as other authors elucidated the following features of anti-G.B.M. antibodies as important for their

pathogenetic role in GN: (1) the pattern of fixation of host IgG, presumably anti-G.B.M. antibody in a linear smooth fashion along the glomerular capillary walls, corresponds precisely to the distribution of heterologous anti-G.B.M. antibody localised in vivo in NTS nephritis and of autologous anti-G.B.M. antibody in autoimmune version of this experimental disease (Lerner et al 1967; Steblay 1968b); (2) the reactivity of serum antibodies or renal eluate with antigens of the G.B.M. was demonstrated by the in vitro reactions. Antibodies from serum or renal eluate react in gel with soluble extracts of homologous or heterologous G.B.M. They also react with G.B.M. of the capillary walls of frozen normal kidney sections giving linear immunofluorescent pattern of staining; (3) when injected to homologous or heterologous recipients the anti-G.B.M. antibodies fix in a linear fashion along the G.B.M. walls of the kidney of the recipient; (4) anti-G.B.M. antibodies fixed in the identical linear pattern to the allotransplanted kidney in a patient who prior to transplantation was shown to have anti-G.B.M. antibodies; (5) the above conclusions demonstrated immunological reactivity of anti-G.B.M. antibodies and their effectiveness in reaching their target organ in vivo. The pathogenicity of anti-G.B.M. antibodies was demonstrated by the fact that following the reaction in vivo with the G.B.M. of allo-transplanted kidney in human, or deposition in the kidney of animal to which they were transferred, the fixation to the G.B.M. was followed by immediate development of nephritis and the disappearance of antibodies from the circulation.

Even if the existence and pathogenic role of anti-G.B.M. antibodies in human GN is established, their incidence remains difficult to assess. This is partly due to the difficulties in detecting circulating anti-G.B.M. antibodies. Conclusions on their incidence gained from the renal biopsies studied by immunofluorescent technique depend upon selection criteria for renal biopsy, and the patients studied may be unrepresentative. The present methods of testing the sera for anti-G.B.M. antibodies underestimates their frequency from the following reasons (Lerner et al 1967): (1) Kidneys with their G.B.M. exposed to the circulation via endothelial pores functionate as an antigen sponge soaking up the anti-G.B.M. antibodies from the circulation and are extremely efficient in removing them from the blood. This efficacy of the kidneys in removing the circulating antibodies was demonstrated in a patient who prior to the transplantation, after nephrectomy, had circulating anti-G.B.M. antibodies detectable even by immunodiffusion, but immediately after transplantation, they were not detectable (Dixon 1968) (2) Sera from nephrectomised end stage chronic glomerulonephritics might well have much lower levels of circulating antibodies because of the lack of continued antigenic stimulation, the kidneys being hyalinised or fibrosed; (3) Patients in advanced renal failure and end stage renal disease might have a low level of circulating anti-G.B.M. antibodies due to the immunosuppressive effect of uremia; (4) It might be that patients with rapidly progressive glomerulonephritis and rapid deterioration of renal function without prolonged period of uremia are the candidates for the detection of circulating anti-G.B.M. antibodies (patients with Goodpasture's syndrome); (5) As anti-G.B.M. antibodies are formed elsewhere, in the immune system and are reaching kidneys via blood, they must be present in the circulation and that present methods are insensitive and underestimate their incidence; (6) Antigens employed so far have not contained all or at least the main antigenic determinants of G.B.M.

4.21. Detection of circulating anti-G.B.M. antibodies by passive haemagglutination method

A great handicap in attempting to study the frequency of anti-G.B.M. antibodies has been the lack of a suitable assay for circulating anti-G.B.M. antibodies. This was a starting point in our efforts to find a practicable, sensitive assay for circulating anti-G.B.M. antibodies.

The presence of IgG and complement in a distinct, smooth linear sharp pattern along the G.B.M. on direct immunofluorescent examination of renal biopsies is presumptive evidence for the presence of anti-G.B.M. antibodies. However, the necessary diseased kidney tissue cannot always be obtained for fluorescent study. Furthermore in patients in whom this pathogenetic mechanism has been established to be responsible for nephritis, there is a need for a sensitive assay in vitro to assess the value of therapy and to help choose the most appropriate time for transplantation. Our efforts to detect circulating anti-G.B.M. antibodies using the double diffusion technique of Ouchterlony proved unsuccessful. Even sera of patients with Goodpasture's syndrome with the direct immunofluorescent evidence for anti-G.B.M. mediated GN, gave no lines of precipitation when tested in agar with soluble G.B.M. antigens.

