The Glomerular Basement Membrane and Nephritis.

Thesis submitted for the Degree of Doctor of Philosophy in the University of Adelaide.

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Abstract

In this study, the binding of antibody and other compounds to the glomerular basement membrane (GBM) was investigated, together with the immunopathology and treatment of anti-GBM-antibody disease.

The binding to GBM of a variety of compounds which are associated with membranous nephropathy was investigated using an *in vitro* binding assay. These included gold, mercury, captopril and cationic IgG with DNA, BSA and C1q used as controls. Cationised IgG and mercury were found to bind to GBM whilst gold and captopril did not. However study of the pI of glomerular IgG from membranous nephropathy patients failed to demonstrate increased amounts of cationic IgG indicating that this mechanism is unlikely to be responsible for the immunopathology of this condition.

A solid phase radioimmunoassay for anti-GBM antibodies was established and used to study the circulating antibody in the diagnosis and monitoring of disease. Although the assay distinguished antibody positive patients from normal controls, several patients with SLE were also positive in the assay and the significance of this is discussed with reference to the antigens of the basement membrane. Further studies of the anti-GBM antibody were performed with the determination of the avidity of the serum antibody showing that the circulating antibody is of low avidity. This implies that the circulating antibody may be of limited relevance to on-going renal disease.

Plasma exchange and chemotherapy are normally performed together in the treatment of anti-GBM disease. The separate use of these therapies was investigated in the Steblay model of anti-GBM nephritis in sheep and they were shown to be effective by different mechanisms. Plasma exchange is probably efficacious by removing non-antibody inflammatory agents from the circulation whilst chemotherapy reduces antibody production. Further investigations of treatment were performed using the prostaglandin PGE_1 since this has been found to be of therapeutic use in an number of different forms of nephritis. PGE_1 was shown to be protective in experimental anti-GBM disease. The most likely effect appeared to be upon inflammatory cells and their products since antibody deposition was not affected.

Statement of Originality

This thesis contains no material that has been accepted for the award of any other degree or diploma in any University with the exception of some of the data included in Chapter 4A. This study was a joint project with members of The Centre for Biomedical Engineering, University of New South Wales (approximate share 50%) and some data from this project have been detailed in the MSc thesis by Allan Pollack in the University of NSW. These data are presented here with the consent of my co-investigators.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

3.9.85

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Many people have assisted with this project. Without their generous cooperation it could not have been possible. In particular I wish to acknowledge with gratitude the role of the following people:

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Chapter 1.

Background and Introduction.

Immunopathology of Glomerulonephritis

Immune damage to the glomerulus has traditionally been thought to arise by one of two separate pathogenic mechanisms. The first of these is the disease caused by circulating immune complexes and the second is that caused by antibodies directed against structural antigens of the kidney.

Circulating Immune Complex Disease

This is the more common of the two disease mechanisms and involves the formation of soluble immune complexes in the circulation. These subsequently become entrapped within the glomerulus and initiate inflammation [1, 2]. Diagnosis of IC generated glomerulonephritis in humans is based on the detection of CIC and on renal immunofluorescence. Characteristically, the latter shows discontinuous, granular immune deposits (Figure 1.1). Much of the immunopathology of CIC disease has been elucidated using animal models, particularly the acute and chronic serum sickness models, (reviewed by Dixon and Wilson [3]) and these studies have shown that a number of factors affect the site and severity of the immune deposits. These include antigen-antibody ratio, reticuloendothelial function, serum IC levels and haemodynamic factors [2]. The sites of immune deposition in human disease vary from mesangial (IgA nephropathy) to subendothelial (SLE, MPGN) and subepithelial (post-streptococcal and membranous nephropathy).

Disease Caused by Antibodies to Structural Antigens

This rarer form of disease involves deposition of antibodies onto structural antigens of the kidney, followed by immune damage. The classic example of this disease is anti-GBM antibody-induced nephritis. The antigen is found in the basement membrane and diagnosis is based on renal immunofluorescence which shows linear glomerular capillary wall staining for IgG and C3 (Figure 1.2). Circulating anti-GBM antibodies which produce linear glomerular immunofluorescence can often be demonstrated on normal kidney tissue.

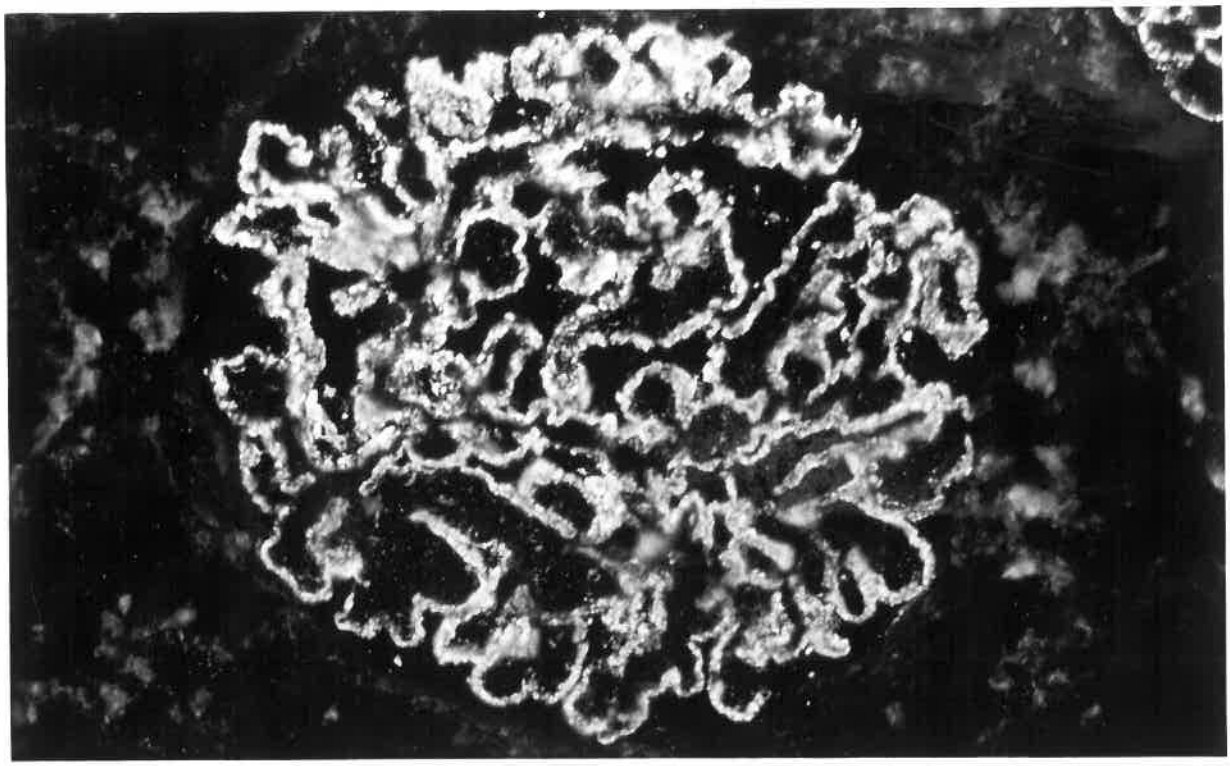
More recently, some other examples of glomerulonephritis resulting from antibodies to structural antigens have been discovered. These are 1; a spontaneous glomerulonephritis in the NZW rabbit in which the antigen is found around the epithelial foot processes [4] and 2; a form of Heymann's nephritis in which the antigen is located in the pits at the base of the foot processes [5]. These two animal models of glomerulonephritis, which display granular immunofluorescence, indicate that some types of human nephritis, previously thought to have a CIC mechanism, may result from antibodies directed against structural glomerular antigens.

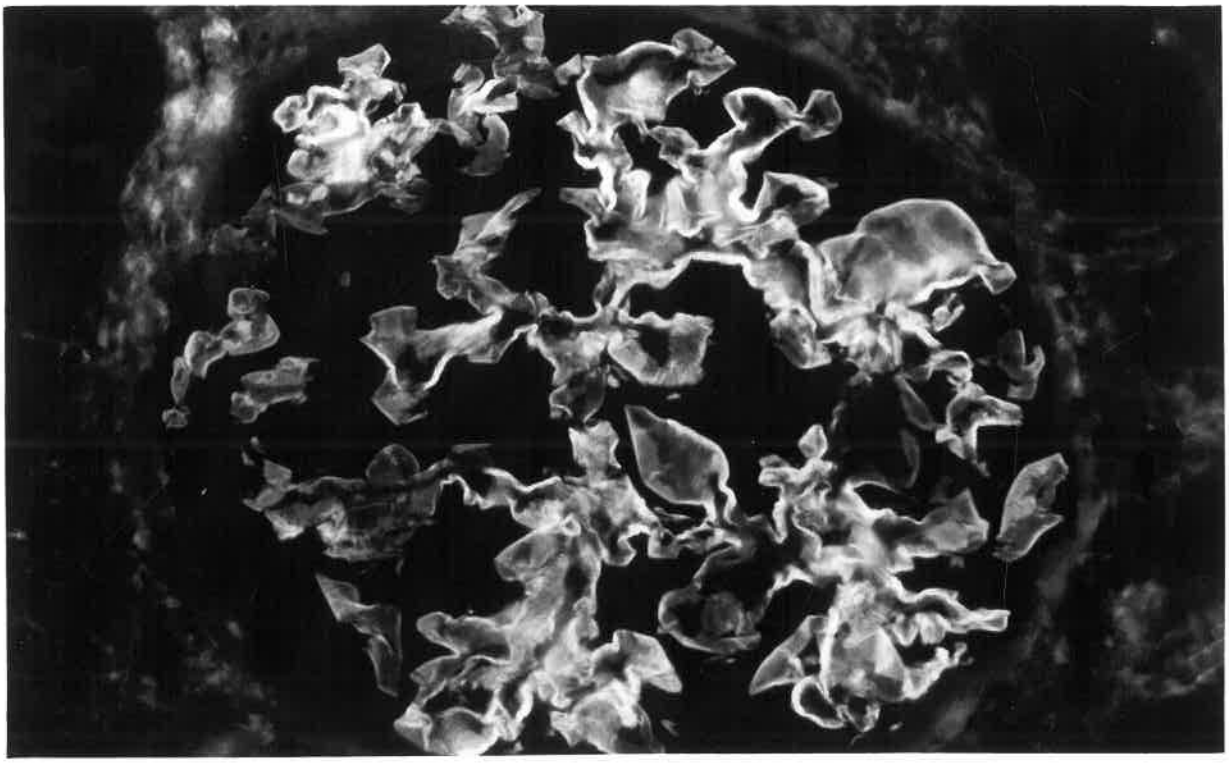
FIGURE 1.1

Direct immunofluorescence demonstration of granular IgG deposits in human glomerulus (x250).

FIGURE 1.2

Linear pattern of anti-GBM-antibody deposits by indirect immunofluorescence using serum from a patient with Goodpasture's syndrome (x250).





"*In Situ*" Immune Complex Formation

Recently, interest has developed in a third pathogenetic mechanism leading to glomerulonephritis in experimental animals. This occurs when antibody reacts with "planted" antigens which have previously become entrapped within the glomerulus [6, 7]. The archetype of "*in situ*" immune complex formation is the autologous phase of experimental anti-GBM antibody-induced nephritis. The heterologous antibody acts as a planted antigen for the recipient animal's (autologous) antibody response [8]. Another model with this pathogenesis was described by Golbus and Wilson [9]. These authors infused the lectin Concanavalin A into rat kidneys. Con A binds to glycoproteins in the basement membrane, and when the lectin was followed with antibody to Con A, the rats developed acute glomerulonephritis. The possibility that a similar *in situ* mechanism operates in some cases of SLE has now arisen, since Izui, Lambert and Miescher [10] showed that free DNA can bind to GBM and subsequently bind anti-DNA antibody.

This further mechanism is likely to be responsible for the formation of subepithelial immune deposits in human membranous glomerulonephritis [6].

The animal models of serum sickness which produce subepithelial immune deposits were originally thought to have a CIC pathogenesis. However, perfusion of pre-formed immune complexes into experimental animals leads to mesangial and subendothelial but not subepithelial deposits [6]. This supports the conclusion that pre-formed ICs fail to penetrate the GBM due to their size.

In some human diseases, and particularly in membranous glomerulonephritis, CIC are absent or present only intermittently. This, together with the existence in membranous glomerulonephritis of subepithelial immune deposits, is consistent with the hypothesis that an *in situ* mechanism is operative [11].

Summary of the Pathogenic Mechanisms of Glomerulonephritis

Three mechanisms for immune damage to the glomerulus have been elucidated: circulating immune complex mediated disease, disease caused by antibodies to structural antigens and the *in situ* or "planted" model. Some of the binding properties of the GBM are investigated in Chapter 2 with a view to study further the mechanisms involved in the *in situ* type of glomerulonephritis. Chapters 3 and 4 are concerned with various aspects of anti-GBM antibody disease.

Anti-Glomerular Basement Membrane Antibody-Induced Disease

Anti-GBM Antibody-Induced Disease in Humans

The symptoms of this disease were first described in 1919 by Goodpasture [12], but the demonstration of linear antibody along the GBM by immunofluorescence was not until 1964 [2]. The disease has a variety of clinical presentations with usually severe, rapidly progressive glomerulonephritis leading to renal failure. In about 70% of cases there is pulmonary haemorrhage, this presentation being known as Goodpasture's syndrome. The remainder of cases have only renal involvement [13]. With recent improvements in diagnosis, a minority of milder presentations have now been recognised. However, dialysis is required in about 75% of all cases and mortality is about 20%.

Experimental Models of Anti-GBM-Antibody Disease

The classic model of anti-GBM-antibody disease is nephrotoxic serum nephritis. This disease is manifested in two phases; first heterologous anti-GBM antibody binds immediately to the GBM leading to damage, this is followed 7-10 days later by an autologous antibody response to the heterologous antibody with further damage [14]. Another model of nephritis can be induced in sheep by immunising them with human GBM and Freund's complete adjuvant [15] (known as the Steblay model). Circulating antibodies capable of transferring the disease were reported by Steblay [16] and Lerner and Dixon [17].

Cross-reactivity between lung and glomerular basement membranes was demonstrated by injecting lung BM into sheep and inducing an anti-GBM antibody response with associated renal disease development [18]. Most heterologous anti-GBM antisera also have antibody reactivity to TBM on indirect immunofluorescence. When injected intravenously into experimental animals these antibodies bind mainly to GBM, presumably due to its greater accessibility. Autologous anti-TBM responses have been demonstrated in a variety of experimental models [19-21].

Normal rabbit urine contains basement membrane fragments and these can induce an antibody response and nephritis when injected into the autologous animal [22]. The significance of these fragments which have also been demonstrated in humans is not known (see Induction of Antibody Formation p. 10).

Diagnosis

The diagnosis of anti-GBM disease is based on the detection by immunofluorescence of immunoglobulin bound in a linear pattern to the basement membrane of renal biopsy tissue [13]. There is usually circulating antibody detectable by indirect immunofluorescence (60-80% of cases) or RIA (85-95% of cases) (see Chapter 3A). About two thirds of patients have C3 deposits on DIF, often in a more irregular pattern than that displayed by IgG [23]. A small number of false positives by DIF occur, particularly in tissue obtained at autopsy or from patients with diabetes mellitus [24, 25]. About 70% demonstrate TBM staining [26]. There are also false positives by RIA,

6/447 in one series with 4 of the positives being patients with SLE [13].

Antigen

Collagenase digestion of glomeruli releases a glycoprotein antigen of molecular weight 26,000 which will bind with anti-GBM-antibodies. Most of the radioimmunoassays developed for measuring anti-GBM antibodies have been based on such non-collagenous antigens [27]. A number of larger antigen-carrying molecules will also survive digestion [28]. Electron microscopy studies reveal the antigenic sites localised within the lamina densa of the basement membrane [29].

Induction of Antibody Formation

The aetiology of anti-GBM antibody disease remains unknown. A number of explanations for the spontaneous development of antibodies has been proposed, based upon the unmasking or release of cross-reactive pulmonary antigens or the introduction of antigenic material into the circulation from the kidney. A few cases of anti-GBM disease have followed influenza A2 viral infections [30] and some others follow exposure to hydrocarbon solvents [31]. A strong association with the DR2 tissue type exists and is clearly important in this and other autoimmune diseases [32]. The detection of GBM antigens in normal human urine has now been reported [33-35] as well as in serum [33]. It seems that basement membrane fragments appear in the circulation as a consequence of basement membrane metabolism and are eliminated in the urine; the role of these fragments in the immunogenesis of anti-GBM-antibodies during the

development of disease is not known. Some cross-reactivity between streptococcal antigens and GBM antigens has been reported [36]; however, an increased incidence of streptococcal infection preceeding the development of disease has not been established [2].

