THE FUNCTIONAL MORPHOLOGY OF BASEMENT MEMBRANES IN THE KIDNEY

by

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CONTENTS

SUMMARY

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Much investigation has been carried out, into the pathogenesis of glomerulonephritis and other renal diseases. The histological and ultrastructural changes in this group of conditions have likewise been analysed, but little attention has been paid to the actual molecular structure of basement membranes and mesangial matrix in glomeruli or in tubules. In this project these structures were examined in normal and diseased kidneys by an FITC lectin technique which identified certain specific sugars in the renal structure. The carbohydrate molecules of basement membranes and mesangial matrix are widespread throughout the membranes and indeed in biochemical analysis are shown to be the major component.

Lectins are specific carbohydrate binding proteins of non-immune origin which have been isolated from both plant and animal kingdoms. Most of the work was done on frozen sections from routine hospital renal biopsies.

The glomerular capillary basement membrane plays a vital role in the filtration of the blood, and is therefore involved in the deposition of any large abnormal molecules circulating in the blood, usually of immunological nature; in addition, its own antigens may induce antibody formation. Several types of glomerulonephritis were

included in the cases examined and some cases of tubular disease, mainly acute focal ischaemic tubular necrosis were also available for study.

The groups of cases (renal biopsies) examined in this way were as follows:

a) Glomerulonephritis cases

- proliferative glomerulonephritis
- membranous glomerulonephritis
- crescentic glomerulonephritis
- focal necrotising glomerulonephritis
- transplantation rejection

b) Tubular disease

- acute tubular necrosis
- myeloma kidney

The results were recorded in negative +, ++ and +++ in glomerular capillary basement membrane and mesangial matrix and in tubular basement membrane and were compared with the pattern of results assessed similarly in a group of normal human kidneys.

The results were plotted as histograms to show the patterns of the diseased kidneys against the normal kidney and also analysed statistically to elicit any statistical

significance in the results. The statistically significant results which emerged were as follows.

In the glomerular disease group (including the transplant rejection cases) the binding of Soy bean agglutin to N-acetyl galactosamine was found to be the only lectin showing significance and was statistically less than that found in the normal in the tubular basement membrane.

When the transplant cases were excluded the binding of Peanut agglutinin to D-gal-β 1:3 galactosamine was statistically more in the mesangium. In the capillary wall and tubular basement membranes Concanavalin A binding to α-D-glucose and α-L-mannose was significantly more than found in the normal.

With the transplants only Soy bean agglutinin binding to N-acetyl galactosamine was significantly less than seen in the normal.

When the tubular disease group's results were analysed the significant results were as follows. Ricin binding to β-D-galactose was significantly more in the mesangium and Concanavalin A binding α-D-glucose and α-L-mannose was significantly more in the capillary wall than in the normal.

In addition a small study was carried out in which the technique was used on sections from paraffin-embedded material. For this project material from two conditions, minimal lesion glomerulonephritis and hereditary nephritis (Alport's syndrome) was compared with the normal.

Differences which were statistically significant were as follows.

In minimal lesion gomerulonephritis the binding of wheat germ agglutinin to N-acetyl-glucosamine was significantly less in the mesangium and the tubular basement membrane. Concanavalin A binding to α-D-glucose and α-L-mannose was also significantly less in both the capillary wall and tubular basement membranes.

On the other hand the only significant result in Alport's syndrome was the binding of Concanavalin A to α-D-glucose and α-L-mannose which was significantly more in tubular basement membrane.

The implications of the statistical results and of the patterns depicted in the histogram were discussed, as far as possible, considering the small number of cases involved.

INTRODUCTION

I James Kenneth Rae hereby certify that this thesis which is approximately ten thousand five hundred words in length has been written by me, that it is the record of work carried out by me, and that it has not been submitted in any previous application for a higher degree.

Date 18/9/84 .....

Signature of Candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of MSc of the University of St Andrews and that he is qualified to submit this thesis in application for that degree.

Date 21/9/84.....

Signature of Supervisor

INTRODUCTION

To sustain life the body has to metabolise food and maintain relatively constant levels of many substances such as sodium, potassium, hydrogen ions, bicarbonate ions and many proteins, in the plasma, and during this metabolic process and the subsequent release of energy, some waste products, especially nitrogenous compounds, are produced. There must therefore be a method for maintaining this constancy, in the face of the absorption of inconstant amounts of food. This is accomplished primarily by the kidneys which are capable of excreting excesses of water and elecrolytes and in so doing regulate the chemical composition of plasma and also extracellular fluid.

A system of urine formation is therefore required which involves a mechanism of ultrafiltration initially, with a further process of selective reabsorption to prevent too large amounts of water and small molecules from being lost to the body.

This system comprises the two kidneys where the functional units, the nephrons, are located, and an excretory system to store and void the excess fluid and solutes. Each mammalian kidney is composed of approximately one million

of these functional units, and proper functioning of the whole system is necessary for the maintenance of life. Additionally the kidneys are involved in the maintenance of blood pressure by the production of the hormone renin and also produce erythropoietin, which stimulates the production of RBCs in the bone marrow. As can be expected, the kidney is very richly supplied with blood vessels. It is estimated that the total volume of circulating blood passes through the kidneys once every five minutes.

The Kidney

In the human they are paired bean-shaped organs about 10-12cms in length, and 5-7cm wide; they weigh together 300gms. Each is enclosed in a thin fibrous tissue capsule which strips easily in normal conditions. When sectioned different regions are readily identified on gross examination. Immediately below the capsule lies the cortex, a zone which includes all the filtering units of the nephron, the glomeruli, the convoluted portions of the proximal and distal tubules, and the early portions of the collecting tubules or ducts (Fig 1).

The rest of the kidney is the medulla. The outer medulla contains the terminal portions (pars recta) of the proximal tubules, loops of Henle and the middle portion of

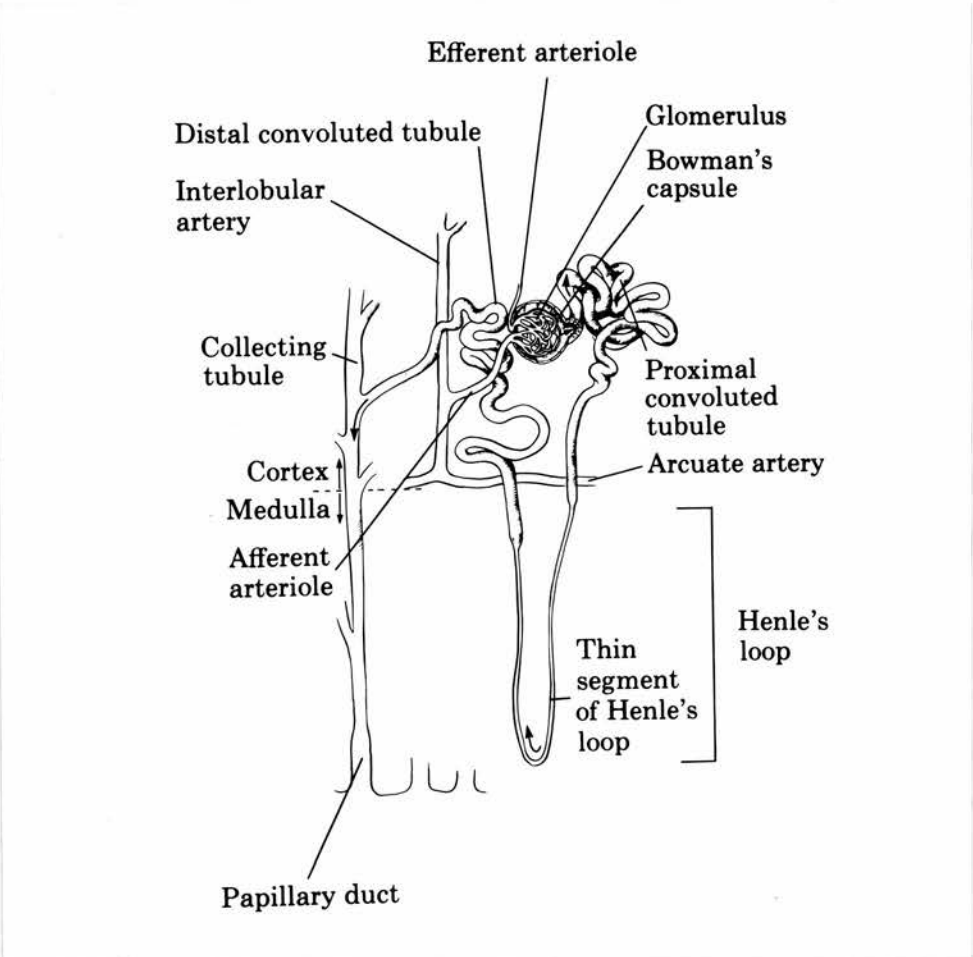


Fig 1 Schematic of Kidney Nephron

From Medical Histology (Bacon and Miles)

the collecting tubules. The inner medulla, farthest from the cortex, contains the descending and ascending limbs of loops of Henle, as well as the terminal portions of the collecting tubules (Fig 1).

This inner medulla borders on the renal pelvis which is a collecting chamber for urine as it emerges from the collecting ducts. The loops and ducts of the medulla are arranged in cone-shaped bundles or pyramids whose tips (papillae) project into the renal pelvis. The medulla as a whole extends into the cortex as fine radially orientated rays called medullary rays.

The Nephron

Each nephron consists of a glomerulus and a long epithelium-lined tube which terminates by joining an excretory duct, emptying into the renal pelvis. It is such a complex structure that it is not possible to describe it histologically as a single entity. It can be divided into two main parts:

I. The glomerulus, essentially a filter,

and II. a long tubule, in which the filtrate is partially reabsorbed and modified.

I. The Glomerulus

This unit is the ultrafilter of the nephron and is located exclusively in the cortex. It is supplied by an afferent arteriole which divides into a series of anastomosing capillary loops which then come together as the efferent arteriole. The two arterioles enter and leave at the vascular pole. The glomerulus is surrounded by Bowman's capsule which consists of a thin layer of epithelial cells (capsular epithelium) supported by a basement membrane (Figs 2 and 3).

The basement membrane of the capillaries is covered on each side by a single layer of cells. On the inner aspect of the basement membrane are the endothelial cells, directly in contact with the blood. Electron microscopy shows their cytoplasm to contain many fenestrations. Epithelial cells (podocytes) lie on the outside of the basement membrane and cover almost all of it with interdigitating cytoplasmic foot processes known as pedicels (Figs 4 and 5). and between the pedicels there are apertures (slit pores) covered over by slit membranes.

Thus, between endothelial and epithelial cells lies the glomerular capillary basement membrane which will be described later (page 12). The exact mechanism by which water and small molecules in the plasma filter through the capillary walls is not completely understood, but find

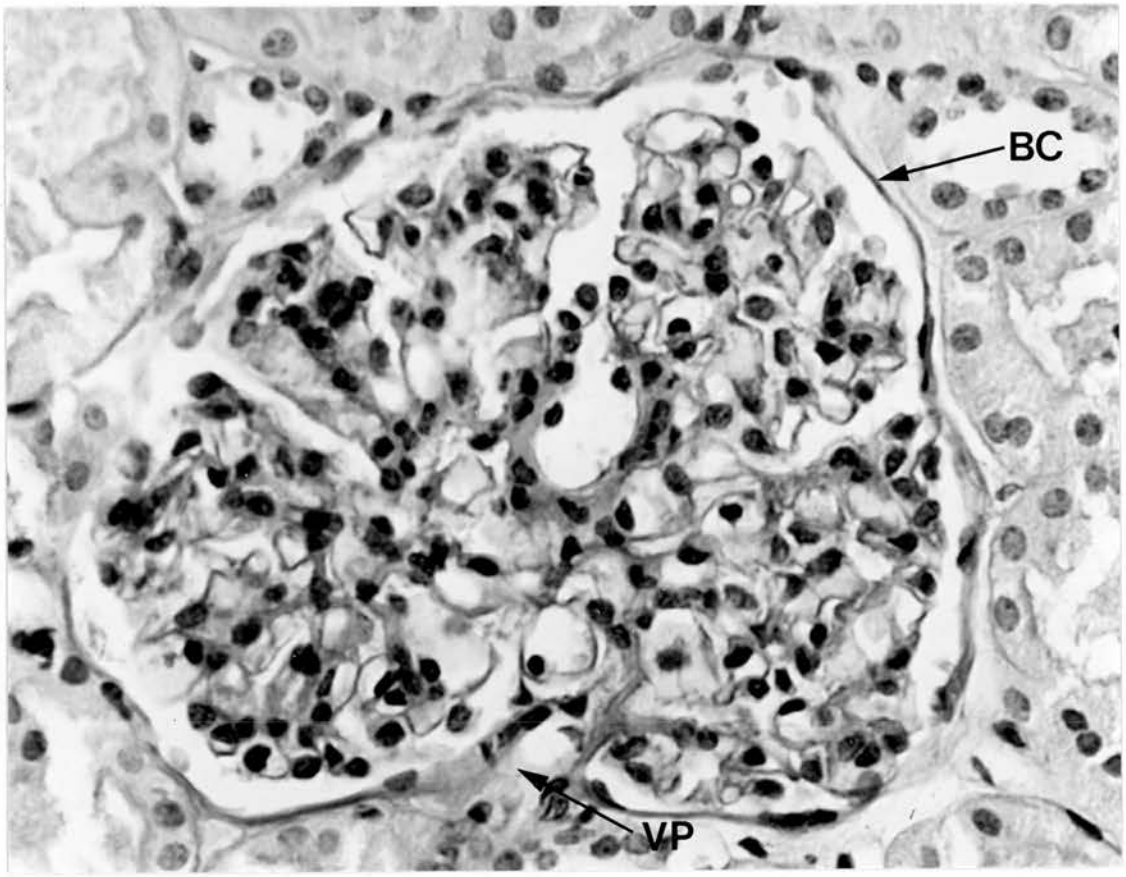


Fig 2 Glomerulus

BC - Bowman's Capsule
VP - Vascular Pole

H & E x40

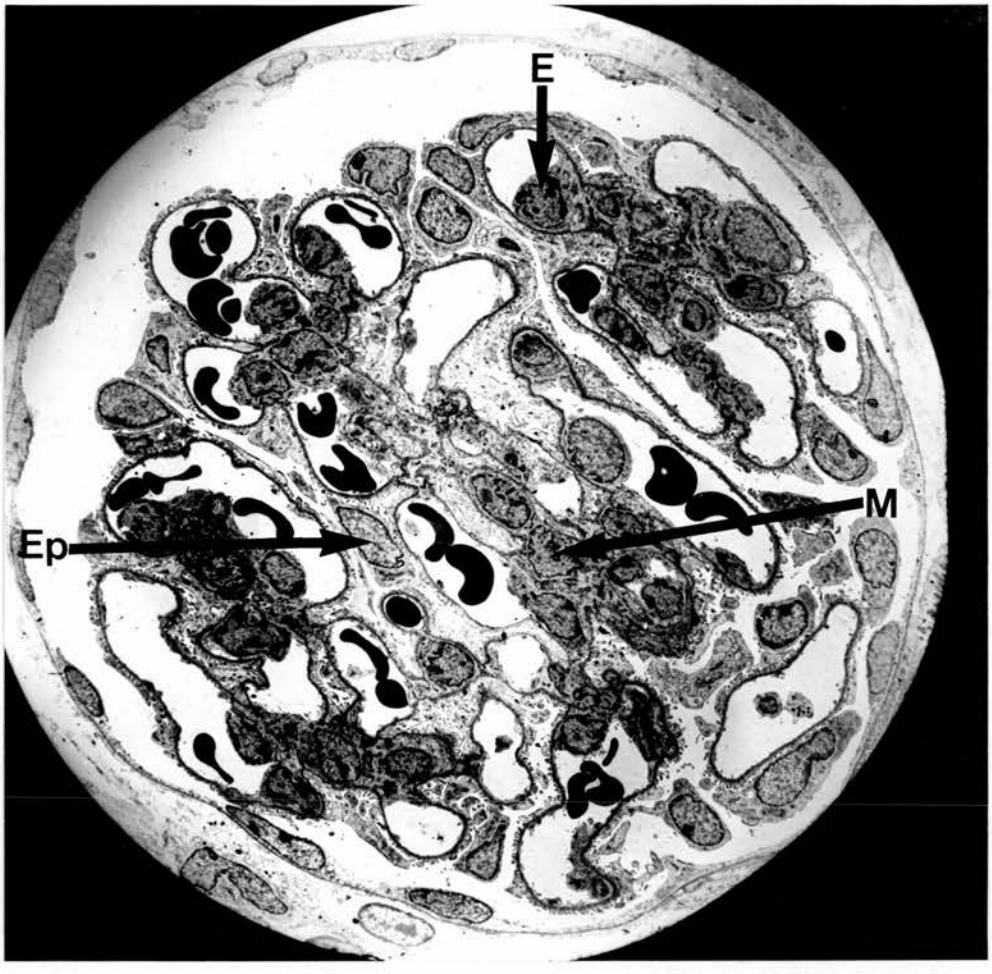


Fig 3 Electron Micrograph of a Section Through a Glomerulus

M - mesangial nucleus
E - endothelial nucleus
Ep - epithelial nucleus

x1,000

(Courtesy of Mr R Donaldson)

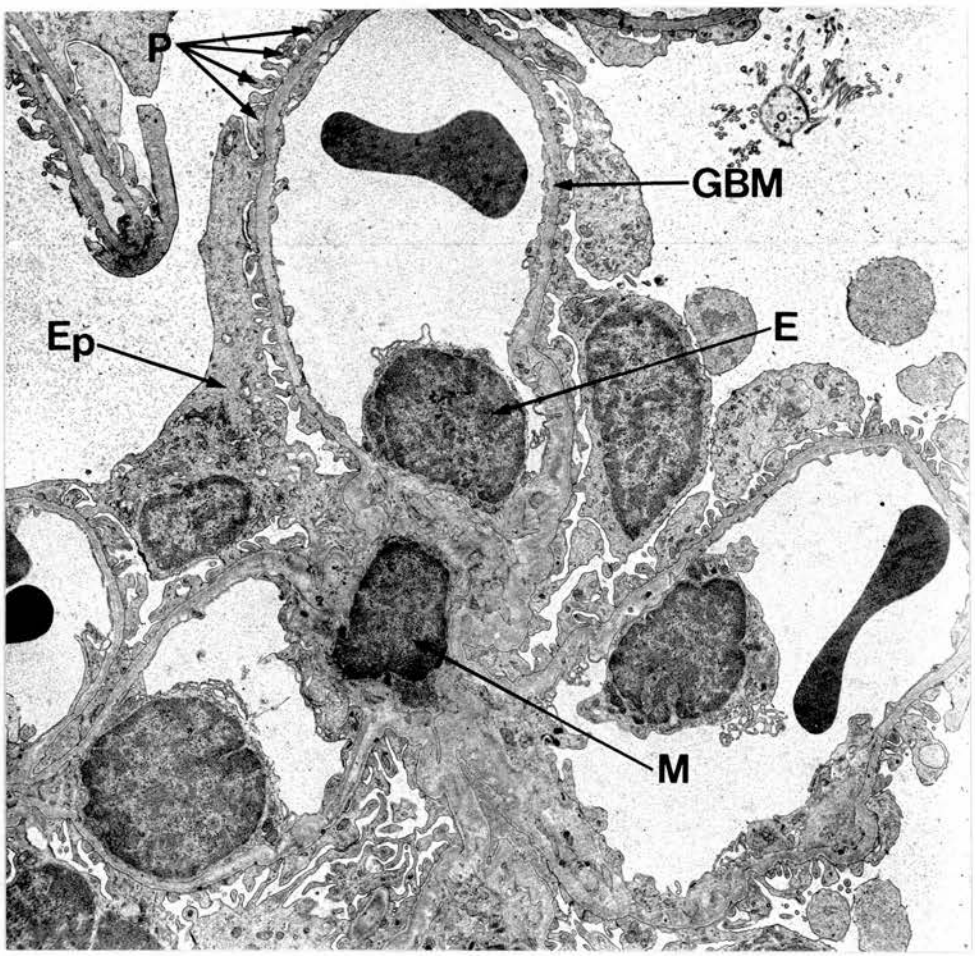


Fig 4 Electron Micrograph of Glomerulus

GBM - glomerular basement membrane
Ep - epithelial cell
E - endothelial nucleus
M - mesangial nucleus
P - pedicels

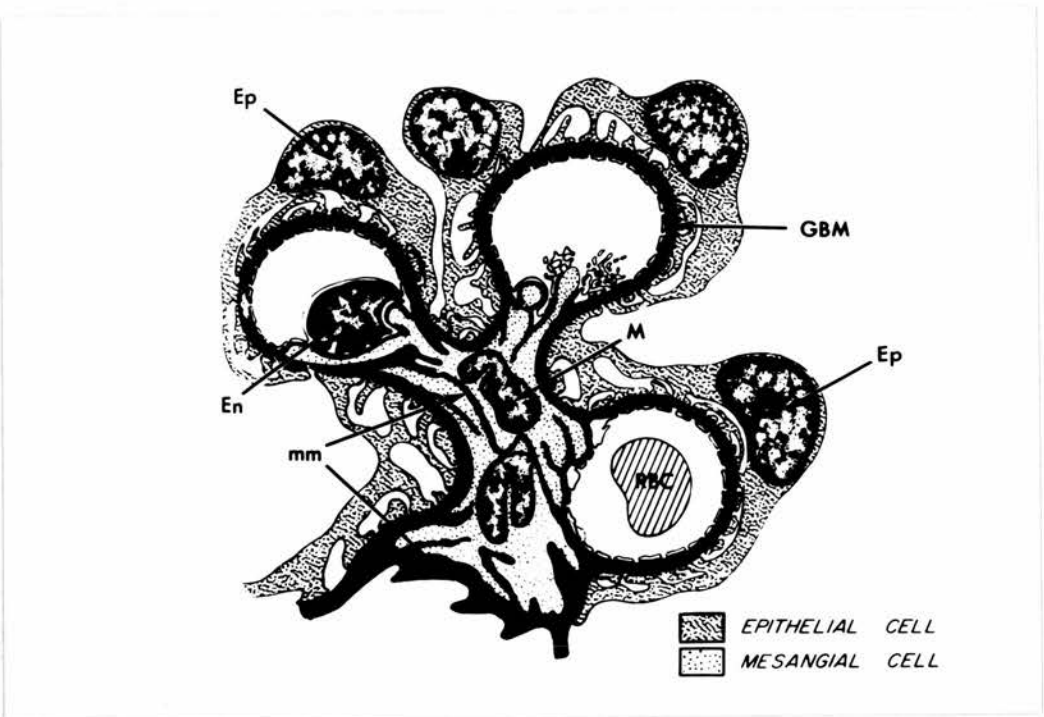


Fig 5 Schematic Drawing of a Glomerulus

E - endothelial nucleus
Ep - epithelial nucleus
M - mesangial nucleus
mm - mesangial matrix
RBC - red blood cells

(after Kefalides, 1978)

their way through the fenestrations of the endothelial cells, through the basement membrane, and finally through the slit pores of the epithelial cells or in some cases through the epithelial cytoplasm. It is not known as yet which is the most important barrier to filtration, although most evidence points to the basement membrane.

The overall effect of these barriers is to allow the passage of small molecules, below 4.2nm in size, whilst retaining anything above, eg red blood cells, white blood cells, platelets, plasma proteins. However there is much evidence to suggest that the passage of molecules through the membrane depends partly on their electrical charge (Rennke et al, 1975) and on their shape as well as on size (Caulfield and Farquhar, 1974).

The resultant ultrafiltrate passes into Bowman's space and thence into the tubule, where it is modified in accordance with the body's needs.

The glomerulus also possesses a mesangium which is a continuous system of cells and matrix, acting as a supporting stalk for the capillaries and also as a very important drainage system (Figs 3, 6a and 6b).

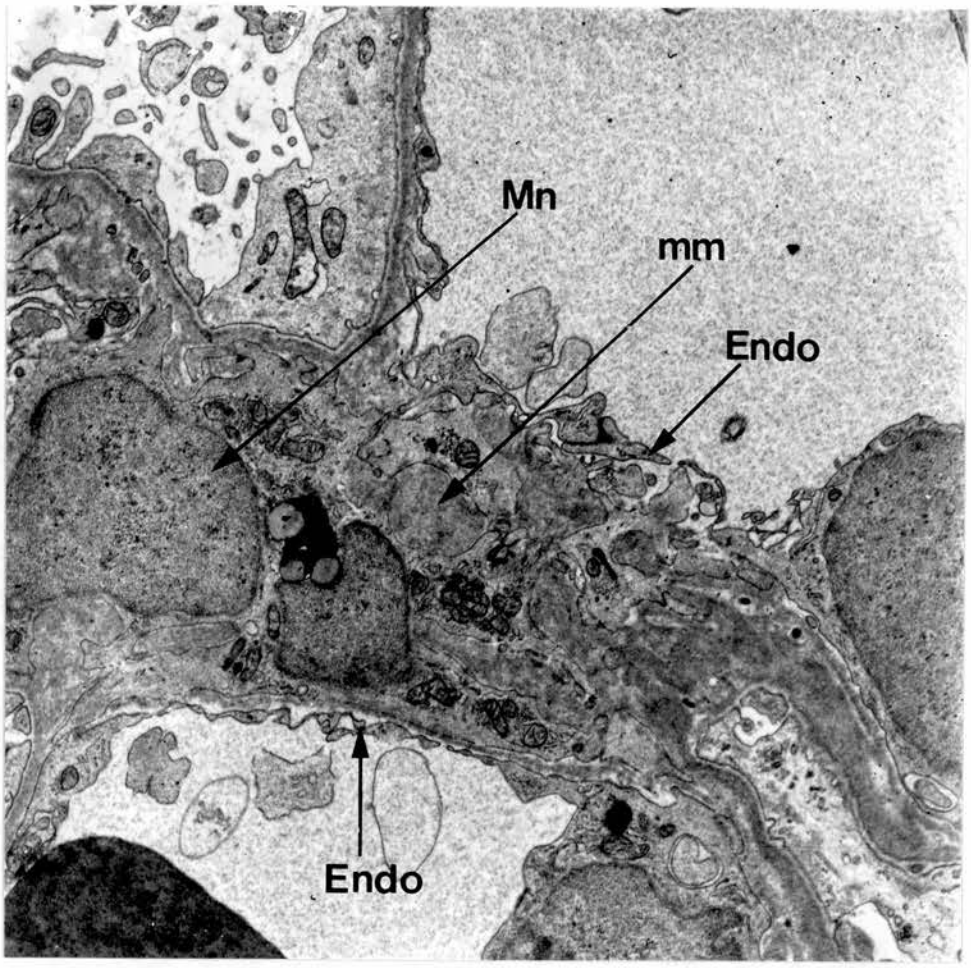


Fig 6 a Low Power Electron Micrograph Showing Mesangium

mm - mesangial matrix
Mn - mesangial nucleus
Endo - endothelium

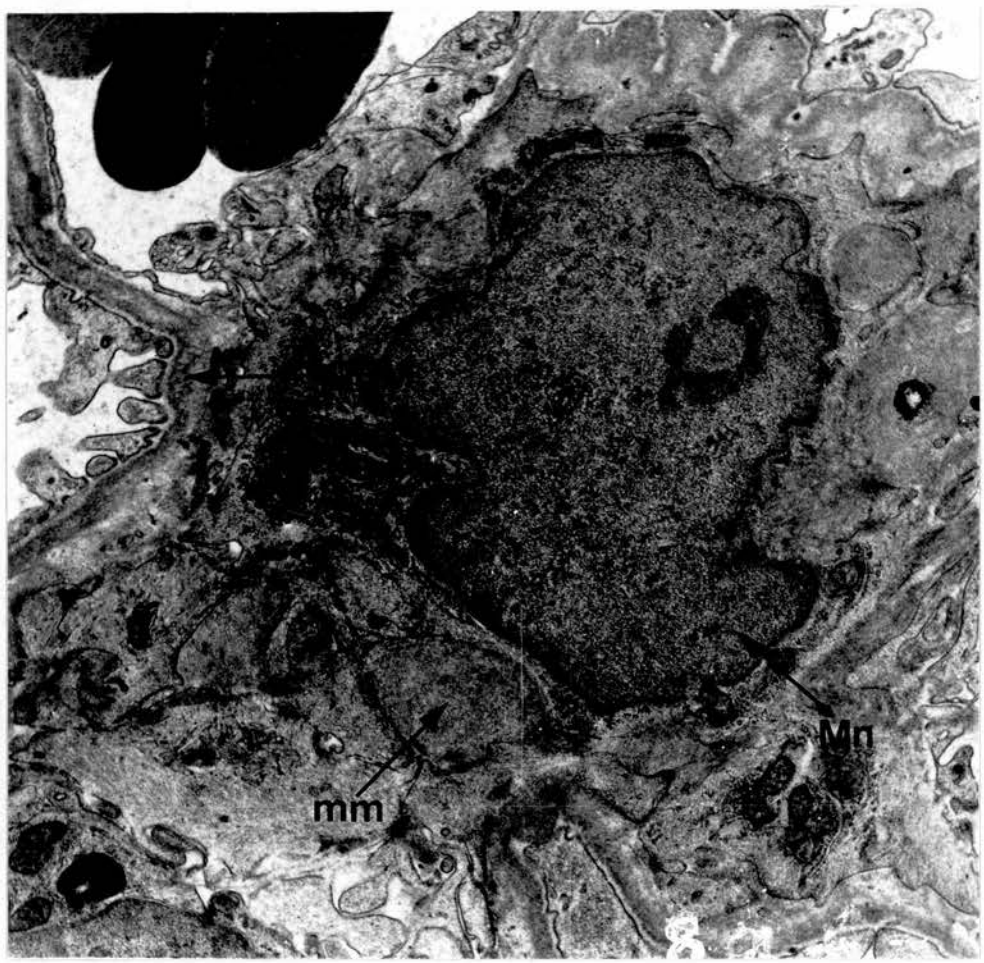


Fig 6 b High Power Electron Micrograph Showing Mesangium

GBM - glomerular basement membrane
Mn - mesangial nucleus
mm - mesangial matrix

Mesangial cells, or at least some of them, are part of the mononuclear phagocyte system and have been shown to take up a variety of tracers, as well as aggregated serum protein, and, very importantly, the immune complexes (Michael et al, 1980). The matrix of the mesangium is a basement membrane-like substance forming continuous columns which pass from the mesangium to the vascular pole and are in continuity with the matrix columns of the juxtaglomerular apparatus (JGA). This matrix stains positively with the PAS stain demonstrating its carbohydrate content (Fig 7). The matrix components include fibronectin, type I, III and IV collagen and glycosaminoglycans, these probably being synthesised by the mesangial cells (Oberley et al, 1979; Striker et al, 1980).

On electron microscopy the matrix has an amorphous structure with an electron density similar to that of GBM but with some different antigenic characteristics. It is aggregated in columns throughout the mesangial region. It also has a fibrillary pattern and occasionally this may lead to difficulties in differentiating the matrix from extraneous fibrillar deposits such as amyloid. The matrix increases progressively with age so that the mesangial regions of glomeruli from older people usually appear more solid than in children.

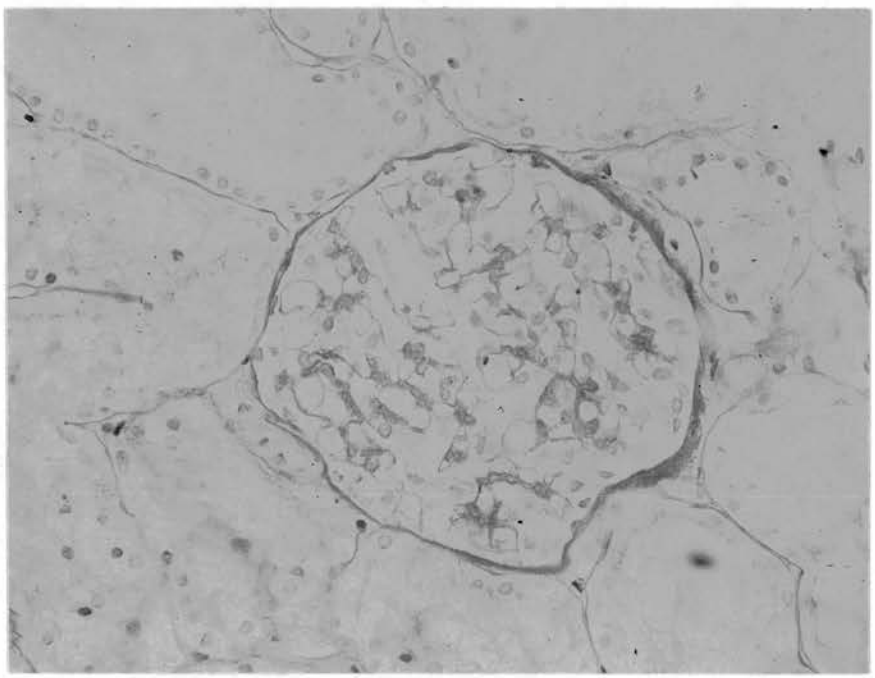


Fig 7 Normal Glomerulus

This stain (PAS) shows well the mesangial matrix which is present in normal amounts

PAS x40

It has been shown (Leiper et al, 1977) that in the mouse circulating macromolecules, which do not pass through the GBM move along the subendothelial space to the mesangium where they are transported to the JGA and distal tubule cells in the matrix columns.

Thus large molecules deposited on or in the membrane from the plasma may be removed into the mesangial matrix and carried down the system to the vascular pole in the region of the juxtaglomerular apparatus (JGA) (Leiper et al, 1977) probably for disposal into the distal convoluted tubule (Fig 8).

In human disease immune complexes deposited in basement membranes are probably removed in this way, incorporated into the mesangial matrix and passed down the matrix columns for disposal in the distal renal tubules.

Recent work by Schreiner et al (1981) has demonstrated a small subset of mesangial cells which are phagocytic and bear Ia antigens. These are membrane proteins coded for the major histocompatibility locus and they enable the cell to present foreign antigen to sensitized T-lymphocytes, thus mediating an immune response.

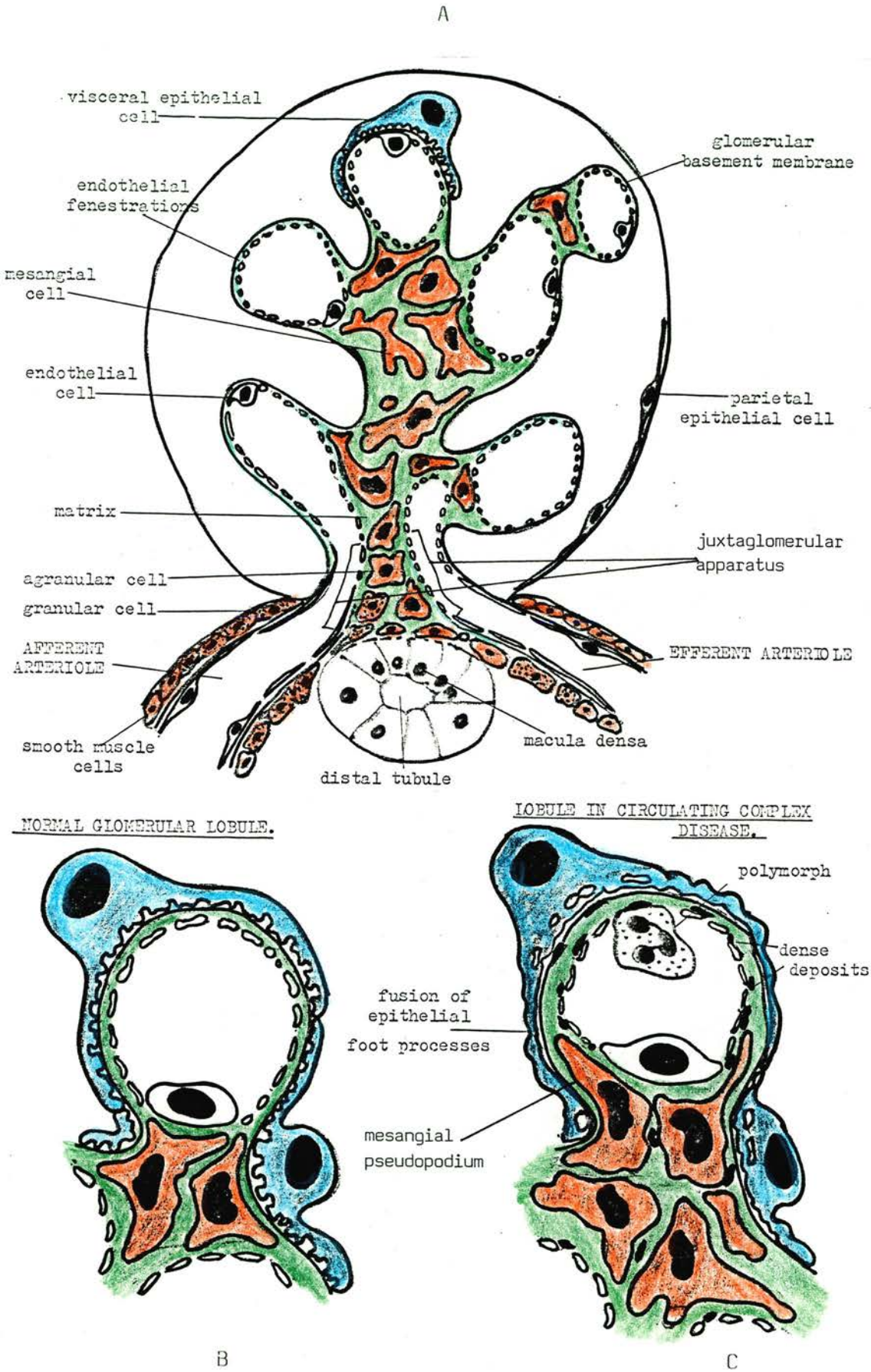


Fig 8 Schematic diagram of glomerulus (after M Leiper)

Basement Membrane Structure

Kefalides (1978) shows that in general basement membranes are extracellular matrices synthesised by a variety of cells which line all epithelial and endothelial surfaces. They are free from lipids and DNA, and are composed of dissimilar protein subunits.

The amino acid and carbohydrate composition of basement membranes varies both in respect to the various sites where they are situated in the body and also from species to species.

Glomerular capillary basement membrane (GBM)

The glomerular capillary basement membrane (GBM) is composed of tightly matted arrays of fine (3-4nm) fibrils embedded in a finely granular matrix (Farquhar et al, 1961). Also present are larger fibrils (11nm), found typically along the luminal surface of the basement membrane between the endothelial (or mesangial) cell membrane and the basement membrane. It has been shown that these membranes are composed of peptide and carbohydrate moieties (Kefalides, 1978) and are approximately 3 times as thick as the basement membranes elsewhere in tbe body. The amino acids found in human glomerular capillary basement membrane include -

hydroxylysine, lysine, histidine, arginine, 3-hydroxy-proline, 4-hydroxyproline, aspartine, serine, glutamine, proline, glycine, alanine, half-cystine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine. The main characteristics are the high content of amino-acids, of the non-polar amino acid glycine and the basic amino acid hydroxylysine.