Many workers have found that passive haemagglutination is one of the most sensitive available methods for the detection of minute amounts of antibody. We have tested several reagents for their ability to link G.B.M. antigens to r.b.c. and assayed the sensitised cells in PH test with specific anti-G.B.M. antiserum or human sera supposed to have anti-G.B.M. antibodies present (sera of patients with direct immunofluorescent evidence of anti-G.B.M. antibody mediated GN). Among the reagents and methods used

were: tannic acid, bisdiazotised benzidine, chromium chloride and glutaraldehyde.

A number of workers have claimed that circulating anti-kidney antibodies could be detected by the use of PH techniques, especially since Lange et al (1949) demonstrated anti-kidney antibodies by the collodion particle technique. However, all the investigators used non-purified kidney antigen (whole kidney homogenate, or supernatant of kidney homogenate) and non-specific reactions contributed to the high proportion of positive results. After the work of Lerner et al (1967) it became apparent how difficult it is to demonstrate circulating anti-G.B.M. antibodies, and only occasional reports on the detection of circulating anti-G.B.M. antibodies in nephrectomised patients appeared.

Recently Mahieu et al (1972) using chemical linkage of G.B.M. antigens to sheep r.b.c. by the $CrCl_3$, demonstrated the presence of circulating anti-G.B.M. antibodies in patients with linear deposits of IgG, and they demonstrated the low level of circulating anti-G.B.M. antibodies in patients with immune complex disease. In our efforts to find a sensitive assay for the detection of circulating anti-G.B.M. antibodies, we tried the same technique, but were unable to confirm the results of Mahieu (1972) and others. Even strong rabbit anti-human G.B.M. serum gave extremely low titre of circulating anti-G.B.M. antibodies when test with $CrCl_3$ sensitised cells, presumably reflecting inadequate coupling of the appropriate antigen determinants.

Our results with the use of tannic acid for coupling G.B.M. antigen to r.b.c. were also disappointing. Although Liu and McCrory, adsorbing whole kidney tryptic digest to TA treated r.b.c. were able to demonstrate

the presence of anti-kidney antibodies in a large group of patients with GN, we were unable to demonstrate the anti-G.B.M. antibodies in any serum tested, and rabbit anti-G.B.M. serum gave just 1:64 titre of circulating anti-G.B.M. antibodies.

In our hands, even the classic bisdiazotised benzidine method was insufficient in demonstrating circulating anti-G.B.M. antibodies present in rabbit anti-human G.B.M. serum or human sera of patients with GN.

Avrameas (1969a) and Onkelinx et al (1969) simultaneously described the method for attachment of proteins to r.b.c. and used the sensitised cells in passive haemagglutination system for detecting specific antibodies. The method detected as little as 0.02 microgram antibody N in a sample (Onkelinx 1969). Free amino groups seem to be the main functional group by which GA links proteins to r.b.c.

We slightly modified the method of Avrameas (1969a) and adapted it for coupling soluble G.B.M. antigens to human r.b.c. It was undoubtedly the best method in our hands and coupling of G.B.M. antigens to r.b.c. with GA gave sensitised erythrocytes possessing the highest haemagglutination titres with known anti-G.B.M. antibodies.

Cells sensitised with GA gave excellent results, small batches of cells prepared at different intervals produced consistent results. Changes in total amount of G.B.M. (from 2 mg/ml to 10 mg/ml) and changes of temperature, have little effect on the final readings. However, much higher concentrations of G.B.M. antigens lead to haemolysis of cells. Similarly Onkelinx et al (1969) noticed haemolysis of cells when too high

concentrations of antigen were used. We found that reaction time of one hour is sufficient for optimal coupling to occur.

The specificity of the method, the specificity for the antibodies detected, was demonstrated by the appropriate inhibition and control experiments. Thus the PH titre became negative if positive sera were pre-incubated with native G.B.M. All control sera (sera from normal subjects) gave negative results. Only in the beginning, when the method was not standardised, occasionally positive results of titre 1:2 were obtained in normal controls. Positive sera gave negative results with uncoated cells, or cells coated with non specific antigen (BSA).

To ensure the specificity of the antibodies detected, antigens were immunologically well defined, preparation of the antigen and solubilisation procedure was shown not to modify the antigenicity.