Pathogenic Significance of Antibody

The recurrence of disease in grafted kidneys has been accepted as evidence of the pathogenicity of the antibody [23], as has the ability of the eluted and circulating antibody to cause disease in monkeys [37]. However, the severity of disease does not correlate well with circulating antibody levels [13], although the level of circulating antibody is higher in acute disease than in the serum of convalescent patients [38-40]. The incidence of pulmonary haemorrhage does not correlate with circulating antibody [13]. See Chapter 3B for further discussion of the significance of the circulating antibody.

Mediation of Damage

The antibody deposited in the basement membrane is complement fixing. Complement activation releases chemotactic factors for PMN's which, on arrival, release proteolytic enzymes which damage the GBM and lead to proteinuria [41]. The coagulation and kinin systems [42], monocytes [43] and platelets [44] are also involved in the mediation of tissue damage in experimental models. Complement depletion blocks damage [45, 46] as does PMN [47] or macrophage removal [48].

Therapy

Therapy of anti-GBM disease has centered around attempts to reduce circulating antibody levels and to reduce inflammatory injury. Treatment consists of aggressive immunosuppression using steroids and azathioprine/cyclophosphamide and intensive plasma exchange regimes [49]. In Chapter 4 investigations of treatment using chemotherapy and plasma exchange separately and using prostaglandin are detailed.

Chapter 2.

The Glomerular Basement Membrane; Binding Properties.

Introduction

Structure and Function of the GBM

The GBM is composed of both collagenous and non-collagenous glycoproteins and also proteoglycans [50, 51]. It functions as a molecular seive, limit 70,000 Daltons, making an ultrafiltrate of the blood for urine production [52]. The membrane consists of 3 layers, the lamina rara interna, lamina rara densa and the lamina rara externa. The lamina densa appears to consist mainly of type IV collagen, whilst the laminae rara are made up of laminin and heparan sulphate proteoglycan [53]. The heparan sulphate provides negative charge which forms the filtration barrier to anions [54].

Antibody Binding to the GBM

Heterologous anti-kidney antibodies were first described by Lindemann in 1900 [55]. The major nephritogenic antigens have since been localised to the GBM [56] and now in human anti-GBM disease to the non-collagenous portion of the type IV collagen [28, 57].

Heterologous serum anti-GBM antibodies (sheep or rabbit) react in a double linear pattern by immunofluorescence [58]. In contrast, the eluted antibody from the kidneys of humans with anti-GBM disease reacts as a single line, as does the antibody eluted from Steblay sheep kidneys [59]. This suggests that the circulating antibody in the sheep disease model has a different specificity from the bound antibody. In humans they appear to have the same reactivity [60]

Binding of Other Compounds to the GBM

Several models of immune responses to non-basement membrane renal antigens have been developed. These include renal tubular antigen (Fx1A) [61] now thought to be an example of *in situ* immune complex formation [62], a form of spontaneous nephritis in the NZW rabbit [4], DNA [10], lectins [9] and charge modified molecules (see next section).

Factors Affecting Localisation of Antigens within the Kidney

The possibility that *in situ* mechanisms of immune damage occur in human disease has led to investigation of the factors affecting the binding of substances to the GBM. It is now well established that molecular charge as well as size affects filtration by the GBM [63-65]. Molecules with a pI greater than 8.5 and a molecular weight between 500,000 and 900,000 will bind *in vivo* [66].

Several experimental models of glomerulonephritis have been developed based on the binding of charge-modified antigens within the kidney. These include cationised ferritin followed by anti-ferritin [67], serial injections of cationic BSA [68], cationised human albumin [69] and cationised IgG [70]. Isaacs and Miller [71] showed that the distribution of immune deposits which developed after infusion of various dextrans was charge dependant. This has also been demonstrated in passive serum sickness [72] and active serum sickness [73] and these studies show that the more cationic the antigen the more nephritogenic and the greater the tendency to form sub-epithelial deposits.

As previously mentioned, post-streptococcal glomerulonephritis is also associated with subepithelial deposits. This raises the possibility that this disease could also have an *in situ* mechanism. Sialic acid depleted IgG can produce immune deposit disease in rats [74]. Patients with post-streptococcal glomerulonephritis may have neuraminidase activity derived from bacterial sources [75] and this might render autologous IgG more cationic by sialic acid removal.

Present Study

An assay to measure the binding of a variety of compounds to human GBM *in vitro* was established. DNA and C1q, which are known to have an affinity for GBM were used as positive controls for the assay and BSA as a negative control. The binding of gold [76], mercury [77] and captopril [78] were measured because these have all been associated with membranous nephropathy:- the prototype form of *in situ* immune deposit glomerulonephritis. In particular, gold has been shown to have an affinity for immunoglobulins and immune complexes [79]. In addition, the binding of cationised IgG together with neuraminidase treated IgG was determined. The study was further extended by measuring the pI of IgG eluted from kidneys of patients with membranous nephropathy and from patients with anti-GBM disease for comparison.

Materials and Methods

GBM Preparation

Normal human kidneys were obtained at autopsy. These were stored frozen at -20° until required. After thawing, cortical tissue was isolated and homogenised using an Ultra-Turrax. The homogenate was seived through a 180μ mesh seive and glomeruli separated using sucrose density sedimentation (1.22 SG, 2000 g, 5 mins). This method was developed from those of Krakowar and Greenspon [56], Spiro [80] and Portis et al [81]. The glomeruli were checked for purity by light microscopy and then sonicated (Branson microprobe, 6x10 sec bursts, 4°). The sonicate was lyophilised for storage.

Reagents

Human gamma globulin	CSL, Victoria
^{195}Au	NEN, Massachusetts
^{203}Hg	NEN, Massachusetts
^{14}C-Captopril	Squibb, New Jersey
^{125}I-C1q	Human, isolated by the EDTA method [82]
^{125}I-BSA	CSL, Victoria
^{14}C-DNA	Plasmid, SSDNA

Cationisation of IgG

Human gamma globulin was cationised using the technique of Danon et al [83]. In this method, carboxyl groups are activated with 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) (Sigma Chemical Co., St Louis, Mo., USA) and then replaced with N,N-dimethyl--1,3-propanediamine (DMPA) (Fluka, Buchs, Switzerland).

Isoelectric Focusing

The extent of cationisation of the IgG was established by isoelectric focusing on polyacrylamide gel slab. A gel of 5%T, 3%C was formed containing Pharmalyte pI range 8.0-10.5. Samples, 5µl (75µg) were applied together with 10µl marker (Pharmacia high pI calibration kit). Focusing was carried out for 2 hours at 2000 volts whilst nitrogen was passed through the apparatus. The gel was then fixed and stained using Coomassie Brilliant Blue.

Radiolabelling

Normal human IgG and cationised IgG were labelled with ^{125}I (NEN, Searle Nucleonics, NSW) by the method of McConahey and Dixon [84].

Neuraminidase Treatment of IgG

Sialic acid residues were removed from human IgG by treatment with neuraminidase [74]. IgG was purified by ion-exchange chromatography and then incubated with neuraminidase (type VI, Sigma Chemical Co., St Louis, Mo., USA) 26 units/g protein. After treatment, the IgG was further purified by 50% ammonium sulphate precipitation and ion-exchange chomatography.

The sialic acid content of the immunoglobulin before and after neuraminidase treatment was measured using a thiobarbituric acid assay [85].

In Vitro Binding Assay

This was based on that developed by Izui et al [10] for testing the binding of DNA to GBM. Lyophilised GBM (300 μg) was sonicated with 0.1 ml 0.15M NaCl and 0.1 ml 0.1M borate buffer pH 8.4 for 1 minute (Branson microprobe, 20% power output).

Control tubes with no GBM were set up. Heat inactivated normal mouse serum (0.1 ml 10%) was added together with 10 μl radiolabelled IgG solution at varying dilutions (10 ng-10 μg) or other radiolabelled compounds. The solutions were shaken at 120 oscillations/minute overnight at 4°. After centrifugation at 1000 g for 20 minute the supernatants were removed and 100μl aliquots were counted in a gamma-counter. The precipitates were also counted.

In Vivo Binding of Modified IgG

Male JC Lewis rats of 200 g were anaesthetised with Nembutal. An incision to expose the left kidney was made and the renal artery located. The kidney was retracted and the artery isolated by blunt dissection. After anchoring the vessel with a loose ligature 1 ml of the appropriate immunoglobulin solution was injected with a 27 gauge hypodermic needle. One hour later, the animals were killed and tissue blocks snap frozen in liquid nitrogen for immunofluorescence.

In an additional experiment, the immunoglobulin solutions were

injected into two rats via the saphenous vein.

Elution Studies

Glomeruli were isolated from kidneys removed at autopsy from patients with membranous nephropathy (2), anti GBM disease (2) and no immune deposits (1). These were eluted with citrate buffer pH 3.2 [86] and the eluates were concentrated and subjected to isoelectric focusing on ultrathin polyacrylamide gel followed by immunoblotting onto nitrocellulose membrane. IgG was specifically located using alkaline phosphatase-anti IgG.

Results

Isoelectric focusing of the cationised IgG showed that all the molecules had a pI > 9.3 compared to a range of 8.0-9.3 for the original immunoglobulin (Figure 2.1).

The thiobarbituric acid assay revealed that neuraminidase treatment reduced the sialic acid content of the IgG from 2.0 to 1.05 μg sialic acid/mg IgG.

In Vitro Binding

Binding of the various compounds to GBM is depicted in Figure 2.2. These data are expressed as follows: the counts specifically bound to GBM are calculated by subtracting counts from tubes without GBM from those with GBM and then expressing them as percentages of the total counts added.

In Vivo Binding

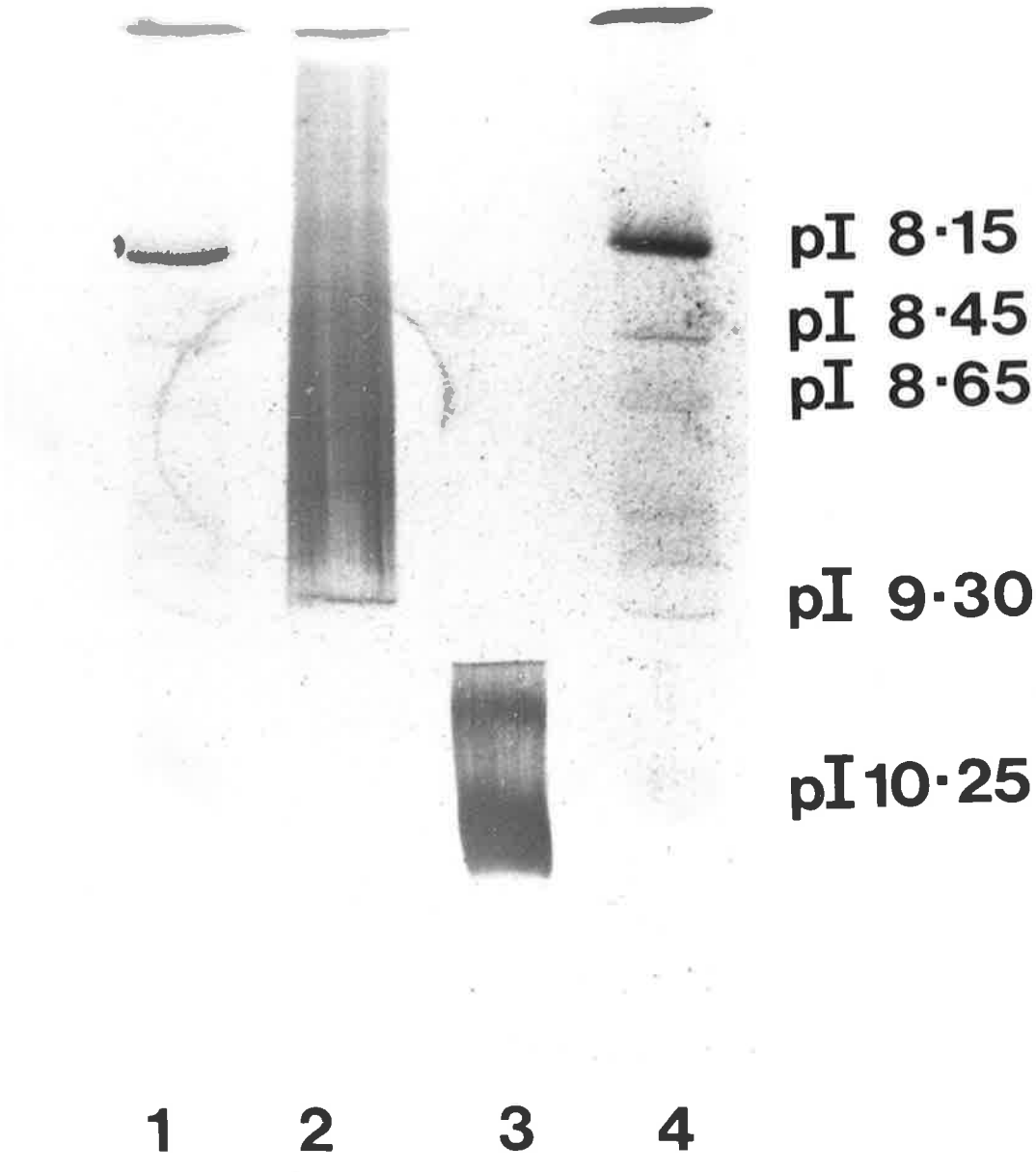
Injection of cationised IgG into the left renal artery caused 1+ glomerular capillary wall fluorescence on direct immunofluorescence examination (Figure 2.3). In contrast, there was no detectable fluorescence following injection of native IgG (Figure 2.4). No fluorescence could be detected following injection of neuraminidase treated IgG either into the renal artery or saphenous vein.

Elution Studies

The results of focusing the glomerular eluates are shown in Figure 2.5. All patient IgG had pI ranges similar to the control values (8-9.3).

FIGURE 2.1

Isoelectric focusing of native IgG (lane 2) and cationised IgG (lane 3). Markers (lanes 1,4) show from the top; lentil lectin (pI 8.15, 8.45, 8.65), trypsinogen (9.30) and cytochrome C (10.25).


pI 8·15
pI 8·45
pI 8·65
pI 9·30
pI 10·25
1
2
3
4

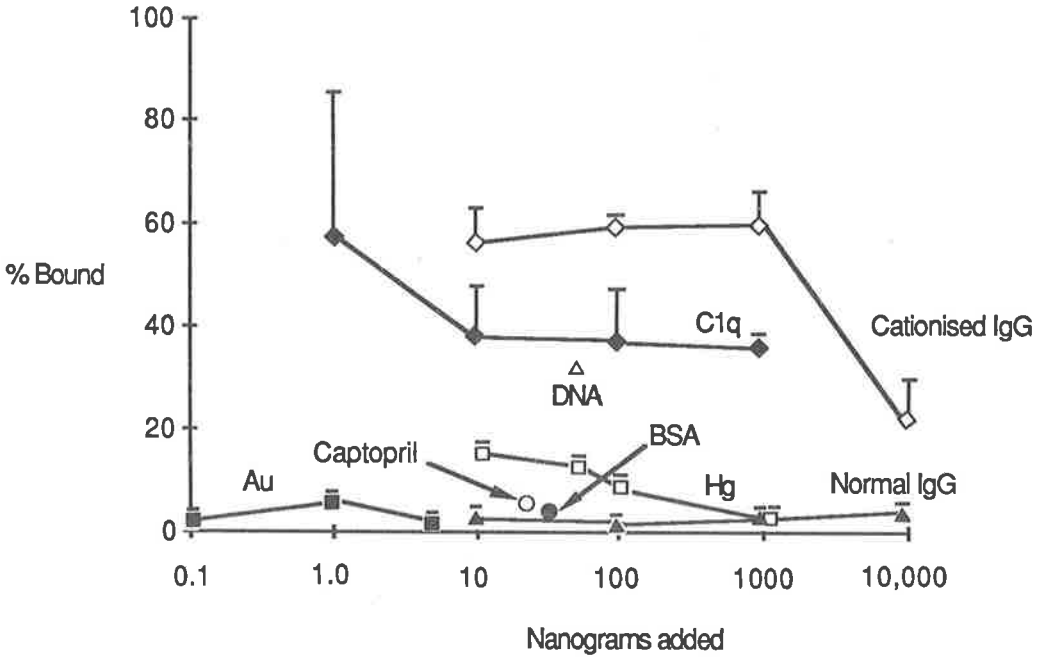


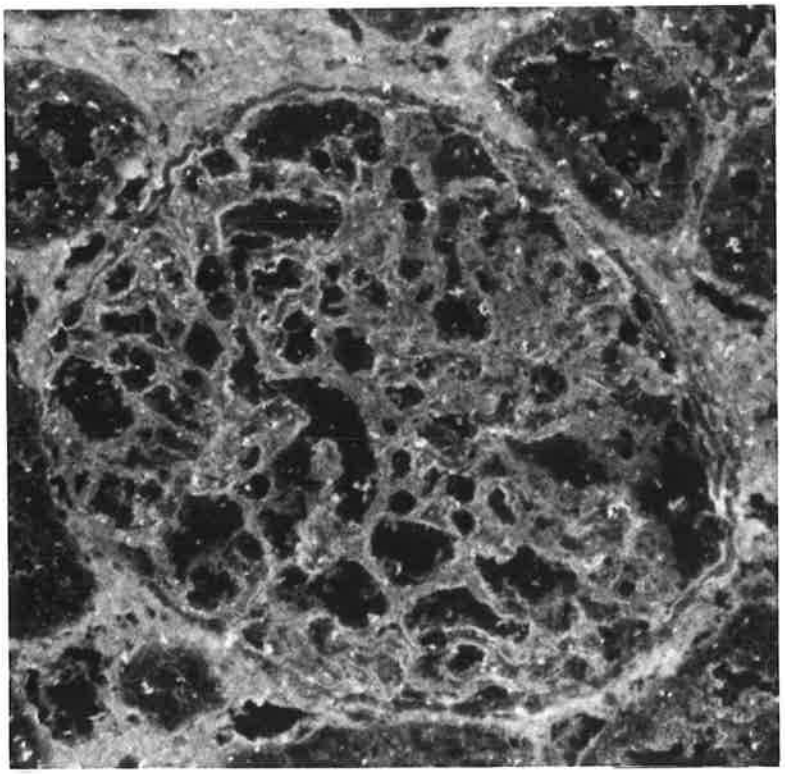
FIGURE 2.2. Binding of various compounds to isolated GBM. Values are mean percent radioactivity specifically bound ± SEM (n=3).