The carbohydrate units which have been identified include hexose, glucose, galactose, mannose, glucosamine, galacto-samine, fucose, sialic acid, hexuronic acid. These terminal sugar residues are components of glycosamino-glycan (GAG) which impart a very strong negative charge to the membrane thereby aiding in the selective filtering process. As the plasma molecules increase in size and net negative charge, the GBM restricts their passage, since the GBM itself possesses regular but apparently discrete anionic sites. The GAGs mainly involved in this process in the kidney are Heparan sulphate, whose residues include N-acetyl-D-glucosamine and N-sulphate-D-glucosamine, and Chondroitin sulphate whose residues include D-galactos-amine (Bancroft, 1982).

The main units with which this present study is concerned are the carbohydrate molecules. Clarification of what actually happens to the GBM constitutents during disease processes may explain to some extent the functional

changes in glomerular disease, and may therefore make more accurate its classification; such processes are always essential preliminaries to more rational treatment, although the actual sequence of events and its results may not be clear at the outset of the investigation.

The membranes consist of a lamina densa (20-50nm) in thickness which runs parallel to the basal cell membranes of the overlying cell type and is separated from the latter by a lighter, approximately 10nm layer, called the lamina lucida or rara. It has been known for some time that included in the basement membranes there is a collagenous component. This is generally accepted to be type IV collagen, in the case of the GBM.

A model for the constituent parts of the glomerulus could therefore be:

	Filtration	Other Factors
Endothelium	controls access of plasma to GBM	shares in ? synthesis of GBM
Basement Membrane	main filter (size, charge and shape of molecules)	
Epithelium	filtrate monitor Recovers lost protein	? synthesis of GBM

	Filtration	Other Factors
Mesangium	phagocytosis of filtration residues	? synthesis and removal of GBM: support for capillary: acting as smooth muscle cells in controlling size of capillary lumina: ? takes part in antigen processing

(Kefalides, 1978)

II. The Tubule

This consists of several segments of differing structure and function each being located in the chain. By common practice this tubule is divided into several specific parts:

a) the proximal convoluted tubule

b) the loop of Henle

c) the distal convoluted tubule

and d) a collecting duct which is shared by a number of tubules. This itself joins an excretory duct.

All tubules are lined with specialised cells, and each type has a role in modifying the tubular fluid passing it (Fig 9).

a). Proximal convoluted tubule

This part is distinguished by long slender microvilli which project into the tubular lumen. These microvilli or brush border provide a large surface area for the exchange

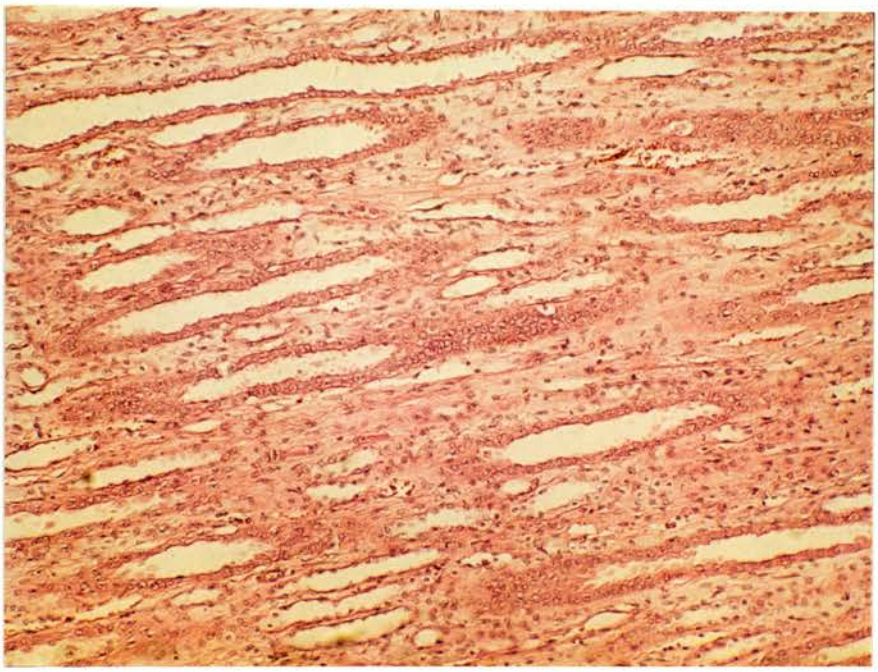


Fig 9a Tubules

Deep renal medulla showing tubules cut longitudinally

H & E x10

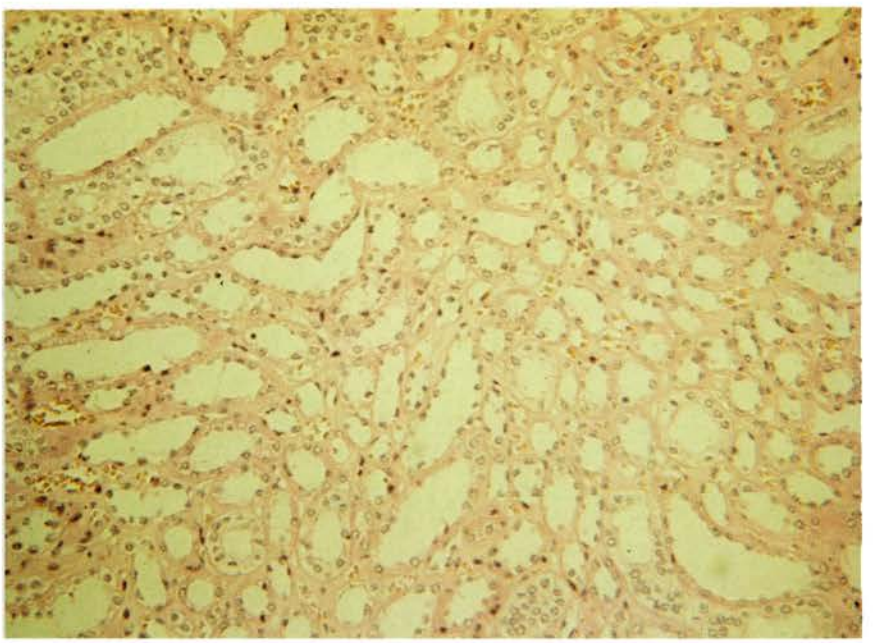


Fig 9b Tubules

Outer renal medulla showing cross-section of tubules

H & E x10

between tubular fluid and cells. The cells are rich in mitochondria, to support a high-level of energy transport. It is in these tubules that many important modifications to the filtrate take place, since only about 1% of the filtrate reaches the renal pelvis as urine. Here much of its sodium, chloride, bicarbonate and about 70% of its water has been reabsorbed. This would leave the tubular fluid very hypo-osmotic were it not for the fact that the proximal tubules are freely permeable to water. Water is reabsorbed passively by osmotic pressure. Many other ions and small molecules whose loss would be very disadvantageous are also reabsorbed here, eg potassium, magnesium, calcium.

b). The Loop of Henle

In the thin segment flat cells with few mitochondria are found, whereas in the thick ascending segment the cells are taller, with more abundant rod-shaped mitochondria. In this region the filtrate is concentrated; the fluid emerging is always hypotonic with regard to normal plasma.

c) and d). Distal Convoluted Tubule and Collecting Duct

The cells in these structures range from cuboidal to columnar in different regions. They have small microvilli and the number of mitochondria diminishes in the late part of the distal convoluted tubule and collecting duct. An important structure is located in a short segment of the

distal tubule when it comes in contact with the arterioles at the vascular entrance to the glomerulus. This distinctive structure is referred to as the macula densa and is in fact part of the juxtaglomerular apparatus, where the hormone renin is produced. The distal convoluted tubule and collecting ducts perform the same function. They are responsible for the final removal of most of the water and sodium left in the filtrate. They are also responsible for the secretion of hydrogen ions, thus regulating the net-acid excretion of the kidney, to preserve the acid-base balance in the body.

Antibodies reacting with TBM as well as immune complexes may induce tubulo-interstitial (TI) disease. It is now evident that the immunopathological mechanisms which are involved in glomerular injury may also cause damage to the tubular basement membrane (TBM).

BACKGROUND TO THE PROJECT

A. Glomerulonephritis

As may be expected in a system whose prime function is the maintenance of the precise concentration of ions and molecules in blood and extracellular fluid necessary for life, damage to the glomerulus by circulating complexes and other immunological materials can be very serious, and

is frequently followed by an attempt on the part of the glomerulus to remove the substances. The resultant morphological and functional changes are known as glomerulonephritis (GN). Large protein molecules other than immunological materials may be involved, but this is less common.

In 1812 a report was presented to the "Society for the Improvement of Medical and Chirugical Knowledge". It was concerned with instances of dropsy after scarlet fever. The author, Wells, reported an association between the oedematous state and renal damage when he wrote "The urine in that disease contains almost always the serous, and sometimes the red matter of the blood." This was perhaps the first suggestion that there is an association between infection and glomerulonephritis; one of the clinical syndromes now associated with glomerulonephritis had been recognised.

Presently two mechanisms seem to play a interacting role.

1. Immunological responses.
2. The coagulation system.

The first is much the more important, but evidence that both are involved has been shown in both clinical investigations and experimental work.

Immunological Responses

Glomerulonephritis has long been associated with factors which initiate immune responses as can be seen in the relationship with prior streptococcal infection. The presence of immunoglobulins in renal glomeruli has been shown by the widespread use of fluorescein Isothiocyanate (FITC) conjugated anti human immunoglobulins on fresh tissue. Complement activation can also be shown by using FITC anti human complement factors. Deposits thus seen can often be further identified as electron dense by the use of the electron microscope.

Experimental Evidence

Models which resemble some forms of human glomerulonephritis can be produced in animals by manipulating the immune mechanism.

1. In 1966 for example, Lerner and Dixon et al, showed that antibodies could be directed against the glomerular basement membrane itself. This produced a GN similar to that found in Goodpasture's Syndrome (anti-glomerular basement membrane disease, Goodpasture, 1919); the antigen in this case is a normal constituent of the glomerulus.

2. As an immune complex model, in 1966 Fish et al showed that there is deposition in the glomeruli of Ag/Ab

complexes formed in the circulation. It appeared that deposition of these particles in the GBM depended on their exceeding a critical size. Those larger than 19S units were entrapped, while smaller molecules were allowed to pass through. Increase in vascular permeability was shown to occur when these complexes were deposited, and if this is true also for glomerular capillaries, it may play a role in glomerular damage.

3. It has been shown experimentally and clinically that activation of immunological processes often triggers off the coagulation mechanism, and fibrin deposition is a common association of immunological glomerulonephritis.

Pathogenesis of Glomerulonephritis

I. We now appreciate that it is the immunological response to antigens, infective agents or otherwise, which is of primary importance in most types of human glomerulonephritis. The antibodies, immune complexes and complement factors, if present in the circulation, may under certain circumstances, which are by no means all clear, be deposited or held up in or on various parts of the glomerular capillary basement membrane, probably as a result of an attempt to filter them through the membrane. The basement membrane may be badly damaged by these substances, particularly complement, and by the enzymes

contained in neutrophil polymorphs (Janoff and Zeligs, 1968; Lazarus et al, 1968), which are often attracted to the site. This damage allows larger molecules than normal to pass through, particularly plasma proteins, thus initiating the process of proteinuria. The mesangial system is stimulated to remove such deposits, if they are on or in the internal part of the basement membrane: if this system is not able to remove all the deposits rapidly enough, permanent damage may result.

When antibodies to exogenous (non-glomerular) antigens are involved in the formation of immune complexes, and the complexes are trapped in or on the GBM, they can be shown in a granular pattern of deposition along the GBM, using immunofluorescent techniques (Koffler and Paronetto, 1965; Seegal et al, 1965).

It is now believed, however, that there may also be an <u>in situ</u> immune complex formation, as shown in the rat mesangium (Mauer et al, 1973) and rat subendothelial space (Couser et al, 1978). This concept proposes that cationic antigens are bound to anionic sites in the GBM and circulating antibodies attach to these antigens so that immune complexes are formed in situ (Fig 10).

II. Antibodies specific for antigens in the GBM itself may be formed by the body. The reason for this auto-

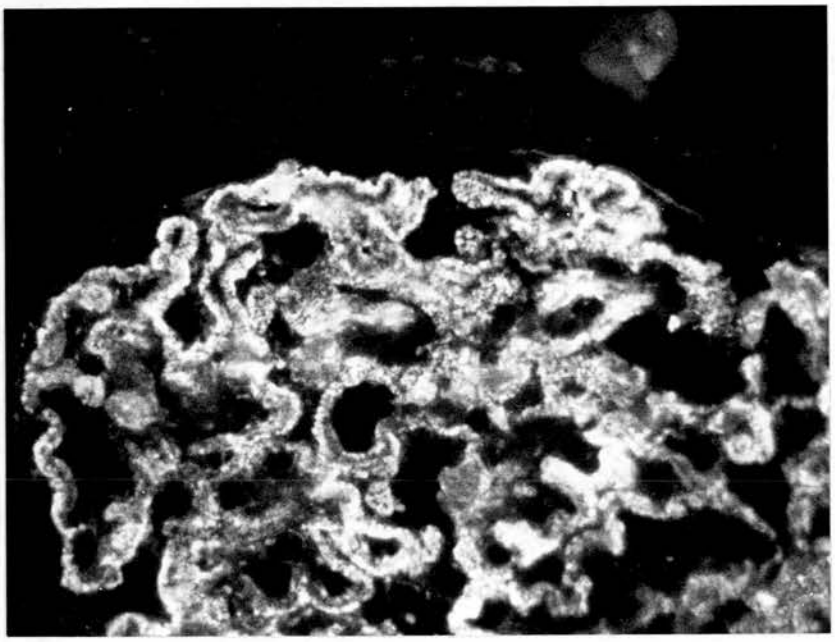


Fig 10 Immunofluorescence in Glomerulus

Granular deposition of immunoglobulins in capillary walls

x40

immune antibody formation is not known, but viral infection, or damage to the membrane by organic solvents may be involved. These bind to the GBM in linear fashion, and show a characteristic linear staining in immunofluorescent studies (Fig 11). This is a rare type of glomerulonephritis in man, and the clinical syndrome is often referred to as Goodpasture's Syndrome.

As early as 1961, Dixon et al showed that it is the soluble complexes formed under moderate antigen excess which initiate circulating immune complex GN. This is because in antibody excess the large insoluble (and more "normal") complexes formed are quickly removed by the mononuclear phagocytes, whereas in antigen excess they are not removed and may remain in circulation for days before eventually becoming deposited in the kidney.

The actual amount of immune complex material which accumulates along the GBM is influenced by:

a) complex size (Cochrane and Hawkins, 1968)

b) vascular permeability (Kniker and Cochrane 1968)

c) antigenic (or complex) electrical change (Border et al, 1981).

The Glomerular Response to Immunological Injury

The most commonly seen response is proliferative glomerulonephritis. This is associated with the sub-

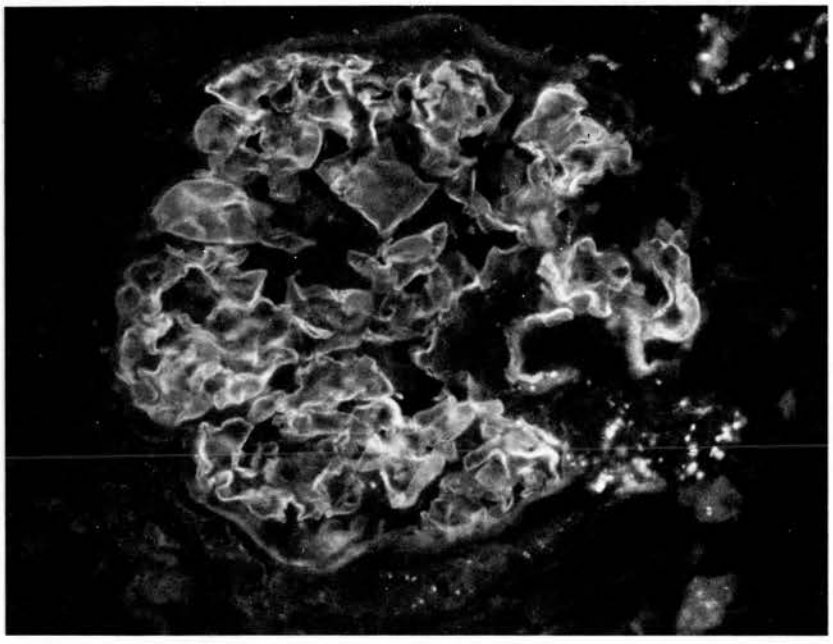


Fig 11 Immunofluorescence in Glomerulus

Linear deposition of immunogloblins in anti-GBM disease (Goodpasture's Syndrome)

x10

endothelial and inner GBM localisation of immune complexes, and there is proliferation of mesangial cells, presumably in an attempt to remove the immune deposits (Vassalli et al, 1963; McLuskey et al, 1966). It is probable that there is also an infiltration of the mesangium by monocytic cells from the circulation (Magil et al, 1981). Neutrophil polymorphs are attracted to the site, and proliferation of mesangial cells is further stimulated by the release of their lysosomal granules. On light microscopy, the glomeruli therefore appear hypercellular (Fig 12).

In membranous glomerulonephritis, the material passes through the GBM, and deposits are formed on its outer aspect. In this case the mesangium is not stimulated, and there is always severe damage to the GBM (Fig 13).

Other types of glomerulonephritis produced by deposition of immunological deposits show a variation in the site of deposition and in the glomerular response. Often the antigen involved is quite unknown.

The transplanted kidney

Several of the cases available for this study were transplanted kidneys. In many cases of chronic and irreversible renal failure, the original cause of the renal condition is glomerulonephritis. In a proportion of

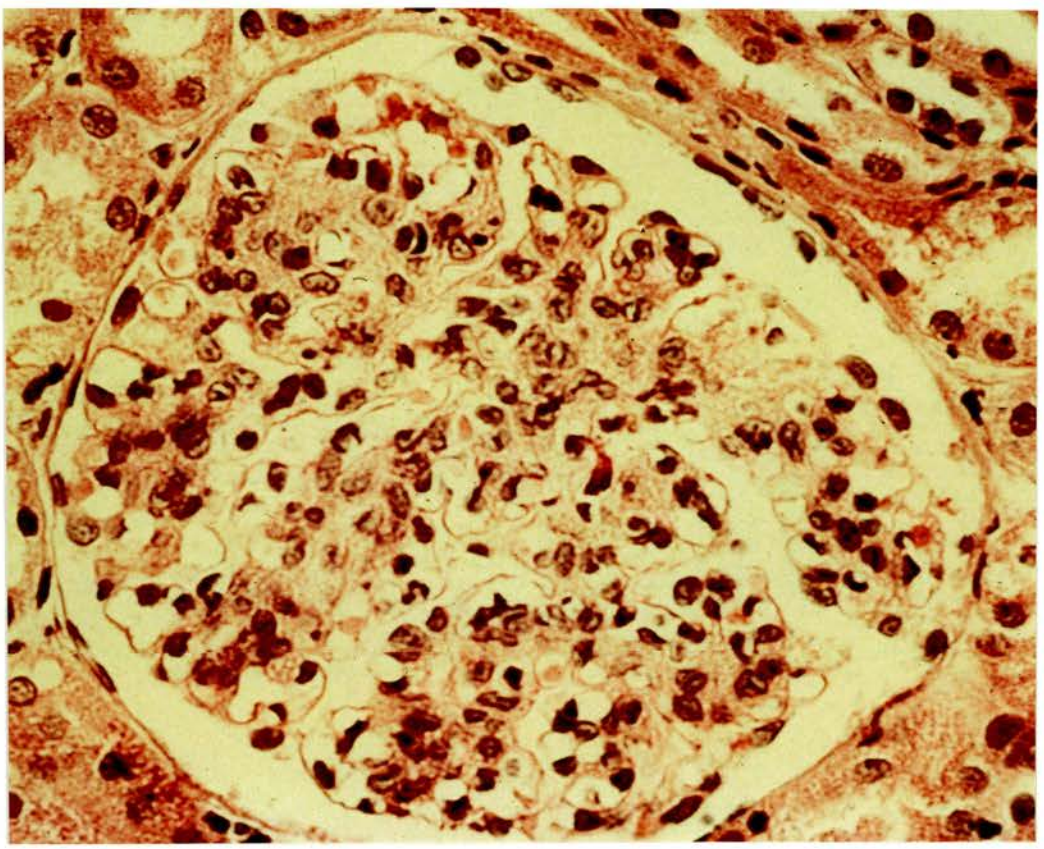


Fig 12 Proliferative Glomerulonephritis

Glomerulus from a case of proliferative glomerulonephritis. The most obvious abnormality is the proliferation of mesangial cells, producing an increase is size of mesangial regions. Capillary walls are of normal thickness.

H & E x240

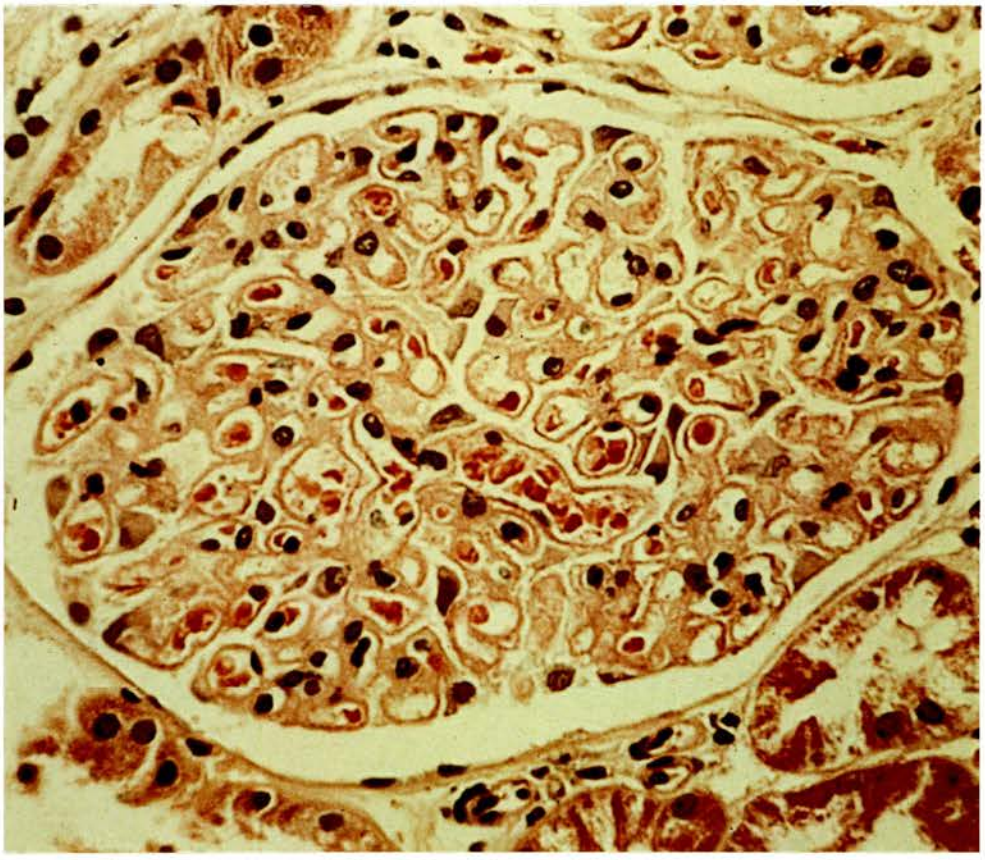


Fig 13 Membranous Glomerulonephritis

Glomerulus from a case of membranous glomerulonephritis. No significant mesangial cell increase is present. Capillary walls are diffusely, though slightly thickened.

H & E x240

cases of end-stage renal failure, treatment is by transplantation of a kidney from a recently dead or living related donor. In such cases, the transplanted kidney may be subjected to the same immunological insults as were the patients' own kidneys. Often, however, the basic immunological condition has subsided by the time the graft is necessary. Every grafted kidney, however, unless it comes from an identical twin, is subjected to the natural immunological rejection process, to a greater or lesser degree. This process affects all renal structures, and is largely of cell-mediated nature, in contra-distinction to the humoral immunological process involved in most cases of human primary GN. It seemed therefore that biopsies from this group would be a valuable addition to the study. Such biopsies are frequently performed after transplantation, where renal function is diminishing, in order to discover whether the diminution is due entirely to rejection, which can be treated, or to some other process.

Minimal lesion glomerulonephritis is a relatively common condition of young people, who develop proteinuria and oedema. Renal biopsy shows little abnormality or a mild mesangial cell proliferation on light microscopy and little or no deposition of immunological material on immunofluorescence microscopy. The pathogenesis of this lesion is still obscure. It is responsive to steroid

therapy, but a few cases relapse, and even may go on to glomerular hyalinisation and renal failure.

Alport's syndrome (hereditary nephritis) is an hereditary condition involving progressive renal damage and deafness. There is a strong hereditary influence, and it is not uncommon to find families who may have more than one sufferer. Characteristic changes are usually seen in the GBM. These include a 'basketwork' splitting of the membrane into multiple interweaving lamellae which enclose electron-lucent areas, and a variable thickening. Again, the pathogenesis of the condition has not been clarified completely, though there is some evidence of loss of normal antigen from the GBM.

The GBM in Glomerulonephritis

The glomerular capillary basement membrane is a highly important part of the blood vessel wall, since it is the layer probably responsible for the filtration of the plasma.

Much of the functional change in the kidneys in GN results from the changes to the GBM by the deposited material including complement factors, immunoglobulins and lysosomal enzymes of polymorphs. If this deposited material is not adequately removed from the glomerulus

permanent damage may occur, leading to irreversible renal failure.

Electron microscopy, transmission and scanning, has shown in greater detail the morphological changes which occur, and the identification of the material being deposited has been made possible by the use of fluorescent tagged anti-human antibodies and by the immunoperoxidase technique. However the nature of the physio-chemical change in the basement membrane, or of the chemical change in its constituents, as a result of the disease processes, has never accurately been demonstrated.

B. Tubular Disease

Included in the study were two cases of acute tubulo-rrhectic tubular necrosis (ATN). This condition occurs usually in association with hypotension or some other peripheral vascular abnormality and the changes are almost entirely of tubular nature. It seems that the lesions, which consist of small foci of discontinuity in tubular basement membranes, with passage of tubular contents into the interstitial tissue, and subsequent inflammatory reaction (Fig 14), are due to generalised ischaemia of the tubular tissue. This ischaemia is frequently caused by constriction of the renal arteries, as a reaction to systemic hypotension. It has been suggested that in this

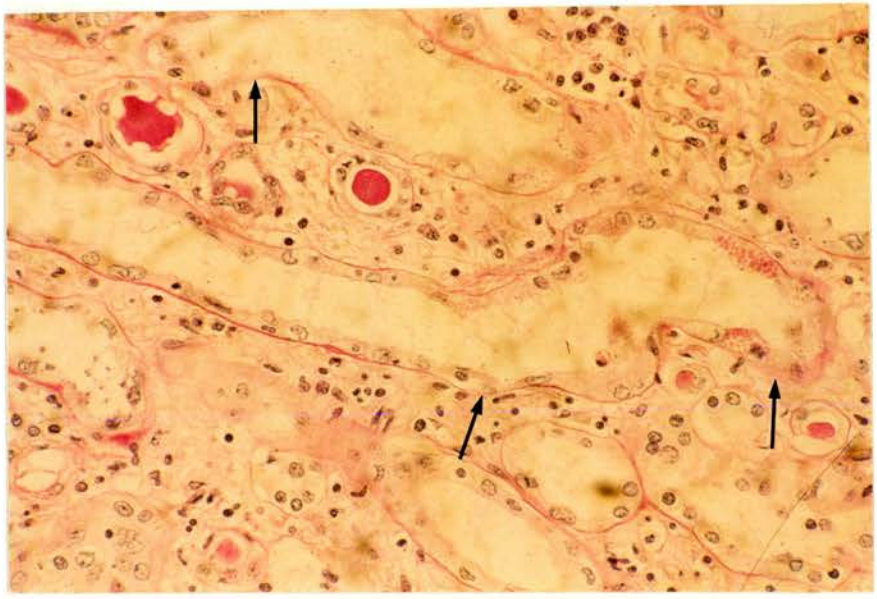


Fig 14a Acute Tubular Necrosis

This field from a case of acute tubular necrosis shows several foci of discontinuity of the tubular basement membrane, some of them identified by arrows, also present is intertubular oedema, and there is a mild interstitial infiltration with inflammatory cells. Some tubules contain hyaline casts.

PAS x40

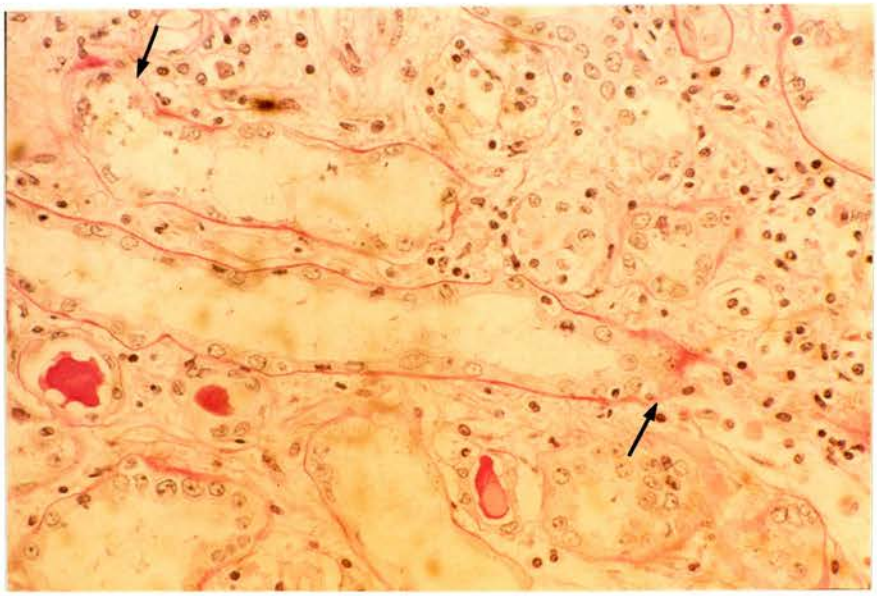


Fig 14b Acute Tubular Necrosis

Another field showing acute tubular necrosis with breaks in basement membrane (arrows)

PAS x40

condition some fibrin deposition occurs in the glomeruli, and that this produces obstruction to blood flow in the tufts, so that even when blood pressure returns to normal, ischaemia of the tubules may continue. It was decided to include these cases in the project, since they would provide two examples of glomeruli which were possibly abnormal but not involved in an immunological insult.

We were able to examine one case of myelomatosis, with typical renal changes, which again are mainly tubular It was felt that this would form a small group with the cases of acute tubular necrosis, with reference to the tubules.

Tubular Basement Membrane

The precise nature of the origin of tubular basement membrane (TBM) is not clear. It is possible that the tubular epithelial cells may have a primary role in its synthesis and maintenance. In contrast to the GBM the fine structure of TBM is characterised solely by a "lamina densa" without contiguous "laminae rarae". The isolation of TBM shows it to contain a collagen moiety and glycosaminoglycans.

C. LECTINS

There are many proteins which have the ability to bind specifically different components, eg enzymes that combine

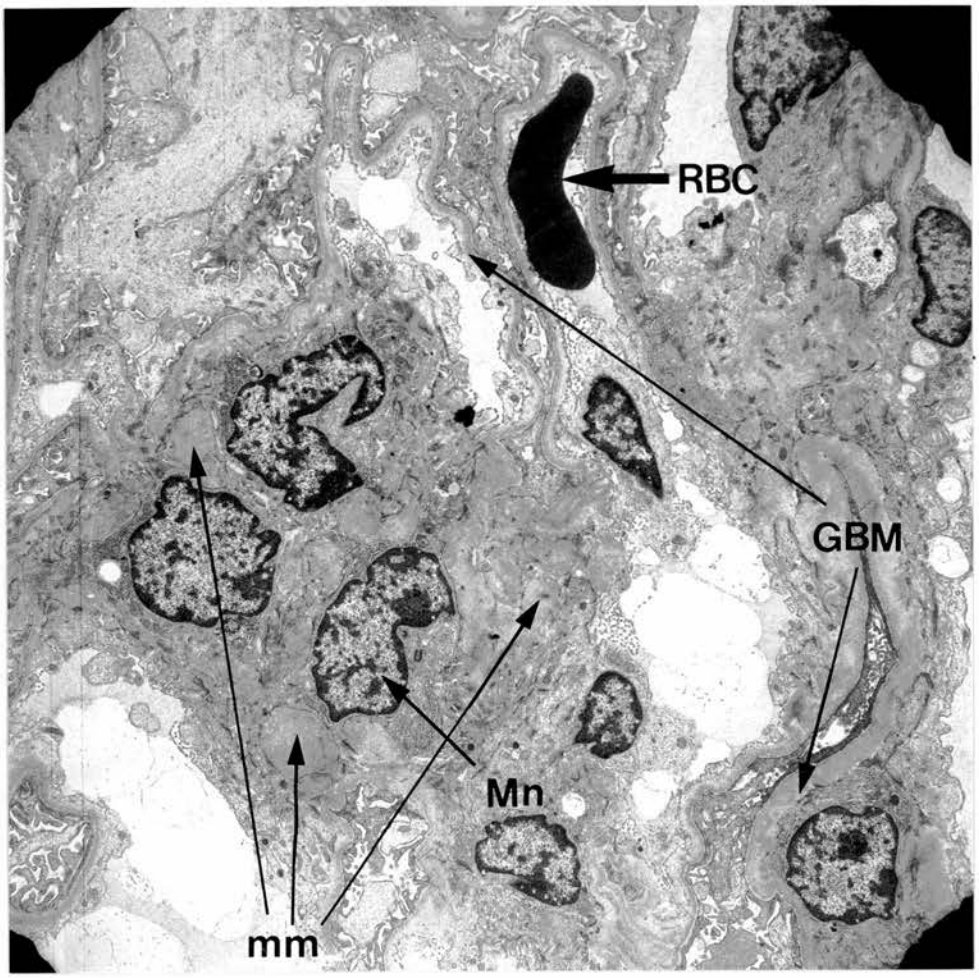


Fig 15 Electron Micrograph of Proliferative Glomerulonephritis

An increase of cells is seen in the mesangial region. Columns of mesangial matrix are identified by arrows and these are also increased in number and are larger than normal. This mesangial reaction is due to the withdrawal into the mesangium of deposits of abnormal material laid down on the GBM.

x3,750

mm - mesangial matrix
Mn - mesangial nucleus
GBM - glomerular basement membrane
RBC - red blood cell

specifically with substrates and inhibitors, and antibodies that bind antigens. In 1888 it was reported that an extract from the castor bean reacted with and agglutinated red blood cells (Lis and Sharon, 1977). Such proteins with specific combining sites have been variously termed agglutinins, haemagglutinins, phytohaemagglutinins and now lectins. They have been found widespread in the plant and animal kingdoms.

The term lectin (coined from the Latin - legere, to select or choose) was based on the observations of Boyd and Sharpleigh (1954) that some seed extracts could distinguish between human blood groups. Specifically, the extract from the lima bean (Phaseolus limensis) selectively agglutinated type A red cells. The reason for this agglutination might seem to be a simple phenomenon, but in fact it has turned out to be a highly complicated event. Basically, for agglutination to occur the lectin must bind to its specific sugar on the cell surface. This will then cause the formation of multiple cross-bridges between opposing cells, in turn leading to agglutination. There are, however, lectins which happily bind to the cell surface but do not cause agglutination. The various factors which have been investigated show that

a) the properties of the lectin

b) the chemical structure of the cell surface receptors

c) cell surface properties and

d) cytoplasmic components all play an interacting part in the agglutination of cells by lectins.

The main characteristics of lectins are their ability to bind sugars, to agglutinate cells, and to stimulate lymphocytes. Through their sugar combining sites lectins react specifically with polysaccharides and glycoproteins to form precipitates.

This reaction is very similar to that seen in an antibody/antigen reaction. Both reactions are specific, exhibit concentration dependence, and may be inhibited, in the case of the antibody/antigen reaction by pre-incubating with its specific antigen and in the case of the lectin by using a low-molecular weight hapten. This is a compound which is identical with or derived from the sugar(s) for which the lectin is specific. However the most important difference is that while antibodies are produced as a result of an antigenic stimulus of the immune system, lectins are formed without a stimulus, being an integral part of the host, whether plant or animal.

Also, although each antibody is specific for its stimulating antigen the range of specificity of antibodies is very wide, but the specificity of lectin is restricted to carbohydrates both simple or complex. Most lectins interact preferentially with a single sugar structure, eg lotus tetragonobulus with L-fucose. Some, however, have a broader specificity and may react with a member of closely related sugars, eg canavalia ensiformis (Con A) with mannose and glucose. Others will interact only with complex carbohydrate structures such as those that occur in glycoproteins or on cell surfaces (Table 1).

It is noteworthy that the sugars with which lectins interact best are those that are typical constituents of glycoproteins or glycolipids. The availability of lectins, their ease of preparation in purified form, the fact that they are amenable to chemical manipulation and that many are inhibited by simple sugars make them a most attractive tool in biological research and investigative procedures.

They have been used for typing blood, and determining secretion status (Boyd and Sharpleigh, 1954) in the detection, isolation and characterisation of carbohydrate-containing macromolecules (Lis and Sharon, 1977), and in the investigation of the distribution and mobility of cell surface glycoproteins in normal and malignant cells.

They have also been tagged or labelled with tracer molecules such as ferritin, isotopes, enzymes or fluorescein, thus allowing them to be utilised in light microscopic, fluorescent microscopic and electron microscopic studies.

It was the ease of the use of lectins tagged with fluorescein that prompted their use in this present study. Since much of the damage caused by immunological and other materials involved in glomerulonephritis affects the basement membrane of the capillary loops in the gomerulus, increasing its permeability, it seems fair to assume that the membrane will be altered physically and/or chemically in some way.

It would not be easy, using microscopic techniques, to assess changes in the <u>protein</u> structure of the BM; however, the ability of lectins to bind specifically to certain sugars, and the development of methods by which these lectins can be identified microscopically, has made it possible to analyse broad changes in the spectrum of alterations in carbohydrate moieties in the membrane.

The standard immunofluorescent (IF) technique using fresh frozen tissue was used mainly because the material does not have to be subjected to the effects of fixatives, dehydrating agents, etc in this technique. However, a

small study of paraffin-embedded material was undertaken, to compare the results of the methods. The groups of cases examined were normal kidneys, and cases of Minimal Lesion GN (Blainey et al, 1960) and of Alport's syndrome (Alport, 1927).