The significance of this study is that it defines a practical sensitive technique for the assay of extremely low titre of circulating anti-G.B.M. antibodies in patients with anti-G.B.M. antibodies present in the circulation. The method gives the reproducible results, is easy to perform, and is quantitative, not only qualitative, and specific for circulating anti-G.B.M. antibodies.

We were also able to demonstrate that it is possible to detect circulating anti-G.B.M. antibodies prior to nephrectomy. The method appears to be more sensitive than indirect immunofluorescence and correlates with the interpretation of direct immunofluorescent findings of renal biopsies; and high titres were regularly obtained when the indirect immunofluorescent method was positive. The differences in the sensitivity of the GA method

as compared to other tried methods of PH might be due to differences in chemical linkage of G.B.M. antigens to r.b.c.

Our data differ from previous reports in that we had fewer positive results in patients with GN, and a higher degree of specificity. Using a GA method it is possible to detect anti-G.B.M. antibodies in rabbit anti-G.B.M. serum diluted more than 1:500,000. It is possible to demonstrate unequivocally the presence of small quantities of anti-G.B.M. antibodies in the sera of all patients with linear deposits of IgG in vivo. The highest titres were obtained in the sera of patients with Goodpasture's syndrome and sera of patients with rapidly progressive GN mediated by anti-G.B.M. antibodies (according to the direct immunofluorescent findings). In such sera, titre of circulating anti-G.B.M. antibodies was as high as 1:64.

The surprising finding in our results is the demonstration of small but significantly raised titre of circulating anti-G.B.M. antibodies in patients with immune-complex nephritis. It appears that glomerular lesions, whatever the primary cause, may give rise to circulating anti-G.B.M. antibodies, but, in such instances, the level of circulating anti-G.B.M. antibodies is low. It is therefore possible that the two immunopathologic mechanisms, anti-G.B.M. antibodies and immune complexes may therefore operate simultaneously in some patients with proliferative GN. In this connection it is of some interest that Gallo (1970) found linear deposits of IgG in individual patients with lupus nephritis and polyarteritis involving the kidney and Landry (1972) reported the presence of basement membrane antibodies in two patients with lupus erythematodes. Koffler et al (1970) demonstrated linear deposits on renal biopsies of IgG in two

patients with lupus erythematodes. Dixon (1971) stated that occasionally anti-G.B.M. antibodies are found in a few cases of membranous glomerulonephritis, acute poststreptococcal GN and malarial nephrosis, all renal disorders thought to be primarily mediated by immune complexes. Hawkins, (1971) commenting on the immunofluorescent findings in patients with subacute and chronic glomerulonephritis, stated that the renal immunofluorescent pattern is not always clearly separarable into either continuous linear or interrupted lumpy granular patterns. Both types may be seen in one biopsy. In conclusion, low titre of circulating anti-G.B.M. antibodies in patients with glomerular damage induced by immune complexes or otherwise, may indicate that both immunopathogenetic mechanisms may be operative in the same patient - the one perhaps triggering off the other. Confirmation of this hypothesis requires elution of anti-G.B.M. antibodies from the kidneys of a patient with immune complex nephritis.

In the meantime it is difficult to evaluate the relationship of the low titre of circulating anti-G.B.M. antibodies to the pathogenesis of GN. The low titre of circulating anti-G.B.M. antibodies may also be regarded as an epiphenomenon, secondary to renal damage and of no pathogenetic significance. Their presence nevertheless indicates that the patient has the capacity to form anti-renal antibodies.

We have so far only limited data on serial determination of anti-G.B.M. antibodies in patients with GN. In one patient with a high titre of circulating antibodies (P.K. St. Hellier Hospital) after bilateral nephrectomy, a very slow decrease in the titre over a 4 month period was demonstrated. Further studies will be directed towards serial determination of circulating anti-G.B.M. antibodies in the course of disease, improvement and relapses.

4.22. Detection of circulating anti-G.B.M. antibodies by indirect immunofluorescent assay

The indirect immunofluorescent assay of serum antibody fixation in vitro to the G.B.M. of normal human kidney section posses a high degree of specificity as the end point is in vitro fixation of IgG to the specific anatomic and presumably antigenic sites to which IgG is bound in vivo (McPhaul and Dixon 1969a). The sensitivity of indirect immunofluorescent method in detecting the levels of circulating anti-G.B.M. antibodies is in the range equivalent to 0.1 to 1.8 microgram/ml of kidney fixing antibody (McPhaul and Dixon 1969a).