FIGURE 2.3

Direct Immunofluorescence study of rat kidney following injection of cationised IgG (x 157).

FIGURE 2.4

Direct immunofluorescence study of rat kidney following injection of native human IgG (x 157).



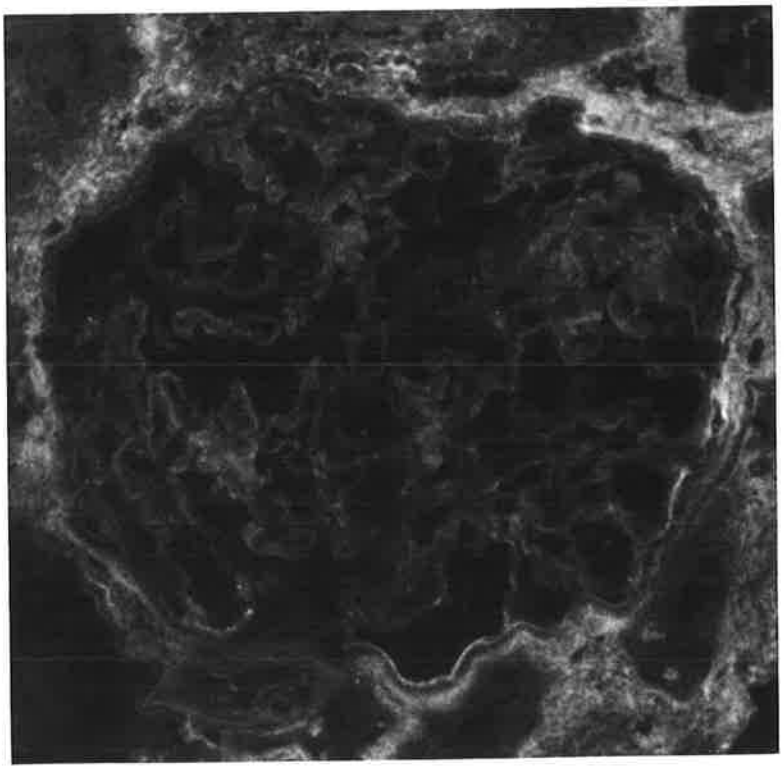
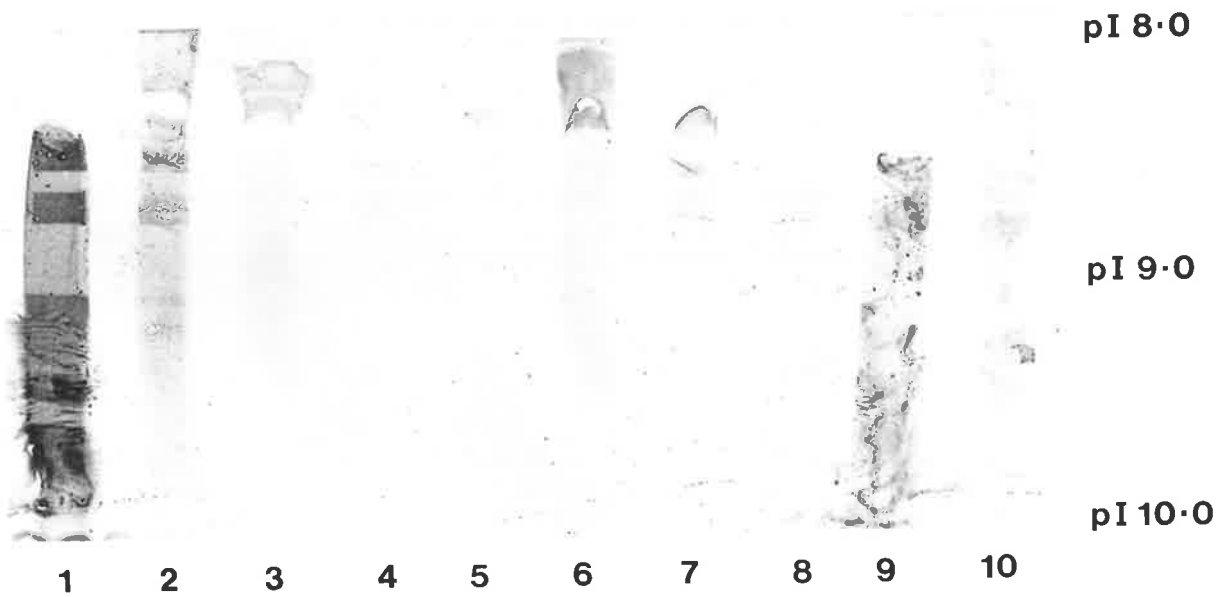


FIGURE 2.5

Immunoblot of human IgG following isoelectric focusing of glomerular eluates on ultrathin polyacrylamide gel.

Lane	
1,9	Cationised IgG
2,10	Native IgG
3,4	Goodpasture's Syndrome Eluates
5	No Immune Deposits Eluate
6,7	Membranous Nephropathy Eluates
8	No Immune Deposits Eluate


pI 8·0
pI 9·0
pI 10·0
1
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10

Discussion

The following general conclusions can be made from the in vitro binding experiments. Cationised IgG, C1q, DNA and possibly mercury bound to GBM. Conversly, gold, BSA, captopril and normal IgG did not bind. Examination of the binding curve of the cationised IgG reveals that between 20 and 60% of the added immunoglobulin could bind to GBM. This compares with less than 5% binding for the native IgG at the same concentrations. The maximum binding reached for the cationised IgG probably reflects that only the most cationic molecules of the range used were able to bind. The decreased proportion bound at the highest dose tested indicates that saturation of the available binding sites had occurred. A maximum of about 2 µg (20% of 10,000 ng) will thus bind to 300 µg GBM. This compares to 12 µg SSDNA which is the maximum that can bind to the same amount of GBM [10]. Recently, Oite et al [87] have also shown that cationised IgG can bind to rat kidney *in vivo.* They found that saturation of one kidney occurred at a dose of about 50 µg.

Removal of sialic acid residues from IgG with neuraminidase did not appear to modify the molecule sufficiently to cause binding. The thiobarbituric acid assay showed that about half the sialic acid residues had been removed; this presumably reflects considerably greater modification of IgG than is likely to occur *in vivo.*

Using the technique described of isoelectric focusing of glomerular eluates followed by immunoblotting, it was not possible to detect IgG of a more cationic nature than normal in glomerular eluates from patients with membranous nephropathy. This argues against such a mechanism in this disease. This of course does not preclude a role for other cations paticularly exogenous antigens in the pathogenesis of membranous nephropathy.

Although granular deposits of IgG and C3 are a feature of gold nephropathy [88], and despite the preferential binding of gold to immunoglobulins and immune complexes [79], gold did not bind to GBM in vitro. This lack of binding is in accordance with the finding by X-ray microanalysis of human renal tissue, which showed that gold localised to proximal tubules and not to glomeruli [76]. The pathogenesis of gold nephropathy is thus more likely to follow either a route similar to that of Heymann's nephritis with the production of autoantibodies to renal tubular epithelial antigens following tubular damage or may result from some other imbalance of the immune system [89].

It seems likely that mercury and captopril-induced membranous nephropathy follow similar immunopathogenic mechanisms since they do not bind directly to the GBM.

Chapter 3.

Anti-Glomerular Basement Membrane-Antibody; Measurement and Properties.

3A. Immunoassay.

3B. Avidity.

Chapter 3A.

Anti-Glomerular Basement Membrane-Antibody; Immunoassay

Introduction

Background

The development of antibodies to the basement membrane is associated with severe, life-threatening disease. The disease is most commonly seen in young males and about 75% require dialysis. Mortality is as high as 20% [13]. Early diagnosis and treatment are vital.

Immunofluorescence

The earliest methods for the diagnosis of anti-GBM-antibody disease utilised direct and indirect immunofluorescence [23, 37]. However, immunofluorescence has the disadvantages of low sensitivity, slow throughput and the requirement for highly skilled operators. There are also a significant number of false positives, particularly from autopsy tissue and from patients with diabetes mellitus [13]. As a result, a number of centres have set up immunoassays for the detection of anti-GBM antibodies.

Immunoassay

The antigen involved in anti-GBM disease has now been shown to be contained in collagenase-resistant fragments of type IV collagen from the GBM [28]. A number of different methods for solubilisation of the GBM to extract the antigenic material have been employed. These include collagenase digestion [90], trypsin digestion [91, 92], heat extraction [93], 8M urea [94] and 6M guanidine-HCl solubilisation [92]. Details of the various

assays published so far are summarised in Table 3.1. Both fluid and solid phase assays have been employed and ^{125}I, ELISA and fluorescence endpoints have been used.

Present Study

In this study, a solid-phase radioimmunoassay for anti-GBM antibodies was set up and used to measure circulating antibody levels in the serum of patients with anti-GBM disease and other forms of glomerulonephritis. The results are compared with those obtained by indirect immunofluorescence.

TABLE 3.1 (after Hunt et al [27])

Authors	Antigen Preparation	Purification
Marquardt et al [95]	3.5M KBr	-
Wilson et al [90]	collagenase	G-200 exclusion
Mahieu et al [93]	heating to 110°	affinity (rabbit anti-GBM)
McPhaul and Mullins [91]	collagenase trypsin	affinity (GPS)
Buffaloe et al [94]	8M urea	G-150 exclusion
Lockwood et al [96]	collagenase	G-200 exclusion
Weislander et al [97]	collagenase	G-200 exclusion

Materials and Methods

Isolation of GBM

GBM from human autopsy kidneys was prepared and lyophilised as described previously (Chapter 2).

Collagenase Purification

Sigma type 1 collagenase was purified using ammonium sulphate precipitation and alumina adsorption to remove non-specific protease activity [98].

Collagenase Digestion

The lyophilised glomeruli (50 mg) were stirred at 37° for 90 mins with collagenase (350µl of 1 mg/ml) in 0.1M tris-acetate buffer pH 7.4 containing 5mM $CaCl_2$. After centrifugation, the soluble material was subjected to Sephacryl S300 gel filtration and the various peaks were analysed for the presence of antigenic material in the assay system.

Iodination

Anti-human IgG was raised in goats by immunisation with human IgG (CSL) and labelled with ^{125}I [84].

Solid Phase RIA

This follows that developed by Lockwood et al [96]. Cooke microtitre plates were coated with CSGBM diluted in 0.15M Phosphate buffer pH 7.0 containing 0.15M NaCl (o/n at 4°). The wells were washed 3 times with buffer and incubated with serum diluted 1:5 with buffer + 0.05% Tween for 3 hours

at room temperature. The plates were washed x3 and radiolabelled anti-IgG diluted in buffer + Tween was added (70,000 cpm) and incubated o/n at 4°. After washing (x3) the wells were cut up and counted in a gamma-counter.

Assay Optimisation

Conditions for the assay were optimised with respect to the molecular size of the antigen (S-300 gel filtration), concentration of antigen in the coating buffer and the pH of the coating buffer.

Patients

Serum from patients with a variety of types of glomerulonephritis was tested using the RIA. Samples were tested using the earliest available sample (usually at first presentation of the patient) and diagnosis was confirmed by a combination of clinical history and immunofluorescence in addition to the RIA.

Results

Glomerular Preparation

The preparation technique yielded about 20% of the kidney as glomeruli, with little tubular contamination. About 20% of the glomeruli retained intact capsules, the remainder being without capsules (Figures 3.1, 3.2).

Purification of the Antigen

Collagenase digestion released a variety of fragments of GBM with differing molecular weights as shown by S-300 gel filtration (Figure 3.3). Investigation revealed that all peaks had antigen present, but that the high molecular weight peak gave the strongest binding (Figure 3.4).

Assay Optimisation

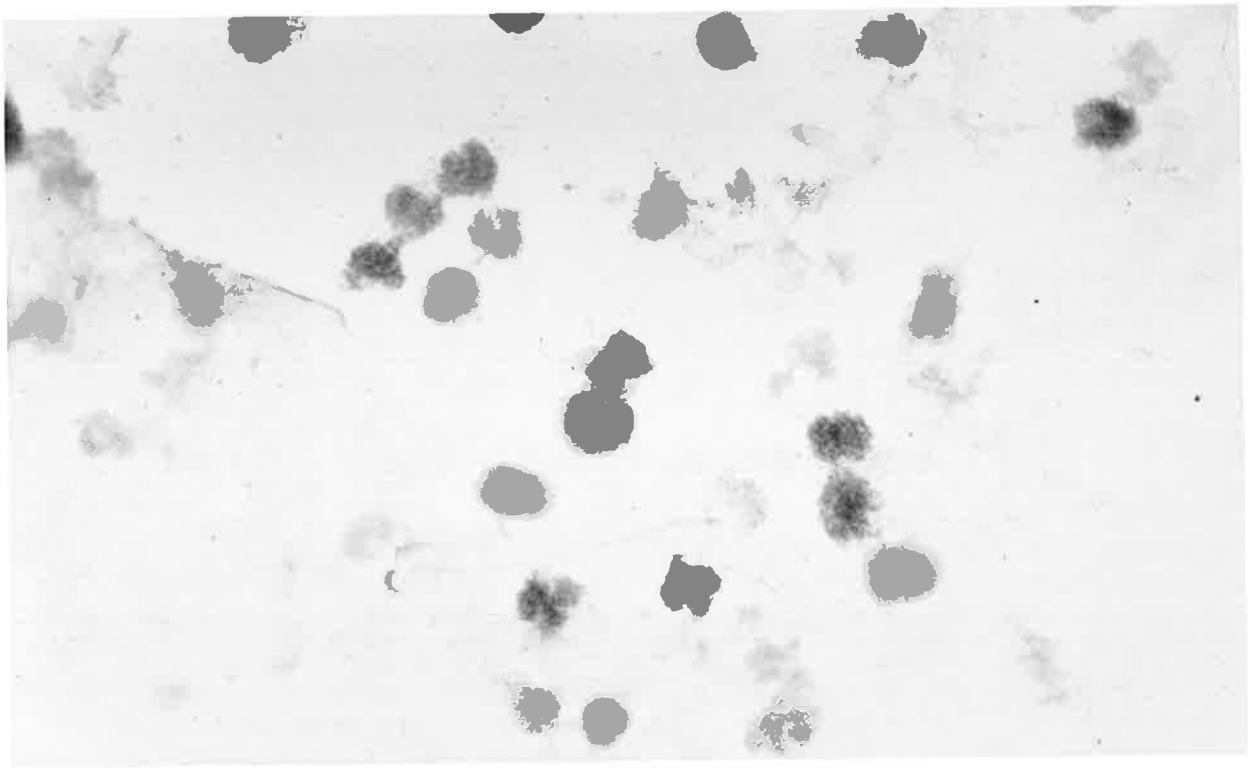
A coating concentration of 50μg/ml and pH of about 7.0 were shown to give best results in the assay (Figures 3.5, 3.6). Using these conditions peak binding of serum from patients with severe anti-GBM disease was about 15% of the total counts added. Serum from normal healthy controls gave binding values of about 2%. There was some inter-assay variation in these figures, presumably due to differences in the binding ability of individual microtitre plates. As a result, both strongly positive and at least 4 negative controls were included in each assay plate.