MATERIALS AND METHODS

MATERIALS AND METHODS

Approximately 200 renal biopsies are submitted to the renal group of the Department of Pathology, University of Edinburgh each year for diagnosis.

The vast majority of these cases are of some type of glomerulonephritis (GN) or transplant biopsies although some cases of acute tubulorrhectic tubular necrosis were also available for investigation and it was decided to apply the lectin technique to any suitable biopsy (ie fresh tissue for immunofluorescence microscopy was provided without further selection). The project was aimed at elucidating what actually happens to the glomerular capillary basement membrane, with regard to its carbohydrate groups, when it is damaged in glomerular disease. Since the author's background and experience is mainly technical it was clear that this project would deal mostly with the methodological, descriptive and analytical aspects of the problem.

Some of the biopsies used were also examined by electron microscopy, in order to investigate any possible association between results of the lectin technique and the actual ultrastructural appearances in the cases.

The aim was therefore to provide data demonstrating changes in glomerular capillary basement membrane then to speculate on the functional results or the pathogenesis of glomerulonephritis.

Material from abnormal renal tissues

Accordingly the material used for the study was that submitted for routine hospital service diagnosis. The renal biopsies in this Department are submitted in three parts, i) in formaldehyde for light microscopy, ii) in glutaraldehyde for electron microscopy and iii) fresh, for freezing and immunofluorescence microscopy. It should be pointed out that not all the biopsies were suitable for examination since the diagnostic service requirements had to be met first and foremost and in some instances little or no fresh tissue could be provided.

The diagnostic groupings were based on the final diagnosis made by the consultant renal pathologist and this diagnosis was not known to the author at the time lectin examination was done.

The carbohydrate component of the membrane was chosen for investigation because of the relative ease with which the lectin technique could be adapted for this purpose. A study of the amino acid components by biochemical methods

would be a rewarding exercise but not possible in the circumstances in which the work was being done.

Transplants

The largest single group in one series were biopsies from transplanted kidneys in which a diagnosis of rejection had been made. While it is not possible to discover the original cause of the patient's chronic renal damage, many such cases are due to GN.

The transplanted kidney is therefore frequently subjected to the same immunological insults as were the patients' own kidneys. In addition of course, the immunological rejection process affects glomeruli, tubules, blood vessels and interstitial tissues, and this process is largely of cell mediated nature in contra-distinction to the humoral immunological process involved in most cases of human primary GN. It seemed therefore that this group would be a valuable addition to the study.

Biopsy Techniques

The tissue was obtained by the percutaneous route usually using a Vim-Silverman needle which provided a core of tissue approximately 10mm by 2mm in diameter.

Normally two cores of tissue were taken and were divided into three parts:

a) formaldehyde fixed material for light microscopy

b) glutaraldehyde fixed material for electron microscopy

c) fresh, unfixed material for immunufluorescence microscopy.

The findings in the renal biopsy material could be relevant only when compared to those elicited from normal renal tissue subjected to the same techniques. To obtain normal tissue, kidneys were obtained from urological surgical theatres after nephrectomy. All of these kidneys were removed because of the presence of carcinomata (four renal neoplasms and one of ureteric origin); no kidney was used in which the renal tissue had been seriously compressed or otherwise damaged.

Only when all diagnostic requirements were met was tissue used for this project and at no time were the patients subjected to biopsy purely for this study. Because the biopsy procedure is relatively "blind" in that it is not possible to be certain that sufficient cortex will be obtained, the cores of tissue occasionally contained few glomeruli. It was decided to eliminate from the study any tissue with fewer than five glomeruli in the sample taken for immunofluorescence microscopy.

I. Light Microscopy

Biopsy tissue was immediately fixed in 4% neutral buffered formaldehyde (pH 7.2). It was fixed for 12-24 hours, then processed through ascending grades of ethanol, chloroform to paraffin wax on a routine tissue processor, embedded in paraffin wax (MP 58-60°C) and sections cut at 2-3 microns. The sections were "floated out" on distilled water at 55°C, mounted on glass slides and dried at 65°C for 1 hour (Appendix).

II. Electron Microscopy

Samples (1mm cubes) of tissue were divided from each end of each cone using a sharp scalpel blade.

1. Fix in 3% glutaraldehyde in cacodylate buffer (pH7.4).
2. Wash in 0.1M cacodylate buffer (pH 7.4) - 15 min x 2 changes.
3. Post-fix in 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.4) - 2 hours.
4. Wash in 10% ethanol - 10 min x 2 changes.
5. Dehydrate in ascending grades of ethanol - 10 min x 2 changes each grade (30%, 50%, 70%, 90%, 95% absolute ethanol).
6. Place in propylene oxide - 20 min x 2 changes.
7. Impregnate in epoxy resin - overnight.

8. Embed in Beem capsules and cure at 60°C for 3-5 days.

Sections were then cut on an LKB III Ultratome.

For orientation and structure identification the standard practice is to cut a thick section about 1 micron thick. This is placed on a glass slide on a droplet of water, flattened by passing a glass rod which has been dropped in chloroform, and dried over a hot plate at 70°C. The section was then stained with 1% toluidine blue in 1% sodium tetroborate for 30 secs at 70°C.

This allows the orientation of the specimen to be made prior to the sectioning.

The sections (50-60nms thick) are cut using a glass knife, floated out on distilled water, flattened by wafting a chloroform moistened rod over them and picked up on Athene old 400 copper grids.

After drying they were stained by the following method (see Appendix).

<u>Uranyl acetate - lead citrate dual stain</u>

1. Place a drop of each stain on a wafer of dental wax.
2. Invert grids on uranyl acetate - 15 mins.
3. Wash gently in 50% ethanol - 10 mins x 2 changes.
4. Distilled water wash - 10 mins.
5. Invert grid on lead citrate solution - 5 mins.
6. Rinse in 0.02N NaOH - 2 mins.

7. Distilled water wash - 5 mins.
8. Blot dry by placing grid on filter paper section side up.

The grids were then viewed in a Jeol 100S transmission electron microscope.

III. Immunofluoresence Microscopy

The core for immunofluorescence studies was placed in a moist chamber for transport to the laboratory. (This chamber was simply a coverslip box with a piece of filter paper which had been moistened with phosphate buffered saline [PBS]). This was essential to prevent the tissue from drying out before it was delivered to the laboratory.

On receipt the tissue was placed on an inert compound OCT (Supplied by Ames Ltd) on a cryostat microtome chuck and snap frozen using solid CO_2. (The OCT allows the sections to be cut very easily while supporting the delicate tissue, it does not react with any of those reagents and is easily removed by washing in PBS).

Sections were cut at 2-3microns on a Bright's Cryostat at -30°C, attached to a microscope slide by direct contact and allowed to dry in air for 2-3 mins.

The standard immunological screen used in this unit is anti-human IgA, IgG, IgM, fibrin/fibrinogen, C3, C4, C1q.

All are raised in rabbits, FITC conjugated and supplied by Dakopatts a/s Denmark.

The method used is as follows:

1. Wash sections in PBS at room temperature - 15 mins.
2. Remove excess PBS and expose to rabbit anti-human FITC conjugated serum diluted 1:20 with PBS - 30 mins in a moist dark chamber at room temperature.
3. Wash well in PBS - 3 changes x 10 mins each.
4. Mount in glycerol:PBS (1:9) mountant.
5. Examine under UV light.

The sections were examined in a Leitz Ortholux microscope with incident light illumination. The system used an HB200W mercury vapour lamp, a BG38 red suppressor filter, KP500 polarising excitation filter with 530nm barrier eyepiece filter. Any reaction was easily seen in the test section. Since FITC was used, this showed as an apple green/yellow colour). It was graded according to 0 - (no reaction), trace +, ++, +++ (very strong reaction).

No attempt was made to distinguish between which tubules were showing activity, and even if only a few were fluorescing, these were graded on the 0 - +++ scale. In the glomerulus it was easier to determine the more precise location of activity, and the site was identified as mesangium and capillary wall.

To record the results the sections were photographed using an Orthomat camera on Kodak Ektachrome 50 Tungsten film.

Method of analysis and presentation of data

In an attempt to perform a statistical analysis of the results, and because some of the diagnostic groups were numerically small, the cases were first arranged in two large groups, 1) those in which there was a major lesion in the glomerulus, the glomerular disease group, and 2) those in which the only major lesion was tubular, the tubular disease group. In group 1), the breakdown of diagnosis was as follows:

proliferative glomerulonephritis	- 3 cases
membranous glomerulonephritis	- 3 cases
crescentic glomerulonephritis	- 1 case
focal necrotising glomerulonephritis	- 1 case
transplant rejection	- 6 cases

In the first statistical analysis, this whole group was compared with the normal. In a further statistical analysis, the transplant cases were removed from the glomerular disease group, and assessed as a separate group.

Group 2 (tubular disease) was made up as follows:

acute tubular necrosis	- 2 cases
myeloma kidney	- 1 case

The criteria used to identify the presence or absence of the carbohydrate were quite simple. Each lectin had a negative control which was a section which underwent the same procedure as the test section except that the lectin was omitted. This showed no specific fluorescent activity when viewed under the UV microscope except for the normal autofluorescence which could be easily discounted.

The data obtained has been presented in two forms, quantitative and qualitative, ie

1) as a statistical analysis,

2) as a histogram, where the degree of positivity of each lectin for each anatomical site is plotted against the positivity seen in the normal controls.

Each form of presentation revealed a different aspect of the results.

Lectin Specificity

All the lectins were conjugated with FITC. The final protein concentration was 1mg/ml based on OD 280.
The working dilution of the lectins was obtained by performing dilution trials. In all cases this was a 1:20 dilution (in phosphate buffered saline pH 7.2) which gave a concentration of 200ugs lectin in each solution.

The purity of the lectins was shown in the technical data accompanying them. By disc gel electrophoresis, using a 7.5% polyacrylamide gel and a Tris-glycine buffer at pH 8.8 a 50µg sample of lectin showed in most cases a single band diffusion when reacted against its specific carbohydrate. The exceptions being Dolichos bifloris agglutinin which had two closely related bands and Limulus polyphemis agglutinin which showed multiple bandings.

For use all lectins were subjected to dilution trials and it was decided that optimally the best results were obtained with 1:20 dilution in PBS. This was then used throughout the study. Also used were two lectins supplied by Miles Ltd. These were used as an indication of activity to try and see if there was any anomalous results obtained by the same lectin supplied by E-Y Laboratories. These lectins were Con A and WGA.

The functional specificity of the lectins can be inhibited by binding with inhibiting carbohydrates.

Lectin	Inhibition
PNA	Galactose
Con A	α-methyl-mannoside
DBA	α-acetyl-α-D-galactosamine
SBA	Galactose
RCA	Galactose

WGA	N-acetyl-glucosamine
UEA	x-L-fucose
LPA	N-acetyl-neuraminic acid

The cryostat sections were incubated to a 1:20 dilution of the FITC lectin conjugate in a moist dark chamber for 30 minutes at room temperature.

Sections were also incubated with PBS to check for autofluoresence. After incubation the slides were carefully rinsed with 0.01M PBS pH 7.2 three times each change of 10 minutes. The surplus was then carefully removed and the section mounted in glycerol/PBS mountant.

RESULTS

RESULTS

From the results obtained in the normal cases, a pattern of lectin binding in the normal kidney was built up, the anatomical sites chosen in the glomerulus being the glomerular capillary basement membrane and the mesangium, and in the tubules, the basement membrane. No differentiation between types of tubules was attempted. The results from the abnormal biopsies were then compared with this normal pattern.

The great majority of the results were obtained from frozen material, and these will be considered first. The small studies on paraffin-embedded material will be described secondly.

In order to illustrate the material on which the assessment of lectin binding was performed, a series of photographs is now appended. This series gives examples of the normal and abnormal pattern in different parts of the nephron, and showing different degrees of positivity. It should be appreciated that the sections were cut, stained, viewed and photographed almost immediately after the individual biopsies were received in the department; since, therefore, they could not be photographed in large batches, and since the most acceptable photographic set-up had to be found by trial and error, there is a certain

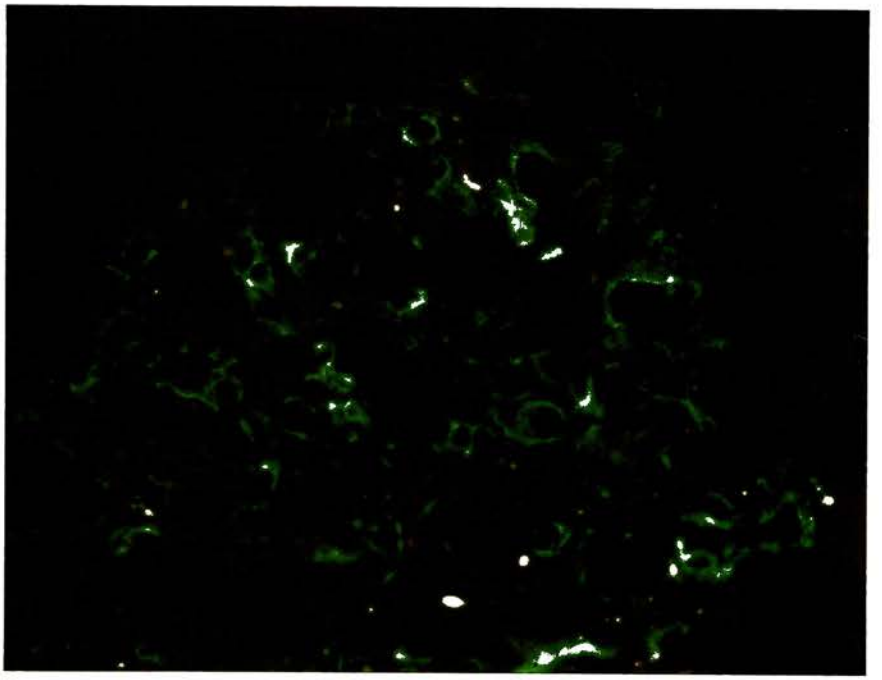
variation in intensity of fluorescence, in contrast, and in general lightness of the illustrations in different cases. When the processed tissue was being photographed it was noticed that the fluorescence of the preparations faded rapidly during the exposure times. This led to some difficulties in obtaining high quality pictures representing the findings. An attempt has been made to choose pictures which illustrate the true differences in fluorescence grading, as they appeared in the microscope.

HISTOGRAMS

The data on which the histograms were constructed are in each case given first, followed by the relevant histogram. Each histogram gives a visual indication of the relationship of individual cases in their disease group to the normal situation, with respect to each lectin.

The data obtained from the lectin binding was expressed in two forms:

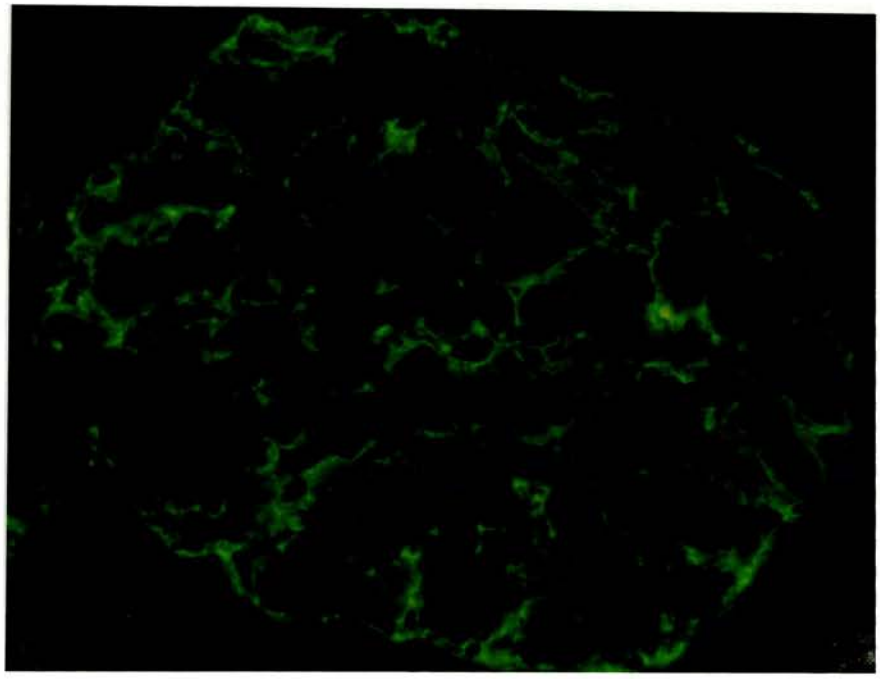
1. Converted to a digitised form and computer analysed, using the Mann-Whitney U statistical test.
2. By histograms which related 1) the amount of positivity on an 0 (no reaction) to +++ (very strong reaction) scale, and 2) the anatomical site, ie mesangium, GBM and TBM.



1. Normal Kidney

Concanavalin A binding showing trace in GBM and mesangium

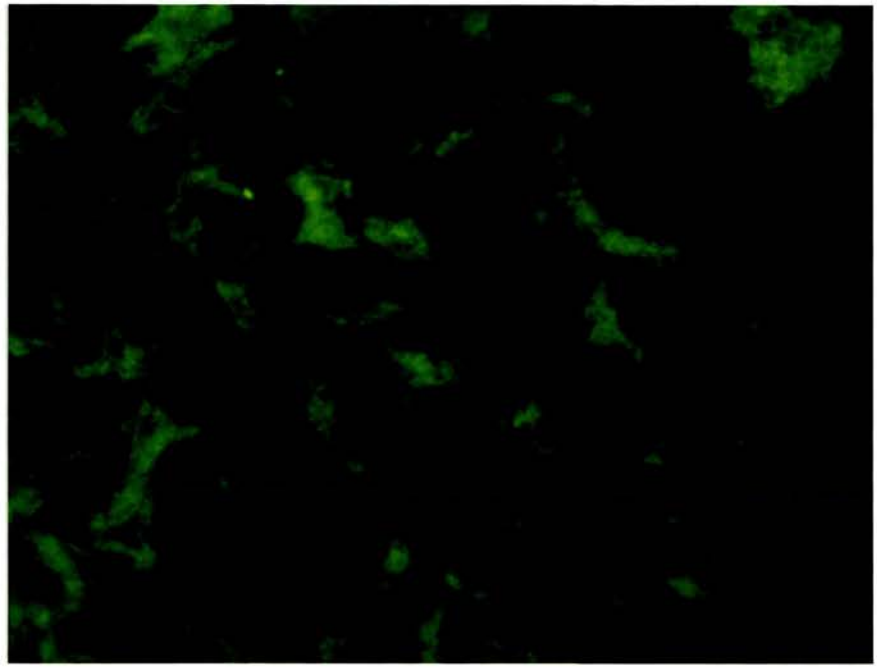
x40



2. Normal Kidney

WGA binding showing + positivity in GBM and trace in mesangium

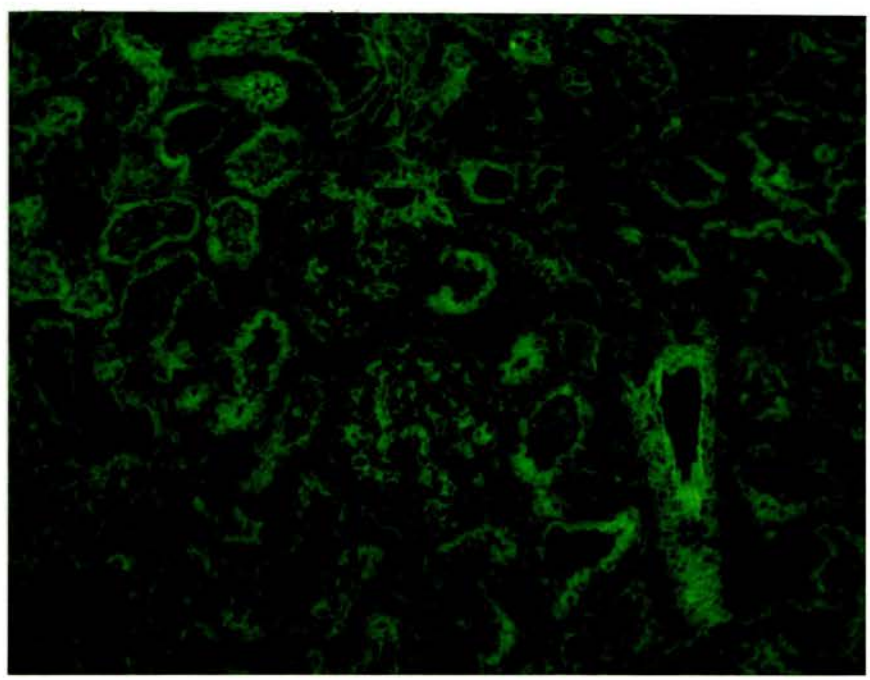
x25



3. Normal Kidney

WGA binding showing + positivity in mesangium

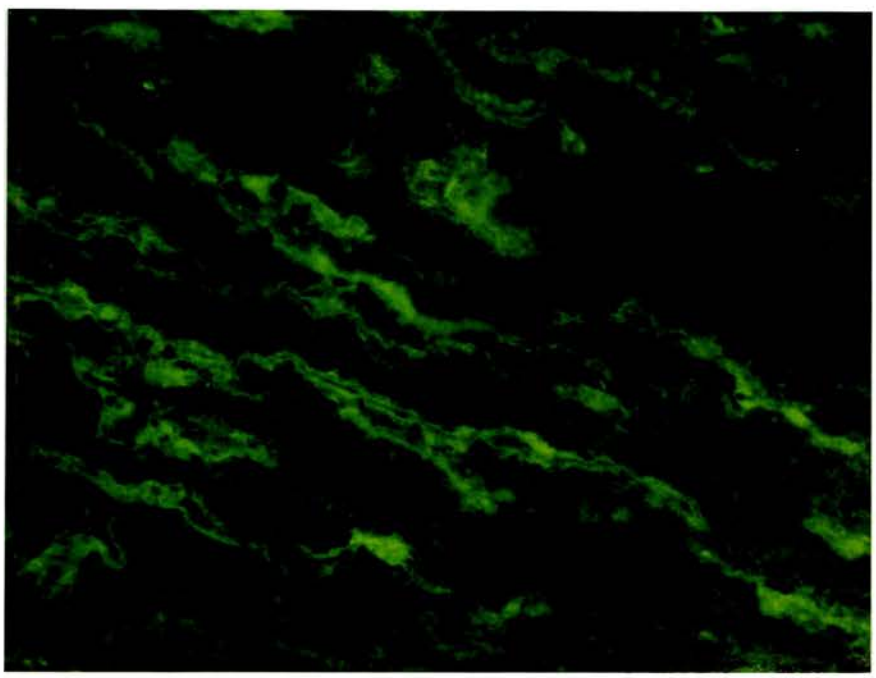
x40



4. Normal Kidney

SBA binding showing variety of positivity on low power

x10



5. Normal Kidney

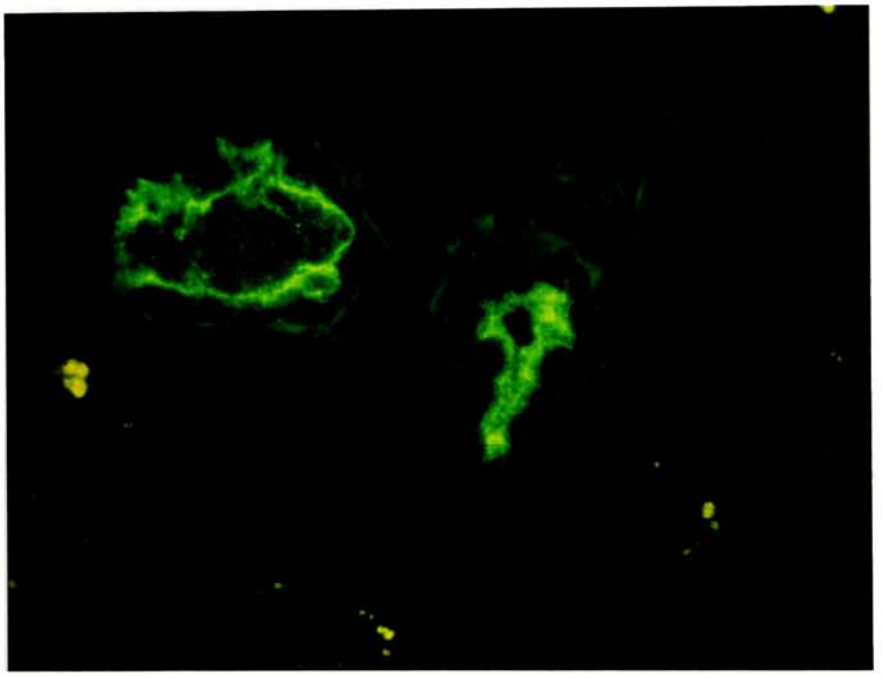
WGA showing + positivity in tubular basement membrane

x25

6. Normal Kidney

PNA binding showing ++ positivity in tubular basement membrane

x25



7. Normal Kidney

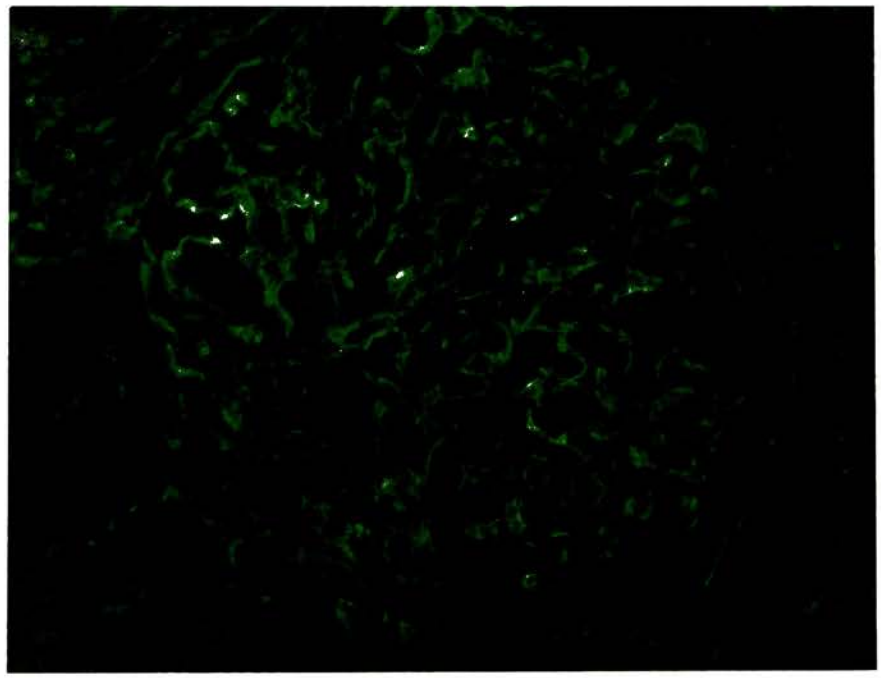
PNA binding showing +++ positivity in tubular brush border