We tested a number of sera in indirect immunofluorescent assay to assess the correlation with passive haemagglutination method. Patients with high titre of circulating anti-G.B.M. antibodies found by PH method and having linear deposits of IgG on their G.B.M. in vivo, exhibited linear pattern of IgG fixation in vitro, with their sera laid over frozen sections of normal human kidney and stained with fluoresceinated anti-human IgG. Apparently the indirect immunofluorescent method is not sensitive enough to detect low titre of circulating anti-G.B.M. antibodies as PH was. When compared to the results of McPhaul and Dixon (1968a) who had approximately 40% of positive results in their patients with anti-G.B.M. mediated GN, our results gave more positive findings. All patients studied, having linear deposits of IgG in vivo, showed positive indirect immunofluorescent assay.

4.23. Anti-G.B.M. antibodies in Goodpasture's syndrome

Patients with Goodpasture's syndrome and some patients with rapidly progressive glomerulonephritis had the highest titre of anti-G.B.M. antibodies recorded in this study. From the previous studies it seems that the anti-G.B.M. antibodies in Goodpasture's syndrome are different from other cases of anti-G.B.M. antibody mediated GN. Goodpasture's syndrome is mediated by potent anti-G.B.M. antibodies with significant reactivity with non-glomerular vascular and other epithelial basement membranes (McPhaul and Dixon 1970a). In addition to the deposits of IgG along G.B.M., immunofluorescent studies have demonstrated fine linear staining for both gamma globulin and complement along the outer aspect of the alveolar septal wall adjacent to the alveolar lumen (Koffler et al 1968; Koffler et al 1969; Markowitz et al 1968). Gamma IgG has been found to be the main Ig present, although traces of IgM and IgA might also be present in immune deposits on alveolar B.M. (Koffler et al 1969). The immune deposits are often located in small groups of alveoli in association with haemorrhage.

The antigenic similarity between lung and G.B.M. is well established (Korngold and Pressman 1953; Baxter and Goodman 1956; Steblay and Rudofsky 1968; Willoughby and Dixon 1970; Dias et al 1970). There is also much experimental evidence to suggest that lung and kidney contain cross-reacting antigens (Koffler et al 1969). Heterologous antisera prepared to either lung or kidney tissue extracts react primarily with kidney (Eisen et al 1950), but antigens responsible for the induction of nephrotoxic antibodies are found in both the lung and the kidney (Koffler et al 1969). Autoimmune GN has been induced in sheep immunised with human lung (Steblay and Rudofsky 1968), but in the same experiment it had been

shown that anti-G.B.M. antibodies, besides inducing autoimmune GN, had strong in vitro affinity for human alveoli and glomeruli. Therefore, in Goodpasture's syndrome, antibodies common to both lung and kidney are mediators of immune lesion in both organs (Proskey et al 1970). The nature and source of immunogen stimulating the production of the common anti-alveolar and anti-G.B.M. antibodies is difficult to establish. The initial immunisation may be triggered off by the viral or bacterial infection with the antigens which cross-react with both lung and kidney (Koffler et al 1969). The cross-reactions reported between streptococcal antigen and G.B.M. may be in favour of this hypothesis (Markowitz and Lange 1964). There is evidence that the primary immunisation may arise in the lung. The clinical history of initial lung involvement followed by renal disease suggests that an infectious agent may release or alter pulmonary B.M. (Koffler et al 1969). However in one patient (Martinez and Kohler series 1971) lung symptoms developed 18 days after nephrectomy.

A number of features might explain pulmonary lesions in other cases of GN mediated by anti-G.B.M. antibodies: (1) not all anti-G.B.M. antibodies have avidity for pulmonary B.M. and pheumotoxic potential; thus Lerner and Dixon (1968a) and Mahieu et al (1972) have established that anti-G.B.M. antibodies in GPS have greater affinity to G.B.M. and lung B.M. than anti-G.B.M. antibodies in cases without lung involvement; (2) better access to glomerular endothelial pores than to lung (endothelial cell cover) basement membranes prevents antibody accumulation in lungs even without the difference in the affinity.