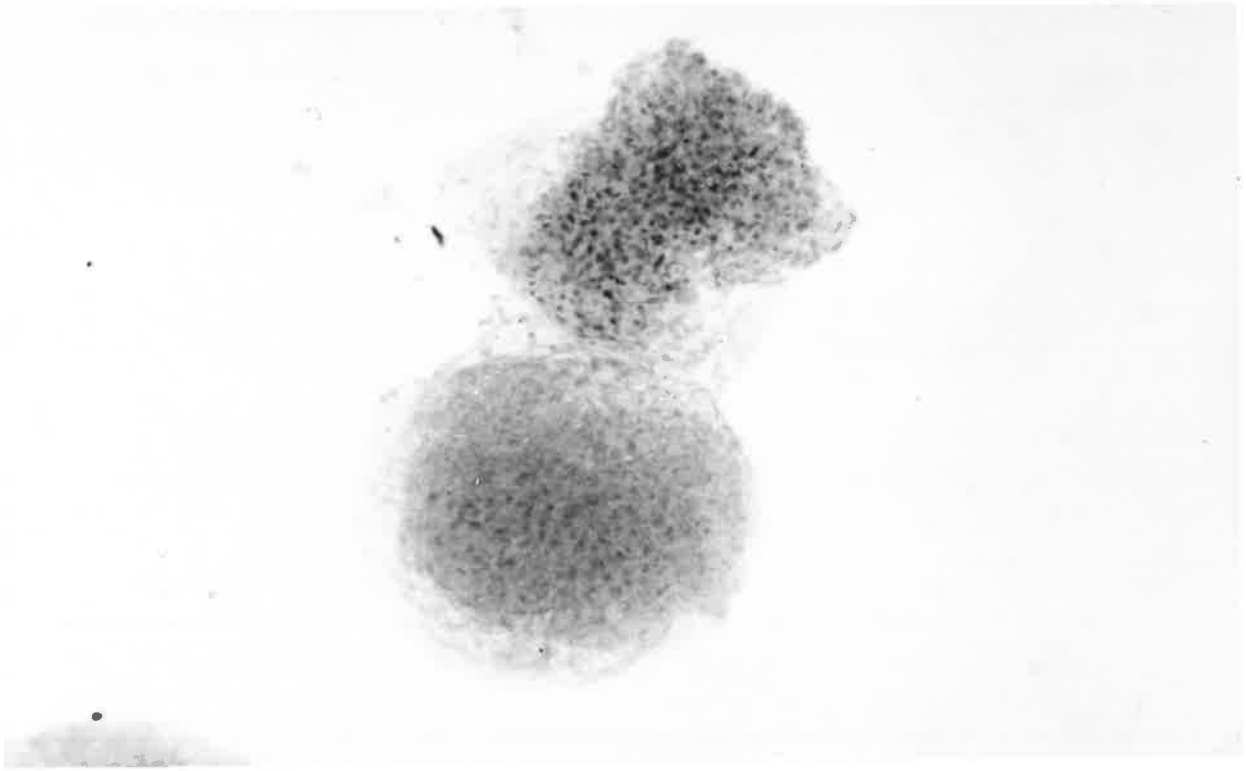
FIGURE 3.1

Light microscopy of isolated human glomeruli stained with haematoxylin and eosin (x15).

FIGURE 3.2

Light microscopy of isolated human glomeruli stained with haematoxylin and eosin (x 70).





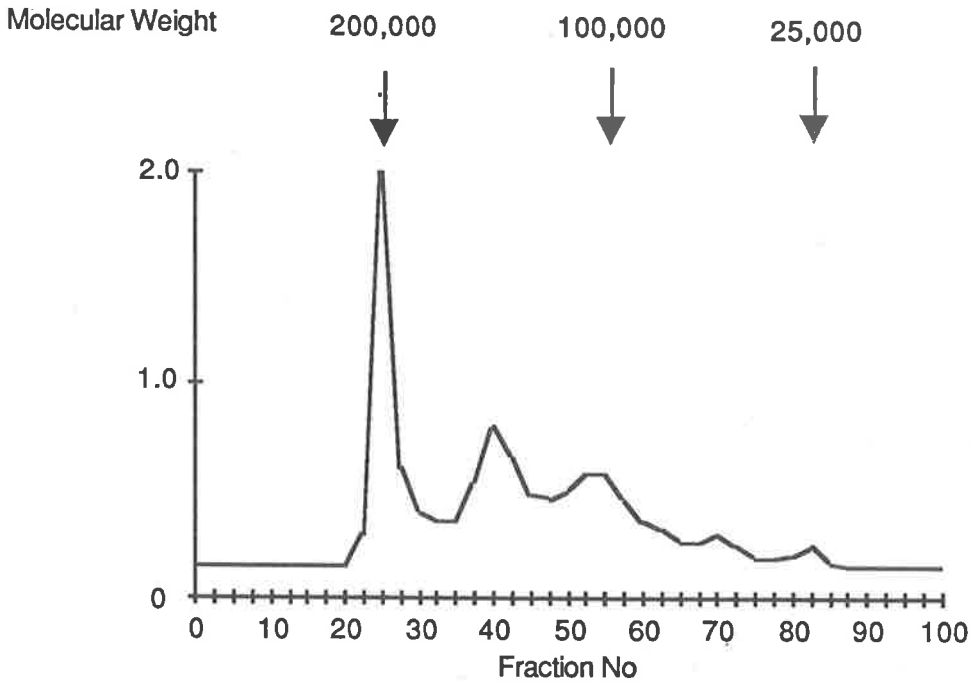


FIGURE 3.3 S-300 Gel filtration of collagenase solubilised GBM.

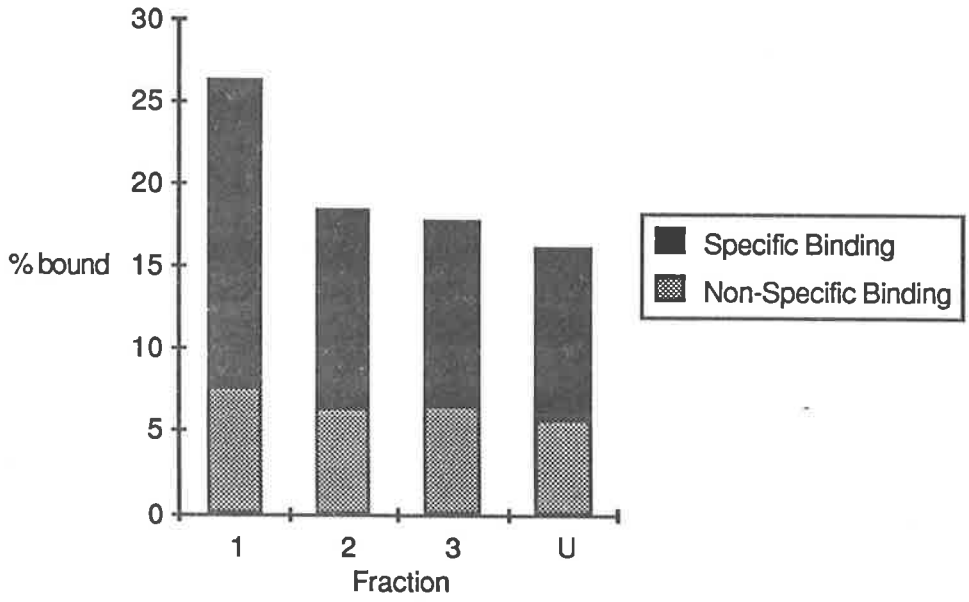


FIGURE 3.4 Binding of anti-GBM antibodies to solid phase antigens (Fraction 1 is the heaviest peak, 2 is the second peak and 3 represents the remaining eluted material. U is the binding of unseparated material)

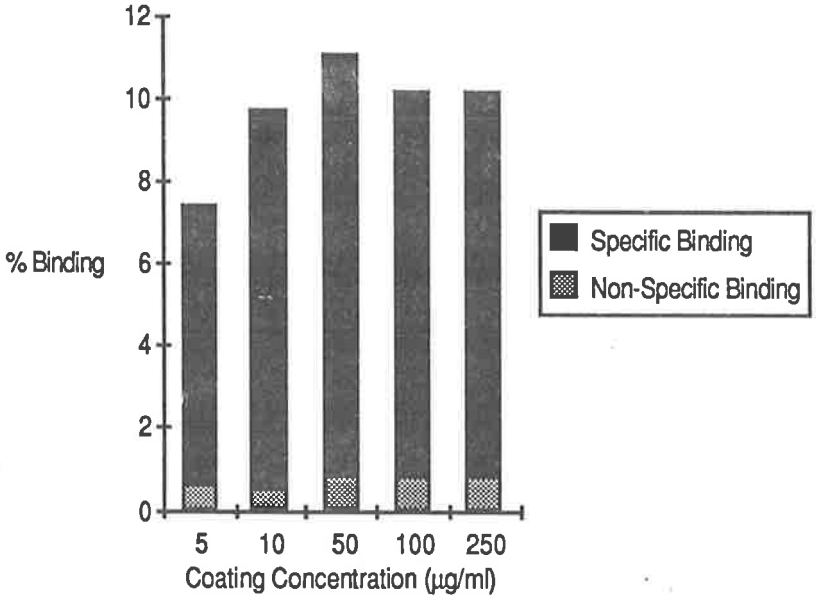


FIGURE 3.5 Effect of increasing concentration of antigen-coating solution on binding in the assay.

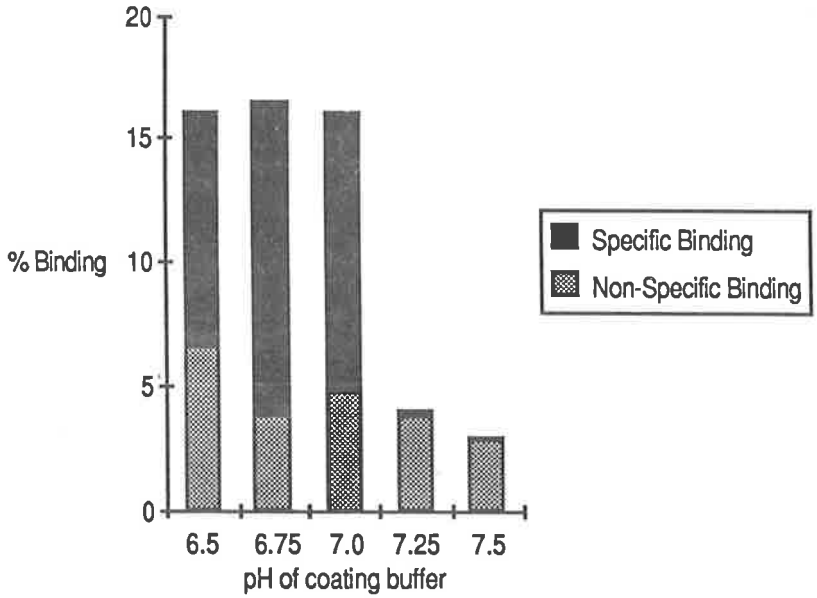


FIGURE 3.6 Effect of pH of coating buffer on binding in the assay.

Assay Results

Titration of an antibody-containing sample gave a curve of reducing binding with increasing dilution (Figure 3.7). Results from 24 Patients with GN are shown in Table 3.2. For comparison 4 serum samples from healthy laboratory staff were measured in each assay. The positive cut-off point was set to exclude all the normal samples.

TABLE 3.2

Diagnosis*	No positive
anti-GBM disease	12/12
SLE	3/9
membranous	1/2
PIGN	0/1

* based on IIF and unequivocal clinical diagnosis

One patient with severe anti-GBM disease was followed with antibody measurements for 7 months during the course of disease. Plasmapheresis was performed on 7 occasions. The antibody binding throughout the course of disease is plotted in Figure 3.8.

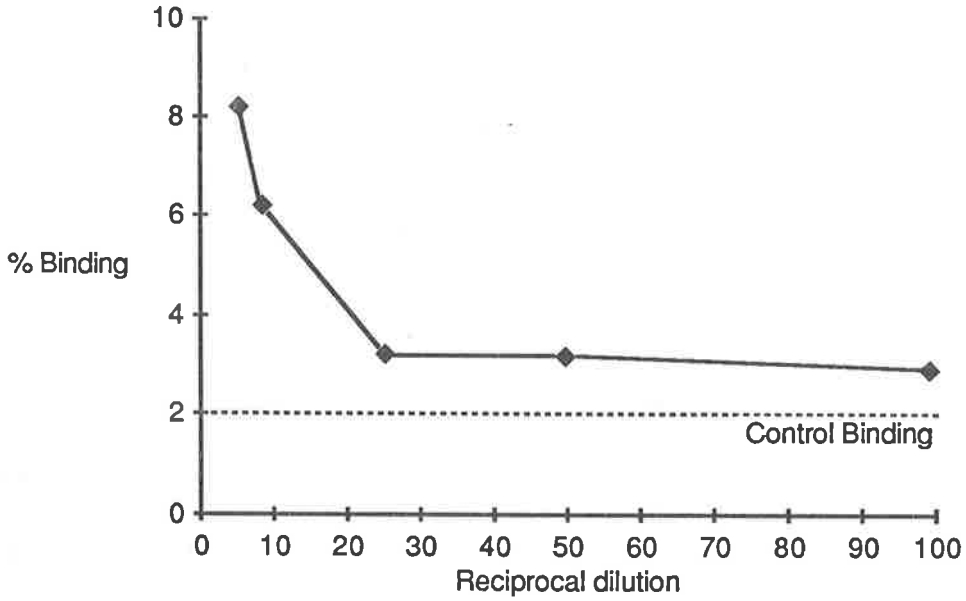


FIGURE 3.7 Reduction of binding with increasing dilution of antibody-containing serum. The binding of undiluted normal control serum in this assay is shown.

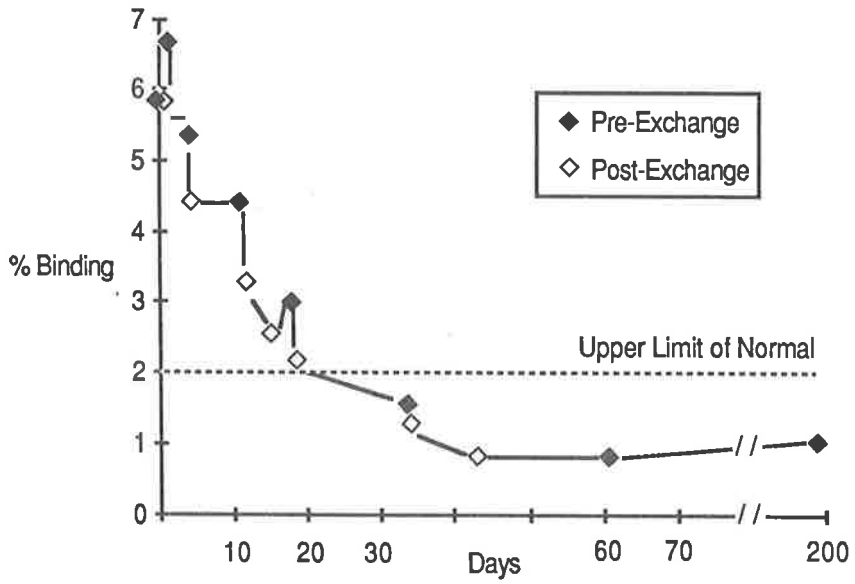


FIGURE 3.8 Time course of disappearance of antibody from serum with treatment

Discussion

A solid phase radioimmunoassay for anti-GBM antibodies was developed. The antigen preparation utilised collagenase digestion which releases non-collagenous glycoproteins. The higher molecular weight fractions were found to give the best figures for binding in the assay, presumably due to there being least modification of these molecules during the extraction procedure and due to their likely better adhesion to the microtitre plate wells in the solid phase assay.