x40

8. Membranous Glomerulonephritis

PNA binding showing trace positivity in GBM and mesangium

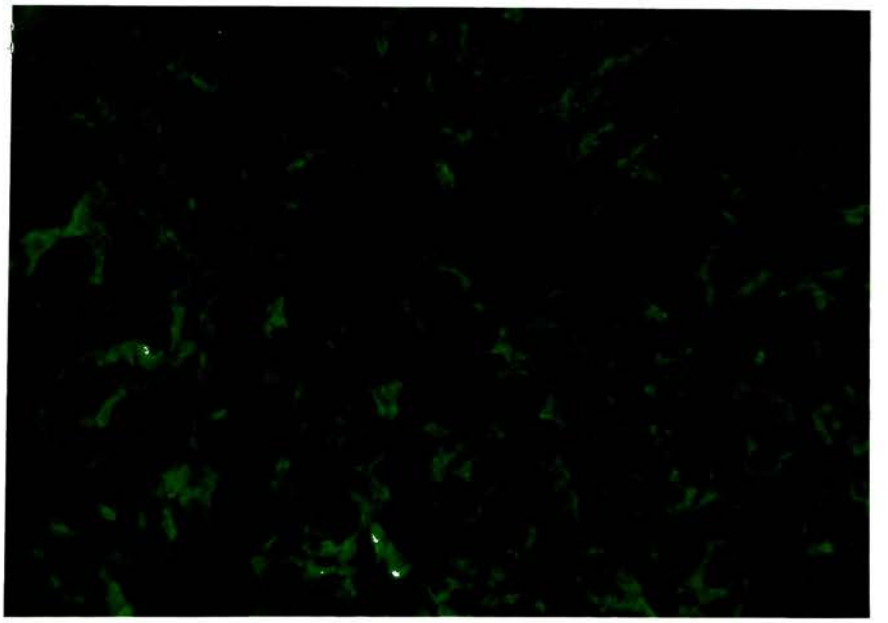
x40



9. Membranous Glomerulonephritis

Concanavalin A binding showing + positivity in GBM and mesangium

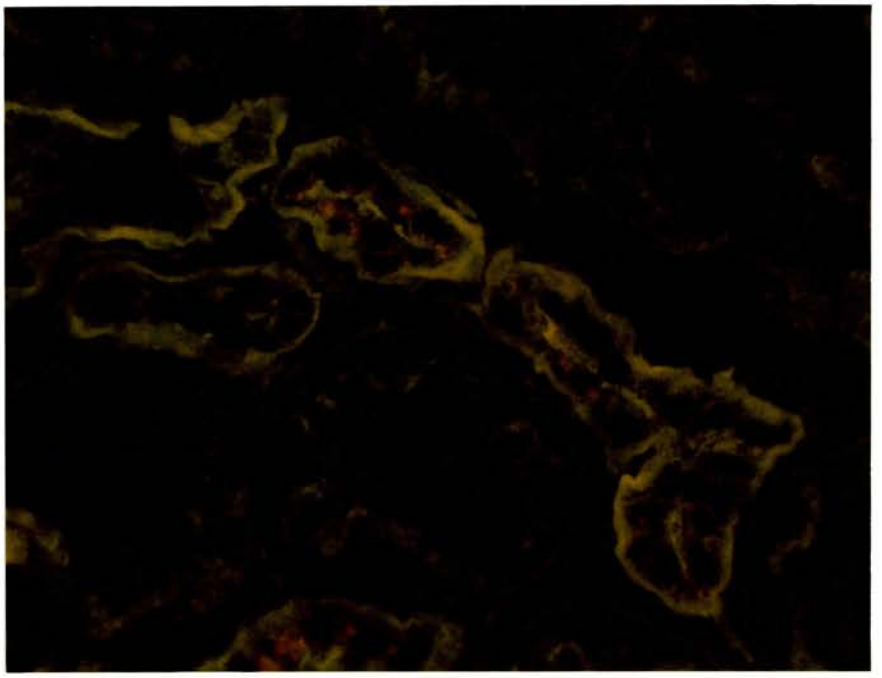
x25



10. Membranous Glomerulonephritis

UEA binding showing ++ positivity in GBM

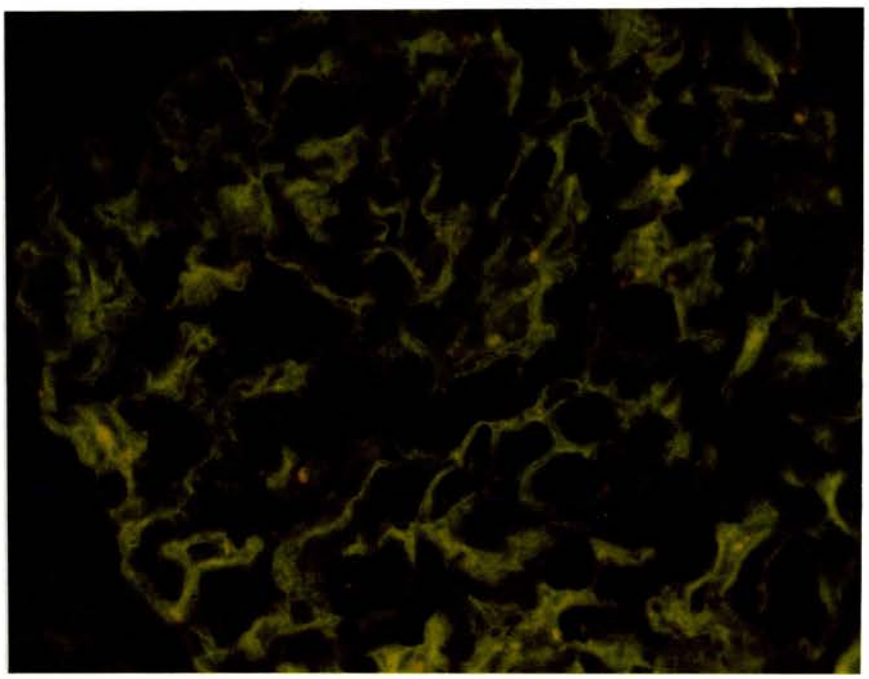
x40



11. Proliferative Glomerulonephritis

WGA binding showing +++ positivity in tubular basement membrane

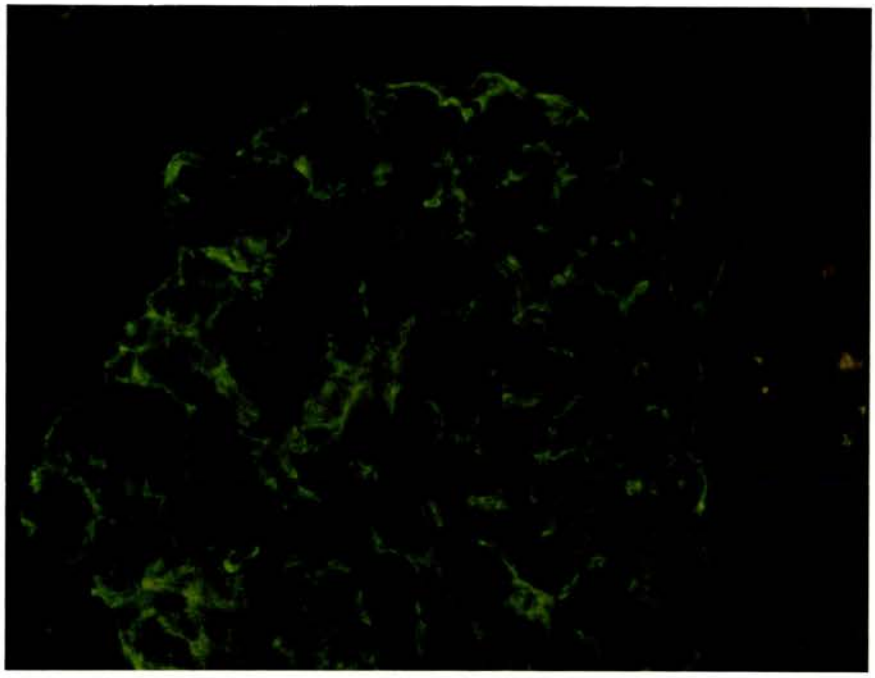
x40



12. Proliferative Glomerulonephritis

WGA binding showing + GBM and trace mesangium positivity

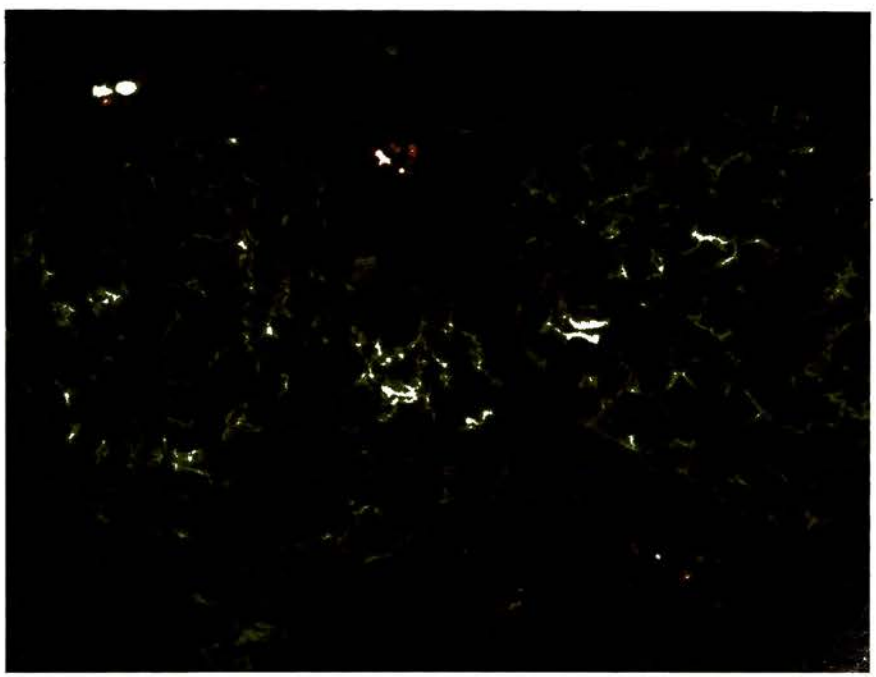
x40



13. Acute Tubular Necrosis

WGA binding ++ positivity in GBM and + positivity in mesangium

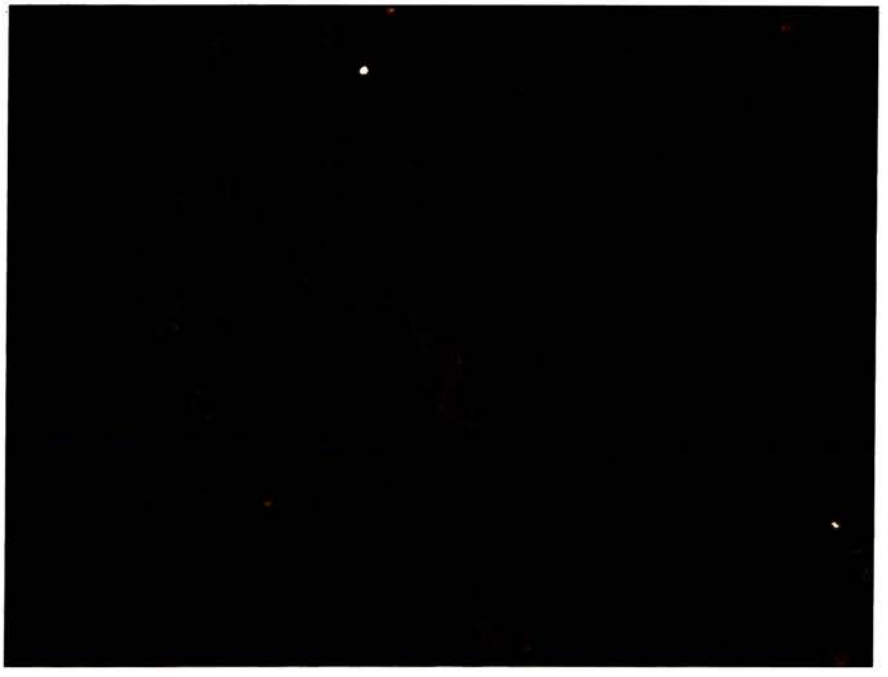
x40



14. Acute Tubular Necrosis

Concanavalin A binding showing + positivity in GBM and + positivity in mesangium

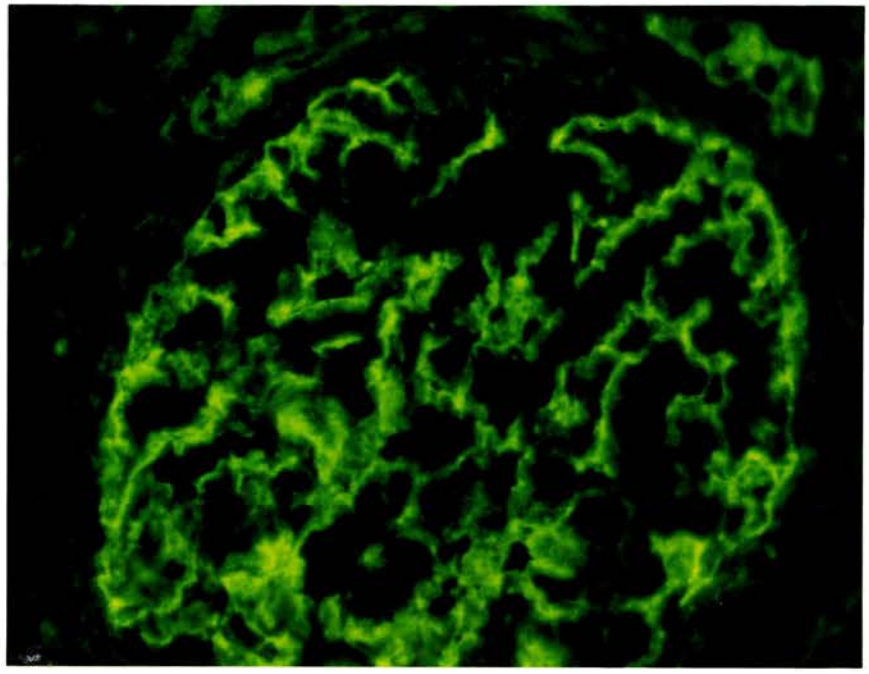
x40



15. Acute Tubular Necrosis

Ricin binding showing trace positivity in GBM and ++ positivity in TBM

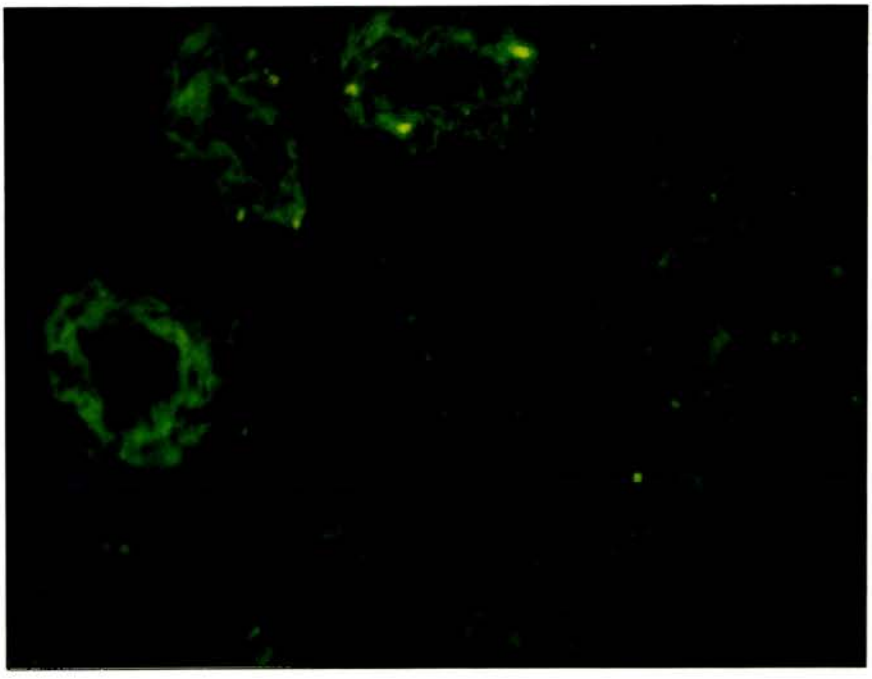
x25



16. Myeloma Kidney

WGA binding showing +++ positivity in GBM and + positivity in TBM

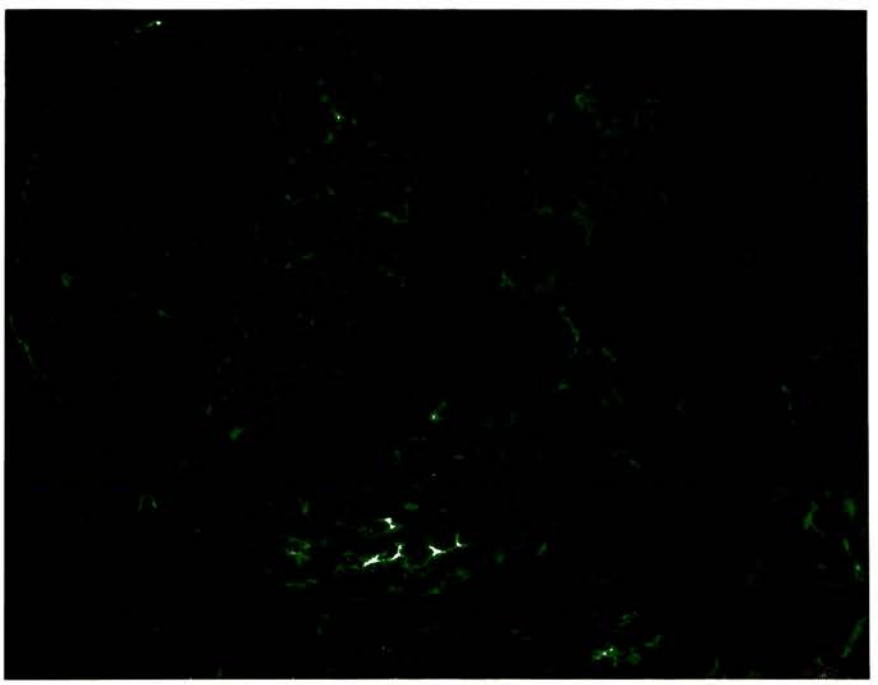
x40



17. Myeloma Kidney

PNA binding showing ++ positivity in TBM

x40



18. Transplant Rejection

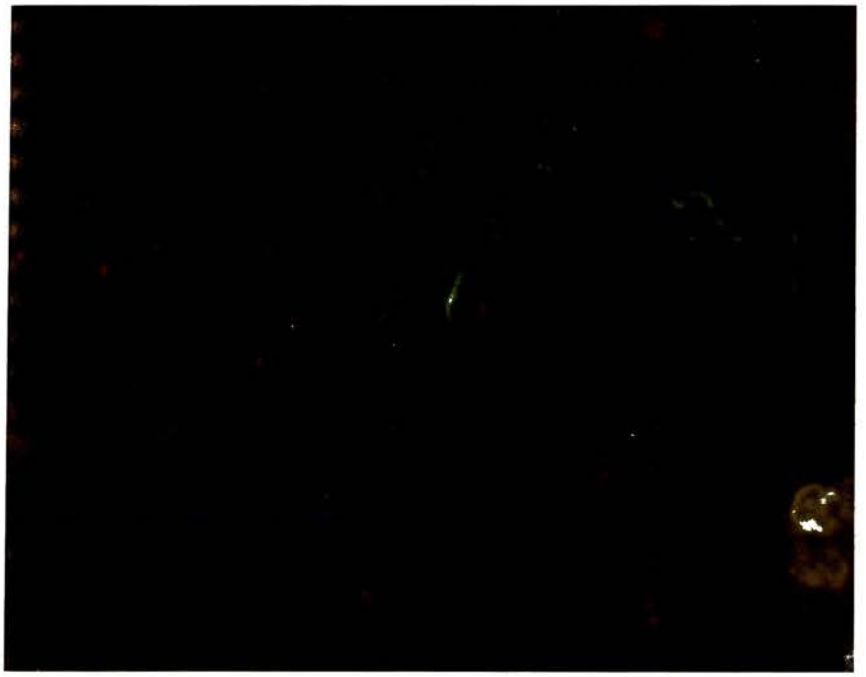
WGA binding showing + in GBM and + in mesangium

x25

19. Transplant Rejection

Immunogenic cells (probably plasma cells) showing ++ positivity with SBA (tubules)

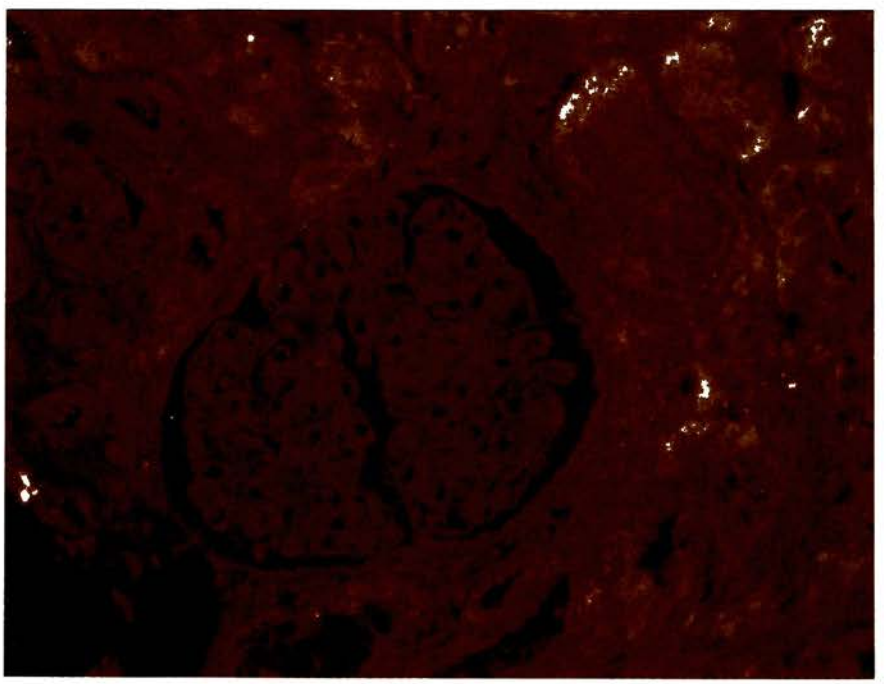
x25



20. Transplant Rejection

Ricin binding showing + positivity in tubular basement membrane

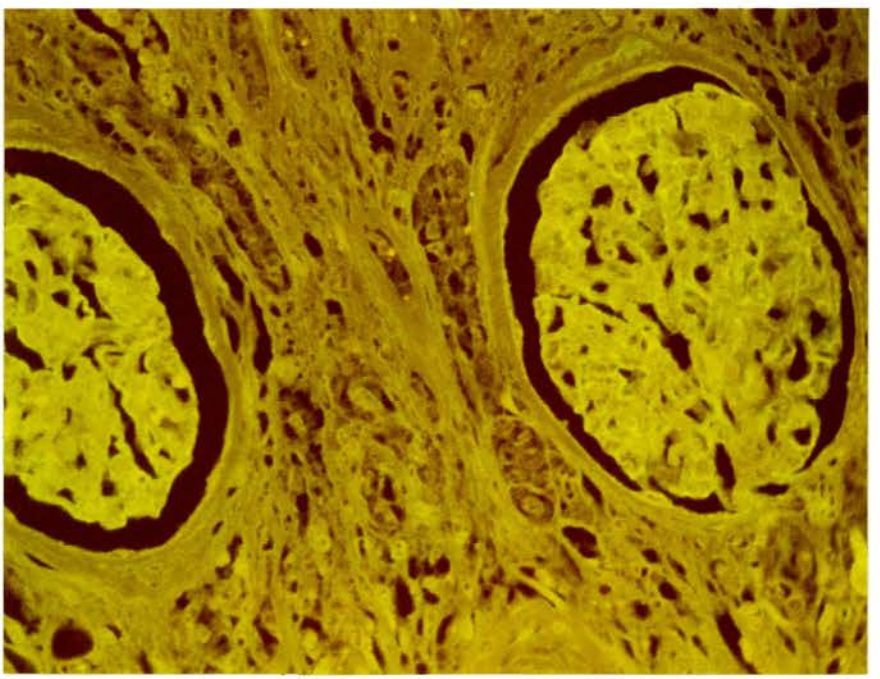
x40



21. Normal Kidney (Wax processed)

Negative control (no specific fluorescence noted)

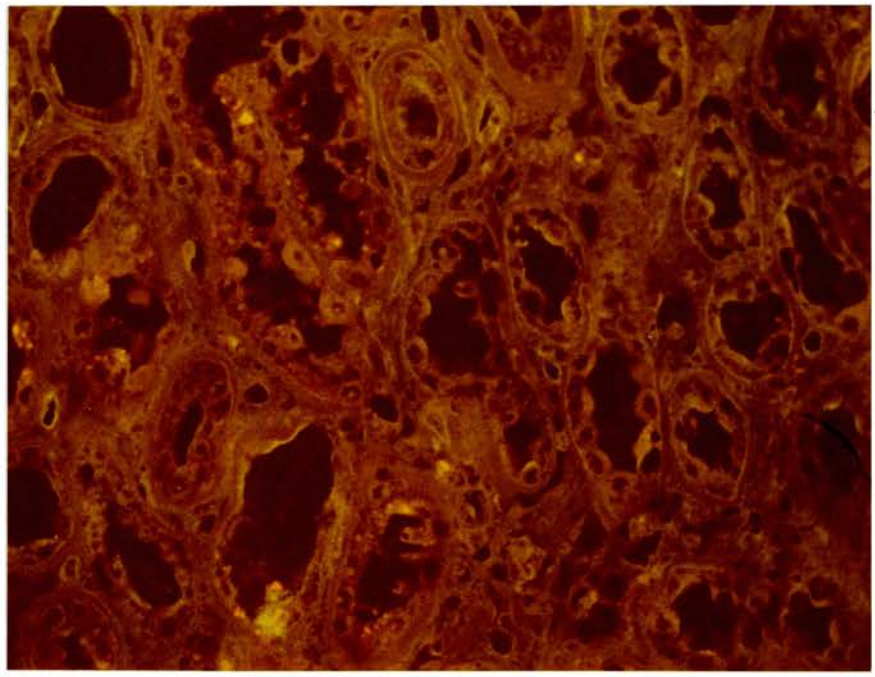
x25



22. Alport's Syndrome (Wax processed)

WGA binding showing +++ GBM + mesangium positivity but showing loss of fluorescence due to fading during photography

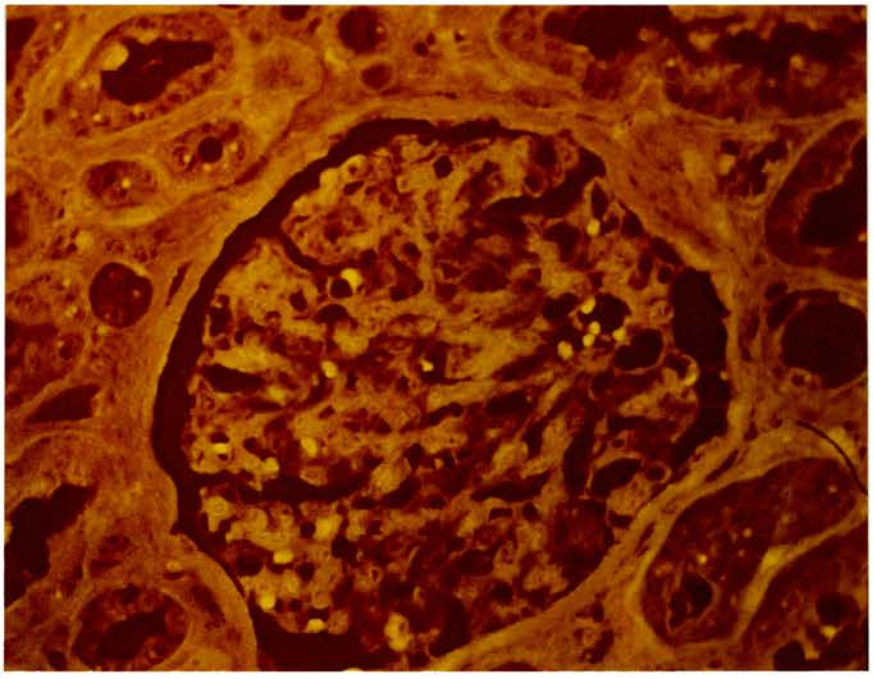
x25



23. Alport's Syndrome (Wax processed)

WGA binding originally showing ++ positivity in tubular basement membrane but showing loss of fluorescence due to fading during photography

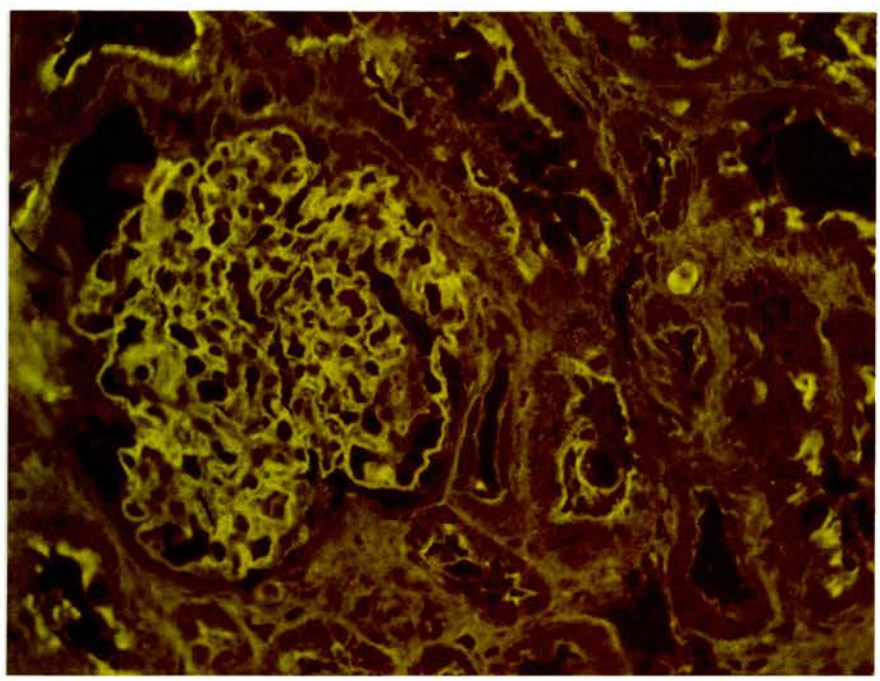
x25



24. Alport's Syndrome (Wax processed)

Negative control - no specific fluorescence seen

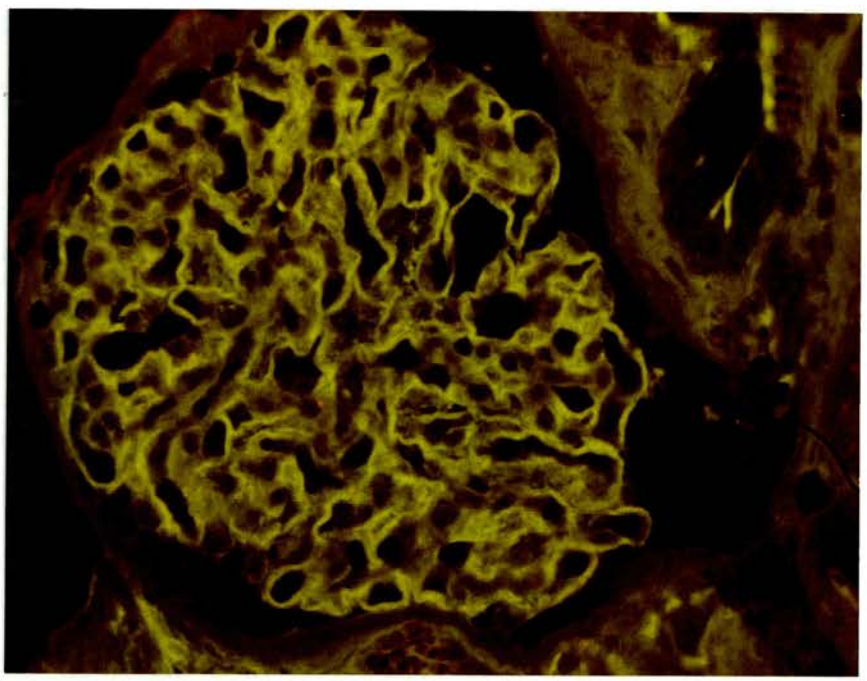
x25



25. Minimal Lesion Glomerulonephritis
(Wax processed)

WGA binding originally showing +++ GBM and tubular cell luminal border, + mesangium, positivity but with loss of fluorescence due to fading during photography

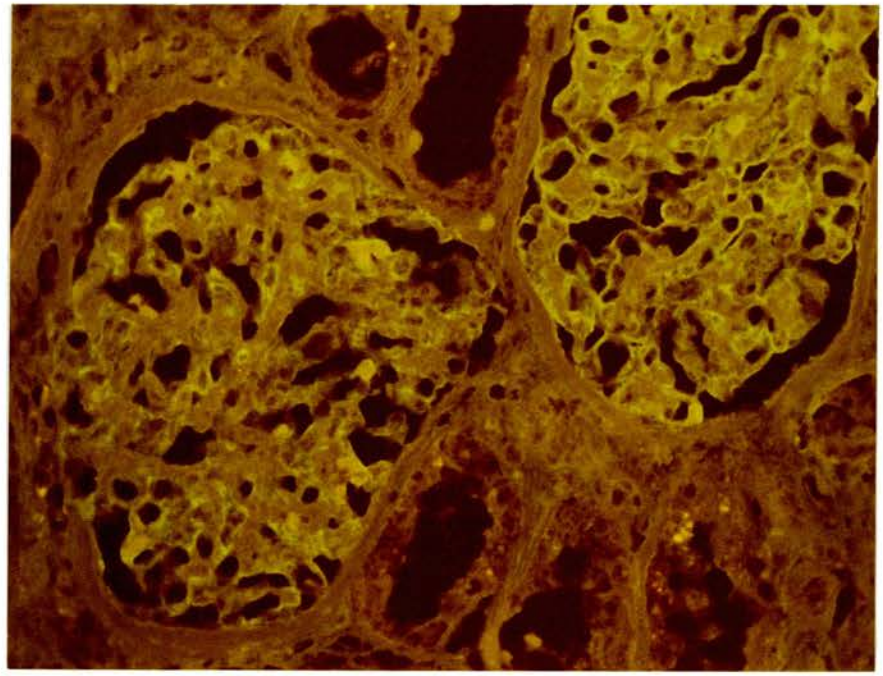
x25



26. Minimal Lesion Glomerulonephritis
(Wax processed)

WGA binding originally showing +++ GBM + mesangium but with loss due to fading during photography

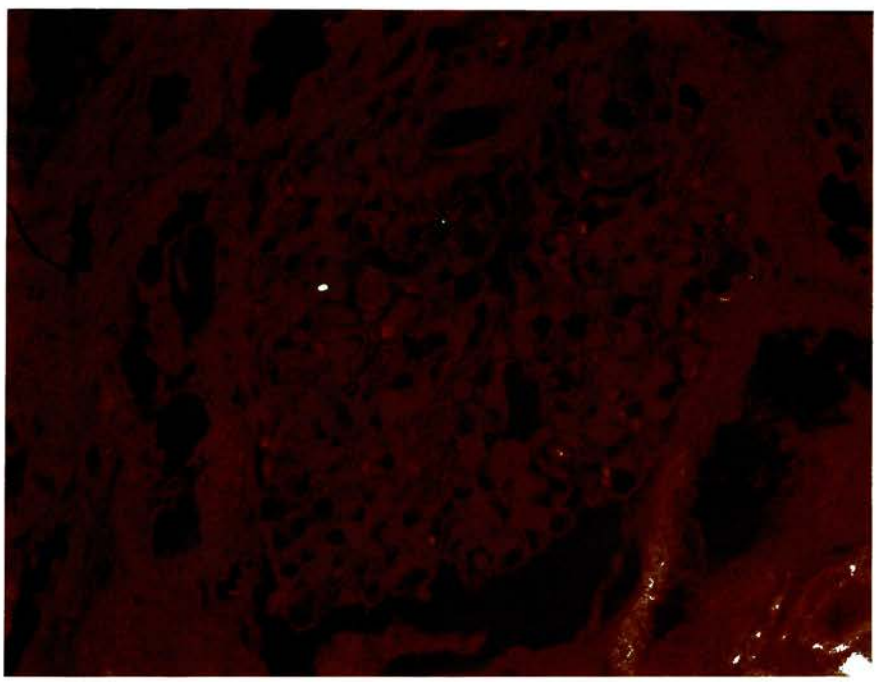
x25



27. Minimal Lesion Glomerulonephritis
(Wax processed)

WGA binding originally showing + mesangium +++ GBM but showing loss of fluorescence due to fading during photography

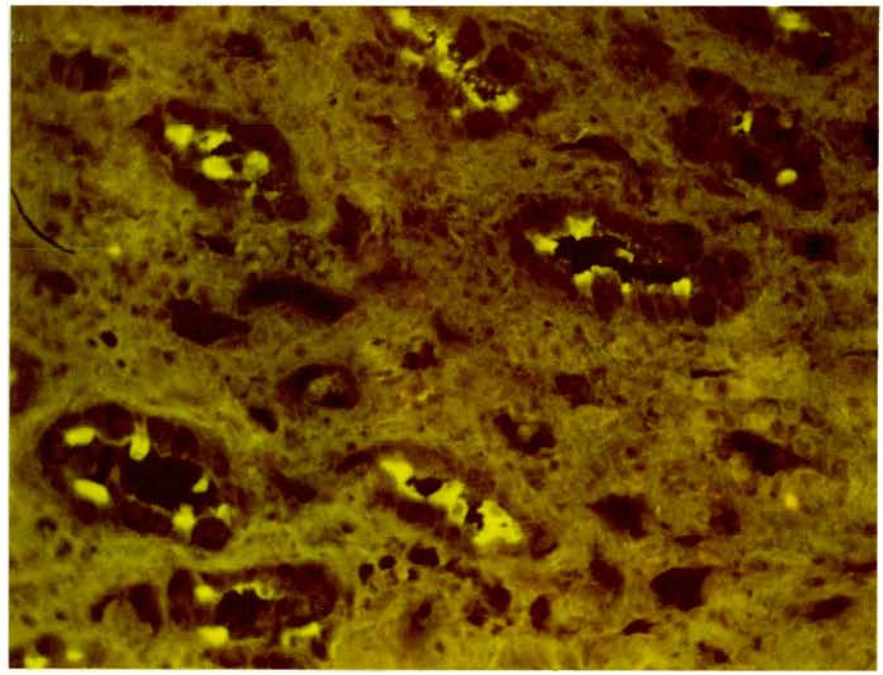
x25



28. Minimal Lesion Glomerulonephritis
(Wax processed)

Negative control - no specific fluorescence seen

x25



29. Minimal Lesion Glomerulonephritis
(Wax processed)

WGA binding tubular cells showing +++ positivity in their luminal aspect (compare with frozen material)

x25

The histograms gave a visual display of the increase or decrease of positivity in lectin binding and proved very useful when looking at the various subgroups comprising the main disease groups.

I. Frozen Material Statistical Analysis

It was felt that, in spite of the small numbers involved, it should be possible to analyse the results statistically to determine if the observations had any significance in this sense. The tabulated forms of the results were converted to a digitised presentation in order that they could be computerised and analysed. The results were converted thus:

0	= 0
trace	= 1
+	= 2
++	= 3
+++	= 4

The data obtained was analysed using the Mann-Whitney U Test (Siegel, 1956). This statistical test is a non-parametric test which can be used to test whether two independent groups have been drawn from the same population. Suppose, for example, we have two populations A and B; the null hypothesis (H_0) is that A and B have the same distribution. The alternative hypothesis (H_1) is that A is stochastically larger than B. Where small sample sizes are concerned, (the larger number N_2 being

less than 20) as in this study, a U value is obtained. If this U value is equal to or less than the critical U value found in the tables, H_0 can be rejected at the level of significance (p) indicated at the head of the table. Since all the data in the study was grouped in series of less than 20, all the calculations obtained a U value. The level of significance was chosen as 5% (p=0.05) and the U value obtained was doubled to obtain a two-tailed test as opposed to a single tailed test.

In this study the majority of the groups were less than 8 in number. Accordingly the statistical computation produced a U-value which was converted directly to a p value, directly from the tables. When the group numbered more than 8, the U value was compared to the values given in the tables and, depending on the level of significance required, the result expressed as significant or not.

Because of the sample sizes two methods were used in expressing the probability (p).

1. When U value = 9, p = 0.002
   U value = 17, p = 0.02
   U value = 22, p = 0.05

   For this study U = 22, p = 0.05 was chosen.

2. The U value was read from the tables (see over) and the level of significance expressed directly. In all cases the p values obtained were doubled to give the level of significance for a two-tailed test.

For samples with populations greater than 8 the first method was employed; with populations less than 8 the second method was used.

In the first analysis, the glomerulonephritis cases and the transplants were grouped together, and their results compared with the normal (Table 2).

It was felt that the pure GN group and the transplant group should now be analysed separately (Tables 3 and 4).

The small group of tubular diseases was analysed in the same way (Table 5).

Table 6 summarises the statistically significant results obtained.

The results of all other lectin bindings proved to be statistically non-significantly different when abnormal was compared with normal, or abnormal with another abnormal. Membranous GN, for example, was not significantly different from proliferative GN. This, of course, may have been at least partly due to the small number of cases involved.

However, the assumption must not be made that the results are in any way inferior or less statistically significant because of the small number of cases.

II. Paraffin Processed Material - Statistical Analysis

In this series two conditions were investigated, minimal lesion glomerulonephritis and Alport's Syndrome (hereditary nephritis). The results were statistically analysed and compared with results from paraffin-embedded material which had been taken from kidneys removed because of the presence of a renal carcinoma. The renal tissue itself was considered to be normal.

The cases of minimal lesion GN and of Alport's syndrome were taken from the routine hospital files of the renal unit in the Department of Pathology. These cases were chosen because a) heavy proteinuria is present in the minimal lesion GN, but there is no real evidence suggesting a pathogenesis involving humoral immunity, as in many other types of glomerulonephritis, and b) there appears to be a hereditary defect in the glomerular capillary basement membrane in Alport's syndrome, though its nature is not clear. It was therefore thought that any further information regarding the biochemical structure of the membrane in these two conditions might be helpful.

The reactions obtained in the normal renal tissue with the lectins were not entirely similar to those seen on the frozen section, but were quite clear. The difference in results may have been due to the effects of fixation and processing. Unfortunately no cases of either of the abnormal conditions were submitted fresh to the laboratory during the course of the project, and therefore it was not possible to control the findings of paraffin processing versus frozen sections in these disease.

The normal tissue examined showed the following binding patterns.

Mesangium: Generally little positivity, the marked exception being WGA, where all seven cases showed a reaction.

GBM: Again, little positivity recorded except in the case of Con A, which showed moderate positivity (in all 7 cases) and WGA which gave an exceptionally strong reaction (in all 7 cases).

TBM: This showed a picture very similar to that seen in the GBM with, in addition to Con A and WGA, a slight positivity in 2 cases with PNA.

TABLE 1

LECTINS

Plants - Legumes	Abbreviated Name	No of Sugar Binding Sites	MW	Sugar
Arachis hyogaea (peanut)	PNA		110,000	gal
Conavalia ensiformis (jack bean)	ConA	4	102,000	Man, Glc
Glycine max (soybean)	SBA	2	120,000	GalNAC
Dolichos bifloris (horse grain)	DBA	4	110,000	GalNAC
Lens culinaris (lentil)		2	60,000	Man, GLc
Lotus tetragonobulus (winged pea)		4	120,000	α-L-fuc
Phaseolus limensis (lima bean)		2	120,000	GalNAC
Phaseolus vulgaris (kidney bean)	PHA		120,000	GalNAC
Plants - others				
Abrus precatorius (jequirty bean)		2	134,000	Gal
Phytolacca americana (pokeweed)	PWM			
Ricinus communis (castor bean)	RCA	2	120,000	Gal
Triticum Vulgare (wheat germ)	WGA	4	36,000	GclNAC
Ulex ureopus	UEA			α-L-fuc
Animals				
Helix pomatia (garden snail)		6	79,000	GalNAC
Limulus polyphemis (horseshoe crab)	LPA	18	335,000	NANAC (Sialic acid)

GlcNAC - N-acetyl glucosamine
Gal - galactose
Man - mannose
glc - glucose
α-L-fuc - α-L-fucose
GalNAC - N-acetylgalactosamine
NaNAC - N-acetyl neuraminic acid (sialic acid)

(After Lis and Sharon, 1977)

TABLE 2

GLOMERULAR DISEASE GROUP INCLUDING TRANSPLANTS

NORMAL VS ABNORMAL: METHOD 1

Lectin	Mesangium	GBM	TBM
	U value	U value	U value
PNA	28	31.5	45.5
Con A	35	24.5	36
DBA	49	45	26.5
SBA	35	45.5	2.5***
Ricin	41	43	39
WGA	42.5	40.5	35
UEA	39.	35.5	38
LPA	45.5	49	49

p = 0.05

*** = very significant result

TABLE 3

GLOMERULAR DISEASE GROUP EXCLUDING TRANSPLANTS

NORMAL VS ABNORMAL: METHOD 2

Lectin	Mesangium		GBM		TBM	
	U	p	U	p	U	p
PNA	10.5	0.04*	14	0.120	22	0.536
Con A	14	0.120	3.5	0.002**	9	0.028
DBA	25	.778	24.5	0.654	17.5	0.232
SBA	20	0.296	25	0.778	2.5	0.002**
Ricin	17	0.232	19	0.338	22.5	0.536
WGA	27.5	0.956	25.5	0.778	18	0.280
UEA	15.5	0.152	14.5	0.120	21.5	0.464
LPA	24.5	0.694	28	1.014	28	1.044

p = 0.05

** = highly significant result

* = significant result

TABLE 4

TRANSPLANT GROUP

NORMAL VS ABNORMAL: METHOD 2

Lectin	Mesangium		GBM		TBM	
	U	p	U	p	U	p
PNA	17.5	0.469	17.5	0.469	18.5	0.531
Con A	21	1.054	21	1.054	15	0.350
DBA	18	0.531	21	1.054	9	0.090
SBA	15	0.350	20	0.946	0	0.001***
Ricin	18	0.531	18	0.531	16.5	0.409
WGA	14	0.294	10	0.120	17	0.469
UEA	18	0.531	21	1.054	16.5	0.409
LPA	21	1.054	21	1.054	21	1.054

p = 0.05

*** = very significant result

TABLE 5

TUBULAR DISEASE GROUP

NORMAL VS ABNORMAL: METHOD 2

Lectin	Mesangium		GBM		TBM	
	U	p	U	p	U	p
PNA	3.5	0.140	7	0.516	9	0.834
Con A	3.5	0.140	0	0.016**	2	0.066
DBA	10.5	0.916	7	0.516	5.5	0.266
SBA	5	0.266	8.5	0.754	10	1.000
Ricin	0	0.016**	5	0.266	3.5	0.140
WGA	6	0.384	10.5	1.082	5	0.266
UEA	9	0.834	9	0.834	6	0.384
LPA	10.5	0.916	10.5	1.082	10.5	1.082

p = 0.05

** = highly significant result

TABLE 6

Lectin activity found to be significant using the Mann-Whitney U Test (ie the H_0 is not proved)

a) Glomerular Disease and Transplants

	GLOMERULUS		TUBULES
Site	Mesangium	Capillary Wall (GBM)	Tubular Basement Membrane
Lectin and Demonstrated	-	-	Soy Bean Agglutinin (SBA) (NAc-α-D-galactosamine)
Compared with Normal	-	-	less than found in normal

b) Glomerular Disease **Excluding** transplants

GLOMERULUS		TUBULES
Mesangium	Capillary Wall (GBM)	Tubular Basement Membrane
Peanut Agglutinin (PNA) (D-gal B (1:3) galactosamine)	Concanavalin A (Con A) (α-D-glucose) and α-L-mannose	Concanavalin A (Con A) (α-D-glucose) and α-L-mannose
More than found in the normal	More than found in the normal	More than found in the normal

/continued ...

TABLE 6 continued

c) Transplants Only

	TUBULES
	Soy Bean Agglutinin (SBA) (NAc-α-D-galactosamine)
	Less than found in normal

d) Tubular Disease

	GLOMERULUS		TUBULES
Site	Mesangium	Capillary Wall (GBM)	Tubular Basement Membrane
Lectin and Sugar	Ricin (B-D-galactose)	Con A (α-D-glucose) α-L-mannose	-
Compared with Normal	More than in the normal	More than in the normal	

TABLE 7

A. MINIMAL LESION GN

NORMAL VS ABNORMAL: METHOD 2

Lectin	Mesangium		GBM		TBM	
	U	p	U	p	U	p
PNA	12	0.788	12.5	0.788	10	0.528
Con A	8	0.206	0	0.006**	0	0.006**
DBA	14	1.076	14	1.076	14	1.076
SBA	14	1.076	12	0.788	14	1.076
Ricin	14	1.076	14	1.076	14	1.076
WGA	3	0.041	9.5	0.412	1	0.012*
UEA	14	1.076	14	1.076	14	1.076
LPA	14	1.076	14	1.076	14	1.076

/continued ...