We eluted the antibodies from the kidneys of a patient who died from Goodpasture's syndrome and studied the antigenic specificity of the eluted antibody. The patient had in vivo fixation of IgG in a characteristic linear pattern along the G.B.M. as demonstrated by immunofluorescence (Peters and Evans 1972). The specificity of the antibodies in eluate was determined by indirect immunofluorescent method and other experiments. The eluted antibodies were found to react with G.B.M. in vitro. Complete inhibition of the immunofluorescence reaction of the kidney G.B.M. with the eluate was obtained by incubation of eluate with native G.B.M. Double diffusion and IEP studies have demonstrated that the eluted antibodies consisted predominantly of IgG. We studied the fixation of ^{131}I labelled eluate to homogenates of different organs and found that it fixed to homogenates of various organs, but predominantly to lung and kidney. This finding is different from previous studies in vivo fixation of the eluted anti-G.B.M. antibodies (McPhaul and Dixon 1970a; Koffler et al 1969). When injected into monkeys, eluted antibodies fixed predominantly to kidneys, in some extent to liver and spleen, but no significant fixation was found in lungs. It has been suggested that such differences between in vivo and in vitro fixation is due to the greater affinity of the injected antibody in vivo for the kidney, so that antibody is preferably deposited in this site.

4.3. Cellular allergic response in glomerulonephritis

On the basis of humoral allergic response alone, it is not possible to explain all aspects of pathogenesis and particularly progressive course of some human glomerulonephritides. Neither of the two pathogenetic mechanisms deposited immune complexes or anti-G.B.M. antibodies have been associated

with characteristic histopathologic lesion in human disease (Lewis et al 1970). Both experimental immune complex or anti-G.B.M. antibody induced nephritis may be associated with either a proliferative or membranous lesion (Germuth et al 1967). In addition, the clinical spectrum often seen in human nephritis associated with anti-G.B.M. antibody may vary from acute, fulminant necrotising glomerulonephritis seen in the majority of patients with Goodpasture's syndrome, to the relatively indolent lesion of chronic glomerulonephritis (Lerner et al 1967).

The above studies suggest that host immune mechanism including cell-mediated immune reactions, may be factors in determining the morphology severity and natural history of a glomerulonephritic lesion. As immune processes involve both humoral as well as cell mediated immune phenomena, we have investigated some aspects of both in human GN, in order to determine whether these processes are selective and correlate with the histology of the glomerular lesion.

In considering the pathogenetic mechanisms involved in the various experimental allergic disorders, it seems possible that immunisation with any tissue antigen may give rise to both circulating antibodies and sensitised cells (Lerner and Dixon 1968; Dixon 1970). There is therefore the possibility that in some circumstances, cell mediated allergic response might be caused by the initial injury, and be responsible for maintenance of disease activity.

The view that there are two populations of lymphocytes, the one concerned with the antigen recognition and the development of cell mediated hypersensitivity and the other with the production of humoral antibody, also provides a reasonable explanation for the presence of both circulating

sensitised lymphocytes and antibodies to G.B.M. (Leading article 1972). The presence of anti-G.B.M. antibodies indicate that organism contains immunocompetent cells specifically equipped to synthesise anti-renal antibodies. It may consequently be supposed that other immunocompetent cells, not necessarily engaged in antibody production but with the same specificity, might possess the ability to react specifically with the antigens in renal structure following the pattern of cellular hypersensitivity reaction. Our present understanding of cellular hypersensitivity indicates that antibody formation and cellular hypersensitivity commonly accompany each other and that a purely humoral or cellular response is an exceptional product of either an abnormal immunologic system or an unusual mode of immunisation.

The exact nature of pathogenetic mechanism involved in progressive human GN is still undefined. As far as the immune complexes are concerned, studies in both experimentally induced GN and acute poststreptococcal GN in humans suggest that they are initiating factors in the one type of renal damage (Germuth et al 1967; Dixon et al 1958; Seegal et al 1965; Andres 1969). However, the evidence for the continuing participation of antigen-antibody complexes in progressive GN is more difficult to obtain. In experimentally induced GN there is a gradual diminuition of glomerular-deposited immune complexes after cessation of administration of the antigen (Dixon et al 1961) and immunofluorescent studies with streptococcal antiserums of renal biopsies specimens in man have often failed to demonstrate streptococcal products in renal lesions of these patients (Seegal et al 1965; McCluskey et al 1966). These studies, therefore, allow the possibility that altered cellular reactivity to exogenous antigens or human G.B.M. or both, might play a role in the pathogenetic process responsible for the continuing disease process in progressive GN.