Circulating antibody was detected in all patients with antibody detectable by indirect immunofluorescence. The sensitivity of the RIA appeared to be approximately the same as IIF as evidenced by the number of positive results by each technique and by the fall in antibody level to normal levels with treatment shown in Figure 3.8. No differences in circulating antibody levels could be detected between patients with and without pulmonary haemorrhage, which is in accordance with other authors' findings [13] and antibody levels fell during the course of disease. A high rate of positive results in patients with forms of GN other than anti-GBM antibody disease was seen (17%). Despite some reports of few such occurences in some series (1% of 447 [13], 0% of 14 [92]), other authors have reported a significant number (8.3% of 120 [93], 13% of 190 [27]). The presence of circulating antibodies to the GBM in these other diseases may reflect antigen release from the GBM secondary to the original renal damage and the anti-GBM antibodies are unlikely to be pathogenic. Alternatively, the

antibodies may reflect cross-reactivity in RIA between different antigens. It now seems probable that the Goodpasture antibody is directed against a single antigen, since a monoclonal antibody reacts in identical fashion to the human serum antibody [99] and studies by Wieslander et al [28] in which the binding in ELISA of the larger collagenase soluble antigens are inhibited by the smallest (26,000 Dalton) antigen tend to confirm this. However, the existence of antibodies to alternative glomerular antigens has been demonstrated in SLE [92]. These authors established that guanidine can liberate such antigens from the GBM. Although their collagenase solubilisation procedure did not release the same antigen (Goodpasture's serum did not react with it) it remains possible that other collagenase digestion procedures do release these antigens. Further evidence for the existence of antibodies to intrinsic GBM antigens in SLE was obtained using a mouse model in which monoclonal anti-DNA antibodies bound directly to glomerular antigens *in vivo* [100]. The different buffers and pHs used for coating the microtitre plates in various anti-GBM antibody assays may be responsible for selectively binding different populations of these glomerular antigens resulting in the different numbers of false positives detected.

The radioimmunoassay is clearly of value in the diagnosis and management of anti-GBM disease. However, in our hands it did not offer any great advantage over indirect immunofluorescence in terms of sensitivity or specificity. Despite this, due to the seriousness of the disease, it seems advisable that a central RIA service should be available in addition to routine

immunofluorescent techniques for the diagnosis of anti-GBM disease.

Chapter 3B.

Anti-Glomerular Basement Membrane-Antibody; Avidity.

Introduction

The Significance of Circulating Antibodies in Human Anti-GBM-Disease

The diagnosis of anti-GBM-disease is based on the detection by direct immunofluorescence of linear deposits of immunoglobulin bound to the glomerular basement membrane [13]. Serum anti-GBM-antibodies are found in at least 60% of patients with proven disease using indirect immunofluorescence [23] and with more sensitive methods of detection nearly all patients have detectable antibody [101]. The antibodies are regarded as pathogenic based on the following evidence: 1, eluted antibody from diseased human kidneys will reproduce the renal damage when transferred to monkeys; 2, the disease may recur in the allograft if transplantation is performed whilst there are still circulating antibodies [37].

Steblay Model of Anti-GBM-Disease

This model is induced in sheep with repeated injections of human GBM [15]. High titres of antibody which bind to human GBM appear in the serum as the disease develops. Linear deposits of sheep immunoglobulin are found along the GBM and in the lung (see Chapter 4A). The existence of serum antibody to sheep GBM in this model is harder to demonstrate. Although Steblay and Rudofsky [102] observed these at a titre of 1:20, we were unable to find reactivity even at 1:10 (see Chapter 4A) nor were Lerner and Dixon [17] able to demonstrate this antibody. Nevertheless, the disease can be transferred from sick to well sheep with serum, although large

volumes (1.5 L) [17], or cross-circulation [16] are required. Clearly there is only limited cross-reactivity between sheep and human GBM and few or no antibodies to sheep GBM circulate. Presumably those antibodies that are reactive with sheep tissue are bound *in vivo* as they are produced.

Antibody Avidity*

In contrast to the sheep model, serum anti-GBM-antibodies are common in the human disease. Two possible explanations are suggested: either that the available glomerular binding sites are saturated, or the antibodies have a low binding ability. The possibility that subpopulations of antibodies with different avidities and specificities exist in the kidneys and circulation seems to warrant investigation. Certainly the serum antibody of the Steblay sheep model which binds in a double linear pattern to the GBM differs in specificity from antibody eluted from the kidney which binds as a single line [59]. Additionally, subpopulations of anti-DNA antibodies with different avidities exist in both the NZB/W mouse model of SLE [103] and in the human disease [104].

These observations make the measurement of the avidity of both serum and kidney-bound anti-GBM-antibody of considerable importance to aid in the interpretation of serum antibody estimations. The measurement of the serum antibody avidity from patients with anti-GBM-antibody disease using a competitive RIA technique is described hereafter.

*Note on terminology.

There is much confusion in the literature between the terms affinity and avidity. Since affinity refers strictly to the reaction between a single antibody-combining site and antigenic determinant whilst avidity includes consideration of antibody and antigen valence [105], avidity has been used in this discussion. More accurately the term functional affinity has been coined by Karush [106] but this does not seem to have found general acceptance.

Materials and Methods

Antibody avidity was determined using a competitive radioimmunoassay technique. Increasing amounts of cold antigen were competed with a fixed amount of labelled antigen for the antibody, causing inhibition of binding of the tracer [107]. The fluid phase RIA for anti-GBM-antibodies described by Holdsworth et al [108] was modified to allow a dose-response to be observed. A brief description follows, detailing the modifications necessary for avidity estimation.

Antigen Preparation

Soluble GBM antigen was prepared from human postmortem kidneys by seiving (180μ) and digesting with collagenase (Worthington USA). The antigen was radiolabelled with ^{125}Iodine to a specific activity of 0.2 μCi/μg and dialysed against tris buffered saline(TBS).

Selection of Serum

Sera from 3 patients with severe anti-GBM-antibody disease (diagnosed by direct renal immunofluorescence) were analysed. Serum taken at the first presentation of each patient (before treatment) was used in each case as this had the highest titre of antibody as demonstrated by indirect immunofluorescence.

Elution of glomerular immunoglobulin.

This was performed on one patient (S.J.) who had bilateral nephrectomy for uncontrolled hypertension. Elution was performed using citrate buffer pH 3.2 [86].

Radioimmunoassay

All measurements were carried out in triplicate. Serum aliquots (50µl) were mixed with 50µl of antigen in TBS and incubated overnight at 4°. The bound antigen was precipitated by incubation at 4° for 1 hour with 250µl goat anti-human IgG. This had previously been shown to be in excess of the amount required to precipitate all the IgG in this volume of serum. The tubes were centrifuged and the precipitates washed to allow counting of specifically bound radioactivity.

Avidity Estimation

A dose of 0.2 µmole ^{125}I-GBM (molarity calculated using a molecular weight of 26000 [28]) was found to give maximum binding. This fixed amount of radioactive antigen was used in each case with increasing amounts (0.2-3.4 µmole) of unlabelled antigen added to compete with the radioactive antigen. Calculation of the dose of unlabelled antigen required to inhibit 50% of the specific binding allowed estimation of the average avidity using the formula described by Müller [107] (see next page).

$$K = 8 / 3([I_t]-[T_t])$$

where T_t is the total tracer concentration

and I_t is the inhibitor concentration when binding is 50% inhibited.

Results

Modification of RIA

In order to measure antibody avidity it was necessary to modify the conditions of the radioimmunoassay so that a dose-response could be observed. This principally involved increasing the concentration of the reactants to drive the equilibrium of the reaction towards the bound state. The amount of serum was increased from 25µl to 50µl and the antigen concentration was increased to 0.75µmole. The increased antigen concentration necessitated reducing the specific activity of the radiolabel on the antigen. The increase in binding with increasing antigen dose is shown in Figure 3.9.

Inhibition of Binding of Serum Antibody

Addition of unlabelled antigen to fixed amounts of radioactive antigen and serum antibody caused inhibition of binding. Results for each patient are plotted in Figures 3.10 - 3.12. The data are shown as Scatchard plots of Bound/Free counts vs. µmole antigen Bound.

The percentage inhibition of binding was calculated using the cpm bound in the absence of unlabelled inhibitor as 0% inhibition and the cpm in the absence of antibody (normal serum) as 100% inhibition. Data from all 3 patients are plotted in Figure 3.13, together with the line of best fit by least squares regression. (This was used due to the high variance of the data, giving a correlation coefficient $r = 0.60$ ($p<0.02$ for 13 degrees of freedom).)

The line of best fit was extrapolated to estimate the dose causing 50% inhibition of binding compared to that bound in the absence of inhibitor. This figure (5.3 μmole) was used to calculate the average avidity of the serum anti-GBM-antibodies which is shown in Table 3.3, together with the individual patient's avidities calculated from the slope of the Scatchard plots.

Eluate Binding Studies.

The quantity of antibody recovered by elution precluded avidity estimation as binding above background could not be demonstrated.

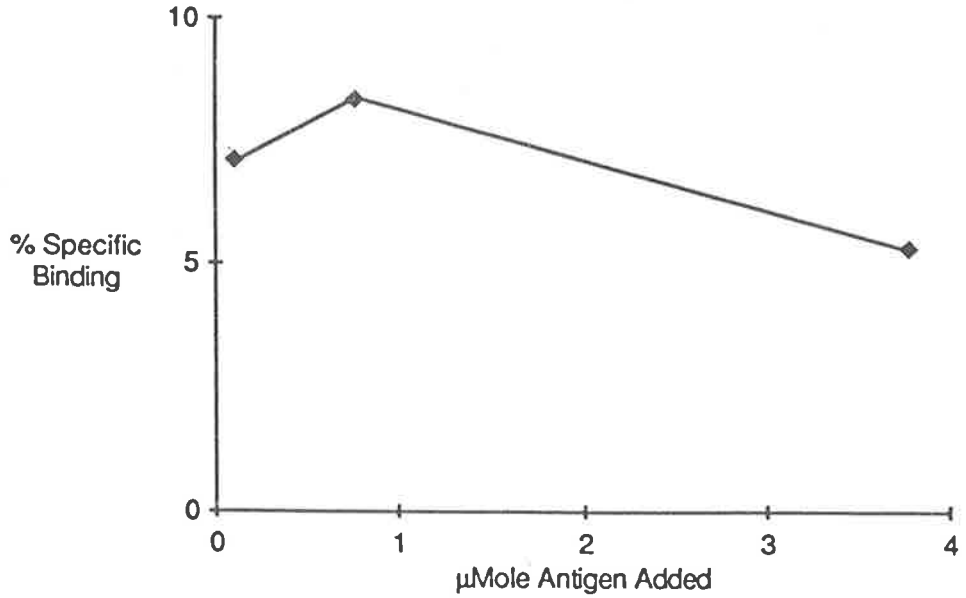


FIGURE 3.9 Number of counts bound with increasing doses of radioactive antigen added to a fixed quantity of antibody-containing serum. Non-specific binding has been subtracted.

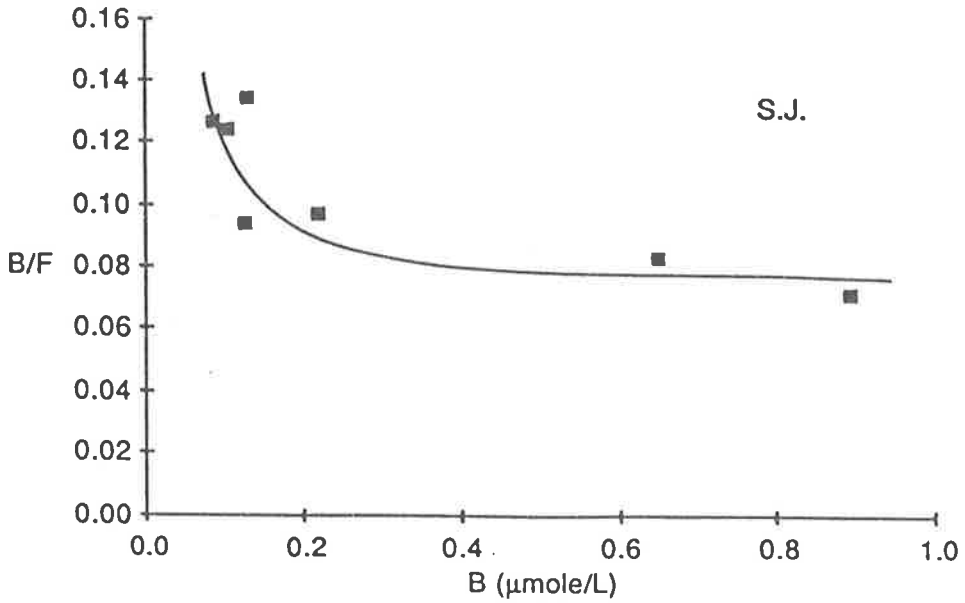


FIGURE 3.10 Scatchard plot of serum antibody from patient S.J.

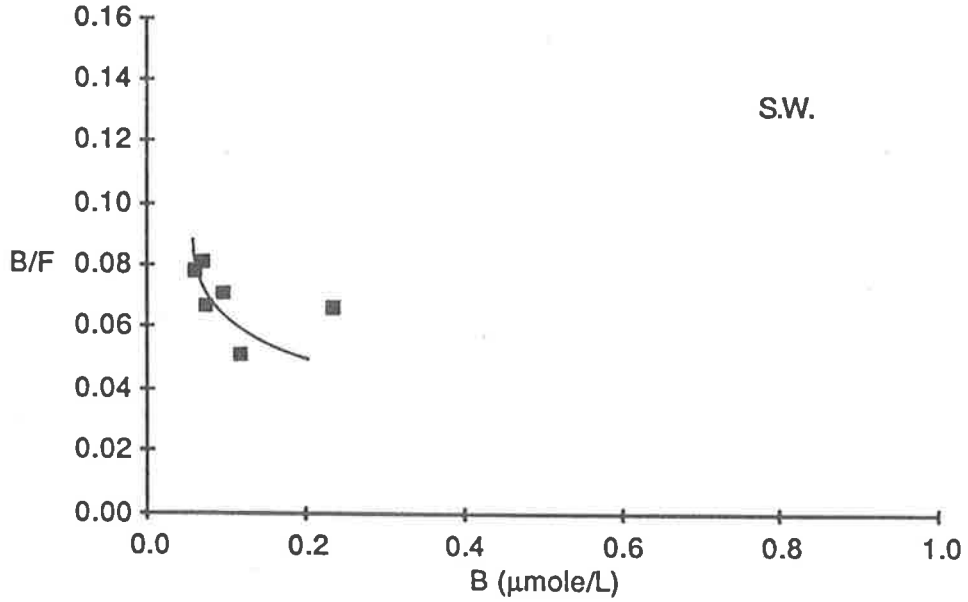


FIGURE 3.11 Scatchard plot of serum antibody from patient S.W.

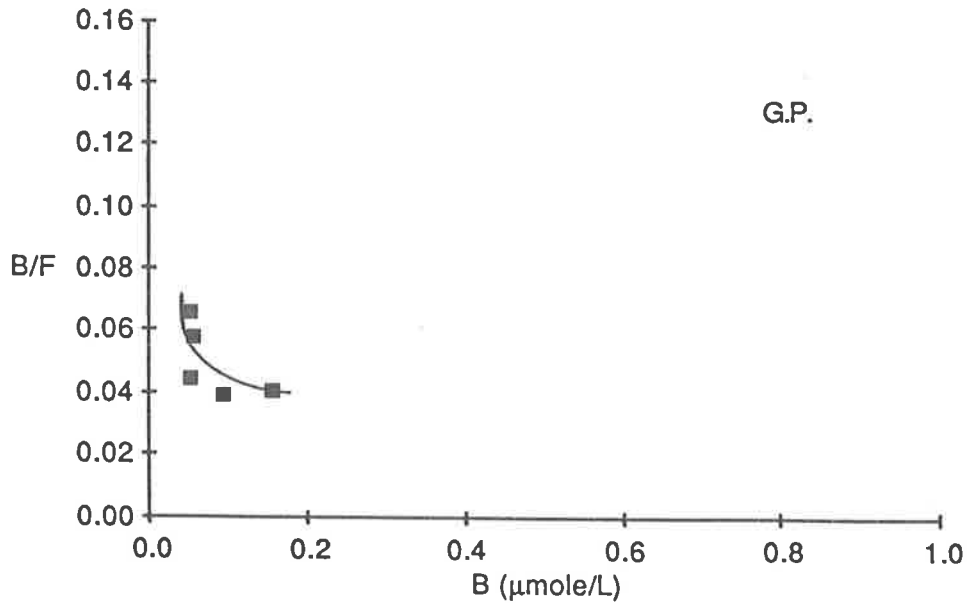


FIGURE 3.12 Scatchard plot of serum antibody from patient G.P.