TABLE 7 continued

B. ALPORT'S SYNDROME

NORMAL VS ABNORMAL: METHOD 2

Lectin	Mesangium		GBM		TBM	
	U	P	U	P	U	P
PNA	12	0.788	12	0.788	14	1.076
Con A	11.5	0.648	6	0.164	0	0.006**
DBA	14	1.076	14	1.076	10.5	0.528
SBA	14	1.076	12	0.788	14	1.076
Ricin	14	1.076	14	1.076	14	1.076
WGA	12	0.788	8	0.306	10	0.528
UEA	14	1.076	14	1.076	10.5	0.528
LPA	14	1.076	14	1.076	14	1.076

p = 0.05

* = significant result

** = highly significant result

TABLE 8

Minimal Lesion GN

	GLOMERULUS		TUBULES
	Mesangium	Capillary Wall (GBM)	Tubular Basement Membrane
Lectin and Sugar	WGA (N-acetyl-β(1.4)D-glucosamine)	Con A (-α-D-glucose) (α-L-mannose)	Con A - (α-D-glucose) (α-L-mannose) WGA (N-acetyl-β(1.4)D-glucosamine
Compared with normal	Less than in normal	Less than in normal	Both less than in normal

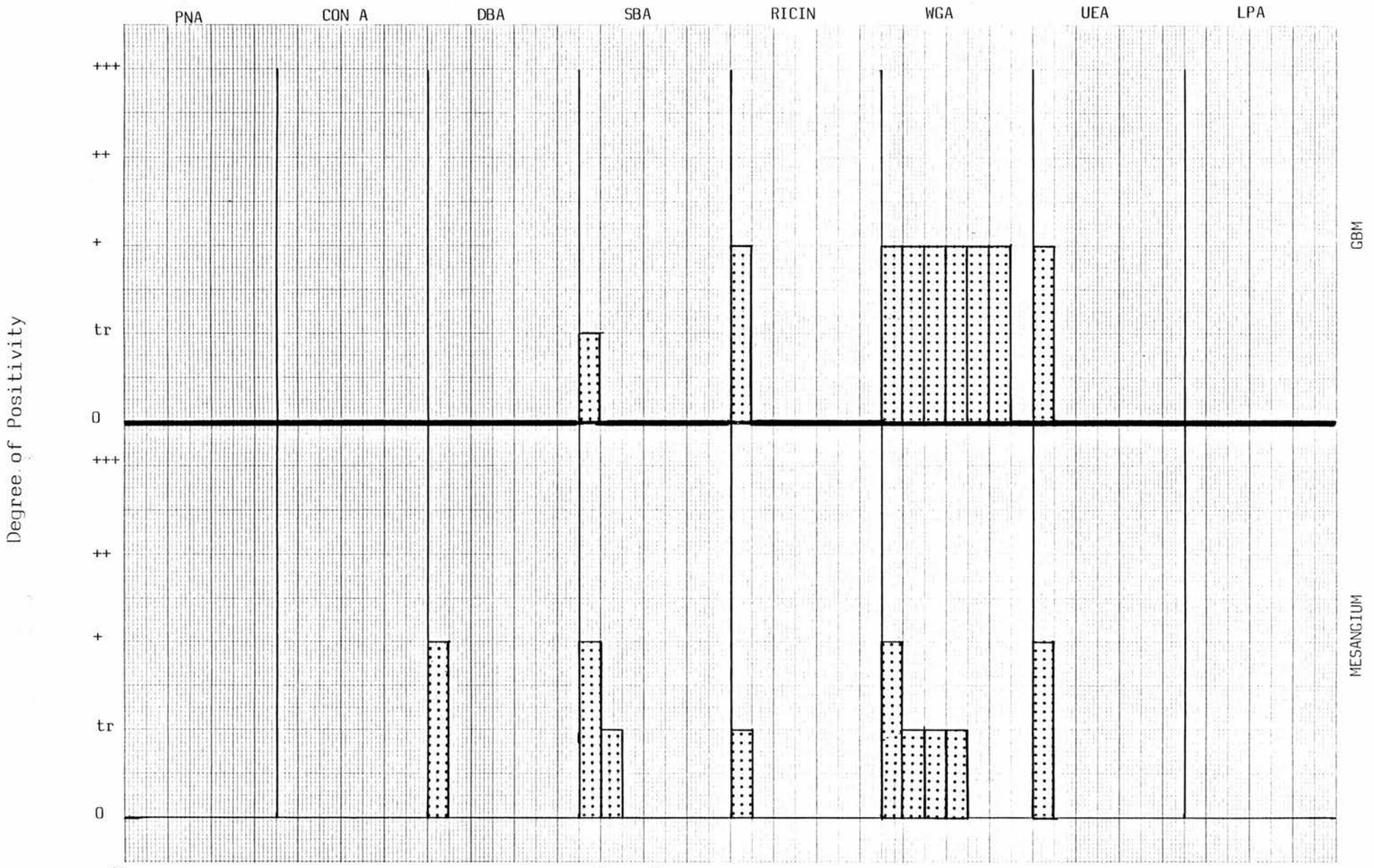
Alport's Syndrome (Hereditary Nephritis

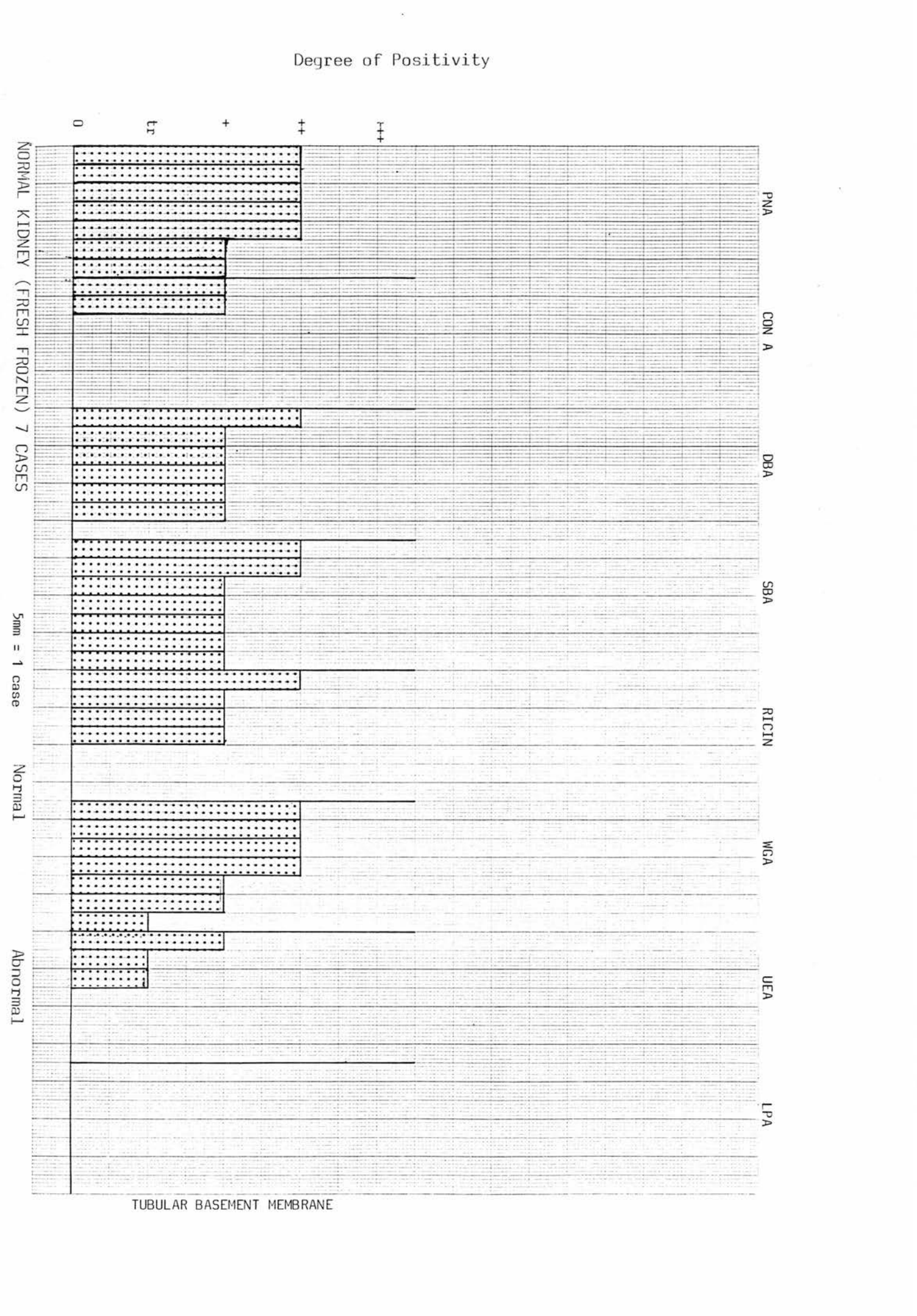
	GLOMERULUS		TUBULES
	Mesangium	Capillary Wall (GBM)	Tubular Basement Membrane
Lectin and sugar demonstrated	-	-	Concanavalin A (-α-D-glucose) α-L-mannose
Compared with normal	-	-	More than in normal

FROZEN SECTION MATERIAL

NORMAL - 7 CASES

Site	No of Cases Positive	Lectin	Sugar Demonstrated	Number of Cases and Degree of Positivity				
				0	tr	+	++	+++
Mesangium	4	WGA	Glc NAc (Neu NAc)		3	1		
	2	SBA	Gal NAc, Gal		1	1		
	1	DBA	Gal NAc			1		
	1	UEA	x-L-Fuc			1		
	1	Ricin	Gal		1			
		PNA	Gal-Gal NAc > Gal					
		Con A	Man, Glc	7				
		LPA	Neu NAc					
GBM	6	WGA	Glc NAc (Neu NAc)			6		
	1	Ricin	Gal			1		
	1	UEA	x-L-Fuc			1		
	1	SBA	Gal NAc (Gal)		1			
		PNA	Gal-Gal NAc > Gal					
		Con A	Man, Glc	7				
		DBA	Gal NAc					
		LPA	Neu NAc					
TBM	7	PNA	Gal-Gal NAc > Gal			2	5	
	2	Con A	Man, Glc			2		
	6	DBA	Gal NAc			5	1	
	7	SBA	Gal NAc (Gal)			5	2	
	4	Ricin	Gal			3		1
	7	WGA	Glc NAc (Neu NAc)		1	2	4	
	3	UEA	x-L-Fuc		2	1		
		LPA	Neu NAc	7				

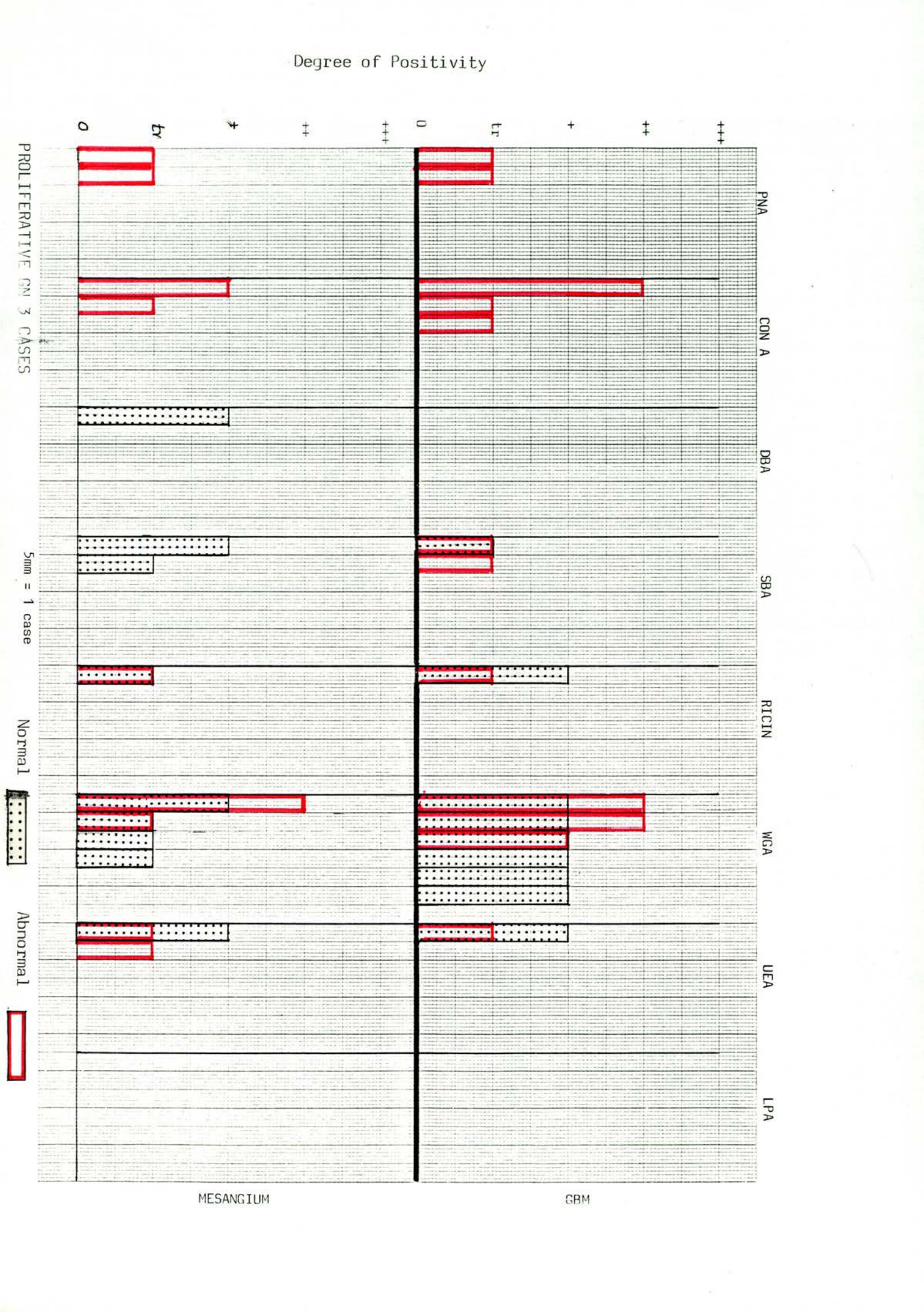

PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
+++
++
+
tr
0
+++
++
+
tr
0
GBM
MESANGIUM
Degree of Positivity
NORMAL KIDNEY (FRESH FROZEN) 7 CASES
5mm = 1 case
Normal
Abnormal

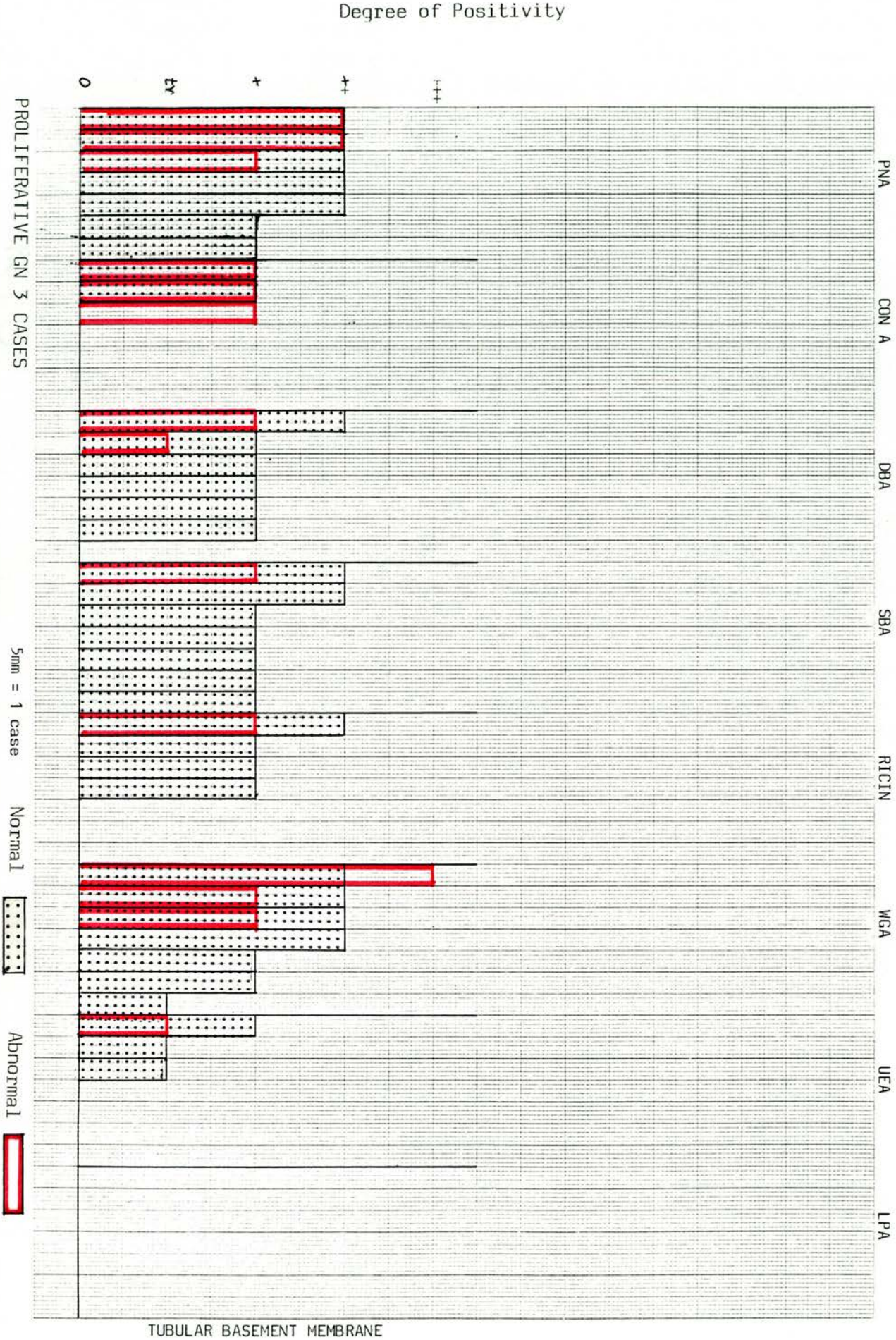

Degree of Positivity
0
tr
+
++
+++
NORMAL KIDNEY (FRESH FROZEN) 7 CASES
5mm = 1 case
Normal
Abnormal
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
TUBULAR BASEMENT MEMBRANE

FROZEN SECTION

PROLIFERATIVE GN - 3 CASES

Site	No of Cases Positive	Lectin	Sugar Demonstrated	Number of Cases and Degree of Positivity				
				0	tr	+	++	+++
Mesangium	2	PNA	Gal-Ga NAc > Gal		2			
	2	Con A	Man, Glc			1	1	
	1	Ricin	Gal		1			
	2	WGA	Glc NAc (Neu NAc)		1		1	
	2	UEA	∝-L-Fuc		2			
		DBA	Gal NAc }					
		SBA	Gal NAc (Gal) }	3				
		LPA	Neu NAc }					
GBM	2	PNA	Ga-Gal NAc > Gal		2			
	3	Con A	Man, Glc		2		1	
	2	SBA	Gal NAc (Gal)		2			
	1	Ricin	Gal		2			
	3	WGA	Glc NAc (Neu NAc)			1	2	
	1	UEA	∝-L-Fuc		1			
		DBA	Gal NAc }	3				
		LPA	Neu NAc }					
TBM	3	PNA	Ga-Gal NAc > Gal			1	2	
	3	Con A	Man, Glc			3		
	2	DBA	Gal NAc		1	1		
	1	Ricin	Gal			1		
	3	WGA	Gcl NAc (NeuNAc)			2		1
	1	UEA	∝-L-Fuc		1			
		LPA	Neu NAc	3				

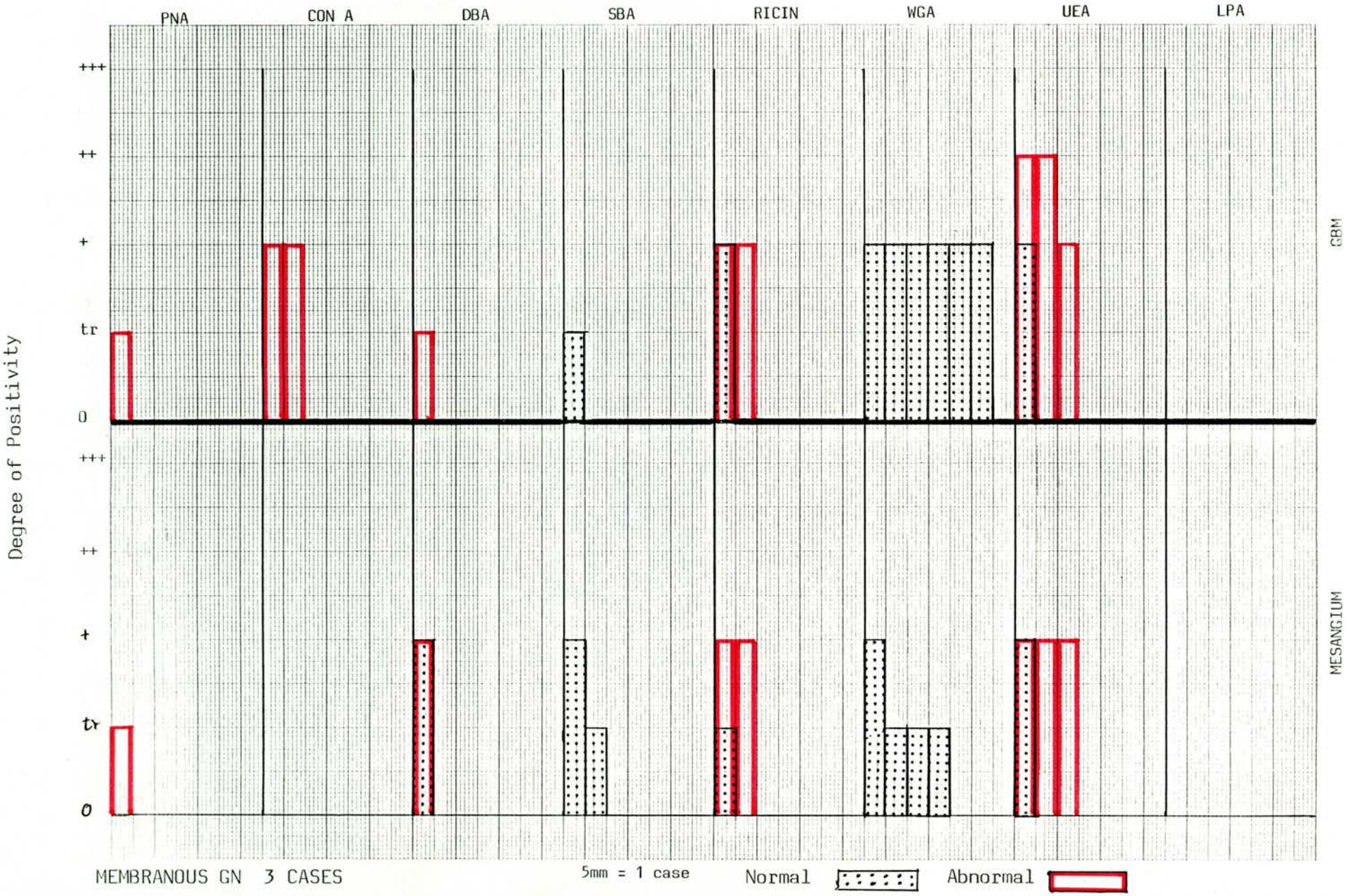

Degree of Positivity
PROLIFERATIVE GN 3 CASES
5mm = 1 case
Normal
Abnormal
MESANGIUM
GBM
0
tr
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA

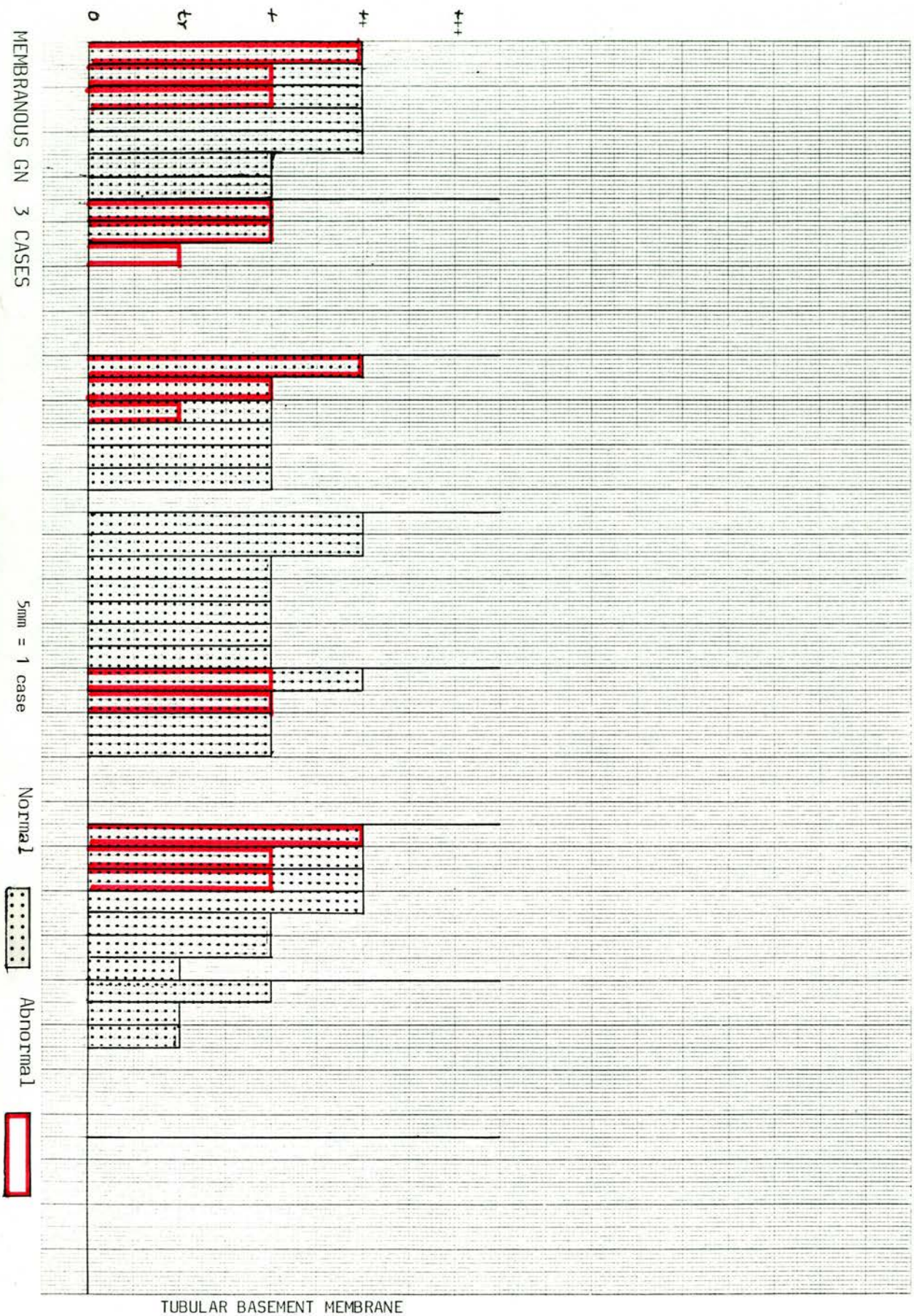

Degree of Positivity
0
tr
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
PROLIFERATIVE GN 3 CASES
5mm = 1 case
Normal
Abnormal
TUBULAR BASEMENT MEMBRANE

FROZEN SECTION

MEMBRANOUS GN - 3 CASES

Site	No of Cases Positive	Lectin	Sugar Demonstrated	Number of Cases and Degree of Positivity				
				0	tr	+	++	+++
Mesangium	1	PNA	Gal-Gal NAc > Gal		1			
	1	DBA	Gal NAc			1		
	2	Ricin	Gal			2		
	3	UEA	∝-L-Fuc			3		
		Con A	Man, Glc	}				
		SBA	Gal NAc (Gal)	} 3				
		WGA	Glc NAc (Neu NAc)	}				
		LPA	Neu NAc	}				
GBM	1	PNA	Gal-Gal NAc > Gal		1			
	2	Con A	Man, Glc			2		
	1	DBA	Gal NAc		1			
	2	Ricin	Gal			2		
	3	UEA	∝-L-Fuc			1	2	
		SBA	Gal NAc (Gal)	}				
		WGA	Glc NAc (Neu NAc)	} 3				
		LPA	Neu NAc	}				

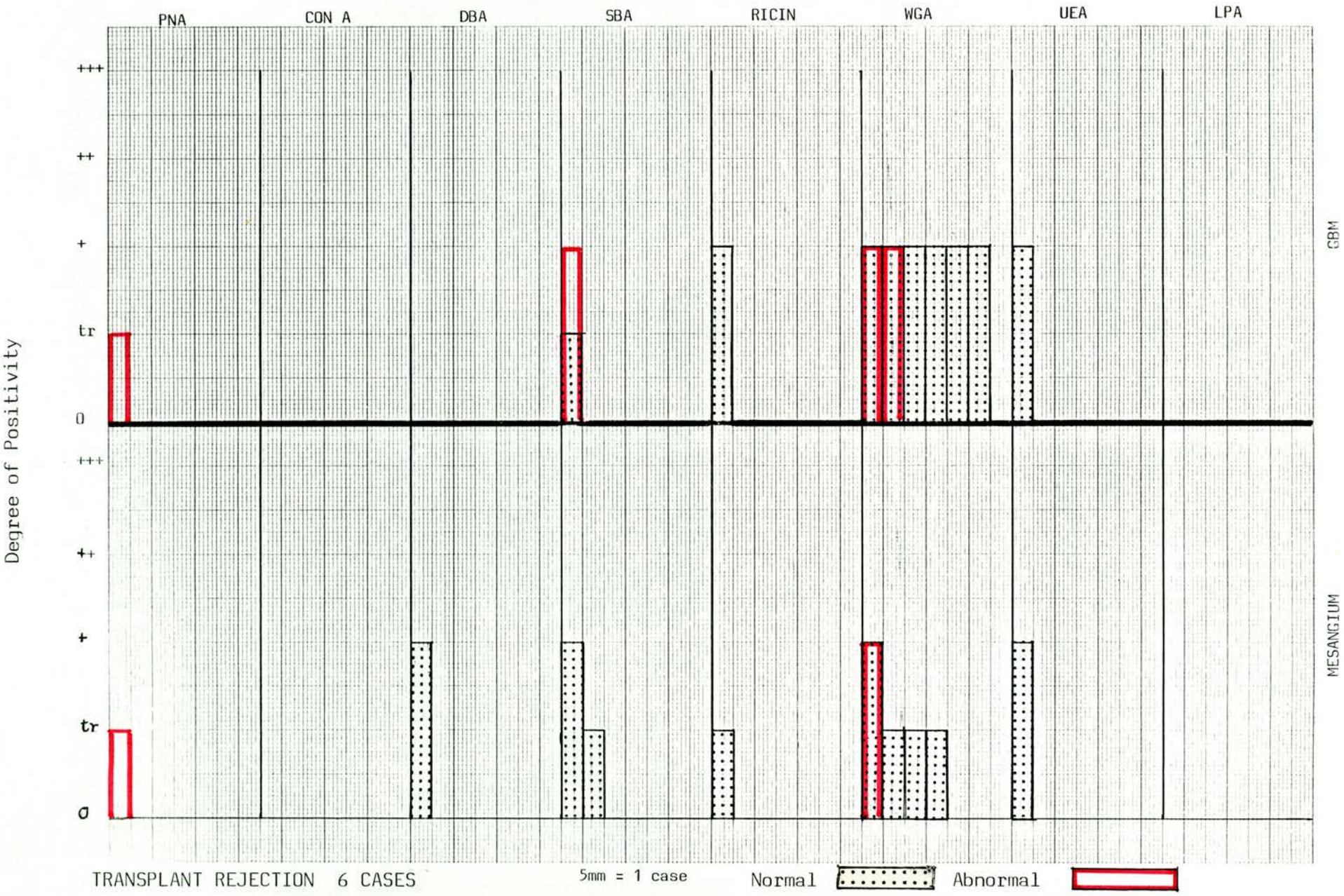

PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
+++
++
+
tr
0
Degree of Positivity
GBM
MESANGIUM
MEMBRANOUS GN 3 CASES
5mm = 1 case
Normal
Abnormal

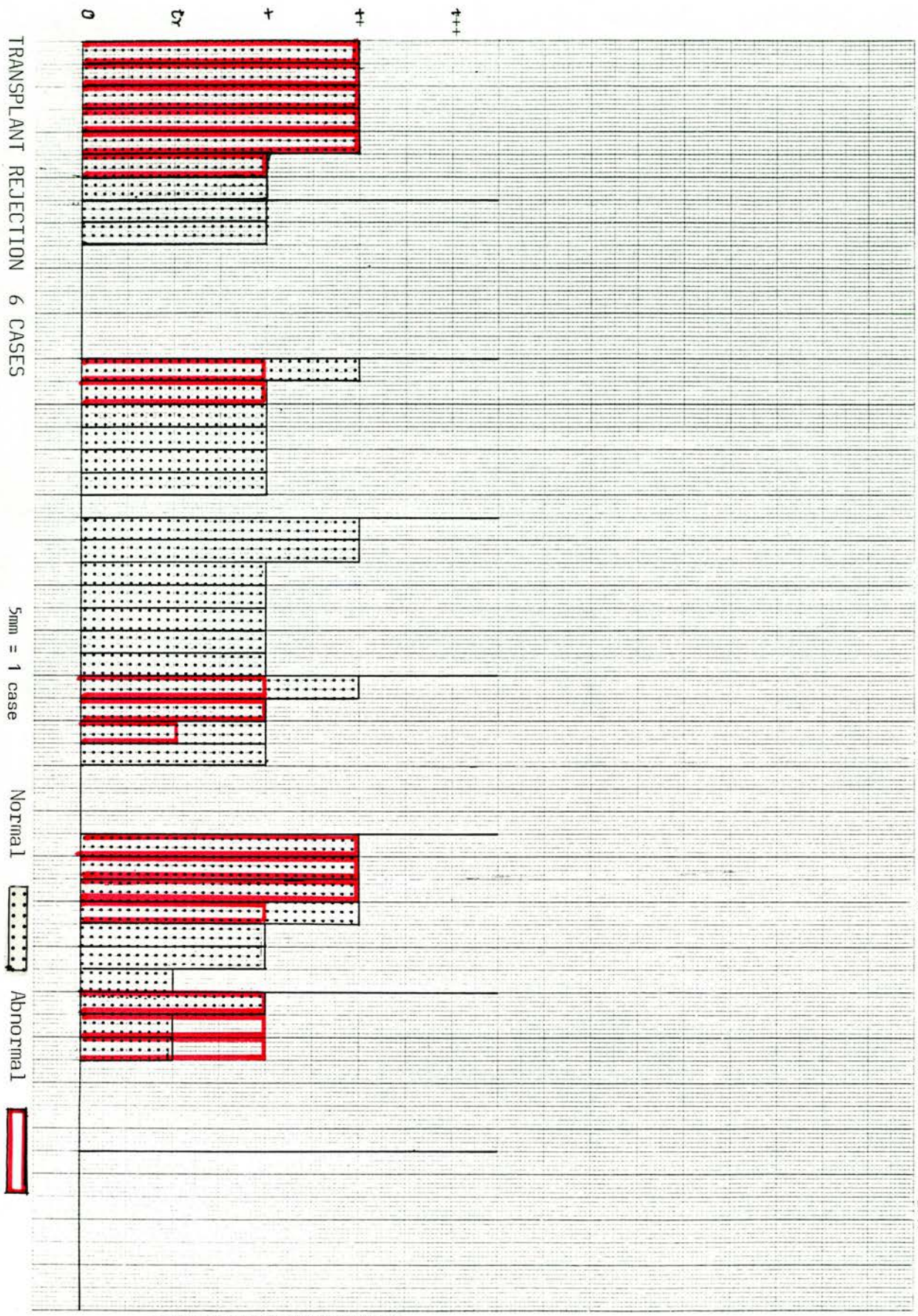

Degree of Positivity
0
tr
+
++
+++
MEMBRANOUS GN 3 CASES
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
5mm = 1 case
Normal
Abnormal
TUBULAR BASEMENT MEMBRANE

FROZEN SECTION

TRANSPLANTS - 6 CASES

Site	No of Cases Positive	Lectin	Sugar Demonstrated	Number of Cases and Degree of Positivity				
				0	tr	+	++	+++
Mesangium	1	PNA	Gal-Gal NAc > Gal		1			
	1	WGA	Glc NAc (Neu NAc)			1		
		All Others	No Reaction					
GBM	1	PNA	Ga-Gal NAc> Gal		1			
	1	SBA	Gal NAc (Gal)			1		
	2	WGA	Glc NAc (Neu NAc)			2		
		All Others	No Reaction					
TBM	5	PNA	Gal-Gal NAc > Gal			1	4	
	2	DBA	Gal NAc			2		
	3	Ricin	Gal		1	2		
	4	WGA	Glc NAc (Neu NAc)			1	3	
	3	UEA	α-L-Fuc			3		
		Con A	Man, Glc }	6				
		SBA	Gal NAc (Gal) }	6				
		LPA	Neu NAc }	6				

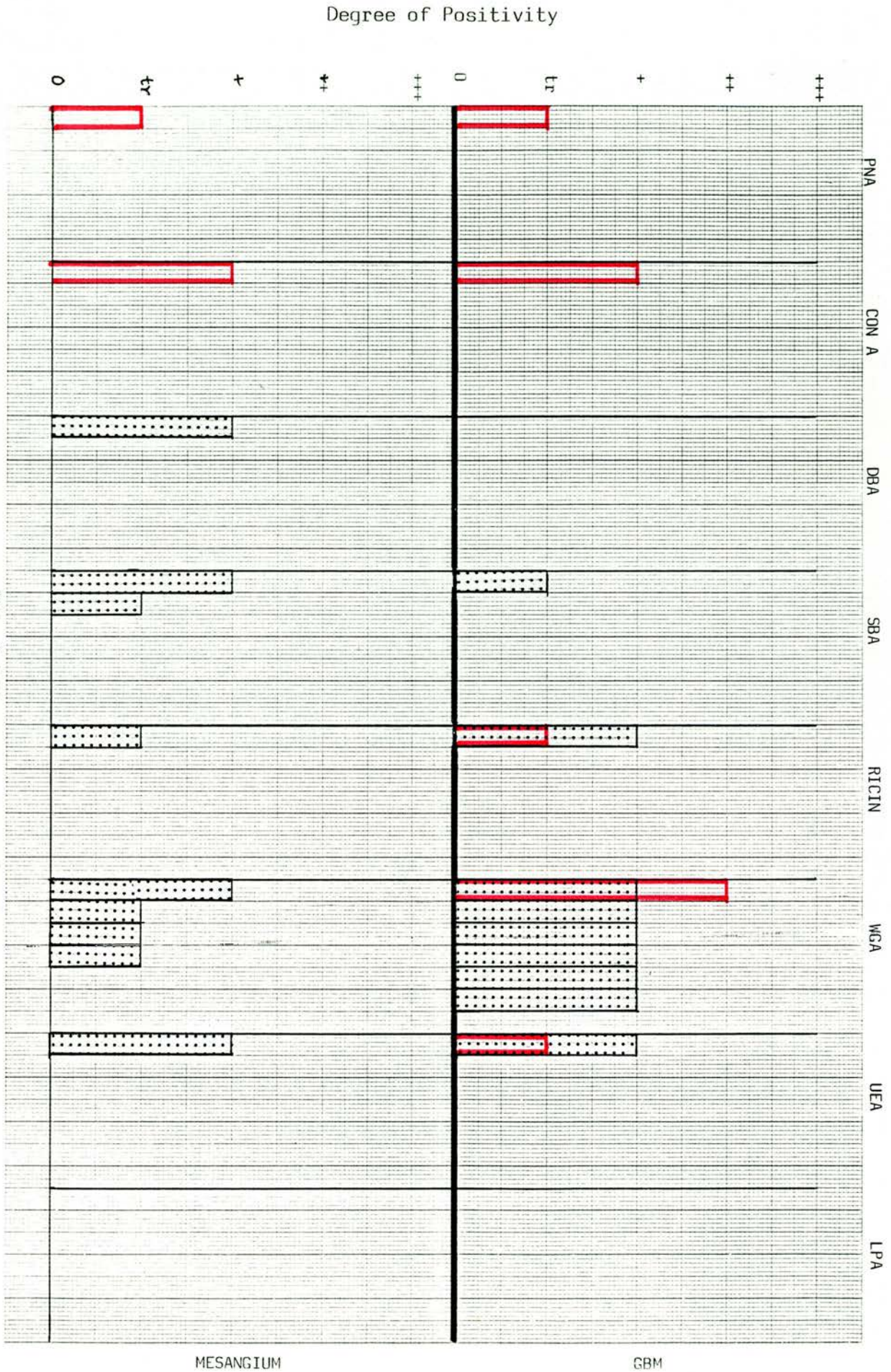

PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
+++
++
+
tr
0
+++
++
+
tr
0
GBM
MESANGIUM
Degree of Positivity
TRANSPLANT REJECTION 6 CASES
5mm = 1 case
Normal
Abnormal