4.31. Cell-mediated allergic response to G.B.M.

As an *in vitro* assay for sensitised cells against G.B.M. we used the direct MIF assay - the peripheral leucocyte migration inhibition test of Søborg and Bendixen (1967a). We demonstrated inhibition of leucocyte migration in the presence of G.B.M. antigens in all non-uraemic patients with proliferative GN. The *in vitro* assay used has been shown to correlate with delayed hypersensitivity *in vivo.* Peripheral white cells from normal subjects did not show cellular hypersensitivity to the antigens used. The results are interpreted as indicating the existence in proliferative GN of a state of organ specific hypersensitivity of the cell type directed to antigenic components of the renal parenchyma - namely G.B.M. There is a possibility that mediators produced by sensitised lymphocytes in contact with G.B.M. *in vivo*, are producing mediators such as MIF, chemotactic factors and others that may result in infiltrating and proliferative changes often seen in both experimental or human light microscopy examination of renal biopsies (Steblay and Rudofsky 1968a; Paronetto and Koffler 1967; Benoit et al 1964; Rocklin et al 1970; Steblay 1963; Steblay 1962; Lerner and Dixon 1966; Lawrence and Landy 1969).

In patients with anti-G.B.M. mediated glomerulonephritis in up to 30% of cases, complement components are not seen together with IgG in linear immune deposits, especially when IgG_4 subgroup is predominant Ig constituting the immune deposits. As IgG_4 does not fix complement, and as almost all cases of anti-G.B.M. mediated GN are rapidly progressive with proliferative changes on light microscopy of kidney tissue, it seems that renal damage and proliferative changes may occur through mechanisms independent of complement mediated chemotaxis. One possibility is that cell-mediated hypersensitivity reactions are involved. It might be that factors secreted by sensitised cells provoke proliferative changes.

Rocklin et al (1970) using the direct MIF assay, found that cellular hypersensitivity accompany the formation of anti-G.B.M. antibodies. We have also demonstrated positive cellular hypersensitivity to G.B.M. not only in GN mediated by anti-G.B.M. antibodies, but also in glomerulonephritis with granular deposits of immunoglobulins.

One difference between our study and some others (Mahieu et al 1972) is that we separated the patients with uraemia, i.e. patients with creatinine clearance below 20 ml/min into a separate group. This might be the reason that we had better clarity in our group of proliferative GN than Mahieu et al (1972) or Dardenne et al (1972). It is well known that patients with uraemia have a state of immunosuppression (Newbery and Sanford 1971); they retain skin grafts for prolonged periods (Damnin et al 1957); the delayed hypersensitivity skin reactions are suppressed (Kirkpatrick et al 1964) and that lymphocyte transformation is decreased when lymphocytes from uraemic patients are studied in mixed lymphocyte culture (Kasakura 1967). Since the positivity of cell mediated allergic reactions and release of mediators from sensitised cells appears to be antigen dependent, the reaction might be self limiting and could be negative in uraemic patients due to kidney tissue destruction, fibrosis etc.

From the results of our study it seems that cellular hypersensitivity reactions to G.B.M. antigens are not operating in membranous or minimal change GN. However, it is too early to gauge the manifestations of the test as a measure of the activity of immunological processes responsible for the disease. One observation is that patients with the syndrome of acute tubular necrosis in our series, and patients with cortical necrosis (Mahieu et al 1972), manifest cellular hypersensitivity to G.B.M. antigens. This phenomenon may be regarded also as a secondary reaction to renal damage,

indicating just a temporary state of hypersensitivity comparable to that observed in myocardial proteins following myocardial infarction. It is difficult on the basis of the small number of observations (three patients) to comment on this possibility.

Because of the limited number of cells obtained, it has not been possible in the present study to test if patients' blood lymphocytes would produce MIF in response to other antigens and whether they were specifically sensitised only to G.B.M. antigens. There might be a possibility that the patients positive to G.B.M. have more generalised hypersensitivity (Holman et al 1959) and would respond to other renal or other tissue antigens as well. However, using the identical in vitro system and comparable groups of patients with renal diseases, Bendixen (1968) tested patients' peripheral blood white cells with renal, but simultaneously with other tissue homogenates, including liver, colonic and ileal mucosa. In all cases showing the inhibition with renal homogenates, migration index (inhibition of cell migration) appeared to be uninfluenced by other antigens. It seems that inhibition of leucocyte migration in the presence of G.B.M. is specific for some groups of patients with kidney diseases.

No correlation was found between circulating anti-G.B.M. antibody titre and the migration indices.

4.32. Direct assay in patients with glomerulonephritis

Bendixen (1968) first reported that leucocytes from patients with active GN were inhibited from migrating from capillary tubes if the kidney antigens were present in tissue culture medium. Leucocytes from normal patients

and from patients with terminal nephropathy or chronic pyelonephritis were unaffected. Previously the same system was used for assessment of hypersensitivity to brucella (Søborg (1967a) and demonstrated to be an excellent correlation of cellular hypersensitivity. By analogy, Bendixen concluded that the positivity of the test to renal antigens also indicates the presence of cellular hypersensitivity in patients with GN, directed towards the antigenic components in normal renal parenchyme.