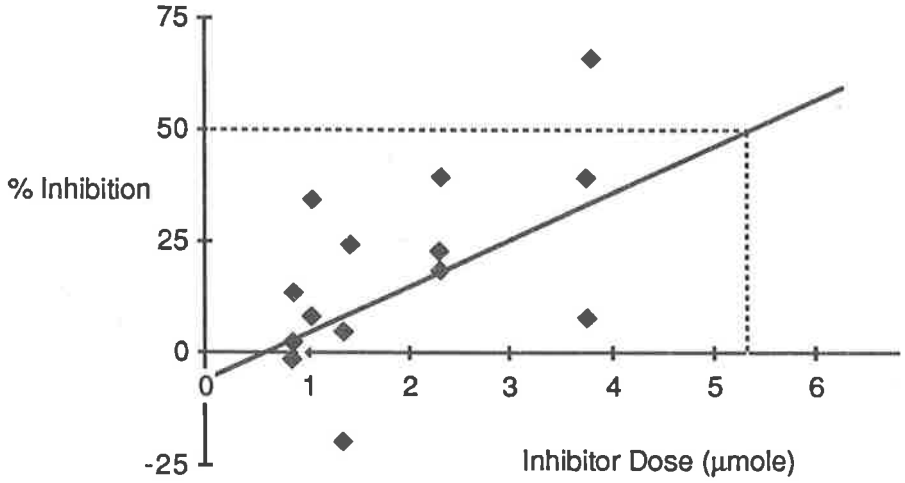


FIGURE 3.13 Inhibition of binding with increasing doses of inhibitor. All points are plotted where 3 estimates of binding at a given dose were obtained. The line of best fit by linear regression is shown with the interpolated dose causing 50% inhibition.

TABLE 3.3

Patient	Serum Avidity K (L/mole)
Mean*	5.9×10^5
S.J.†	3.1×10^5
S.W.†	6.1×10^5
G.P.†	2.7×10^6

*Average avidity calculated from 50% inhibition of binding (mean of 3 sera).
†Calculated from maximum slope of Scatchard Plots.

Discussion

Serum anti-GBM-antibodies were shown to have avidities in the region of 10^5. This is the low end of the range 10^5-10^{11} for anti-protein and anti-carbohydrate antibody avidities [106].

Estimation of the avidity of low avidity antibodies has inherent problems. There is non-linearity of binding curves and inhibitions of more than about 30% are unobtainable [107]. Additionally some of the mathematical assumptions are not entirely valid. This means that K values in the region of 10^5 are at best only estimates. Despite this, the avidity of serum anti-GBM-antibody is clearly of this order.

A number of assumptions have been made in this work. The possibility exists that the antigen used for RIA is grossly altered by the extraction procedure and may bear little resemblance to the *in vivo* antigen. In addition, different patients may have antibodies of different specificity as well as avidity. The avidity of antibodies against such different antigens could also differ. Examination of the Scatchard plots indicates considerable heterogeneity of the antibodies within a patient's serum shown by the non-linearity of the plots. The individual curves also indicate some differences of the binding behaviour between patients. In the calculation of avidity, it is assumed that the antibody is initially free, that is, it is not bound to antigen. GBM antigen certainly exists in serum [33, 110] and is therefore presumably in equilibrium with it. It seems unlikely though, that this will

markedly alter the measured avidity since the amount of circulating antigen is negligible compared to the amount added in the assay.

Despite the above qualifications, the low avidity found here indicates that the serum antibody binds poorly to GBM. It lends support to the hypothesis that it is low binding avidity rather than saturation of glomerular binding sites that is responsible for the appearance in the circulation of antibody. This is in agreement with the data for anti-DNA-antibody in SLE [104]. The implication is that antibodies of high avidity bind immediately as they are formed whilst the low avidity antibodies circulate. This could explain the imperfect correlation of serum anti-GBM-antibody levels with severity of disease in humans [13]. The fact that nephrectomy does not lead to rapid increases in circulating antibody levels [13] is also in accordance. This hypothesis also offers an explanation for the high circulating anti-human GBM-antibody titre found, together with the low or absent circulating anti-sheep GBM-antibody titre, in the Steblay disease model.

Although these observations question the role of circulating anti-GBM antibody measurements in the pathogenesis of anti-GBM disease, the estimation of circulating antibody levels remains valuable for diagnostic purposes and for indicating when antibody production (both relevant and irrelevant) during the course of anti-GBM-disease has ceased.

Chapter 4.

Experimental Investigations in the Treatment of Anti-Glomerular Basement Membrane Antibody-Induced Disease.

Background.

4A. Separate Use of Plasma Exchange and Chemotherapy.

4B. Use of Prostaglandin PGE_1.

Chapter 4. Experimental Treatments of Anti-GBM Disease.

Background

The early attempts at the treatment of anti-GBM-antibody disease were largely unsuccessful. Before the advent of haemo-dialysis, mortality was as high as 80% [110]. Removal of the supposed antigen source by performing bilateral nephrectomy was at one time practised [111], but these heroic measures are no longer favoured. High doses of steroids appear to help arrest glomerular damage and remain in use today. In addition to steroids, combined immunosuppressive therapy and plasmapheresis, which were advocated by Lockwood et al in 1975 [112] and later by others [113], now form the modern approach to the treatment of the disease, and are the subject of a controlled trial currently being performed in the USA. The overall mortality has now fallen to less than 25% [13].

In this study, two approaches to the treatment of anti-GBM-antibody disease have been investigated. These are first, the separate use of plasma exchange therapy and chemotherapy (chapter 4A), and second the use of the prostaglandin PGE_1 (chapter 4B).

Chapter 4A.

Experimental Treatment of Anti-GBM Disease; Separate Use of Plasma Exchange and Chemotherapy

Introduction

The apparent success of combined plasma exchange (PE) therapy and chemotherapy (CT) has made it impossible to perform a controlled trial in humans of these treatments separately. An animal study was therefore required. One of the classic animal models of the disease caused by circulating antibodies to the GBM was first described by Steblay [15]. The disease is induced by repeated immunisation of sheep with human GBM and Freund's complete adjuvant. There is progressive renal failure and death follows 2-3 months after the first injection.

This model has since been the subject of extensive study to elucidate the pathogenesis involved (reviewed by Steblay [114]). In addition to the work from Steblay's group, Dixon and others from Scripp's Clinic and Research Foundation have also studied this model. Both groups performed experiments to show that the disease could be transferred from sick to healthy animals using serum [17, 115]. Later, Steblay and Rudofsky [18] eluted antibody from the diseased sheep kidney and found it bound to both sheep and human kidney (and to lung BM).

Thus this animal model appears useful for studying the treatment of human anti-GBM-antibody disease. The model is induced by circulating antibody which is reactive against the animal's own kidney. The size of the sheep's blood vessels allows suitable access for shunting to allow PE to be performed.

Materials and Methods

Immunogen

Human glomeruli were isolated and sonicated as previously described in Chapter 2.

Immunisation

Six month old sheep (approximate wt. 25 kg) fed a standard diet of 2 kg lucerne chaff per day were used for the study. The human glomerular sonicate (300 mg/ml), emulsified with an equal volume of Freund's complete adjuvant, was injected into multiple sites. The majority was given intramuscularly, with smaller quantities injected subcutaneously and intradermally. The immunisation schedules were varied slightly when difficulty in inducing disease was experienced. The exact details are given in Table 4.1. Essentially each animal received 150 mg doses of glomerular sonicate at 2 weekly intervals until it was moribund.

Plasma Exchange Procedure

Plasma exchange was performed via an arterio-venous shunt inserted between the right common carotid artery and the right external jugular vein. The operation was performed once renal clearance had deteriorated to 50% of normal; 1 day later PE therapy was initiated. Three daily exchanges, followed by 2 rest days, followed by 3 daily exchanges were performed. In each case 1 litre of plasma was exchanged with 1 litre Haemaccel using a Plasmaflux filter (Fresenius). 5000 units of heparin were introduced before

the exchange and then a further 2000 units of heparin in the Haemaccel. Blood was taken before and after the exchange from the arterial line.

Chemotherapy

Stock solutions of cyclophosphamide (Sigma Chemical Co., St Louis, Mo, USA) and prednisolone (Glaxo, Boronia, Victoria) were prepared at 2.5% (w/v) in normal saline and filtered using a 0.22μ Millipore filter. They were stored at 4°.

TABLE 4.1

SHEEP CODE NUMBER		TREATMENT
135, 160, 129, 143, 067 211, 038, 062, 013 + 7 others which did not develop severe renal failure	(PE) (CT)	150 mg sonicated glomeruli in 1 ml FCA biweekly for 20 weeks, then 150 mg in 7.5 ml FCA biweekly
1797 + 3 others which did not develop severe renal failure	(control)	150 mg sonicated glomeruli in 1 ml FCA biweekly for 13 weeks, then 150 mg in 7.5 ml FCA biweekly
36,32 + 1 other which did not develop severe renal failure	(control)	150 mg sonicated glomeruli in 1.6 ml FCA biweekly
147 + 2 others which did not severe renal failure	(control)	50 mg sonicated glomeruli in 1.6 ml FCA biweekly

After renal clearance had fallen to 50% of normal, daily intravenous injections of 2 ml (50 mg) cyclophosphamide and 4 ml (100 mg) prednisolone were given. This corresponds to a dose of 2 mg/kg/day of cyclophosphamide and 4 mg/kg/day of prednisolone. Injections were continued until the sheep were considered moribund, whereupon they were killed. The sheep were also given 5000 units of heparin on the days when paired PE sheep were plasma exchanged.

Renal Clearance

This was measured using ^{51}Cr EDTA (Australian Atomic Energy Commission). Four ml of the isotope containing 2.4 mBq diluted in isotonic saline were injected into the right external jugular vein. Two ml blood samples were collected 0, 2.5, 3, 3.5 and 4 hours later. The plasma was separated from the cells and 1 ml aliquots were counted in a gamma-counter with the appropriate spectral settings for 20 minutes. The renal clearance of ^{51}Cr EDTA was calculated from the line of best fit of the plot of $\log_e$ plasma concentration vs. time.

Immunofluorescence

Indirect immunofluorescence was performed on 2μ frozen sections of normal human or sheep kidney. The sections were overlaid with 20μl sheep serum for 30 minutes. After washing with PBS, bound immunoglobulin was detected using 20μl FITC goat anti-sheep IgG. Fluorescence was visually

assessed and graded 0-3+ by a "blind" operator.

Direct immunofluorescence was performed on frozen sections of kidney tissue removed at autopsy and mounted in Tissue-Tek. Immunoglobulin deposits were visualised using FITC-labelled anti-immunoglobulin.

Elution

Sample blocks from the kidneys of several sheep were eluted using the method of Woodroffe and Wilson [86].

Results

Disease Development

Thirteen out of 26 sheep injected with human GBM did not develop severe renal disease (as defined by a decrease in ^{51}Cr EDTA clearance to less than half of the original value). The remainder of the sheep developed severe renal disease over a varying time course, from 7-29 weeks.

Direct immunofluorescence performed on kidney sections from sheep with disease showed strong linear GBM (3+) (and TBM) staining (Figure 4.1, 4.2). Indirect immunofluorescence on sheep which developed renal failure demonstrated at least 2+ GBM staining on human tissue targets in all cases at some stage of the experiment. In addition 9 of 10 sheep which did not develop renal failure had positive GBM staining.

Survival

Once disease had developed, survival times varied in the different experimental groups as shown in Table 4.2. The increased survival of both PE (8.2 weeks) and CT (6.5 weeks) groups compared to control animals (2.2 weeks) was statistically significant ($p < 0.05$ Mann-Whitney U test).

Renal Clearance

^{51}Cr EDTA clearances are shown in Table 4.2 and in Figures 4.3, 4.4 and 4.5 for controls, PE and CT groups. Renal clearance in the control and PE groups declined to figures approaching zero just before death. In contrast, the clearances of the CT group remained stable or increased

following treatment.

TABLE 4.2

	Initial Clearance (ml/min)	Pre-terminal Clearance (ml/min)	Disease Onset Time (weeks)	Survival After Onset (weeks)
Control	67.2 ± 9.0	3.2 ± 0.9	13.2 ± 2.4	2.2 ± 0.8
PE	55.8 ± 3.0	5.0 ± 3.2	17.8 ± 5.0	8.2 ± 1.2
CT	52.2 ± 5.8	37.8 ± 5.3	13.8 ± 3.5	6.5 ± 0.6

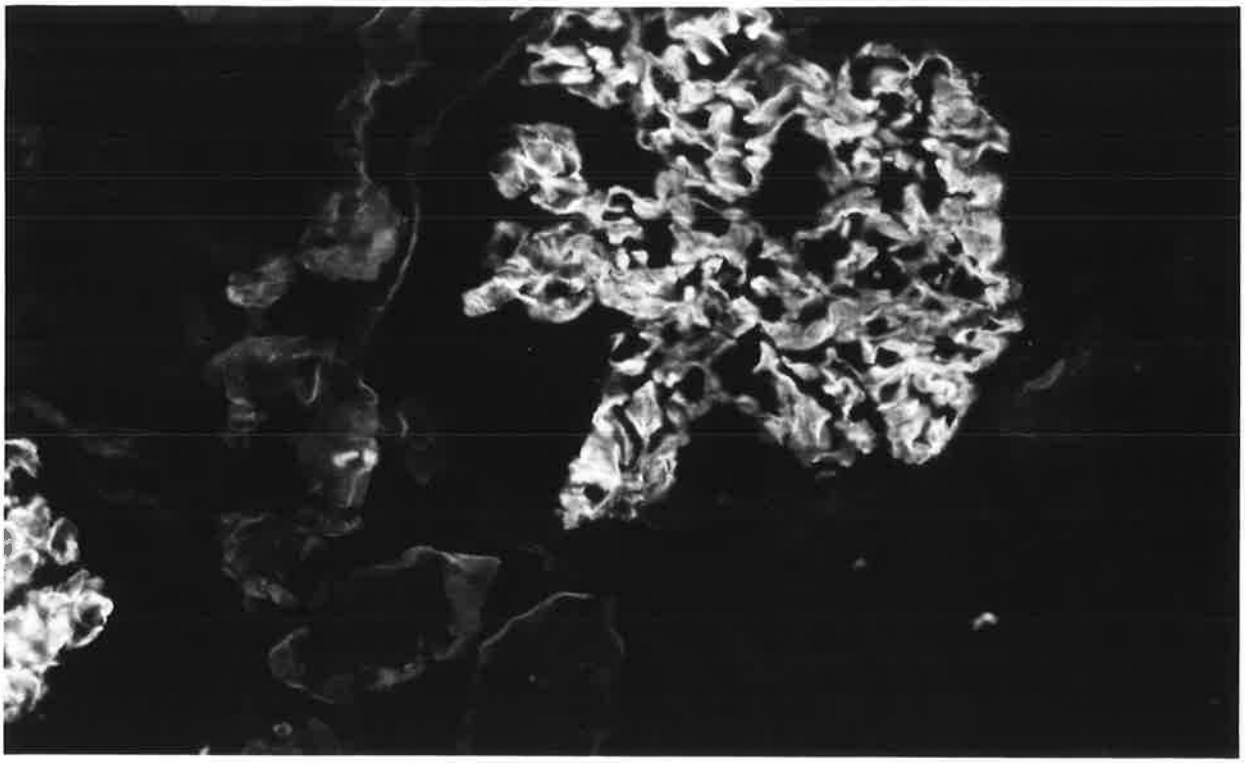
all values are mean ± SEM (n = 4)

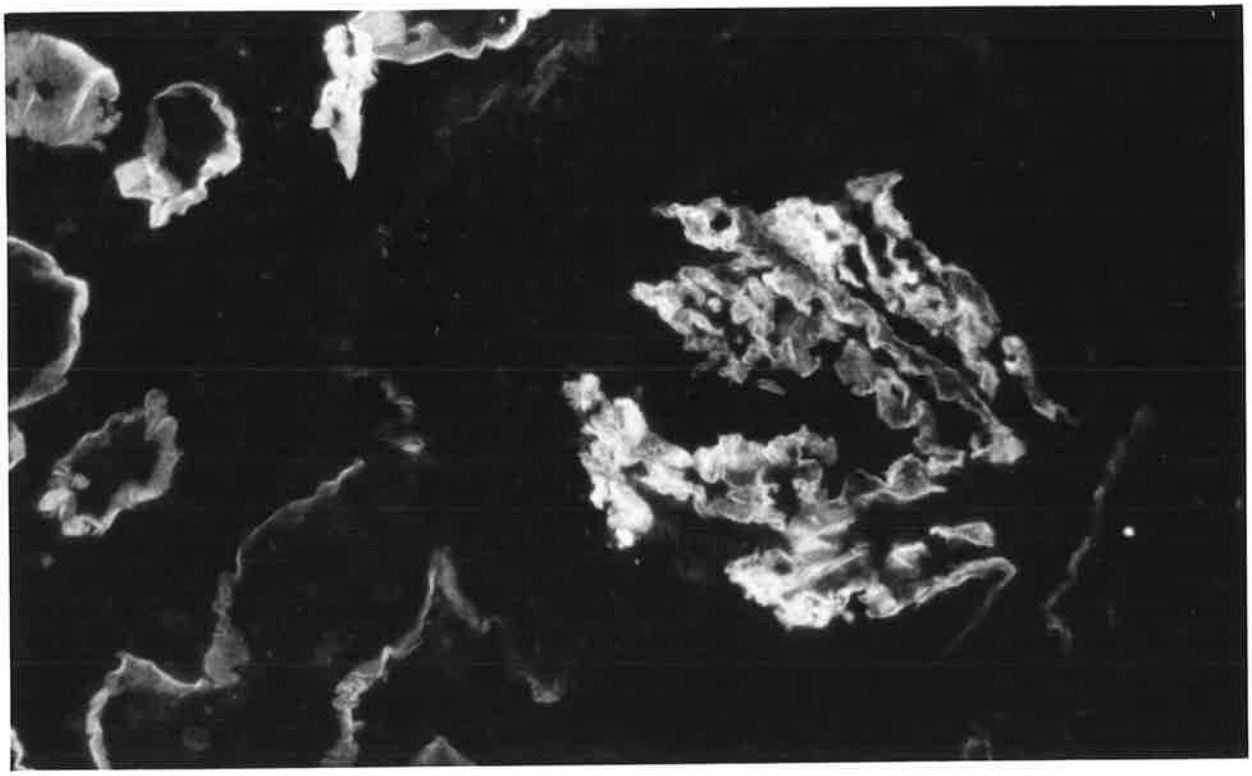
FIGURE 4.1

Direct immunofluorescence of sheep glomerulus showing 3+ linear GBM staining for IgG (x 250).