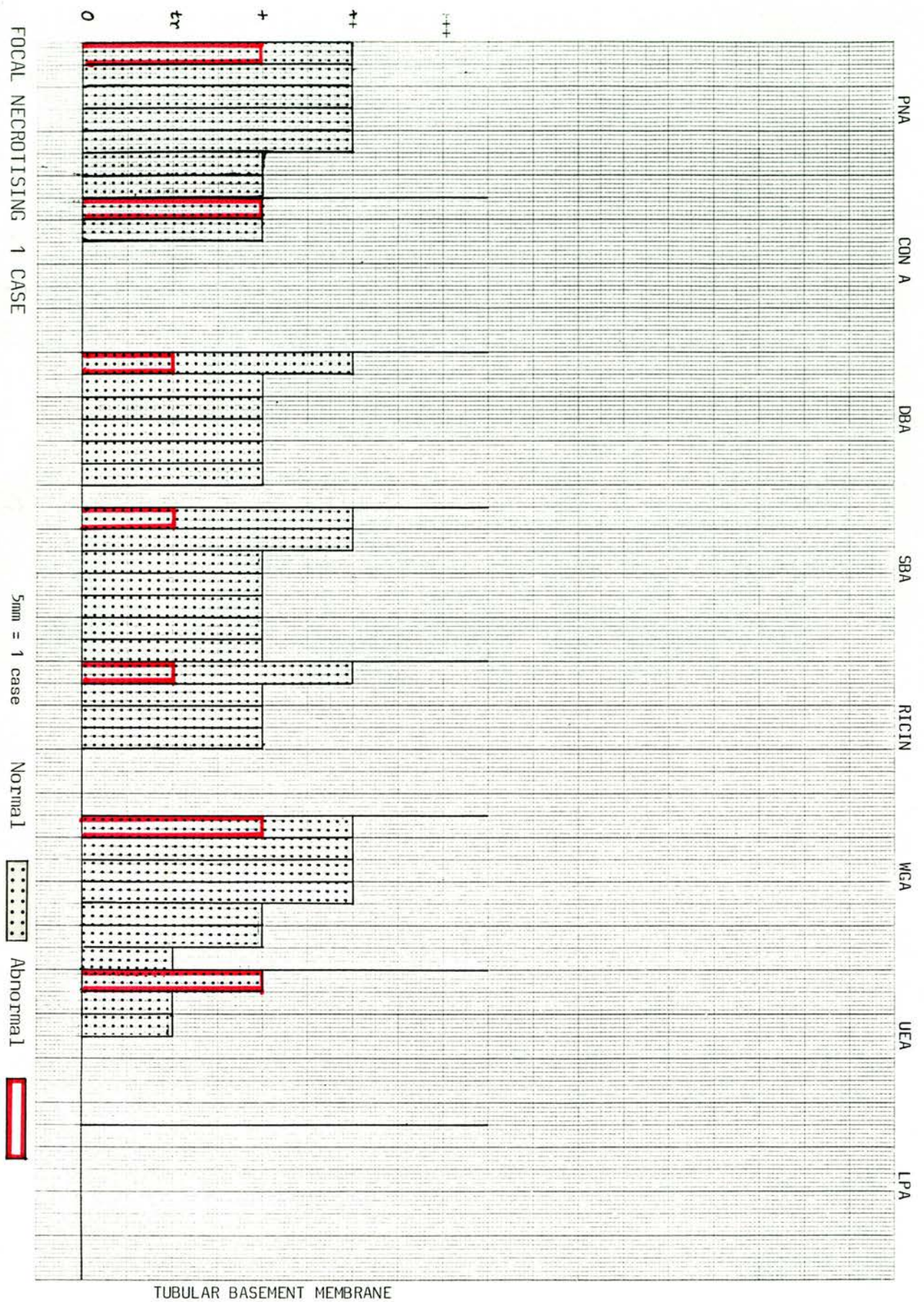

Degree of Positivity
0
±
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
TRANSPLANT REJECTION 6 CASES
5mm = 1 case
Normal
Abnormal
TUBULAR BASEMENT MEMBRANE

FROZEN SECTION

FOCAL NECROTISING GN- 1 CASE

Site	No of Cases Positive	Lectin	Sugar Demonstrated	Number of Cases and Degree of Positivity				
				0	tr	+	++	+++
Mesangium	1	PNA	Gal-Gal NAc > Gal		1			
	1	Con A	Man, Glc			1		
		All Others	No Reaction					
GBM	1	PNA	Ga-Gal NAc > Gal		1			
	1	Con A	Man, Glc			1		
	1	Ricin	Gal		1			
	1	WGA	Glc NAc (Neu NAc)				1	
	1	UEA	∝-L-Fuc		1			
		DBA	Gal NAc }					
		SBA	Gal NAc (Gal) }	1				
		LPA	Neu NAc }					
TBM	1	PNA	Ga-Gal NAc > Gal			1		
	1	Con A	Man, Glc			1		
	1	DBA	Gal NAc		1			
	1	SBA	Gal NAc (Gal)		1			
	1	Ricin	Gal		1			
	1	WGA	Glc NAc (Neu NAc)			1		
	1	UEA	∝-L-Fuc			1		
		LPA	Neu NAc	1				

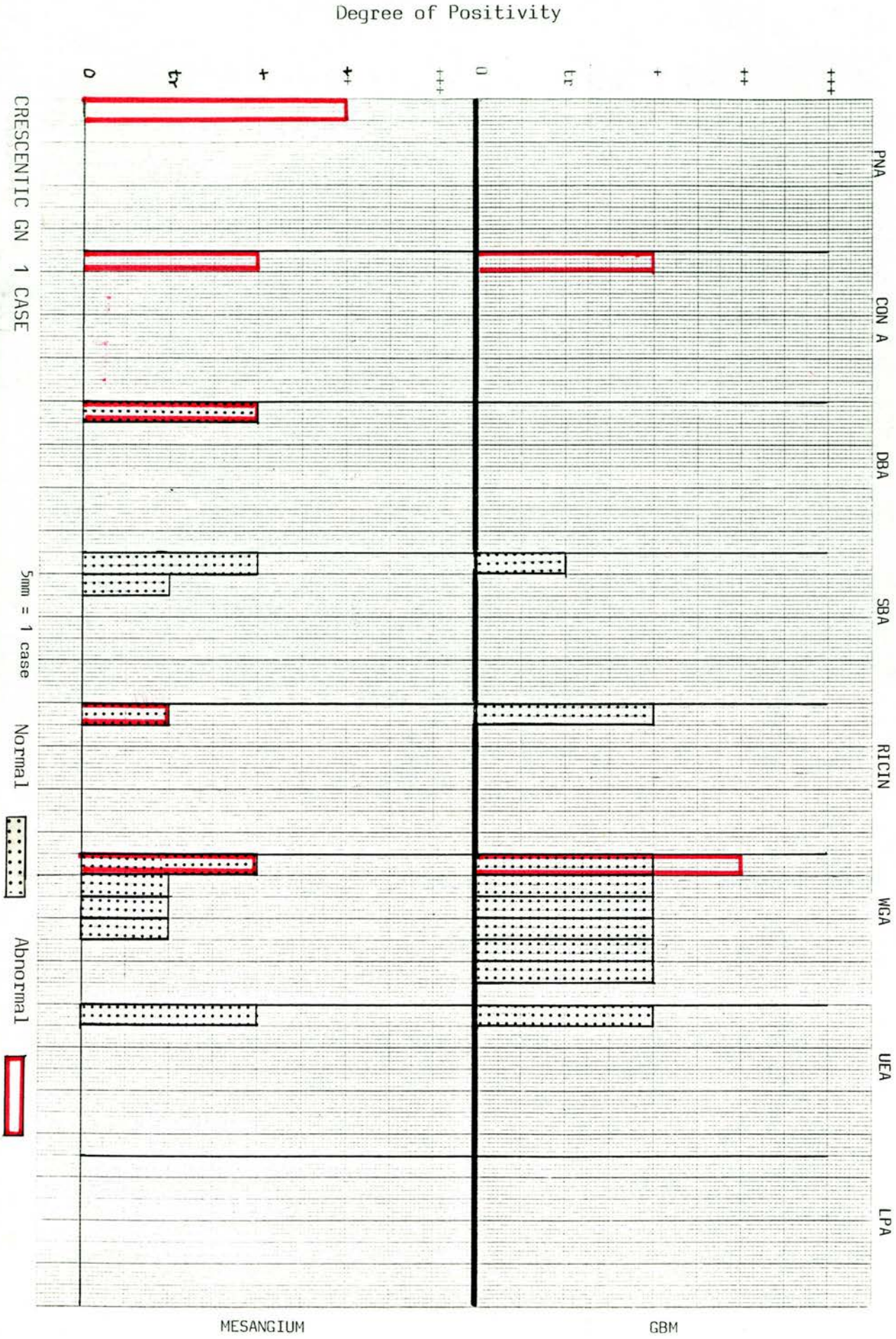

Degree of Positivity
0
tr
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
MESANGIUM
GBM
FOCAL NECROTISING GN 1 CASE
5mm = 1 case
Normal
Abnormal

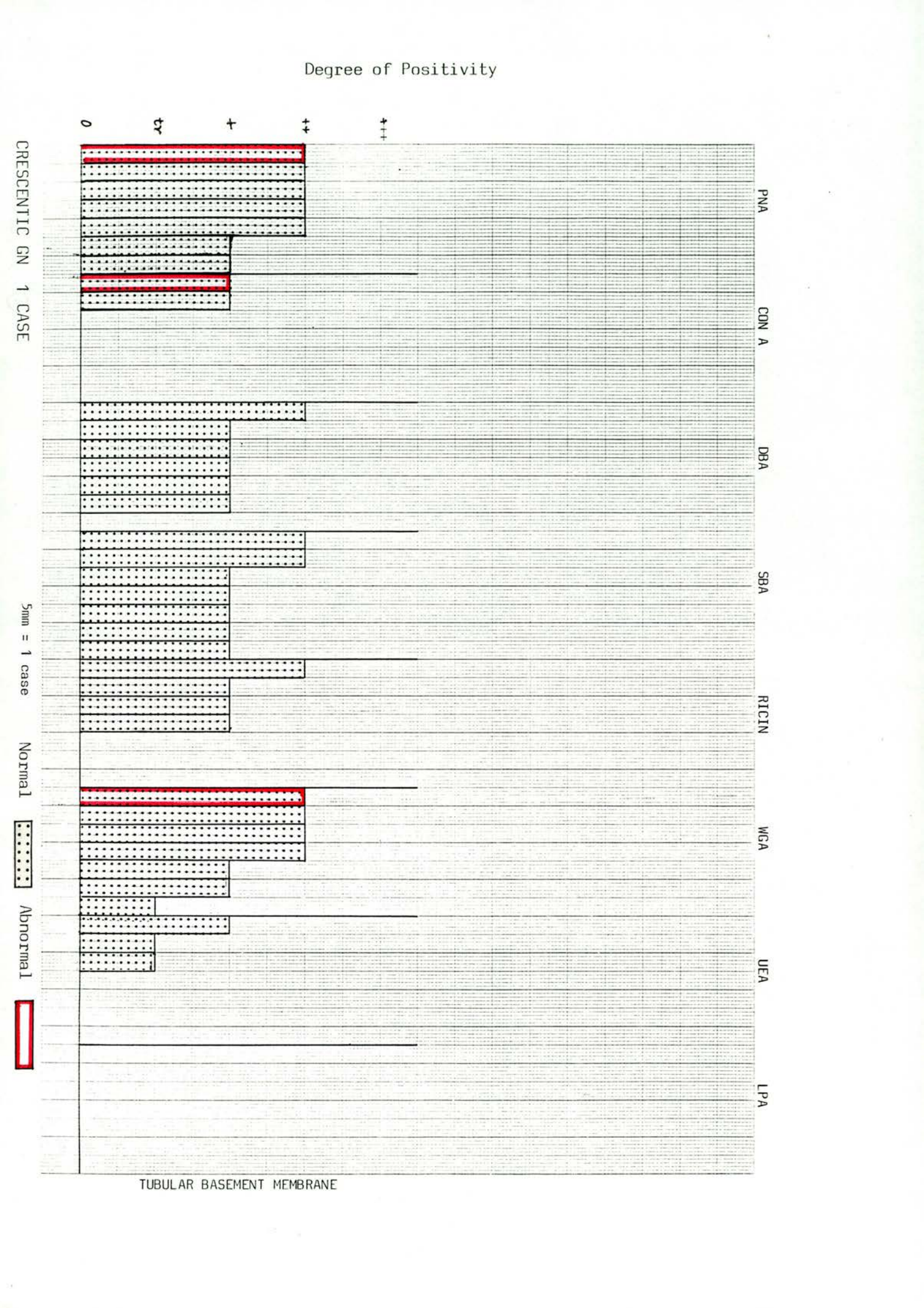

Degree of Positivity
0
tr
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
FOCAL NECROTISING 1 CASE
5mm = 1 case
Normal
Abnormal
TUBULAR BASEMENT MEMBRANE

FROZEN SECTION

CRESCENTIC GN - 1 CASE

Site	No of Cases Positive	Lectin	Sugar Demonstrated	Number of Cases and Degree of Positivity				
				0	tr	+	++	+++
Mesangium	1	PNA	Gal-Gal NAc > Gal				1	
	1	Con A	Man, Glc			1		
	1	DBA	Gal NAc			1		
	1	Ricin	Gal		1			
	1	WGA	Glc NAc (Neu NAc)			1		
		SBA	Gal NAc (Gal) }					
		UEA	α-L-Fuc }	1				
		LPA	Neu NAc }					
GBM	1	Con A	Man, Glc			1		
	1	WGA	Glc NAc (Neu NAc)				1	
		All Others	No Reaction					
TBM	1	PNA	Gal-Gal NAc > Gal				1	
	1	Con A	Man, Glc			1		
	1	WGA	Glc NAc (Neu NAc)				1	
		All Others	No Reaction					

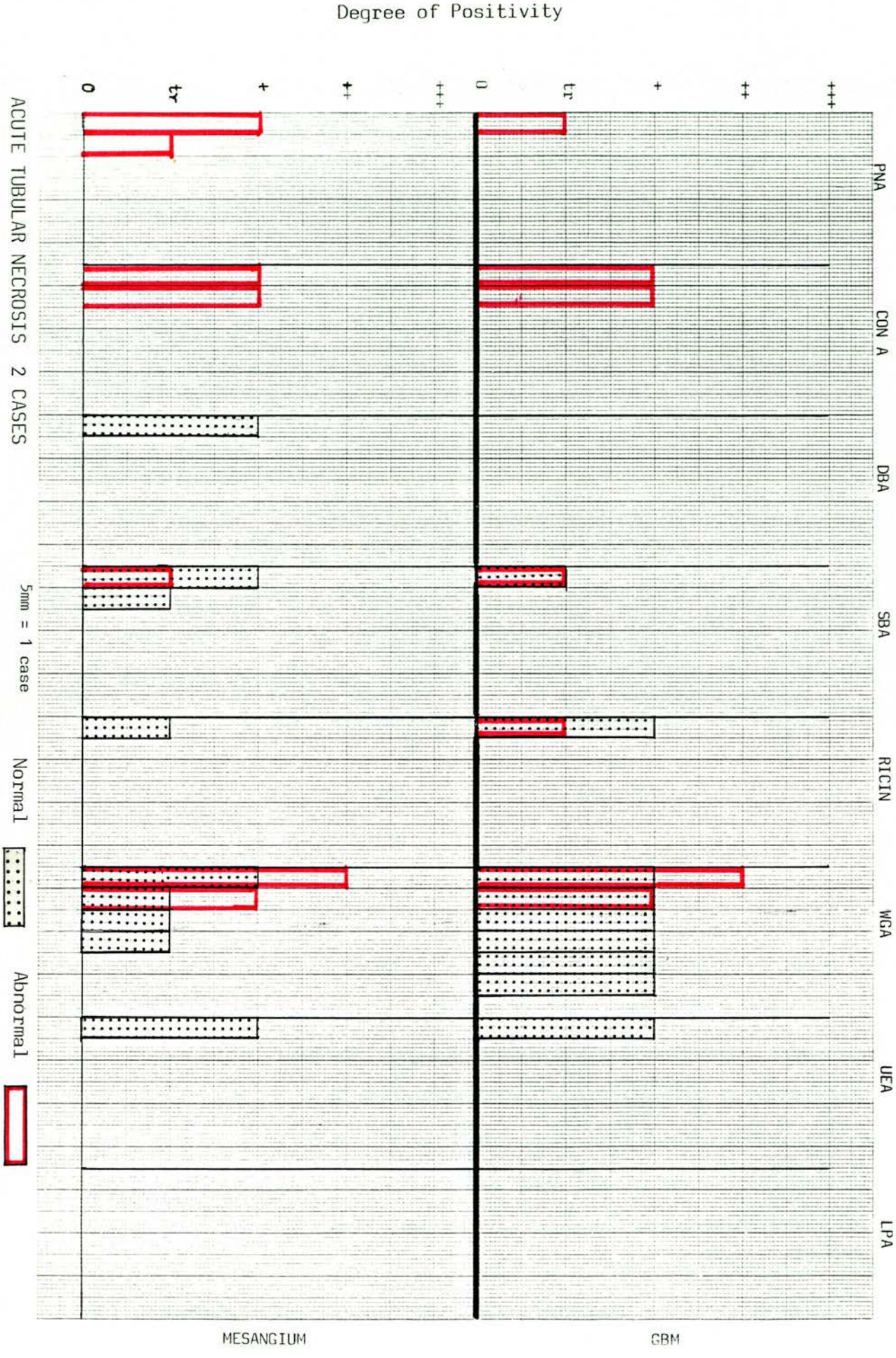

Degree of Positivity
0
tr
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
MESANGIUM
GBM
CRESCENTIC GN 1 CASE
5mm = 1 case
Normal
Abnormal

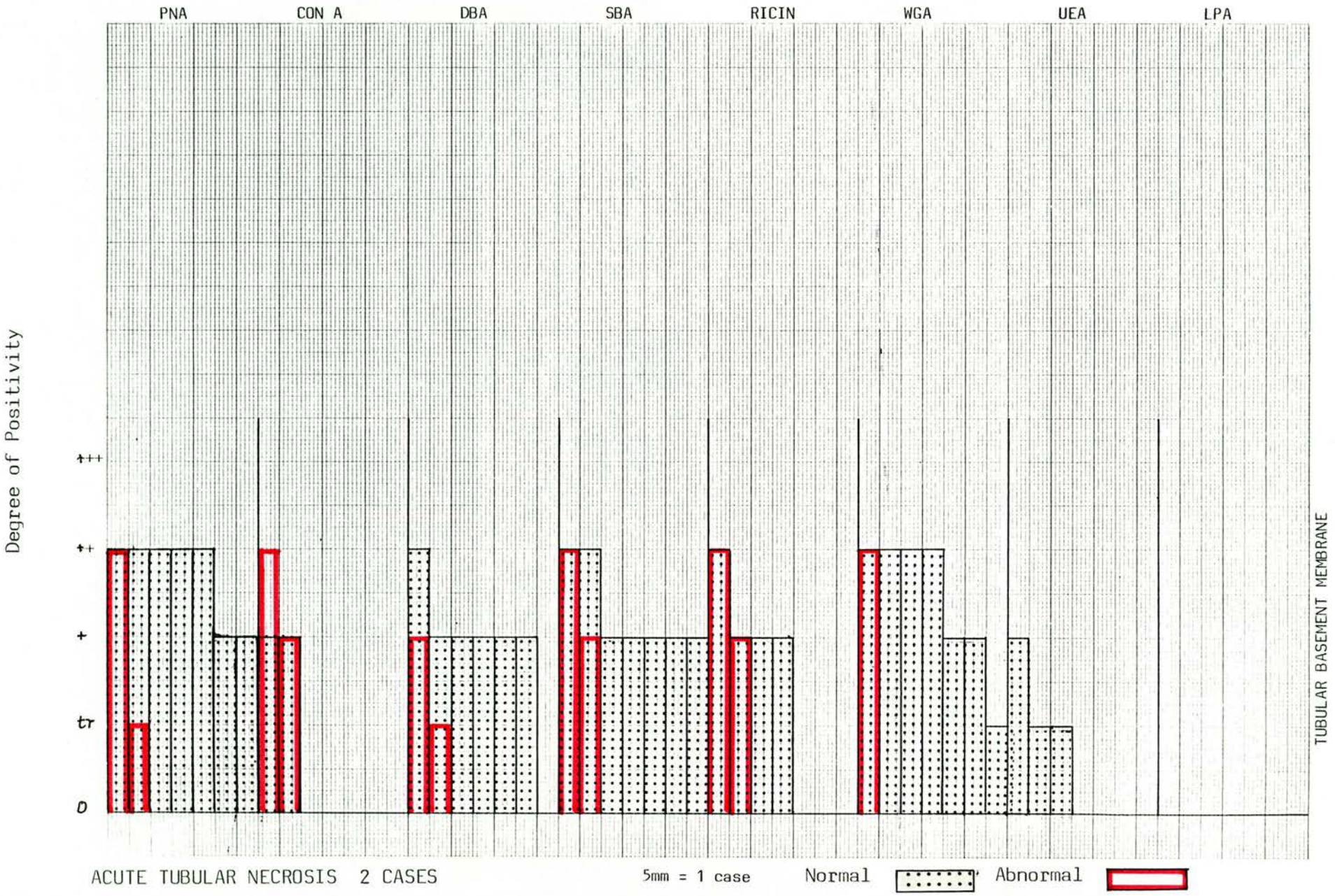

Degree of Positivity
0
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
CRESCENTIC GN 1 CASE
5mm = 1 case
Normal
Abnormal
TUBULAR BASEMENT MEMBRANE

FROZEN SECTION

ACUTE TUBULAR NECROSIS - 2 CASES

Site	No of Cases Positive	Lectin	Sugar Demonstrated	Number of Cases and Degree of Positivity				
				0	tr	+	++	+++
Mesangium	2	PNA	Gal-Gal NAc > Gal		1	1		
	2	Con A	Man, Glc			2		
	1	SBA	Gal NAc (Gal)		1			
	1	Ricin	Gal		1			
	2	WGA	Glc NAc (Neu NAc)			1	1	
		DBA	Gal NAc }					
		UEA	∝-L-Fuc }	2				
		LPA	Neu NAc }					
GBM	1	PNA	Gal-Gal NAc > Gal		1			
	2	Con A	Man, Glc			2		
	1	SBA	Gal NAc (Gal)		1			
	1	Ricin	Gal		1			
	2	WGA	Glc NAc (Neu NAc)			1	1	
		DBA	Gal NAc }					
		UEA	∝-L-Fuc }	2				
		LPA	Neu NAc }					
TBM	2	PNA	Ga-Gal NAc > Gal		1		1	
	2	Con A	Man, Glc			1	1	
	2	DBA	Gal NAc		1	1		
	2	SBA	Gal NAc (Neu NAc)			1	1	
	2	Ricin	Gal			1	1	
	1	WGA	Glc NAc (Neu NAc)				1	
		UEA	∝-L-Fuc }	2				
		LPA	Neu NAc }					

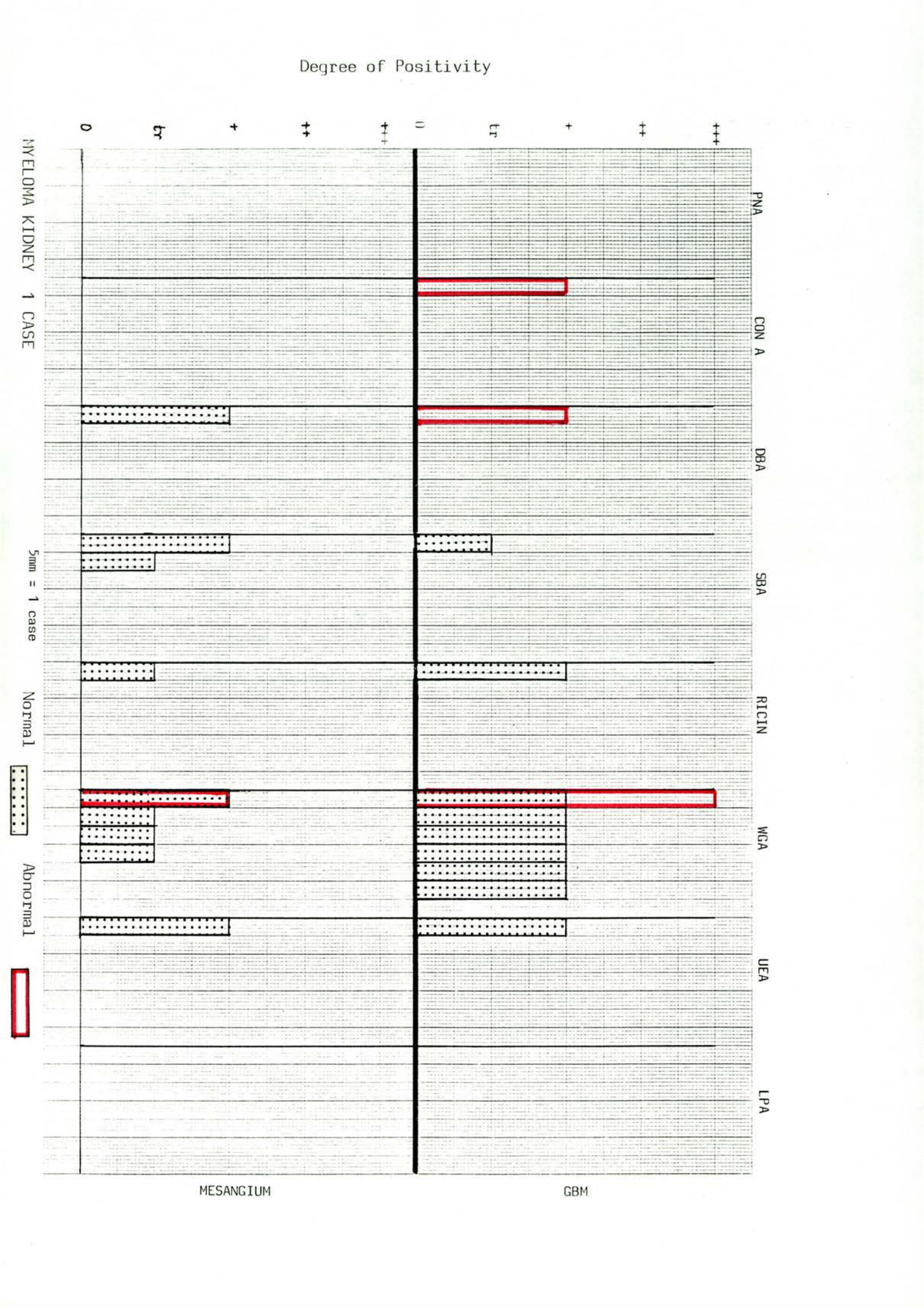

Degree of Positivity
0
tr
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
MESANGIUM
GBM
ACUTE TUBULAR NECROSIS 2 CASES
5mm = 1 case
Normal
Abnormal

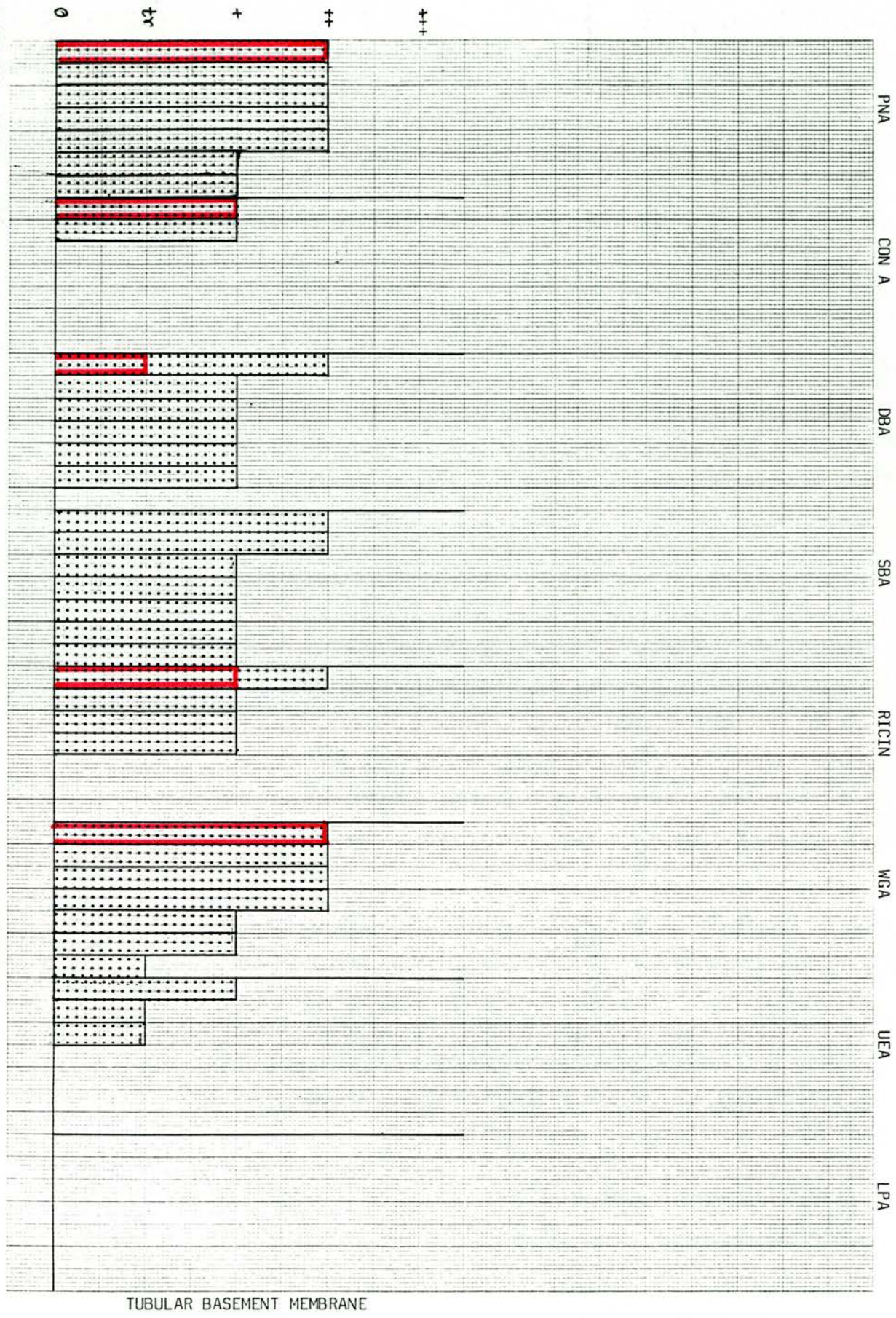

PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
Degree of Positivity
+++
++
+
tr
0
TUBULAR BASEMENT MEMBRANE
ACUTE TUBULAR NECROSIS 2 CASES
5mm = 1 case
Normal
Abnormal

FROZEN SECTION

MYELOMA - 1 CASE

Site	No of Cases Positive	Lectin	Sugar Demonstrated	Number of Cases and Degree of Positivity				
				0	tr	+	++	+++
Mesangium	1	WGA	Glc NAc (Neu NAc)			1		
		All Others	No Reaction					
GBM	1	Con A	Man, Glc			1		
	1	DBA	Gal NAc			1		
	1	WGA	Glc NAc (Neu NAc)					1
		All Others	No Reaction					
TBM	1	PNA	Gal-Gal NAc > Gal				1	
	1	Con A	Man, Glc			1		
	1	DBA	Gal NAc		1			
	1	Ricin	Gal			1		
	1	WGA	Glc NAc (Neu NAc)				1	
		SBA	Gal NAc (Gal) }					
		UEA	α-L-Fuc }	1				
		LPA	Neu NAc }					

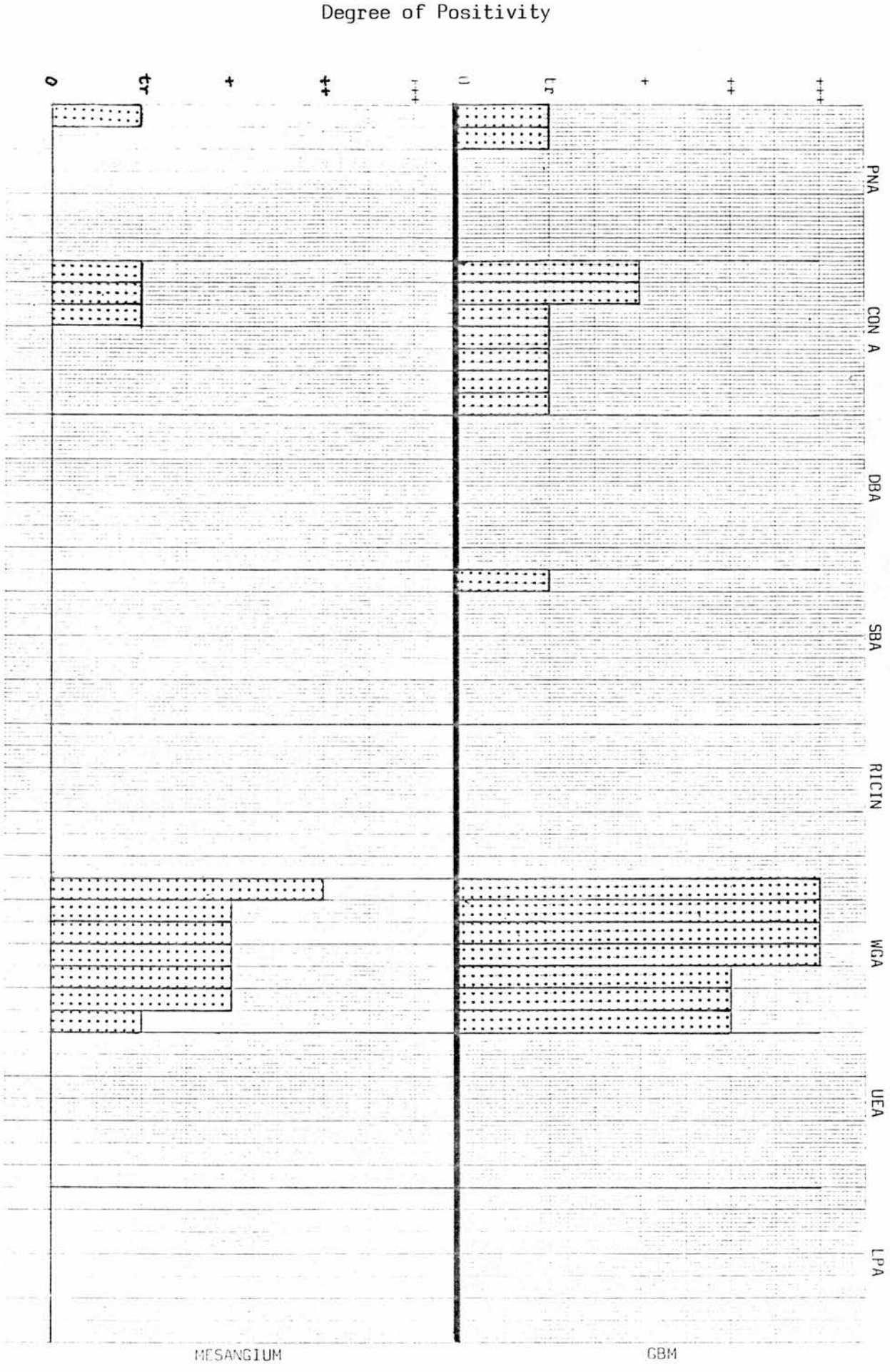

Degree of Positivity
0
tr
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
MESANGIUM
GBM
MYELOMA KIDNEY 1 CASE
5mm = 1 case
Normal
Abnormal

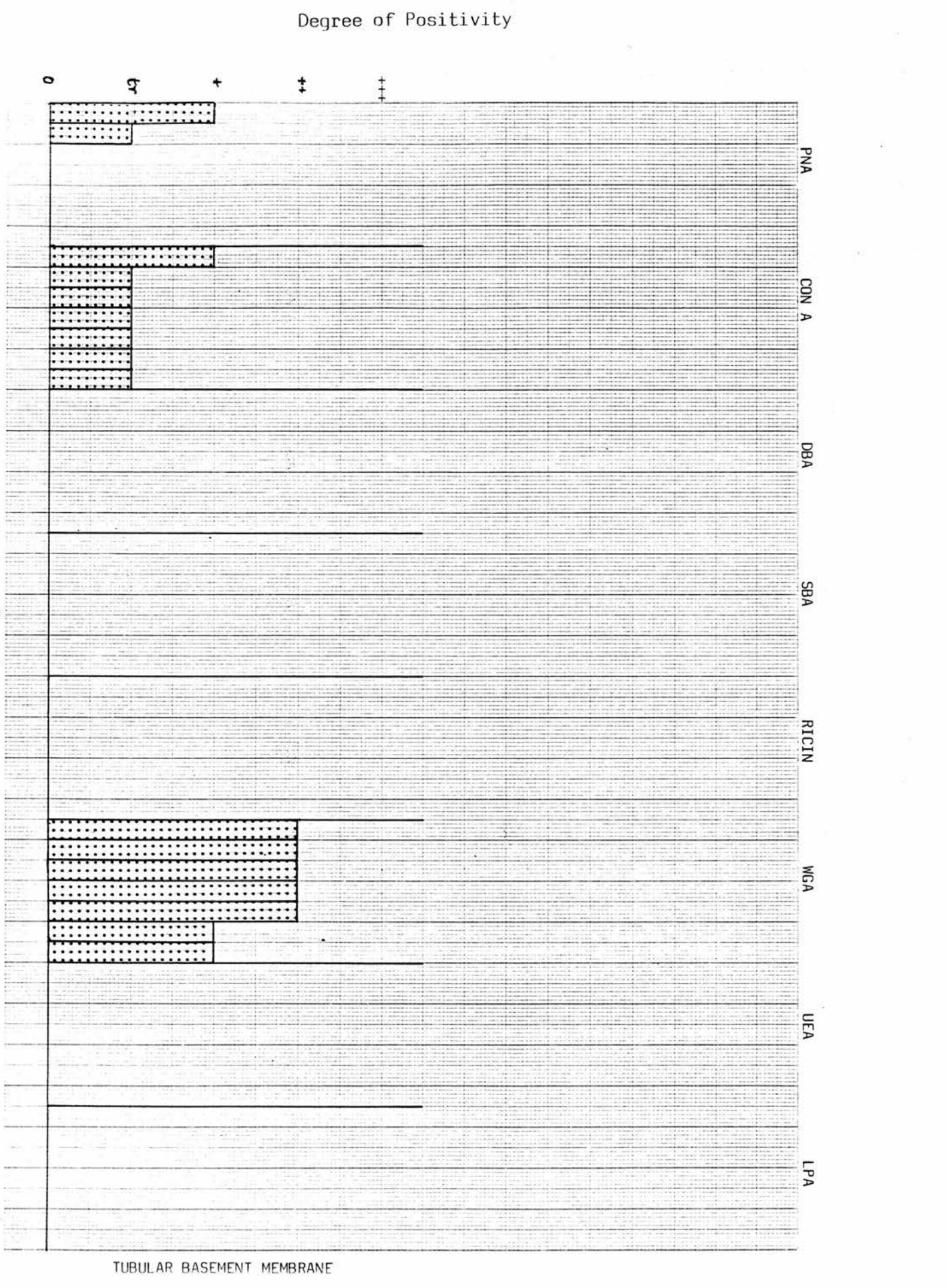

Degree of Positivity
0
tr
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
MYELOMA KIDNEY 1 CASE
5mm = 1 case
Normal
Abnormal
TUBULAR BASEMENT MEMBRANE