Mallick et al (1972) used the direct assay for evalidating the cell mediated hypersensitivity in patients with various renal diseases and found that the test was positive in 20 out of 27 patients . The antigen used was foetal kidney homogenate. In contrast to our finding of negative direct assay in patients with minimal change GN, they found positive test in all 8 patients studied with mimimal change GN.

Anti-renal cellular hypersensitivity was studied by the direct MIF assay in patients after renal transplantation (Weeke et al 1970; Ellis et al 1971; House et al 1972). These groups found a high percentage of positive results during rejection.

4.321. Technical data on the direct assay

It is now known that the lymphocytes represent the immunocompetent cells of the organism. Consequently it would be desirable to use preparations containing the highest possible percentage of mononuclear cells. Among numerous techniques for the separation of lymphocytes from the peripheral blood (Agranoff et al 1954; Gray and Russell 1956; Jensen et al 1962), a suitable method had to be selected. Such a method should

interfere as little as possible with the vitality and immunological qualities of the cells, and at the same time be comparatively simple and reproducible. Søborg (1967a) tried several methods for separation of lymphocytes (sedimentation with polyvinylpyrrholidone, dextran and simple sedimentation). With spontaneous sedimentation the risk of inducing non-specific changes in the cellular reaction capacity was eliminated and the erythrocyte contamination minimal.

The leucocyte migration test was carried out using sterile procedures. Cell migration could be observed within a few hours, and after 24 hours a well defined area of migrating cells surrounded the opening of the capillary tube. In measuring the cell migration, we measured the outer halo, as we observed that if inhibition occurred, both inner halo (consisting of mono-nuclear-polymorphs mixture) and the outer halo (consisting predominantly of polymorphs) were inhibited. The direct assay has the advantage of speed and the results are available in 24 hours. This time is too short for antibody production to occur as is the case in lymphocyte transformation. Like other workers, we also noticed that large doses of antigen, when soluble antigen was tested, were necessary for the inhibition to occur.

We incubated cells of normal subject in the serum of a patient who exhibited positive cellular reactivity to G.B.M. antigens. But our study showed that the serum from a positive patient did not sensitise normal cells. This experiment indicates that cytophilic antibodies were not responsible for the inhibition found in our system.

4.322. Cell cooperation and mediators in the direct assay

In the direct assay we used, a mixture of approximately 50% of lymphocytes and 50% of peripheral leucocytes was used. Peripheral lymphocytes are immunocompetent cells, but it is difficult to state which cells correspond to peritoneal macrophages of guinea pigs of indirect assay, whether PMN or monocytes. But it seems that the change in migration which occurs after antigenic confrontation is in the whole cell population, not only mononuclear. This observation has led to the hypothesis that the immunologically committed cells on confrontation with the specific antigen, release an active substance which affects the whole cell population (Falk et al 1969). This substance has been found in the supernatants of sensitised cells cultured for 24 hours with the specific antigen (Bloom and Bennett 1966). Extracts of killed sensitive cells were unable to inhibit the migration of normal cells, thus indicating that the specific interaction between the hypersensitive cells and the antigen is intimately bound up with the viability and normal biological activities of the cells (Søborg and Bendixen 1967a).

4.33. Cellular allergic response to streptococcal antigens

We demonstrated that lymphocytes from patients are not only sensitive to G.B.M. but also to streptococcal membrane antigens. The significant inhibition of cell migration was shown in the presence of group A particulate streptococcal membrane antigens of type 5 and 12. The finding of almost identical distribution of positivity to G.B.M. antigens and streptococci is probably best explained on the basis of the known antigenic cross reactivity between G.B.M. and streptococcal membranes.

Our observations confirm and amplify the findings of Zabriskie et al (1970) and Dardenne et al (1972), who also demonstrated the presence of cellular hypersensitivity to streptococcal membrane antigens in similar groups of patients. The fact that streptococcal membranes from both a nephritogenic and non-nephritogenic strain gave essentially similar results is surprising in view of the small number of streptococci inducing GN. However, it is conceivable that all streptococcal strains possess nephritogenic characteristics, and it is only the ease with which nephritogenic antigens or toxins are exposed in vivo which separates nephritogenic and non-nephritogenic strains (Zabriskie et al 1970; Zabriskie 1971).