FIGURE 4.2

Direct immunofluorescence of sheep glomerulus showing 3+ linear GBM and TBM staining for IgG (x 250).





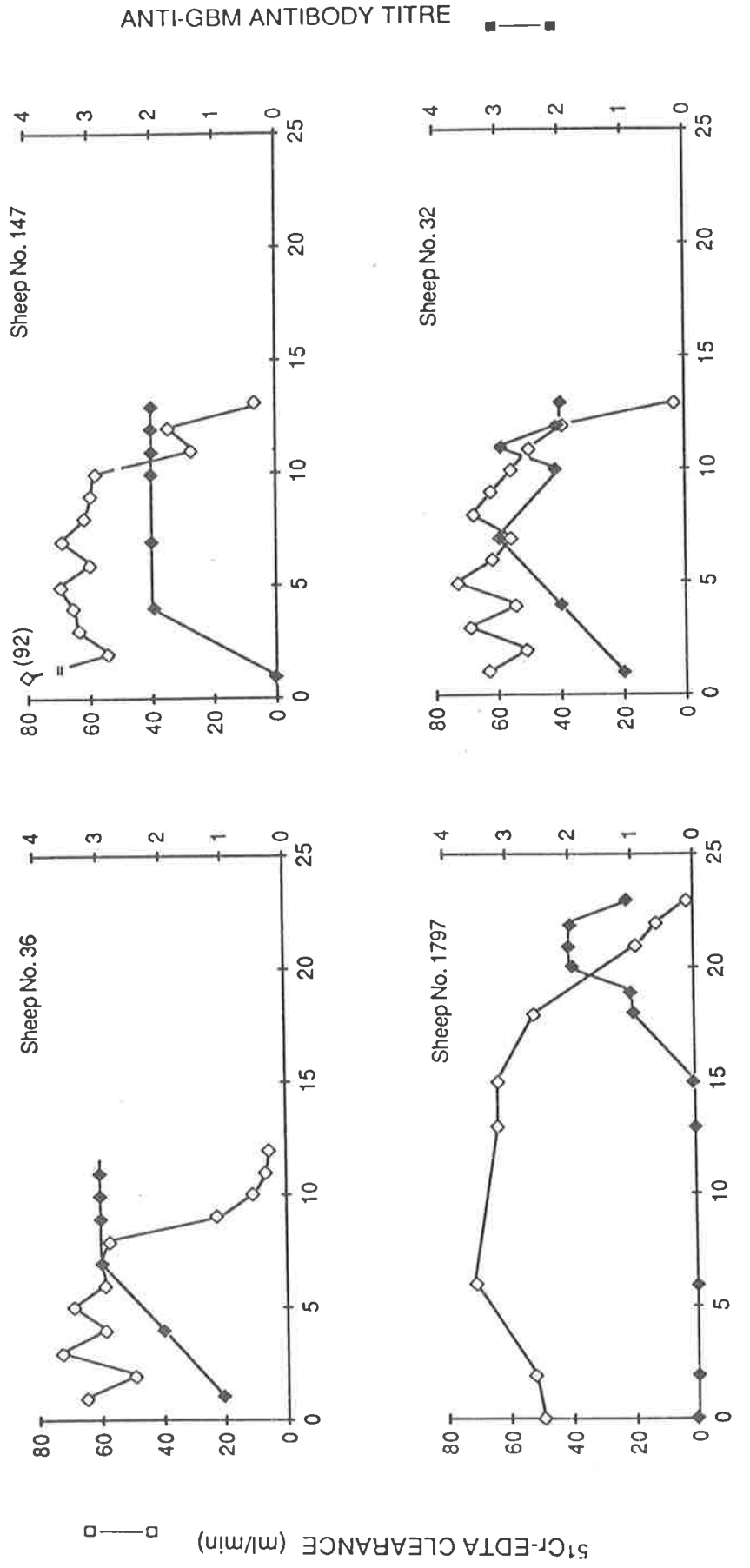


FIGURE 4.3 (Control)

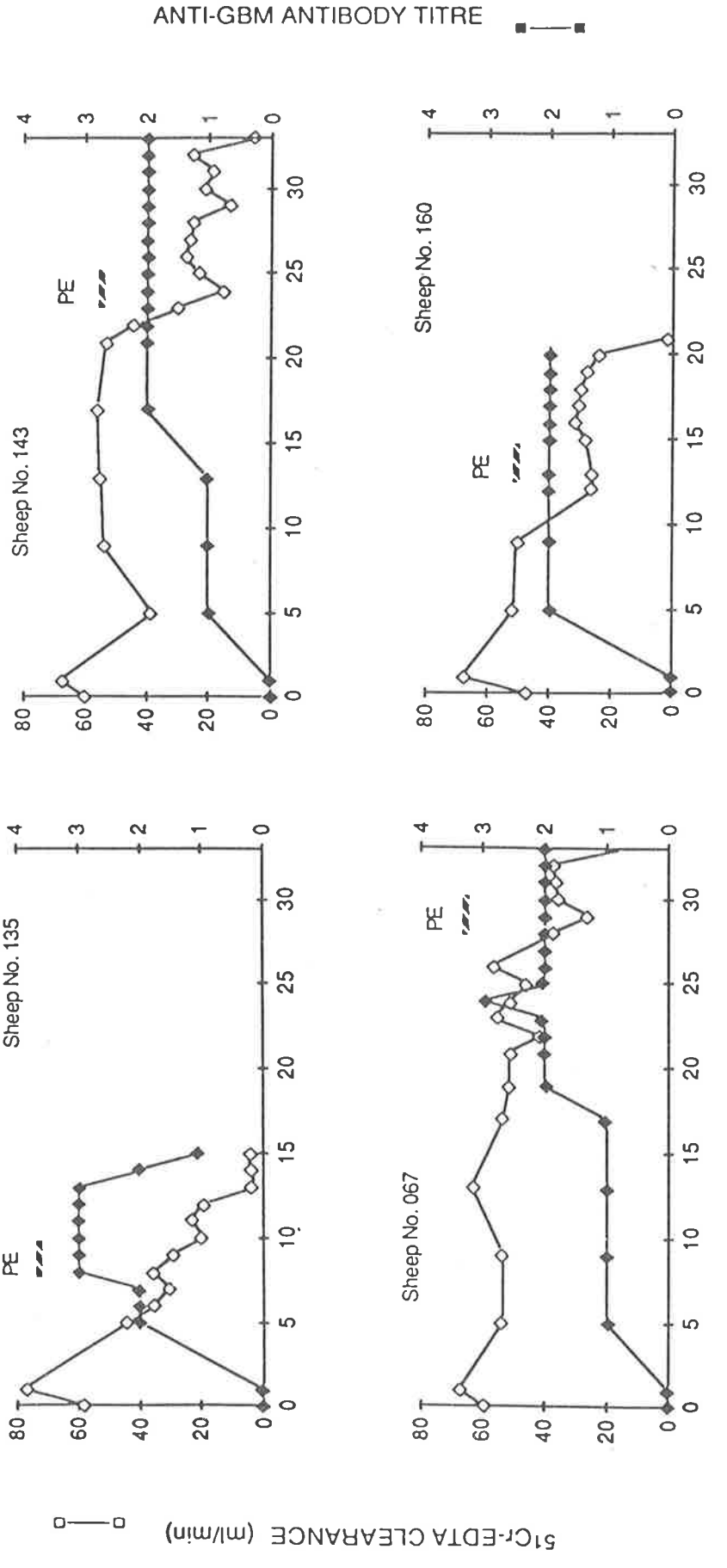


FIGURE 4.4 (Plasma Exchange)

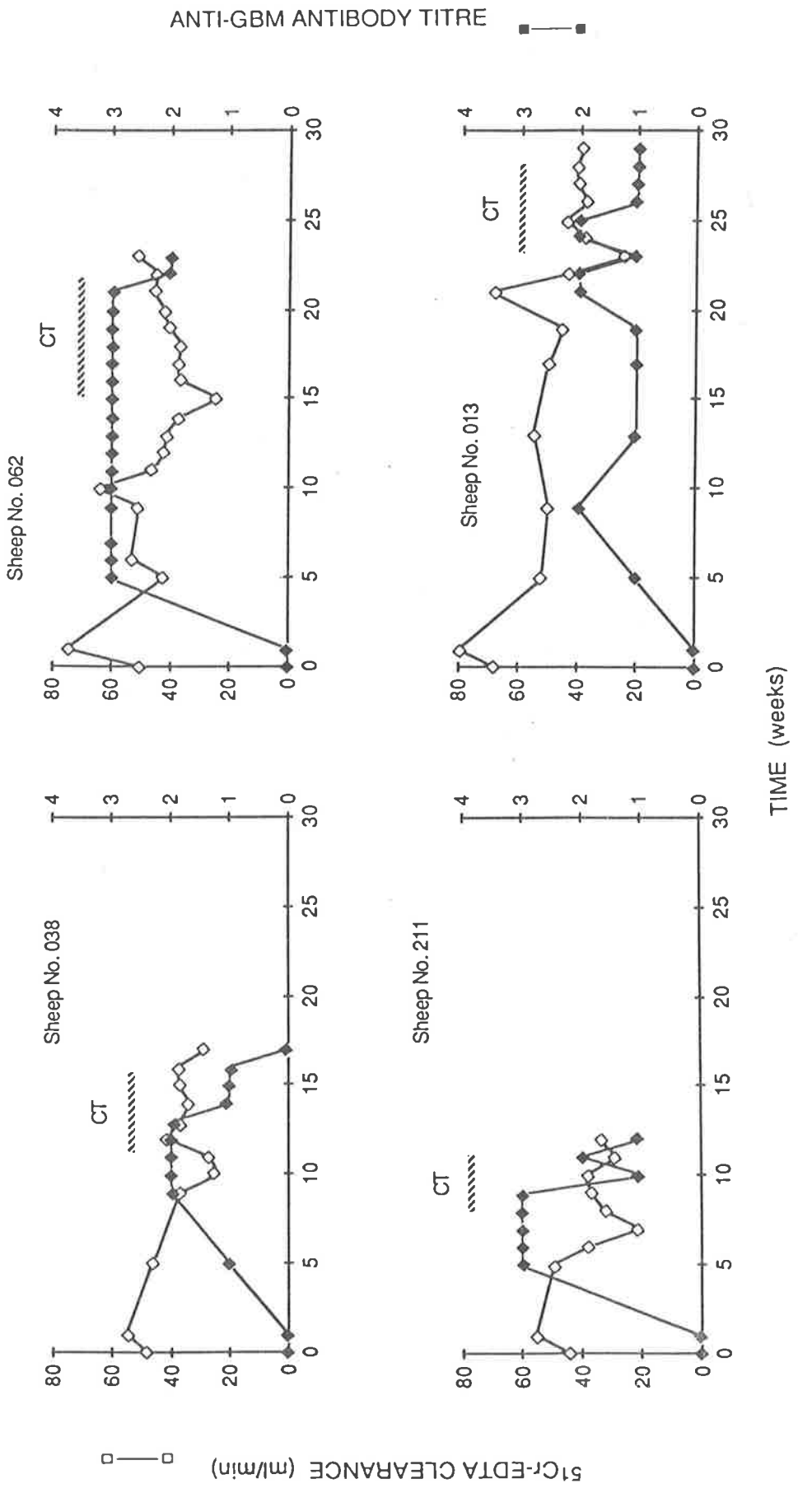


FIGURE 4.5 (Chemotherapy)

Anti-Human-GBM-antibody levels

These are plotted in figs 4.3, 4.4 and 4.5. Antibody titres in the figures are expressed as; 1:50 =1, 1:250=2, 1:1000=3. Serum antibodies reacting with human kidney tissue appeared 5 weeks after the initiation of injections. There was linear staining of GBM and also of TBM (Figure 4.6). The titres in most of the sheep rose to 1/250; in a few cases 1/1000 levels were seen.

Each plasma exchange caused an immediate decline in antibody levels (Figure 4.7) and antibody was detectable in the filtrates. However, there was a rapid return to higher titres after each exchange and consequently no permanent decrease in antibody titres was evident. Chemotherapy caused a decline in anti-human-GBM antibody titres in all 4 cases.

Anti-Sheep-antibody levels

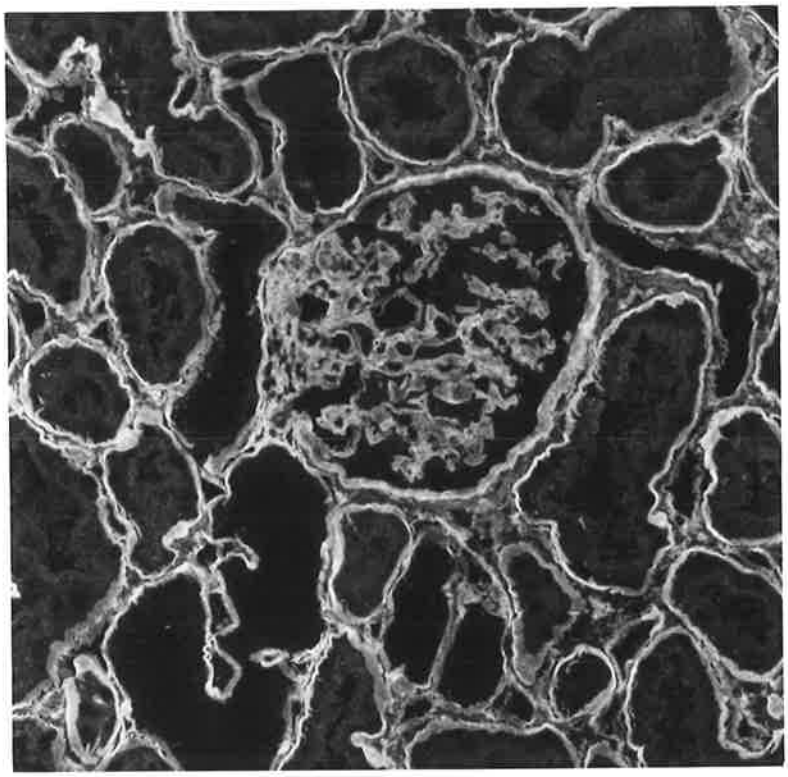
Anti-GBM antibodies reactive with normal sheep kidney targets could not be detected (Figure 4.8). All sera from sheep 1797 were tested at 1:10 and were negative. One sample from each of the twelve sheep was selected (when the anti-human GBM titre first reached its highest value) and was tested undiluted and at 1:5. GBM fluorescence was not observed in any case.