PARAFFIN-EMBEDDED TISSUE

NORMAL - 7 CASES

Site	No of Cases Positive	Lectin	Sugar Demonstrated	Number of Cases and Degree of Positivity				
				0	tr	+	++	+++
Mesangium	1	PNA	Gal-Gal NAc > Gal		1			
	3	Con A	Man, Glc		3			
	7	WGA	Glc NAc (Neu NAc)		1	5	1	
		All Others	No Reaction					
GBM	2	PNA	Gal-Gal NAc > Gal		2			
	7	Con A	Man, Glc		5	2		
	1	SBA	Gal NAc (Gal)		1			
	7	WGA	Glc NAc (Neu NAc)				3	4
		All Others	No Reaction					
TBM	2	PNA	Gal-Gal NAc > Gal		1	1		
	7	Con A	Man, Glc		6	1		
	7	WGA	Glc NAc (Neu NAc)			2	5	
		All Others	No Reaction					

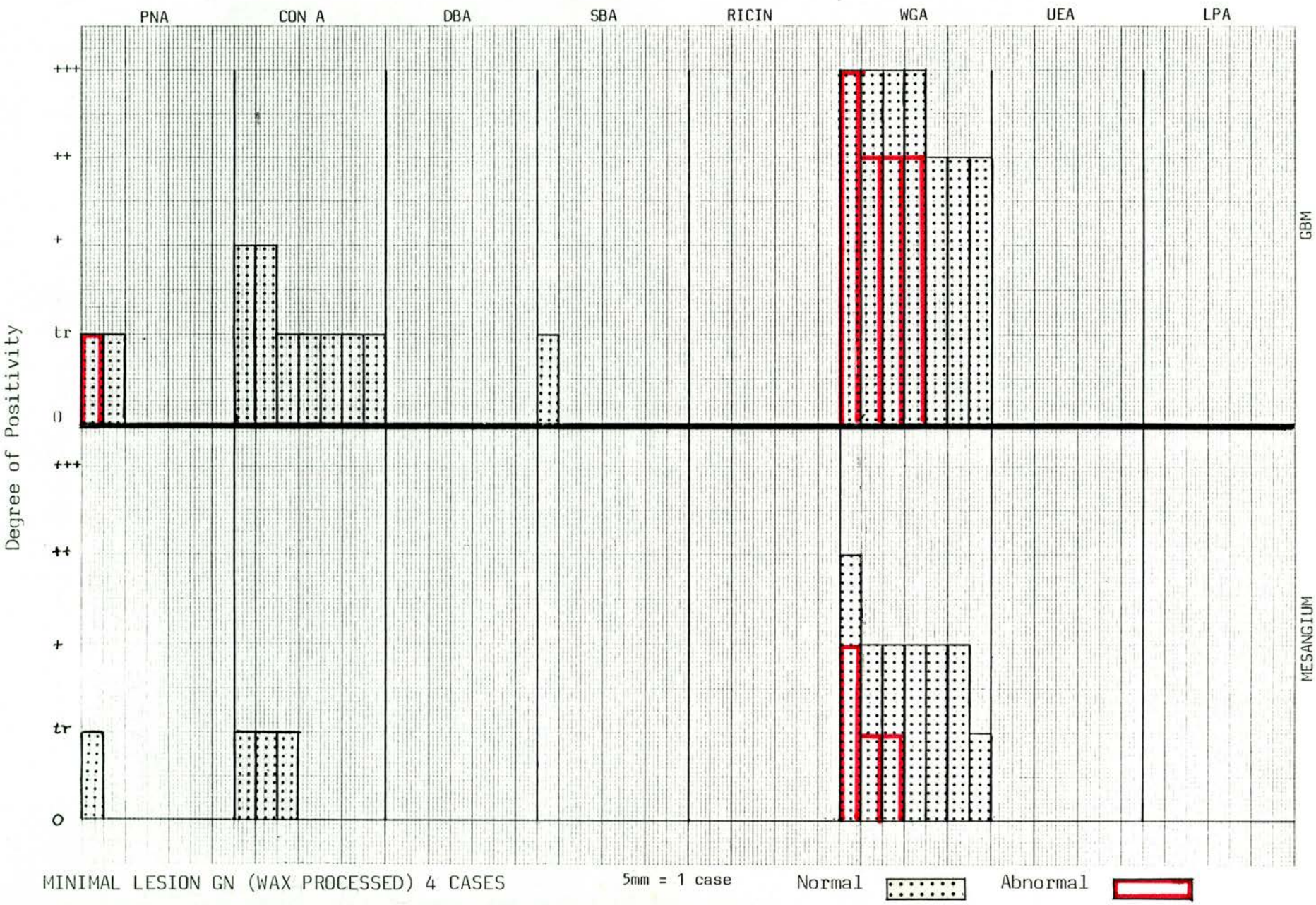

Degree of Positivity
NORMAL KIDNEY (WAX PROCESSED) 7 CASES
5mm = 1 case
Normal
Abnormal
0
tr
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
MESANGIUM
GBM

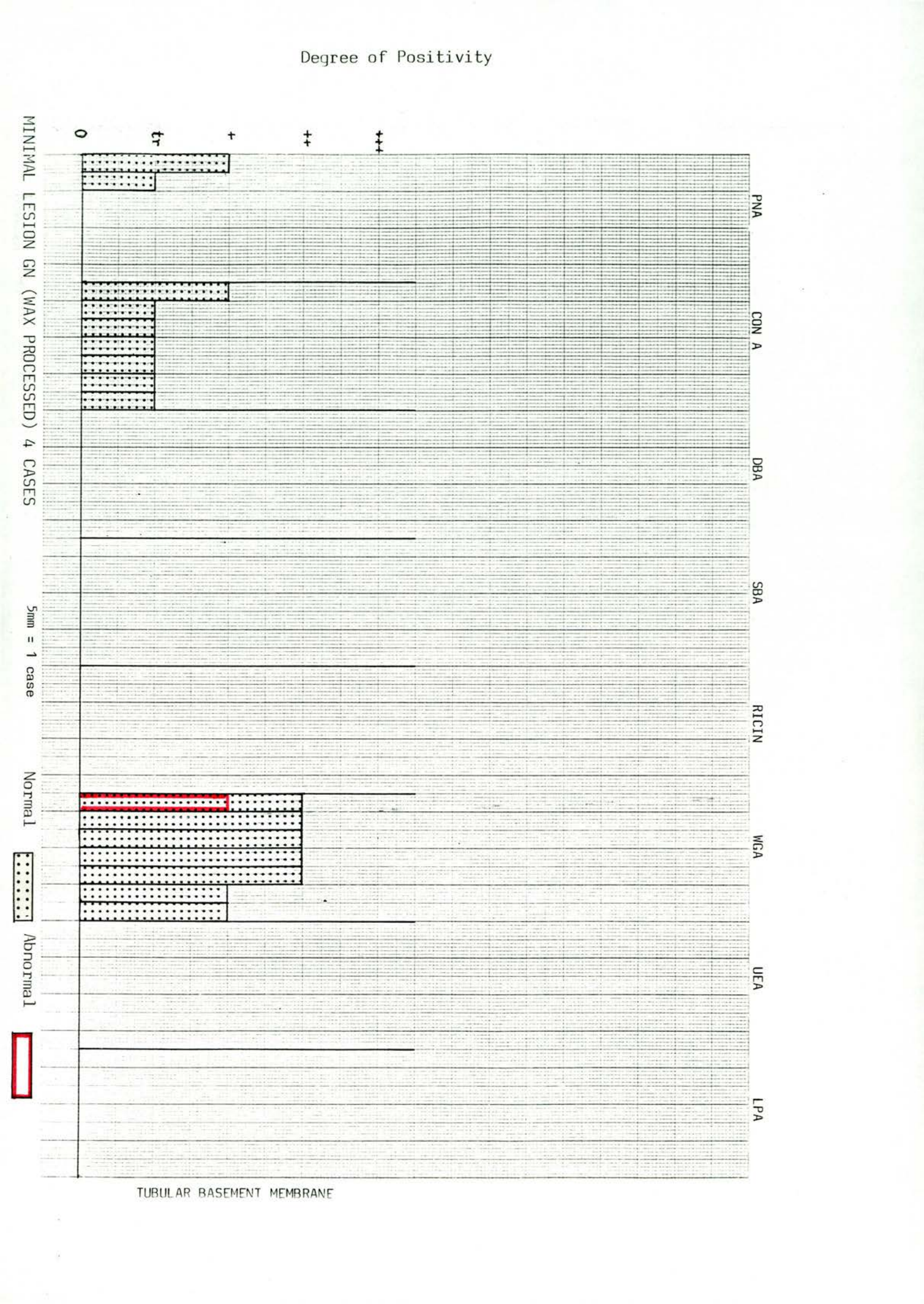

Degree of Positivity
0
tr
+
++
+++
NORMAL KIDNEY (WAX PROCESSED) 7 CASES
5mm = 1 case
Normal
Abnormal
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
TUBULAR BASEMENT MEMBRANE

PARAFFIN-EMBEDDED TISSUE

MINIMAL LESION GN - 4 CASES

Site	No of Cases Positive	Lectin	Sugar Demonstrated	Number of Cases and Degree of Positivity				
				0	tr	+	++	+++
Mesangium	3	WGA	Glc NAc (Neu NAc)		2	1		
		All Others	No Reaction					
GBM	1	PNA	Gal-Gal NAc > Gal		1			
	4	WGA	Glc NAc (Neu NAc)				3	1
		All Others	No Reaction					
TBM	1	WGA	Glc NAc (Neu NAc)			1		
		All Others	No Reaction					

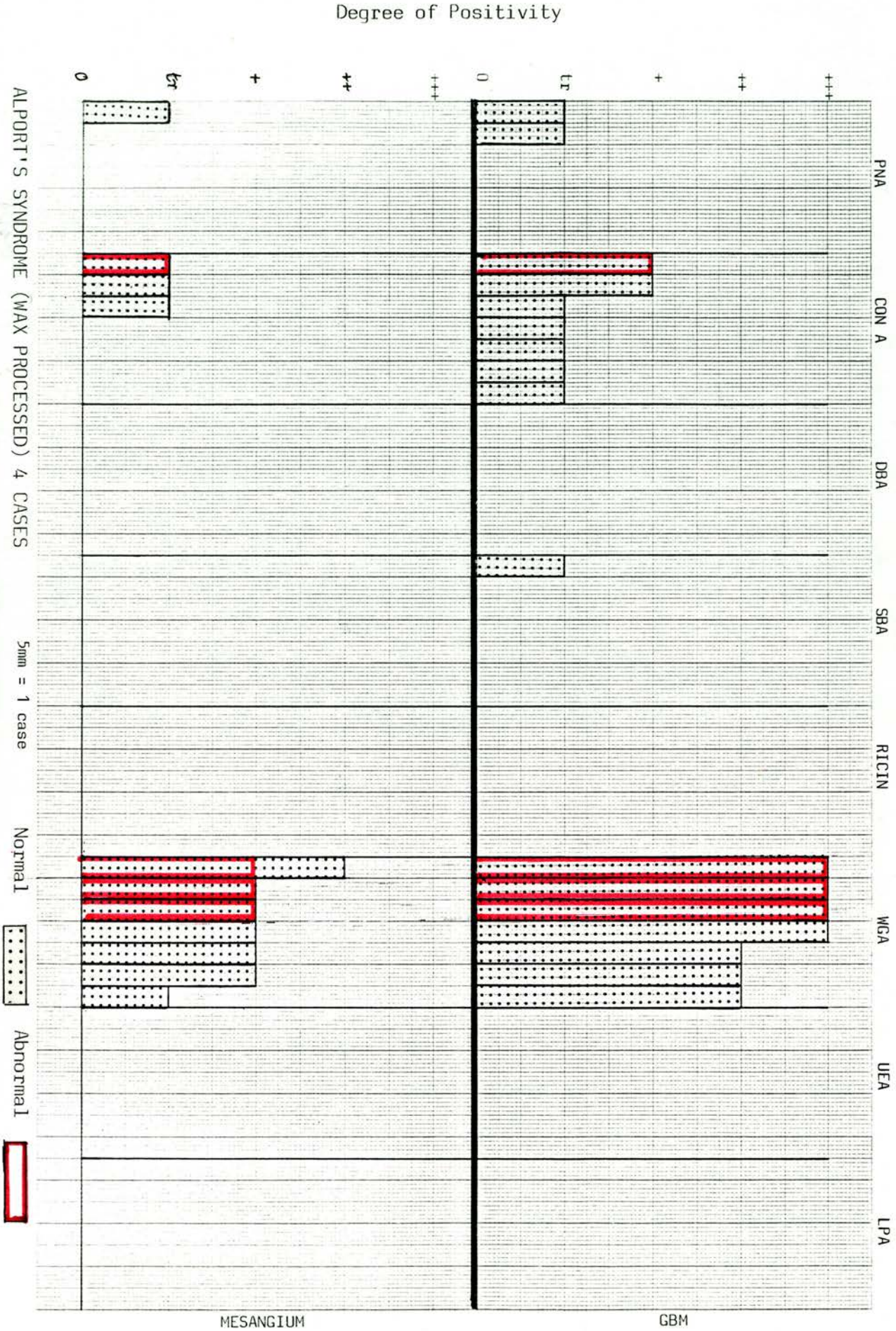

PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
+++
++
+
tr
0
Degree of Positivity
GBM
MESANGIUM
MINIMAL LESION GN (WAX PROCESSED) 4 CASES
5mm = 1 case
Normal
Abnormal

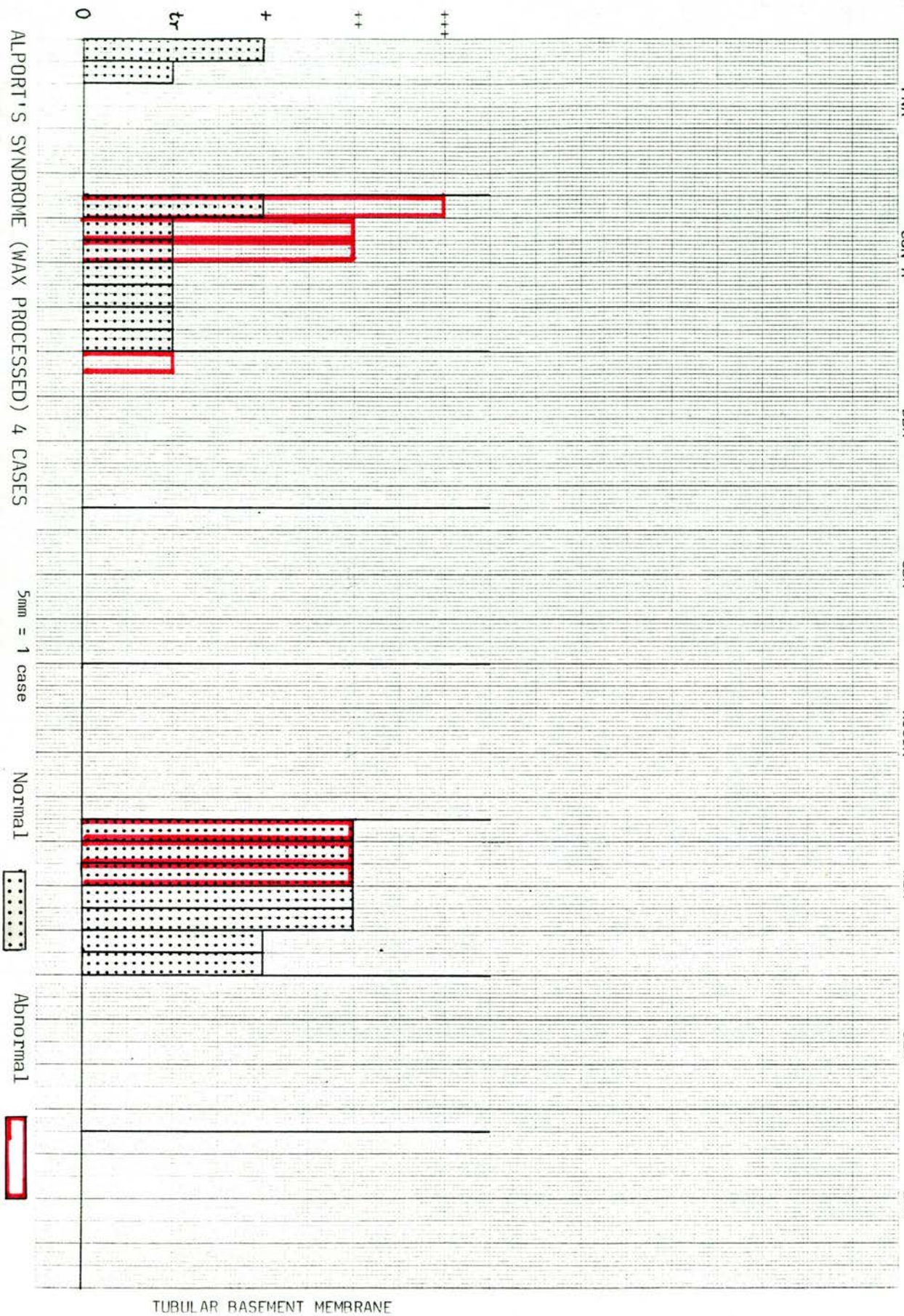

Degree of Positivity
0
tr
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
MINIMAL LESION GN (WAX PROCESSED) 4 CASES
5mm = 1 case
Normal
Abnormal
TUBULAR BASEMENT MEMBRANE

PARAFFIN-EMBEDDED TISSUE

ALPORT'S SYNDROME - 4 CASES

Site	No of Cases Positive	Lectin	Sugar Demonstrated	Number of Cases and Degree of Positivity				
				0	tr	+	++	+++
Mesangium	1	Con A	Man, Glc		1			
	3	WGA	Glc NAc (Neu NAc)			3		
		All Others	No Reaction					
GBM	1	Con A	Man, Glc			1		
	3	WGA	Glc NAc (Neu NAc)					3
		All Others	No Reaction					
TBM	3	Con A	Man, Glc				2	1
	1	DBA	Gal NAc		1			
	4	WGA	Glc NAc (Neu NAc)				4	
		All Others	No Reaction					


Degree of Positivity
0
tr
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
MESANGIUM
GBM
ALPORT'S SYNDROME (WAX PROCESSED) 4 CASES
5mm = 1 case
Normal
Abnormal


Degree of Positivity
0
tr
+
++
+++
PNA
CON A
DBA
SBA
RICIN
WGA
UEA
LPA
ALPORT'S SYNDROME (WAX PROCESSED) 4 CASES
5mm = 1 case
Normal
Abnormal
TUBULAR BASEMENT MEMBRANE

DISCUSSION

The results of the lectin binding in these two groups are given in Table 7, and the significant results summarised in Table 8.

There is an obvious decrease in wheat germ agglutinin in mesangium and in Concanavalin A activity in the GBM as compared with normal tissue.

In tubular basement membranes, both lectins showed less binding. The carbohydrate moieties showing apparent loss are α-D-glucose and α-L-mannose in the case of Concanavalin A, and N-acetyl β-(1.4)D-glucosamine (sialic acid) in the case of wheat germ agglutinin.

The only lectin showing a change of statistical significance was Concanavalin A which binds to α-D-glucose and α-L-mannose. This was found to be increased only in the tubular basement membranes as compared to the normal.

Ultrastructural Data

The renal biopsy cases used in this work were examined by the electron microscope, where glomeruli were present in the resin-embedded material. While no new findings of significance were extracted from this study, a few electron micrographs are appended (Figs 15,16,17,18a,18b,19,20 and 21) to show the structural appearances of the glomeruli and tubules in the disease processes which were investigated by the lectin technique.

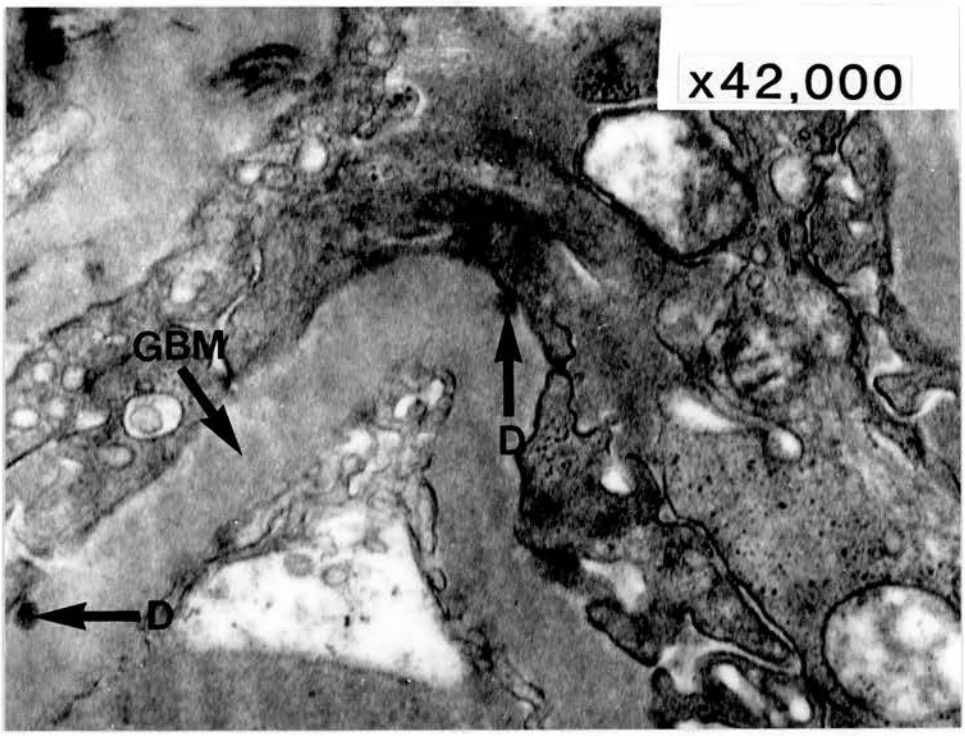


Fig 16 Profliferative Glomerulonephritis

This is a higher power photograph of a glomerular capillary (cut rather tangentially) in a case of proliferative GN. The GBM looks rather thick because of the tangential cutting but otherwise shows no structural abnormality. Two small rounded deposits, presumably of immunological material, are seen in the outer part of the GBM, and have presumably passed through from the endothelial aspect. Pedicels are very broad based, and their normal structure almost completely lost.

x42,000

GBM - glomerular basement membrane
D - deposits

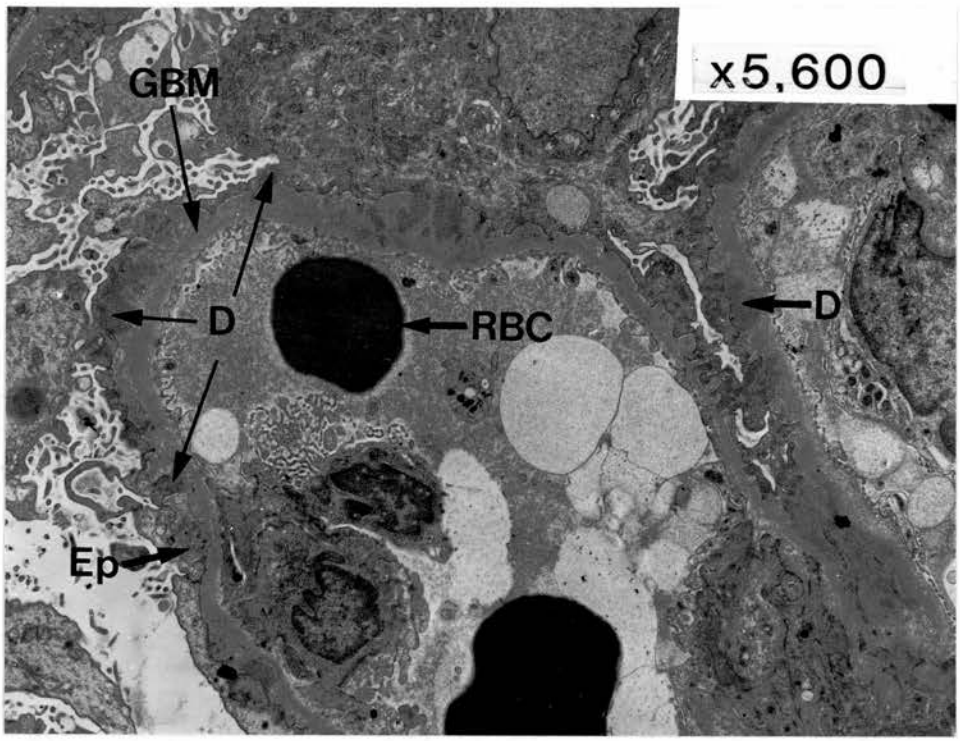


Fig 17 Electron Micrograph of Membranous Glomerulonephritis

This micrograph shows the characteristic electron dense small deposits of immunological material on the subepithelial aspect of the GBM. Some of these are indicated by arrows. It is not known whether the antigen in these cases of membranous GN is circulating exogenous, such as a virus or bacterium, or whether it is a fixed antigen, either planted from the circulation or part of the membrane itself.

x5,600

D - deposit
GBM - glomerular basement membrane
Ep - epithelial cell
RBC - red blood cell

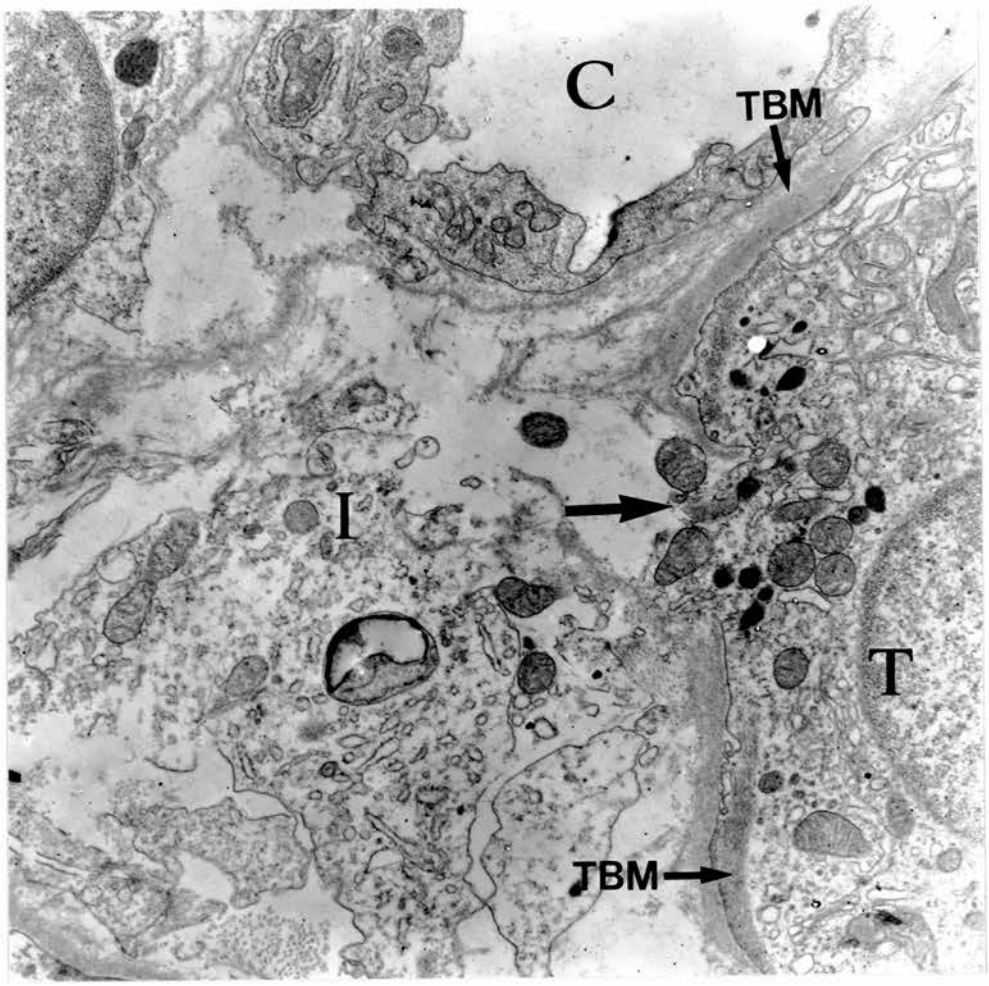


Fig 18 Electron Micrograph of Acute Tubular Necrosis

This micrograph shows, (arrow), a discontinuity in the basement membrane of a renal tubular, through which the mitochondria and other cell organelles of a degenerate tubular cell are passing into the interstitial space. This picture is not actually from one of the cases included in this study because such foci of tubulorrhexis are sparse and are very difficult to find on electron microscopy. The cause of the break in the basement membrane in such cases is probably, usually ischaemic damage to the cell itself

x16,000

T - tubule
C - capillary
I - interstitial space
TBM - tubular basement membrane

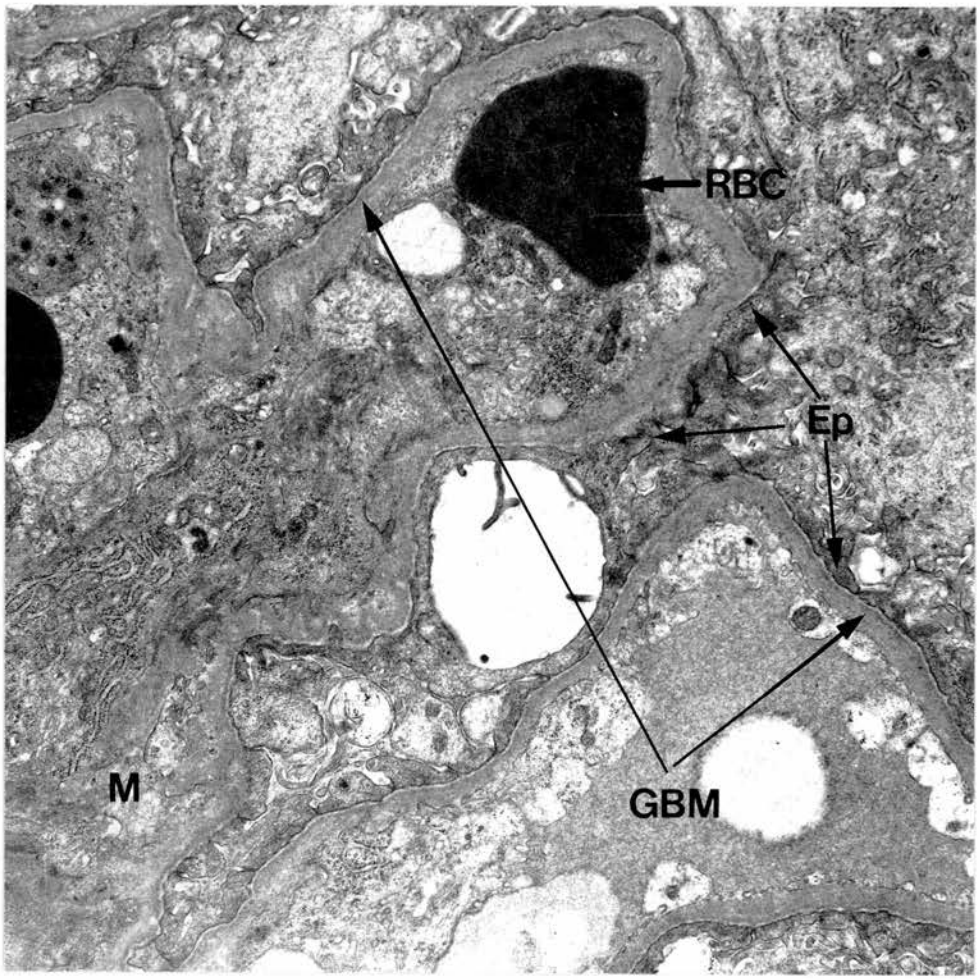


Fig 19 Electron micrograh of minimal lesion glomerulonephritis

This micrograph shows very marked loss of epithelial pedicel structure and perhaps a little swelling of the endothelium. The GBM appears virtually normal, only a small portion of mesangium is seen and appears normal.

x10,000

E - epithelial cell
GBM - glomerular basement membrane
RBC - red blood cell
M - mesangium

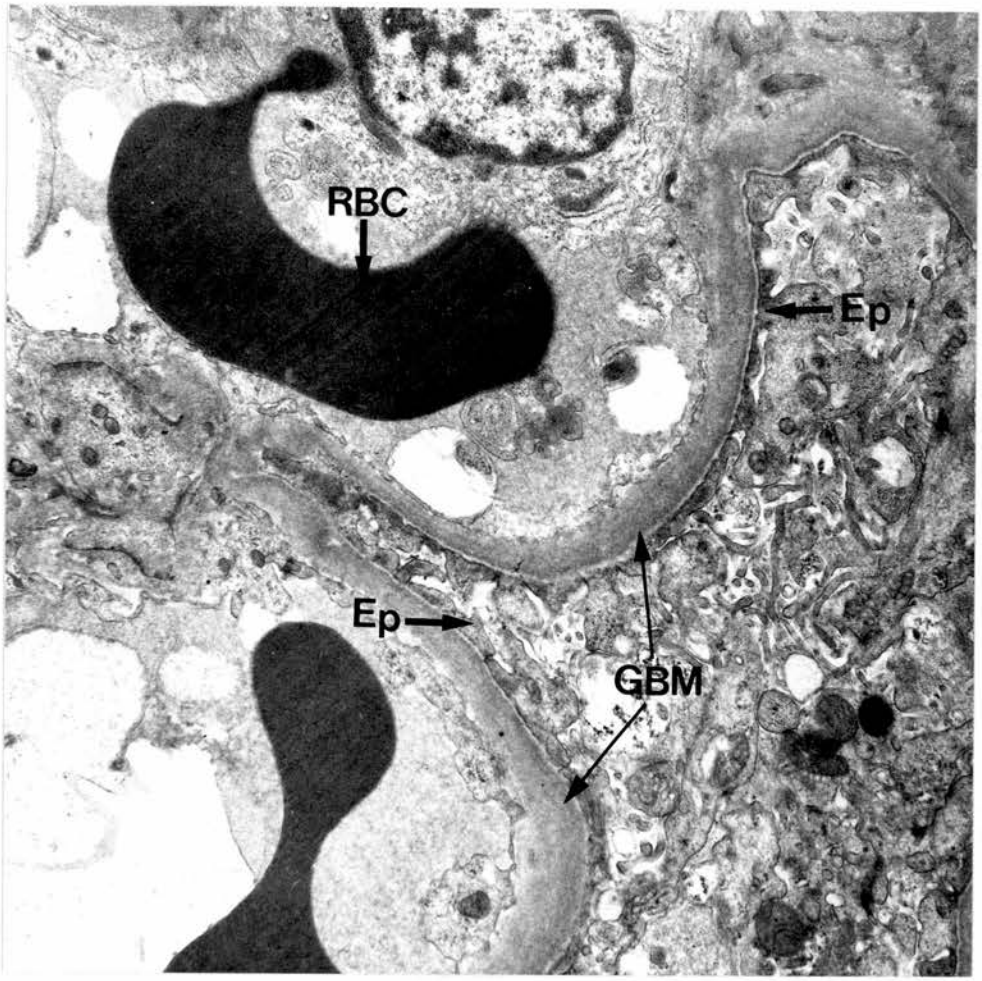


Fig 20 Electron Micrograph of Minimal Lesion Glomerulonephritis

This is a higher power picture of minimal lesion GN and shows the broad based pedicels with, in some places, complete loss of pedicel structure. The GBM is not noticeably abnormal.

x15,000

Ep - epithelial cell
GBM - glomerular basement membrane
RBC - red blood cell

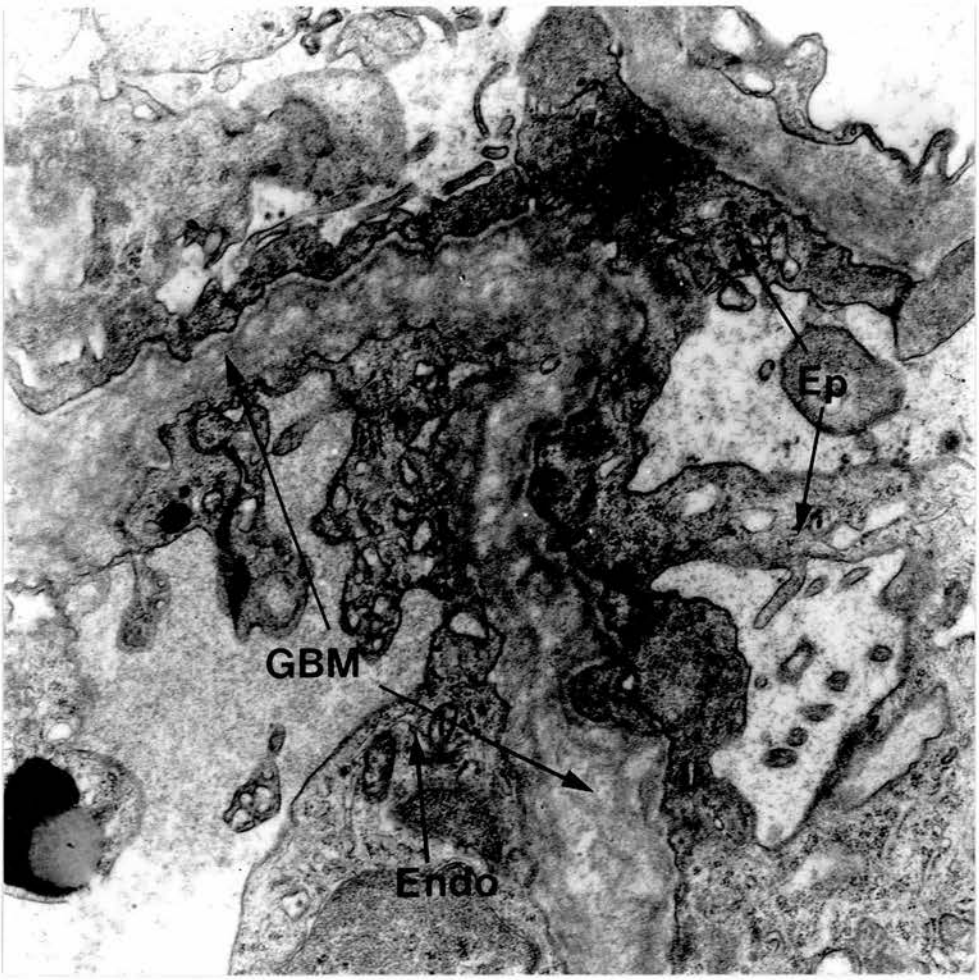


Fig 21 Electron Micrograph of Alport's Syndrome

The GBM in this micrograph shows the typical laminated "basketweave" pattern common in cases of Alport's Syndrome. The exact nature of the deficiency is unknown but may well be a congenital lack of one or more antigens from the membrane.

x27,000

Ep - epithelial cell
GBM - glomerular basement membrane
Endo - endothelium

DISCUSSION

There are a great many pathological processes which may attack and damage the kidneys and, depending on the causative agent, the severity and duration of the disease, the consequences cover a wide range of damage, from mild dysfunction to chronic renal failure.