Dardenne et al (1972) using the direct MIF assay, demonstrated the sensitivity to streptococcal membrane antigens in 46% of cases of chronic proliferative GN. Non-proliferative diseases, as well as control subjects did not demonstrate such hypersensitivity. The difference in our results and theirs might be partly due to the fact that we excluded proliferative GN patients with creatinine clearance below 20 ml/min.

The observations from our study and some previous studies (Zabriskie 1971; Dardenne et al 1972) indicate that the sensitisation to streptococcal membrane antigens exist in chronic progressive GN. Epidemiological, immunofluorescent and electron microscopy data (Zabriskie 1971; Andres 1969) have underlined the responsibility of streptococci in the genesis of acute GN. In view of the paucity of the information regarding the nature of the immunising antigens in chronic progressive GN, it is conceivable that the sensitisation to the streptococcal membrane antigens might be the factor contributing in the pathogenesis of renal damage, whether or not related to antigenic cross reactivity with G.B.M. (Zabriskie et al 1970).

5. CONCLUSION

Isolation, purification and immunochemical characterisation of normal human glomerular basement membrane and assessment of humoral and cellular allergic response to the soluble G.B.M. antigens were the objects of this study.

A detailed method for the isolation and purification of glomeruli from the normal human kidneys was described. G.B.M. was isolated after sonication of glomeruli and soluble G.B.M. antigens were obtained by proteolytic digestion of G.B.M. using collagenase. Normal rabbits were immunised with native or soluble G.B.M. and specific antiserum was produced. Immunochemical characterisation of G.B.M. antigens was performed using the methods of immunodiffusion, immunoelectrophoresis, antigen antibody cross electrophoresis and chromatography on Sephadex G-150 and Sepharose 6B. These studies demonstrated that it was possible to separate the main antigenic components of soluble G.B.M. One component was of a high molecular weight of more than 200,000, the other was of molecular weight in the 20,000 - 100,000 range. These two high molecular weight fragments retained the antigenic properties, a third fraction of approximately 10,000 m.w. had been degraded to small non-antigenic fragments of the original G.B.M. Gel fractionation studies showed that isolated fractions of soluble G.B.M. either obtained after chromatography on Sephadex G-150, or Sepharose 6B were not uniform, but composed of multiple components of different molecular weight and size.

The patients studied were categorised in regard to clinical, light microscopy and immunofluorescent findings. In the assessment of humoral allergic response to G.B.M. antigens the following methods were used: indirect immunofluorescence, double diffusion, passive haemagglutination methods and elution and subsequent characterisation of eluted antibodies from the kidneys. IgG from the sera of 10 patients with direct immunofluorescent evidence for anti-G.B.M. mediated glomerulonephritis fixed in vitro in a linear pattern to frozen sections of normal human kidney. The indirect immunofluorescent method was found to correlate well with direct immunofluorescence. Double diffusion was found to be too insensitive for the detection of circulating anti-G.B.M. antibodies. Among four passive haemagglutination methods tried, it was found that only glutaraldehyde coupling of G.B.M. antigens to human erythorcytes was sufficiently sensitive to detect circulating anti-G.B.M. antibodies. Under optimal conditions established, it was possible to detect anti-G.B.M. antibodies in rabbit anti-G.B.M. serum diluted more than 1:500,000. This test allowed the detection of circulating anti-G.B.M. antibodies in the sera of patients with glomerulonephritis, prior to nephrectomy. Evidence was obtained indicating that the test was specific for anti-G.B.M. antibodies. Circulating anti-G.B.M. antibodies were regularly found in high titre in patients with linear deposits of IgG on the basement membrane. Patients with granular deposits of IgG had low but significantly elevated titres of anti-G.B.M. antibodies. Antibodies were eluted from the kidneys of a patient with Goodpasture's syndrome and characterised to be mainly IgG and to fix in vitro to homogenates of various organs, but predominantly to kidney and lung. The fixation studies were done using previously labelled antibodies by ^{131}I.

For the in vitro assessment of cellular allergic response to G.B.M. and streptococcal membrane antigens, the peripheral blood leucocyte migration inhibition test was used. Evidence for cell-mediated allergic response to G.B.M. and streptococcal membrane antigens was found in patients with proliferative glomerulonephritis independently of whether the immunofluorescent appearances suggested nephritis due to immune complexes or anti-G.B.M. antibodies.

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