This project is concerned mainly with the range of conditions known as glomerulonephritis (GN). The most common cause of GN is now thought to be the deposition and resultant effects of immune materials in or on the glomerular capillary basement membranes. These immune materials may be circulating complexes, antibodies combining with 'planted' or fixed antigens, (ie exogens), antigens already present in the basement membrane or other components of the immune reaction such as complement. The localisation of complexes in the glomerular capillary basement membrane (GBM) is not fully understood, but may be related to the size of the complex and/or its electrical charge, and the ability of the GBM either to retain or to allow the passage of such complexes.

Not all types of glomerulonephritis are caused by an immunopathological mechanisms, as in the case of minimal lesion GN, which at present has not been shown to have a firm immunological basis and whose pathogenesis has not

been elucidated. Other factors such as fibrin deposition, platelet adherence, increased intracapillary pressure, increased or disturbed blood flow, and changes in the phagocytic cells in the glomeruli have all been implicated as initiating or influencing the course of glomerular disease.

Also included in the study was tissue obtained from patients who had had a renal transplant and whose grafts were showing clinical signs of rejection.

Transplantation rejection is believed primarily to be a cell mediated immunological response with a secondary humoral immunological effect. In addition to these factors, fibrin is often found in glomerlar capillary walls and lumen, and there is no doubt that rejection often involves the activation of the coagulation system. The effects of rejection may be seen in both the glomeruli and tubules, but often to a much greater extent in the latter.

Glomerular capillary basement membrane

There is evidence that the glomerular basement membrane is the important entity in the glomerular filtration process. The membrane itself is a three layered structure, each layer having a different chemical structural composition. Analysis of the membrane by a variety of methods shows it

to consist of collagenous protein (Type IV collagen), glycoproteins, of which the best characterised is laminin, and proteoglycans.

The mesangial matrix has a fibrillary pattern in EM and appears to consist of glycosaminoglycan (GAG) moieties and a protein component similar to that found in the GBM.

It was decided to study the GBM because of its obvious functional importance, to shed some light, if possible, on any changes in composition which may take place in abnormal conditions. Since the scope of the project was limited to a study of those substances which could be examined by microscopical means, it was decided to look at the glycosaminoglycan (GAG) units of the proteoglycans. Biochemical assays by Kefalides (1978) have shown that the following sugars can be isolated from GBM: hexose, glucose, galactose, mannose, glucosamine, galactosamine, fucose, hexuronic acid and sialic acid. It is interesting to note, however, that Farquhar et al (1982) in a study of the architecture of the GBM, found that sialic acid cound not be detected by their isolation and extraction methods. This finding is contrary to that of Spiro and Parthasarathy (1982) who found in their studies of bovine GBM that sialic acid is present. It is important to point

out that sialic acid is present in the plasma membrane of epithelial and endothelial cells.

The GAG molecules impart a very strong negative charge to the basement membrane and the fact that they are present suggests that their role may be important in maintaining the charge barrier properties of the GBM. Heparan sulphate is the GAG present in greatest abundance in the GBM. Another important property of GAGs is that they exert a steric exclusion effect on proteins in solution (Comper and Laurent 1978; Lindahl and Hook 1977). It would therefore be fair to assume that they could have an effect on the selective filtering properties of the GBM.

Tubular basement membrane

This study included three patients whose primary renal disease affected the tubules; two of these patients suffered from acute tubulorrhectic tubular necrosis (ATN) and one was a case of myelomatosis, with the features of "myeloma kidney". It had been hoped that more cases of tubular disease would be available; however, while the small numbers made positive conclusions about the disease represented impossible, the cases acted as a comparative control group for the cases of glomerular disease.

In these cases, the primary lesion affects the tubules but does so in a focal and isolated manner. The main cause of acute tubular necrosis is ischaemia of the tubules which damages the tubular cells initially and leads to basement membrane damage. As the lesions are focal, some biopsy material may in fact appear normal.

To study the GAGs in glomerular and tubular basement membranes, the use of fluorescein tagged lectins was explored. Lectins are plant and animal ligands which are naturally occurring, and have among their actions the ability to bind specific carbohydrate moieties such as those found in the GBM and mesangial matrix. By tagging them with a fluorescent tracer, fluorescein isothiocyanate (FITC), lectins can be used to identify specific sugars, which can then be easily visualised using a fluorescence microscope.

The larger part of the work was done on tissue which had been submitted fresh initially for the identification of immunoglobulins by the immunofluorescent technique. The use of this method, rather than of immunoperoxidase on paraffin sections, cut out any possible chemical effect of fixatives or dehydrating and clearing agents on the tissue.

However, as a small pilot study, some cases were subjected to fixation in 4% buffered formaldehyde and processed to paraffin wax. These cases were examined by the same lectin method as the frozen material, and as perhaps could be expected, several of those which reacted reasonably well on frozen tissue reacted less well on fixed tissue. The decrease in lectin staining on fixed tissue may be due to a cross-linking of the GBM molecules by the fixative, masking available combining sites, or conversely it may show that the GBM has lost material, through the action of fixatives etc, exposing carbohydrates such as sialic acid not normally available to the lectins. The one exception is WGA where there is increased binding. This is of interest since this lectin is specific for sialic acid (see below)

Two diagnostic groups were chosen for this retrospective survey on paraffin embedded tissue, a) <u>minimal lesion</u> GN, because no satisfactory pathogenesis has yet been formulated for this disease, but there can be no doubt that the GBM is more permeable than normal to protein, and b) <u>Alport's syndrome</u>, in which it is known that an abnormality of the GBM exists, said to be due to an antigenic defect (McCoy, 1982).

Assessment of Results

A. STATISTICAL ANALYSIS

When the findings in the glomerular disease group as a whole are analysed against the normals, the analysis shows that SBA binding is the only lectin to give a significant change, and this only in tubular BM. It shows that N-acetyl-β-D-galactosamine is present in lesser amounts than in the normal cases. However, when the transplant cases are removed from this group and the cases of primary glomerular disease, were compared with the normal cases, an increase in the binding of Con A in the GBM and of PNA in the mesangium is found. These findings suggest that there is an increase in the corresponding sugar molecules, α-L-mannose and α-D-glucose in Con A and D-gal-β (1.3) galactosamine in PNA.

It is interesting to note that galactosamine is present in chondroitin sulphate. This anionic substance is present in GBM, and in cases of glomerulonephritis it is possible that breakdown of the structure of the GBM by immunological materials might set free excess galactosamine which might then be carried into the mesangium by the normal processes. Passage into the mesangium could also account for the fact that it was not found in excess in the GBM.

The α-D-glucose and α-mannose increase found in the GBM could be the result of damage to the membrane, releasing these molecules; the small molecules would probably not be picked up by the mesangium, but would pass through the GBM. Reabsorption by the tubules could possibly have led to the appearance of excess α-D-glucose in tubular BM, but this is highly speculative and unsupported by any other evidence. Although these molecules are not thought to be constituents of GAGs, they are components of collagen, which is present in basement membranes. The lectin binding may be picking up damage to the membrane at a different structural level, ie to collagen itself, and therefore possibly damage of a different degree of intensity.

As already seen, when the transplant group is included, neither of these changes is found, and this would suggest that the relative normality of the glomerular structure in the graft rejection process has masked the abnormalities referred to above in the true GN cases.

When <u>transplants</u> alone are compared with the normal, the only significant change found is the same as that obtained with the combined GN and transplant group, that is, a loss of N-acetyl-galactosamine in the tubular BM. This strongly suggests that the main structural damage in graft rejection lies in the tubules, not in the glomeruli. The

loss of this carbohydrate must indicate an alteration in the integrity of the tubular basement membrane although, at present, this is very difficult to prove. It must be borne in mind that these changes are brought about by an acute rejection episode of relatively short duration, and also that the kidney, before and during transplantation, is subjected to a fair degree of ischaemia which must have an effect on the tubules.

The results in the tubular disease group were statistically significant with only two lectins, and unexpectedly, in the glomerulus, not in the tubular basement membrane. The lectins were a) ricin, which is specific for β-D-galactose, and b) Concanavalin A, which is specific for α-D-glucose and α-L-mannose. Both these lectins were found to be increased in comparison with the normal. Mesangial increase of β-D-galactose may be related to the fact that this sugar is a residue of N-acetyl β-D-galactosamine, which is a component of the GAG chondroitin. The increase in α-D-glucose in the GBM is the same change as found in pure glomerular disease. It may seem strange and unlikely that glomerular changes should be found in cases of tubular disease. It should, however, be noted that previous work has suggested glomerular abnormalities in acute tubular necrosis, based on intraglomerular coagulation and glomerular ischaemia.

In contrast, however, to these findings in the glomerulus, no significant change was found in the tubules. The explanation of this unexpected result may lie in the the fact that in tubular necrosis and in myelomatosis the significant histological finding is the presence of focal and very small discontinuities in the tubular basement membrane. It is possible that the biochemical changes in the membrane are strictly limited to the foci in which breaks are seen, and that these are not numerous enough to affect the overall relative normality of the tubular BM.

Another related factor to consider is that these lesions are thought to be produced by ischaemia which may be of a transient nature. This ischaemia is obviously sufficient to cause functional damage but may be of such short duration that it does not cause identifiable change in the carbohydrate content.

B. HISTOGRAMS

These were drawn to show the relationship of the amount of lectin binding in the mesangium, the GBM and the TBM, in the normal and the abnormal cases. The statistical analysis showed which lectins were significantly increased or decreased in the various anatomical sites in each group of cases, but is was felt that because of the small numbers of cases there might be other interesting possible

differences which could have been statistically significant had more cases been available. Presentation in the form of histograms reveals these differences. The histograms were drawn for each of the disease groups, in every case against the results found for normal tissue.

i) FRESH FROZEN TISSUE

Glomerular Disease Group

Membranous GN

The findings in this group show that there is a complete loss of both SBA and WGA binding in mesangium and GBM compared to the normal, with the appearance of Con A, PNA and DBA binding in GBM, and more cases showing ricin and UEA binding. In the mesangium, PNA binding appeared, and ricin and UEA binding were again increased.

The loss of SBA binding (N-acetyl-α-D-galactosamine), and WGA binding (N-acetyl-D-glucosamine) would seem to indicate that there is a loss in chondroitin and heparan sulphate GAGs. This is extremely interesting because of the fact that proteinuria, which is heavy in membranous GN, is believed to be at least partly due, as indicated previously, to loss of anionic sites in the GBM which consist largely of heparan sulphate, with some chondroitin sulphate.

GBM: The appearance of Con A which binds α-L-mannose and α-D-glucose, may indicate damage to the collagenous component of the membrane, setting free these molecules.

TBM: The results of the TBM are similar to that seen with the normals, the exceptions being loss of SBA (N-acetyl-α-D-galactosamine) and UEA (α-L-fucose) binding. This is difficult to interpret, though it should be said that in well established cases of membranous GN, severe glomerular damage results in atrophy of dependent tubules, with changes in the tubular basement membrane.

Proliferative GN

The histogram shows that in the GBM there is an appearance of Con A and PNA binding with one more case showing SBA binding. The rise in Con A (mannose and glucose) again may be due to the membrane collagen component being altered.

TBM: The findings are very similar to those of normal tissue.

Mesangium

In the mesangium, DBA and SBA binding are not present, while PNA and Con A binding have appeared in 2-3 cases. The Con A increase may indicate that the mesangium, like the GBM, contains more mannose or glucose, which may be due to alteration in the collagen component. It should be

said that Type IV collagen fibrils may be seen in the mesangium in some cases of GN, and not uncommonly in proliferative GN.

Transplants

There seemed to be a general loss of binding in several cases in GBM and mesangium as compared to the normal. with one case showing a little PNA.

The findings suggest a general loss of structural material from the glomerulus, but this was not sufficiently marked to be statistically significant. The reason for this, if true, would be a matter of speculation.

TBM: The results here show a complete loss of SBA and of Con A binding and reduction in cases showing DBA binding.

The loss of N-acetyl-α-D-galactosamine and of glucose/mannose is presumably due to a change in the membranes leading to loss of structural components. It is relevant to say that in transplant rejection, unlike cases of primary glomerulonephritis, tubules are damaged at least as much as, and usually more than, the glomeruli.

Focal Necrotising GN

PNA and Con A binding appeared in both the GBM and in the mesangium, and all other lectin binding disappeared in the

mesangium. Since we had only one case, no really relevant comments can be made, but it might be said that in this condition, actual necrosis occurs in segments of some glomeruli, and this may have accounted for the loss of lectin binding in those glomeruli.

TBM: This was very similar to the normal.

Crescentic GN

Mesangium: Con A appeared in GBM and mesangium of this one case, and PNA in the mesangium. SBA and UEA were absent in both sites. Ricin biding was absent in the GBM. It is not possible to comment on this on the basis of one case, though the findings may be accurate; glomeruli are badly damaged in this type of glomerulonephritis.

TBM: There was disappearance of DBA, SBA, ricin and UEA, all of which reacted to a reasonable extent with normal tissue. The tubular changes, with losses in N-acetyl-α-D-galactosamine, β-D-galactose and α-L-fucose would seem to indicate rather severe tubular damage. This is, in fact, quite commonly seen in crescentic GN on light microscopy, since pressure from the crescents cuts down the blood flow through the glomeruli to the tubules. In this particular case, definite tubulo-interstitial changes were present.

Tubular Disease Group

Acute Tubulorrhectic Tubular Necrosis (ATN)

PNA and Con A binding appeared in both GBM and mesangium. No binding was seen with DBA, ricin or UEA in the mesangium. UEA binding was lacking also in the GBM. The apparent increase in glucose/mannose, and in D-gal B (1:3) galactosamine in glomeruli is admittedly difficult to interpret. Some damage does sometimes occur to glomeruli in acute tubular necrosis, due to fibrin deposition.

TBM: The findings were, surprisingly, very similar to the normal, apart from a lack of UEA binding (α-L-fucose). It must be pointed out, however, that acute tubular necrosis is a very focal disease, only small scattered foci of TBM damage being seen on light microscopy.

Myeloma Kidney

SBA, ricin and UEA had disappeared from both GMA and mesangium, and DBA from the mesangium. Con A and DBA had appeared in the GBM. This applied, of course to only one case, but glomerular changes may occur in cases of multiple myeloma, because circulating abnormal proteins of immunological nature may be deposited in the glomeruli. The lack of reactivity of the mesangium might have been accounted for by its being full of abnormal materials; indeed in this case some mesangial enlargement was seen.

TBM: The findings were essentially normal, except for the loss of SBA and UEA binding. There is more marked TBM damage in this condition than in acute tubular necrosis, and such damage may account for the apparent loss of N-acetyl-B-D- galactosamine and α-L-fucose.

ii) PARAFFIN EMBEDDED TISSUES

It will be remembered that the lectin-binding pattern in normal tissue differed from that found when fresh frozen tissue was used, possibly because of the action of materials used in processing on the components of glomeruli and tubules.

Minimal Lesion GN

Mesangium and GBM: The results were very similar to those obtained in the normals, but there was complete loss of Con A in both mesangium and GBM. SBA was not present in the GBM, nor PNA in the mesangium. The loss of α-L-mannose and α-L-glucose from the glomeruli could indicate a true finding in minimal lesion GN, but this canot be stated with certainty because no fresh biopsy was available during the time of this project. However, if this finding was shown to be reproducible, it might be of real diagnostic importance, since the histological differentiation of minimal lesion GN from a mesangial

proliferative GN is not really possible, the diagnosis depending largely on the clinical features. The finding could, of course, indicate a fundamental abnormality in GBM and mesangial matrix structure in this condition.

TBM: Here only one case reacted at all with any of the lectins, viz WGA. This is difficult to explain, since there is no suggestion of any significant tubular abnormality in minimal lesion GN.

Alport's Syndrome

Mesangium and GBM: Both showed reactions very similar to those obtained in the normal, the only findings of note being that only one case reacted with Con A in mesangium and GBM, and a possible loss of PNA binding in both sites. Whether these data fit with the suggested lack of a GBM antigen in this hereditary condition, it is impossible to say.

TBM: Here also the findings are very similar to normal, except for an increase in Con A binding, and again a lack of PNA binding.

This small pilot study of the use of the FITC lectin technique on paraffin wax-embedded material has suggested some interesting points for investigation, but it does seem that such changes are produced by the chemicals used in the processing of the tissue that no fundamental

conclusions on the pathogenesis of disease would be justified from the results of this technique. However, it is not impossible that use of paraffin-embedded material examined in this way could provide a practical diagnostic test in the differentiation of types of renal disease.

There has been an increasing awareness of the possibility of using lectins on fixed tissues. In this project the results obtained showed that whilst the technique was possible the overall amount of binding was much less than that obtained with fresh frozen material. This was possibly due to the loss of carbohydrate residues during processing.

Fletcher and Walker (1981) state that the glomerulus may lose 80% of its polyanions due to processing; they called it the "jellied glomerulus". Rittman et al (1983) used various fixatives on oral mucosa and skin, to ascertain if there was any change in the lectin binding. Comparing the results with these in fresh material they found there was a weaker but similar pattern in the basement membranes of fixed tissue when fixed with acetone, alcohol and Bouin's fixative. As in the present study, they found that tissue fixed in formaldehyde gave very weak general binding.

An interesting finding was that of West et al (1982) who used an enzyme (trypsin) to pre-digest thin sections before exposure to lectin. They used formaldehyde-fixed tissue and state that with the digestion method the lectin binding is as satisfactory as in non-digested frozen material. Trypsin is used commonly as an antigen unmasker in other histochemical studies, and future developments in this field should obviously include an investigation of this technique.

Other lectin-binding studies in kidney

Recent work (Holthofer, 1983) used FITC-tagged lectins to identify renal binding sites in the 14 different species including human. The tissue used in the human study was obtained from the non-involved poles of kidneys which had been removed from renal carcinoma.

The findings in his study were very similar to the results of the present project in that Holthofer showed strong WGA binding in the glomerulus and WGA, RCA, SBA and PNA binding in the tubules. The results of the present study showed strong binding with PNA, SBA and WGA and slightly less with ricin in the glomerulus and tubular basement membrane. One interesting point in Holthofer's study is that it showed the lectins to be capable of binding in many species. Findings similar to Holthofer's have been reported by Murata et al (1983).

Murata et al (1983) showed different binding between the glomerulus and the tubules. This study showed that WGA reacted with the glomerulus strongly and that PNA, DBA, SBA and ricin reacted with the tubules. The present study showed binding with WGA, SAB and ricin in the GBM, and PNA, DBA, SBA and ricin in the tubules.

However, both these studies were performed on normal tissue only, purely as an exercise to identify specific lectin-binding sites in different parts of the nephron and not, as in this project, on diseased kidneys to identify changes in the membranes and matrix.

Le Hir et al (1982) has also employed lectins to identify the different cell types of the renal tubules. They found that the cell cytoplasm, and also in certain instances the brush border of the tubular cells, reacted with a lectin-peroxidase conjugate. The study showed binding with WGA and SBA in "the glomeruli" and with SBA, PNA and WGA in the tubules.

In the present project, while most attention was paid to the lectin-binding sites in basement membranes, it was noted that some tubular cells were also positive, especially with WGA, and this was particularly true of the luminal tips of cells, mainly in the medulla. Brush borders of proximal tubules also sometimes stained

positively with WGA. The results, however, were not sufficiently clear cut to be quantitatively analysed. This was largely due to the variability of cellular and brush border staining throughout the sections examined. It was felt, therefore, necessary to exclude these results from the project. Interest has also been shown recently in lectin-binding in other tissues (Freeman et al, 1980; Golbus and Cutis, 1979; Vierbuschen et al, 1980; Leathem and Atkins, 1983; Rittman and MacKenzie, 1983).

Sialic Acid

It is clear that among the binding patterns found in this study, the lectins used to identify the sugar residues N-acetyl-B-D-galactosamine; B-D-galactose and N-acetyl-B-D-glucosamine, often showed strong positivity. These sugar residues are constituents of the GAGs chondroitin sulphate and heparan sulphate. One of the residues, N-acetyl-B-D-galactosamine, is also the terminal sugar residue of a group of heteropolyaminosaccharides which comprise the neuraminic acids. These neuraminic acids are naturally occurring, N-acylated, and are commonly known as sialic acid.

It may be, therefore, that in the identification of heparan sulphate and chondroitin sulphate, two GAGs known to exist in GBM (Farquhar et al, 1982), sialic acid is reacting also.

Farquhar et al (1982) however, state that in the isolation procedure used by them, neither galactosamine or sialic acid are detectable in rat GBM, although Spiro and Parthasarathy (1981) do show the presence of both galactosamine and sialic acid in bovine GBM.

The present study has shown that N-acetyl-α-D-galactosamine can be identified in human kidney, using SBA and DBA lectin. However, one of the lectins Limulus polyphemus (LP) was stated (by the manufacturers) to be specific for sialic acid. This particular lectin failed to show any reaction at all. This may have been firstly due to the fact that neuraminic acid (sialic acid) is a chain of residues (Lehringer, 1975), the terminal residue normally being N-acetyl-β-D-glucosamine, and the lectin, while being specific for neuraminic acid, was unable to bind with it because the exposed residue on the chain may have been non-selective. Secondly, the addition of magnesium ions to the buffer used to dilute the lectin (Morris, 1983) has proved beneficial in aiding the binding of this lectin. (This was not done in the present study.)

It would seem therefore that in using WGA and SBA the terminal sugar residues of both sialic acid and the GAGs may be simultaneously demonstrated.

Sialic acid, among other sugar residues, has been implicated recently (Glass et al, 1981; Weir et al, 1981; Weir et al, 1982; Weir et al, 1984) in an important fundamental physiological reaction, that of phagocyte recognition of bacterial cell wall membrane. It has been shown that the phagocytes recognise the bacterium and bind to it because bacterial cell wall possesses sugar-residue molecules, particularly sialic acid. The phagocyte itself possesses "lectin-like" receptors and thus an attachment is mediated similar to an antibody-antigen reaction.

If this is so, it is tempting to suggest that part of the removal of material deposited in the glomerular capillary basement membrane in immunological diseases of the kidney might be removed by mesangial cells of macrophage type, through the medium of reaction between lectin receptors on mesangial cell membrane and sialic acid sugar residues in the antigens to be removed. It is known that a glomerulus involved in this type of reaction, ie in proliferative GN, shows on electron microscopic examination pseudopodia of mesangial cytoplasm interposed between basement membrane and endothelium of the glomerular capillary wall (see Fig 8c).

It has been thought that the pseudopodia development was possibly in order to produce excess matrix into which the antigens (or complexes) were drawn, to be removed, as in a

Sephadex column, into the main columns of mesangial matrix. It may, however, be that "lectin-like" receptors on the cell membranes of the mesangial pseudopodia bind to sialic acid molecules in the antigen/complexes, and thus carry them into the mesangial matrix.

It is therefore interesting to note that in the results of proliferative GN, the histogram shows an increase in WGA binding in two cases in the GBM, which might indicate an increase in the sialic acid residues possibly included in antigens/complexes on the GBM (endothelial side) or even concentrated along the mesangial pseudopodia cell membrane, and already bound to the naturally occurring lectins on this membrane. Likewise, the histograms show a complete loss of SBA in the mesangium, as compared with the normal. Could this in any way be associated with an increase of natural lectins on the increased surface area of cell membrane present, competing for the sialic acid residues normally present in the mesangium? This speculation would, of course, depend on the possibility that sialic acid residues from bacterial wall membranes could accompany the antigenic component present in the glomerular deposits.

A point of peripheral interest to the present work arises in this connection. Farquhar and Kanwar (1983) have shown that by enzyme digestion methods the anionic sites contain heparan sulphate since they are removed by heparinase, while procedures which are specific for other GAGs and sialoproteins leave the sites relatively unaffected.

To test the hypothesis that sialic acid is present in the anionic sites, Weir (1984) suggests an interesting, but simple, experiment. By exposing the GBM to a monolayer of suitable human macrophages any sialic acid containing sites might be identified by the attachment of these macrophages due to the lectin receptor on the macrophage cell membranes. This attachment could then be visualised by using a macrophage marker such as chloroacetate esterase (Filipi et al, 1983) which in turn would indicate the presence of sialic acid in the anionic sites.

CONCLUSION

CONCLUSION

This project has shown that changes identifiable by the lectin-binding technique do occur in glomerular and tubular basement membrane in disease processes. It is therefore possible that by examining these patterns on a routine basis in renal biopsies, a diagnostic test might be added to those already used in the investigation of human renal disease. Obviously, more studies of normal and abnormal tissue must be performed before such a test could be implemented, but it is hoped that this project may have helped to lay a foundation for this development.

REFERENCES

REFERENCES

ALPORT AC (1927) Br Med J 1: 504

BANCROFT JD, STEVENS A (1982) Eds. Theory and Practice of Histological Techniques, 2nd Edition. p183

BLAINEY JD, BREWER DB, HARDWICKE J, SOOTHILL JF (1960) Quart J Med 29: 235

BOYD WC, SHARPLEIGH E (1954) Science 119: 419

BORDER WA, KAMIL ES, WARD HJ, COHEN AH (1981) Lab Invest 45: 442

BRETTON R, BARIETTY J, GROSSETETE J (1976) 1st International Symposium on Immunoenzymatic Techniques INSERM Symposium no 2. Feldmann et al (eds). p501

CAULFIELD JP, FARQUHAR MG (1974) J Cell Biol 63: 883

COUSER WG, STEINMULLER DR, STILLMONT MM, SALANT DJ, LOWENSTEIN LM (1978) J Clin Invest 62: 1275

COCHRANE CG, HAWKINS D (1968) J Exp Med 127: 137

COMPER WD, LAURENT TE (1978) Physiol Rev 58: 255

DIXON FJ, FELDMAN JD, VAZQUEZ JJ (1961) J Exp Med 113: 899

FARQUHAR MG, WISSIG SL, PALADE GE (1961) J Exp Med 113: 47

FARQUHAR MG, COURTOY PJ, LEMKIN MC, KANWAR YS (1982) In: New Trends in Basement Membrane Research. Kucher K, Schoene, H, Templ R (eds). Raven Press, p9

FARQUHAR MG, KANWAR YS (1983) In: Immune Mechanisms in Renal Disease. Cummings NB, Michael AF, Wilson CB (eds). Plenum Medical

FILIPI IM, LAKE BD (1983) Eds. Histochemistry in Pathology. Churchill Livingstone, p329

FISH AJ, MICHAEL AF, VERNIER RL et al (1966) Am J Path 49: 997

FLETCHER A, WALKER F (1981) J Pathol 134: 336

FREEMAN HJ, LOTAN R, KIM YS (1980) Lab Invest 42: 405

GLASS E, STEWART J, WEIR DM (1981) Immunology 14: 599

GOODPASTURE EW (1919) Am J Med Sci 158: 863

GOLBUS SM, CURTIS BW (1979) Kidney Int 16: 148

HOLTHOFER H (1983) J Histochem Cytochem 31: 531

HSU S-M, REE HJ (1983) J Histochem Cytochem 31: 538

JANOFF A, ZELIGS JD (1968) Science 161: 702

JONES DB (1951) Am J Path 27: 991

KEFALIDES NF (1978) Ed. Biology and Chemistry of Basement Membranes. Academic Press

KNIKER WT, COCHRANE CG (1968) J Exp Med 127: 119

KOFFLER D, PARONETTO F (1965) J Clin Invest 44: 1665

LAZARUS GS, BROWN RS, DANIELS JR, FULLMER HM (1968) Science 159: 1483

LERNER RA, DIXON FJ (1968) J Immunol 100: 1277

LEATHEM A, ATKINS N (1983) J Clin Path 36: 747

Le HIR M, DUBACH UC (1982) Histochem 74:521, 531

LEHNINGER AL (1975) In: Biochemistry - The Molecular Basis of Cell Structure and Function, 2nd Edition Worth.

LEIPER JM, THOMSON D, MACDONALD MK (1977) Lab Invest 37: 526

LENDRUM AC, FRASER DS, SLIDDERS W, HENDERSON R (1962) J Clin Path 15: 401

LINDAHL U, HOOK M, BACKSTROM G et al (1977) Fed Proc 36: 19-23

LIS H, SHARON N (1977) In: The Antigens. Chapter 7. Sela, M (ed). Academic Press (New York)

MAGIL AB, WADSWORTH LD, LOEWEN M (1981) Lab Invest 44: 27

MAUER, SM, SUTHERLAND DER, HOWARD RJ, FISH AJ, MAJARIAN JS, MICHAEL AF (1973) J Exp Med 137: 553

MAZZULA M, LHERMITTE M, LAFITTE J-J, ROUSSEL P (1982) J Histochem Cytochem 30: 956

McCOY RC, JOHNSON HK, STONE WJ, WILSON CB (1982) Kidney Int 21: 642

McLUSKEY RT, VASSALLI P, GALLO G (1966) New Eng J Med 274: 695

McMANUS JFA (1948) Nature (Lond) 158: 202

MICHAEL AF, KEANE WF, RAIJ L et al (1980) Kidney Int 17: 141

MORRIS RG (1983) MSc Thesis, submitted to Heriot-Watt University

MURATA F, TSUYAMA S, SUZUKI S, HAMADA H, OZAWA M, MURAMATSU T (1983) J Histochem Cytochem 31: 139

OBERLEY TD, BURKHOLDER PM, BARBER TA, HWANG CC (1979) Invest Cell Pathol 2: 27

RENNKE HG, COTRAN RS, VENKATACHALAM MA (1975) J Cell Biol 67: 628

RITTMAN BR, McKENZIE IC (1983) Histochem J 15: 467

SCHREINER GF, KIELY JM, COTRAN RS, UNANUE ER (1981) J Clin Invest 68: 920

SEEGAL BC, ANDRES GA, HSU KC et al (1965) Fed Proc 24: 100

SIEGEL S (1956) Non Parametric Statistics for the Behavioural Sciences. Int Student Edition. McGraw-Hill, p116

SIMIONESCU M, SIMIONESCU N, PALADE GE (1982) J Cell Biol 94: 406

SPIRO RG, PARTHARATHY N (1981) In: New Trends in Basement Membrane Research. Kuehn K, Schoene H, Timpl C (eds). Raven Press (New York). p87

STRIKER GE, KILLEN PD, FARIN RM (1980) Transpl Proc 12 (suppl 1): 88

VASSALLI P, SIMON G, ROUILLER C (1963) Am J Path 43: 579

VIERBUCHEN M, KLEIN PJ, UHLENBRUCK G, KLEIN HO, SCHAEFER HE, FISCHER R (1980) Recent Results in Cancer Research 75: 68

WEIR DM, GLASS E, STEWART J (1981) Ann Immunol (Inst Pasteur) 132D: 131

WEIR DM, STEWART J, GLASS E (1982) Immunobiol 161: 334

WEIR DM, STEWART J, GLASS EJ et al (1984) In: Tissue Culture and Research. Rohlich P, Bacsy E (eds). Akademiai Kiada, Budapest

WEIR DM (1984) Personal Communication

WEST KP, PLATTS HA, FLETCHER A, WALKER F (1982) J Clin Path 35: 239

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APPENDIX

APPENDIX

Periodic Acid Schiff Reaction (McManus 1948)

1. Periodic Acid Solution

Periodic acid	1gm
Distilled water	200cms^3

2. Schiff's Reagent

Dissolve 1gm basic fuchsin in 200cm^3 of boiling distilled water. (Remove flask from heat before adding basic fuchsin.)

Allow solution to cool to 50^{o}C and add 2gms potassium metabisulphite. Allow to cool to room temperature then add 2cms^3 of concentrated hydrochloric acid, mix and leave overnight in the dark, add 2gms activated charcoal, shake well and filter. Store at 4^{o}C.

Method

1. Dewax sections and bring to distilled water.
2. Treat with periodic acid - 5 mins.
3. Wash well in several changes of distilled water - 10 mins.
4. Cover with Schiff's reagent - 15 mins.
5. Wash well in running tap water - 10 mins.

6. Stain nuclei with haematoxylin - (differentiating in 1% acid alcohol and blueing up in 1% lithium carbonate as usual).
7. Dehydrate in ascending grades of ethanol.
8. Clear in xylene.
9. Mount in a synthetic mountant.

Results

Periodate-reactive carbohydrates	-	magenta
Nuclei	-	blue

Martius yellow, brilliant crystal scarlet, soluble blue (MSB) (Lendrum et al 1962)

a) Martius yellow (acid yellow 24)

Martius yellow	- 0.5gms
Phosphotungstic acid	- 2.0gms
95% ethanol	- 100cms^3

b) Brilliant crystal scarlet (acid red 44)

Brilliant crystal scarlet	- 1.0gms
Glacial acetic acid	- 2.0cms^3
Distilled water	- 100cms^3

c) Phosphotungstic acid

Phosphotungstic acid	- 1gm
Distilled water	- 100cms^3

d) Soluble Blue (Acid blue 93)

Soluble blue	- 0.5gms
Glacial acetic acid	- 1.0cms^3
Distilled water	- 100cms^3

e) Glacial Acetic Acid

Glacial acetic acid	- 10cms^3
Distilled water	- 100cms

Method

1. Dewax sections and bring to water.
2. Stain nuclei with celestine blue - haematoxylin sequesence.
3. "Blue up" in 1% lithium carbonate and differentiate in 1% acid alcohol as required.
4. Wash well in tap water.
5. Rinse in 95% ethanol.
6. Stain in Martius yellow solution - 2 mins.
7. Rinse in distilled water.
8. Stain in brilliant crystal scarlet - 10 mins.
9. Rinse in distilled water.
10. Treat with 1% phosphotungstic acid until no red remains in the collagen.
11. Stain in soluble blue until collagen 30-60 secs.
12. Rinse in 1% acetic acid.
13. Dehydrate through alcohols.
14. Clear in xylene and mount in synthetic resin.

Results

Nuclei	- blue
Red blood cells	- yellow
Fibrin	- red
Collagen	- blue

Jones Periodic Acid - Methenamine Silver Method (PASM)

a) Periodic Acid

Periodic acid	-	0.5gms
Distilled water	-	100cms^3

b) Methenamine Silver Solution

i)	5% sodium tetraborate	-	5cms^3
	Distilled water	-	25cms^3
ii)	3% methenamine	-	25cms^3
	5% silver nitrate	-	3cms^3

Solution i) is added to solution ii).

A precipitate forms which redissolves spontaneously on gentle agitation. Must be prepared fresh and pre-heated to 60°C for use.

c) Gold Chloride

Gold chloride	-	0.2gms
Distilled water	-	100cms^3

d) Sodium Thiosulphate

Sodium thiosulphate	-	3gms
Distilled water	-	100cms^3

Method

1. Dewax sections and bring to distilled water.
2. Place in 0.5% periodic acid - 10 mins.
3. Rinse in distilled water.
4. Place in pre-warmed methenamine silver solution at 60°C (check regularly after 15 mins until GBM appears black).
5. Rinse in distilled water.
6. Tone in 0.2% gold chloride until sections appear blue black - 1 min.
7. Rinse in distilled water.
8. Fix in 3% sodium thiosulphate - 5 min.
9. Wash well in tap water.
10. Counterstain other tissue structures with a light haematoxylin and eosin stain.
11. Dehydrate in ethanol.
12. Clear in xylene and mount in a synthetic resin.

Results

Nuclei	-	blue
Background	-	light pink
Basement membrane	-	black

Renal Biopsies - Standard Methods

4% Neutral Buffered Formaldehyde

36-40%	formaldehyde	$100cm^3$
	distilled water	$900cm^3$
	sodium di-hydrogen orthophosphage (monohydrolate)	4gms
Disodium hydrogen orthophosphate (anhydrous)		6.5gms

The standard staining methods employed are:

1. Haematoxylin and eosin.
2. Periodic acid Schiff reaction (PAS).
3. Martius yellow, brilliant crystal scarlet, soluble blue (MSB).
4. Periodic acid, methenamine silver method of Jones.*
5. Methyl green - pyronin (MGP).*

Method 4 applied only to membranous GN, and method 5 to transplant biopsies.

Electron Microscopy

Fixative	Glutaraldehyde (25%)	$5cms^3$
	0.2M Cacodylate	$15cms^{3PT}$
	Distilled water	20cms

Buffer	Sodium Cacodylate	42.8gms
	NHCl	6.9cm$_3$
	Distilled water to 1 litre	

Epoxy resin

Solution A

C212 resin - 200cms$_3$

Duodecynyl succinic anhydroxide hardener (DDSA) - 200cms$_3$

rinse well together

Solution B

Di-butyl phethalate - 20cms$_3$

HY 960 (Accelator) - 5cms

mix well together

Working solution

Solution A - 19cms$_3$

Solution B - 1cm$_3$

Uranyl acetate - lead citrate dual stain

1. Uranyl acetate — saturated solution of urayl acetate in 50% ethanol

2. Lead citrate

lead citrate	0.04gms (in a dry cone tube)
distilled water	5cms$_3$
ION NaOH	0.1cms$_3$

This is then made up to 10cm with distilled water and to aid the solution of the lead citrate the tube is placed in an ultrasonic cleaner (50 cycles/sec) until all is dissolved.