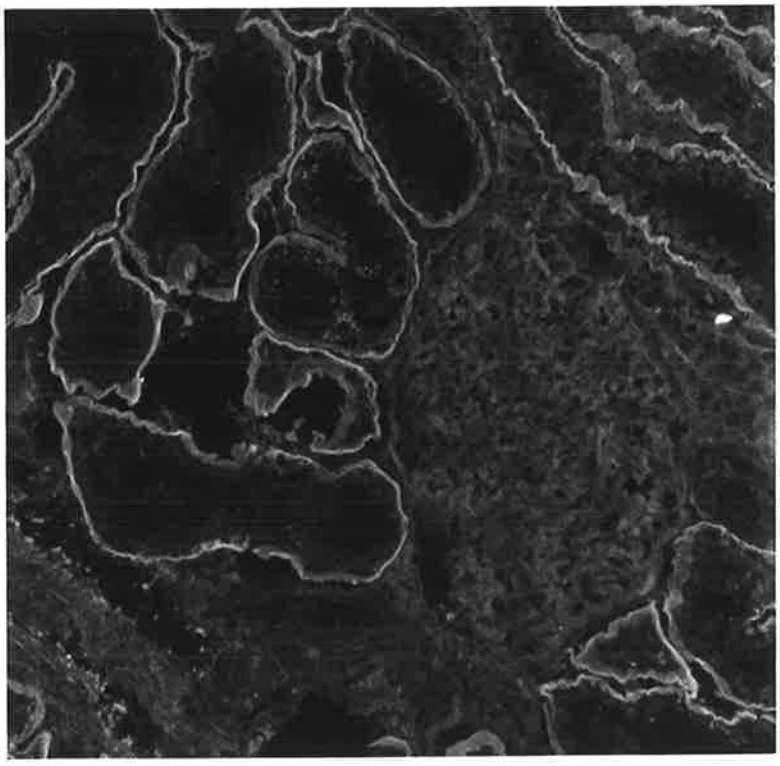
FIGURE 4.6

IIF using Steblay sheep serum on human kidney target with FITC conjugated goat antisheep IgG (x 157).

FIGURE 4.8

IIF using Steblay sheep serum on sheep kidney target. This shows TBM staining only (x 157).





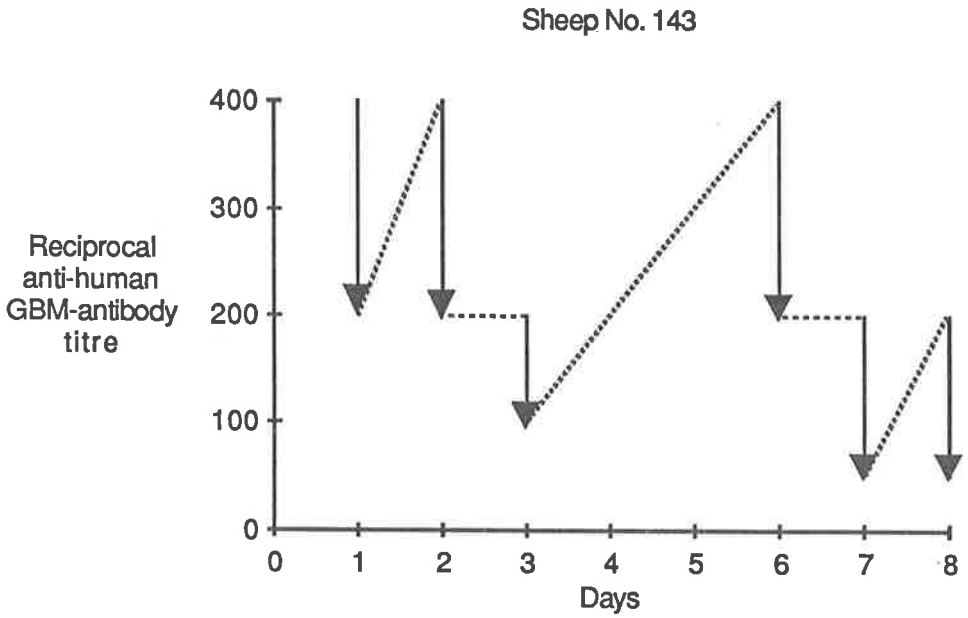


FIGURE 4.7 Effect of plasma exchange on anti-GBM-titre.

Eluted Antibody

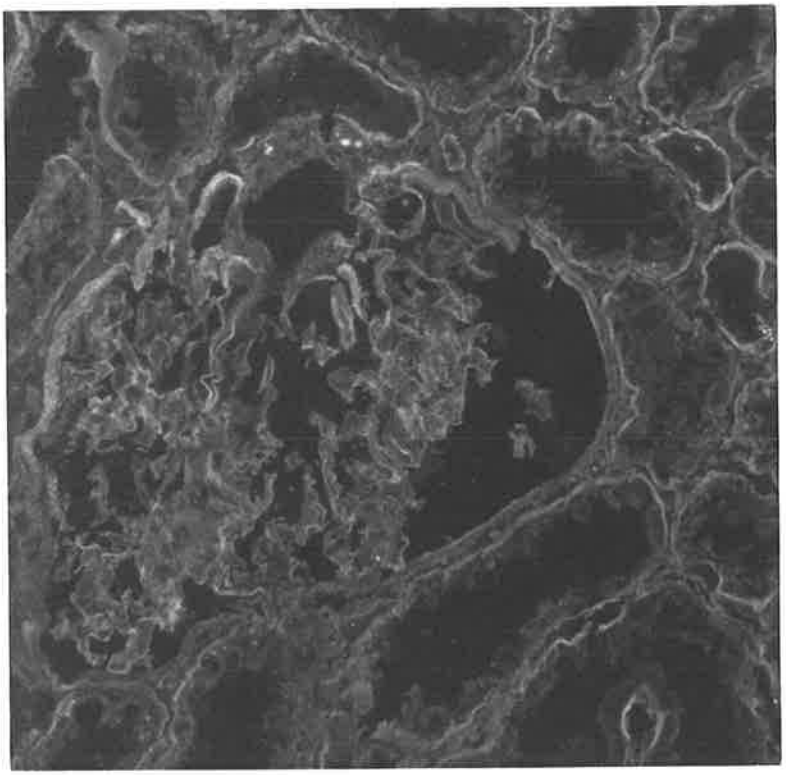
Antibody eluted from pooled kidney tissue obtained from sheep after death was reactive with human GBM (Figure 4.9 (1+) fluorescence). In addition the eluate showed a weaker reaction with sheep GBM (Figure 4.10 (±) fluorescence).

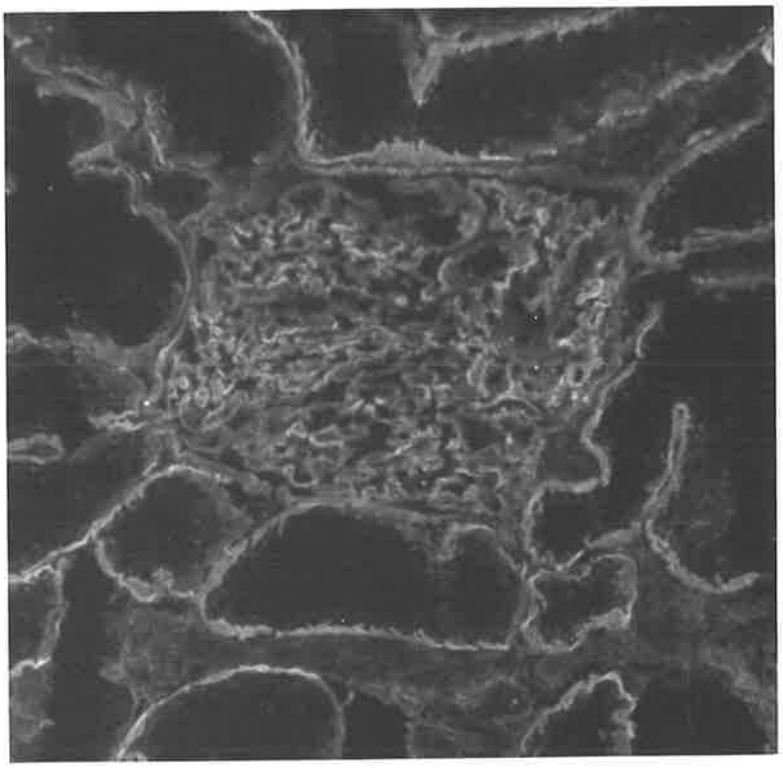
FIGURE 4.9

IIF using eluted antibody from Steblay sheep kidney on human kidney target (x 157).

FIGURE 4.10

IIF using eluted antibody from Steblay sheep kidney on sheep kidney target (X 157).





Discussion

Renal disease was induced in sheep following immunisation with human GBM. Some difficulty in initiating the disease was experienced and it was necessary to increase the amount of FCA used from 1 ml to 7.5 ml. The antigen dose remained 150 mg. In Steblay's original work [15], 150 mg was injected in approximately 6 ml FCA into sheep of 50-60 kg; all the sheep immunised died of renal failure, 3 out of 4 within 6 weeks and the remaining animal at 13 weeks. In contrast, only half the sheep in our study ultimately developed disease as defined by reduced renal clearance. This may reflect inter-strain differences in the response to immunisation.

There was considerable variability in the time before onset of the disease. Once induced, the disease was progressive and fatal within 4 weeks. Chemotherapy preserved renal function and extended the mean survival time after development of renal failure from 2.2 to 6.5 weeks. In each of the four animals it lowered the titre of circulating antibody to human GBM. The animals succumbed, however, to the toxic effects of the drugs used, each of the 4 animals in the CT group having pulmonary oedema at autopsy. It appears that this is a direct effect of the CT, as none of the other sheep had pulmonary oedema at death. Plasma exchange prolonged mean survival from 2.2 to 8.2 weeks although it did not cause an improvement in renal function or a long-term lowering of circulating antibody levels. PE therapy proved inadequate to preserve life indefinitely.

Antibodies reactive with normal sheep kidney were absent from the circulation despite being present in the glomeruli and in the kidney eluates. It seems that anti-sheep-GBM antibodies become entrapped within the sheep kidney as rapidly as they are produced. This observation is supported by data obtained by Lerner and Dixon [17]. Using the Steblay model, they transferred disease from sick to healthy sheep using serum immunoglobulin, but found it necessary to nephrectomize the donor sheep one week earlier for the transference to be effective. In contrast, Steblay and Rudofsky [116] were able to demonstrate circulating antibody to both autologous and homologous tissue by indirect immunofluorescence. Additionally, Steblay and Rudofsky [102] found that 2 out of 6 sheep injected with human lung basement membrane had low (1:20) titres of antibodies reactive with sheep GBM (compared to 1:10,240 against human GBM). They also demonstrated that injection of 1500 ml of the serum i.p. into healthy sheep led to linear GBM staining and disease. Clearly, circulating antibody to sheep GBM is either absent or present in vanishingly small quantities.

These data imply that the circulating anti-GBM antibody levels are of limited relevance to the disease process. The human GBM appears not to be highly immunogenic to sheep. The adjuvant plays a vital role, described by Steblay [117]. In this paper, unpublished work is reviewed, showing that disease transference with serum from nephrectomised donors only occurs after pre-injection of the recipient with FCA. From these observations it may be concluded that there is limited cross-reactivity between sheep and human

GBM. Only a small proportion of the antibodies produced are reactive with sheep GBM and most or all of these are bound to the sheep's kidney as they are produced.

How plasma exchange and chemotherapy are effective in anti-GBM-antibody disease is by no means certain. Discussion of their modes of action follows.

Plasma exchange of the volume of serum used for this study would be expected to remove about 50% of the circulating IgG [118]. Over the next 1-2 days re-equilibration with the extravascular compartment would occur. The effect of removing antibody may be a direct one, reducing the deposition of circulating antibody in the kidney. Alternatively depletion of circulating inflammatory mediators such as complement and clotting factors may be of primary importance. Naturally, these effects may be complementary.

Chemotherapy may reduce antibody synthesis by a direct cytotoxic effect on B cells or by an effect on helper T cells. The maintenance of circulating antibody levels is complex. Removal of circulating antibody leads to rebound and increased levels [119]. This is prevented by cyclophosphamide and suggests that feedback regulation of circulating levels through antibody synthesis occurs [120]. However, these data were obtained after a single primary immunisation with antigen. When repeated after secondary immunisation the rebound phenomemon did not occur [121].

Our results using PE and CT indicate that both treatments are effective separately. CT appeared to lead to suppression of antibody synthesis shown

by the reduced circulating antibody to the human immunogen. PE prolonged survival despite there being no demonstrable circulating antibody to the sheep's own kidney. This favours an explanation of PE being effective through removal of other inflammatory mediators. Despite this, the possibility that small quantities of anti-sheep-antibody, too small to be detected, are being removed by PE cannot be completely discountenanced. Whether the combination of PE and CT treatments gives enhanced therapeutic efficacy cannot be concluded from this study: further experiments using combined therapies would be necessary.

Chapter 4B.

Experimental Treatment of Anti-GBM Disease; Use of Prostaglandin PGE_1.

Introduction

Prostaglandins have been used as therapeutic agents in a variety of experimental animal models of immune disease. These include adjuvant arthritis [122, 123], immune complex vasculitis [123, 124], chronic serum sickness [125] and the NZB/W hybrid mouse model of lupus disease [126-128]. In each of these models, PGE_1 was administered in pharmacologic doses, and was seen to have a protective effect.

How this protective effect is manifested remains uncertain. Prostaglandins have a wide variety of effects on immune reactions and the inflammatory process.

Effect of Prostaglandins on the Cell-Mediated Immune Response

Prostaglandins can both suppress and enhance the cell-mediated immune response and appear to perform in a regulatory role. Thus prostaglandins can inhibit mitogen stimulation of lymphocytes [129], inhibit lymphocyte mediated cytotoxicity [130] and prevent specific T-dependent antibody formation [131, 132]. PGE can be shown to activate suppressor cells that modulate antibody synthesis [133, 134]. It also modulates the plaque-forming cell response *in vitro* and *in vivo* [132].

Effect of Prostaglandins on PMNs

Prostaglandins have been shown to reduce chemotaxis of polymorphonuclear leukocytes (PMNs) [135]. Stimulation of neutrophils leads to degranulation of lysosomal contents and generation of free radicals

(reviewed Weissman et al [136]). The lysosomal enzymes act on a variety of substrates leading to inflammation. PGE modifies the release of these enzymes through stabilisation of the cell membrane and increases in endogenous cAMP [137]. In addition, the products of arachidonic acid metabolism, prostaglandins and thromboxanes, have an important role in the generation and feedback regulation of the microbicidal and tissue-damaging free radicals such as superoxide anion and hydroxyl radical [138], and thus exogenously administered prostaglandins are potentially anti-inflammatory.

Effect of Prostaglandins on Vasopermeability

Vasopermeability is an important factor in IC deposition [139]. Prostaglandins will prevent IgE-induced histamine release from lung fragments and basophils [140] and cause alterations to platelet and granulocyte function [124]. Prostaglandins inhibit the vasopermeability changes induced by histamine, bradykinin and C3a [141] and consequently renal deposition of IC's may be altered as a result of an alteration to renal haemodynamics [142]. PGE does not alter reticuloendothelial cell clearance of circulating immune complexes [143].

Effects of Prostaglandins on the Humoral Antibody Response

Prostaglandins can enhance and suppress the humoral antibody response. The evidence obtained for a quantitative difference in antibody response following provocation has been conflicting. Kelley et al [127] and Zurier et al [126] did not show alterations to serum autoantibodies in NZB/W

mice following PGE_1 treatment. However, Izui et al [144], using the same model, demonstrated decreased antibody to retrovirus gp70, whilst the anti-DNA antibodies remained unaltered. In their model of immune complex glomerulonephritis, McLeish et al [145], found that PGE_1 decreased the circulating anti-apoferritin antibody in mice receiving daily injections of apoferritin.

Summary of the Effects of Prostaglandin on Experimental Immune Disease

In the different models of experimental immune disease a number of modes of action for the protective effect of prostaglandin can be postulated. There could be alterations, either qualitative or quantitative to the antibody response, either directly or indirectly via the cellular immune response. There could be changes to vascular permeability affecting IC deposition and changes in the release of the mediators of inflammation.

Nephrotoxic Nephritis

Nephrotoxic nephritis (NTN) is the classic model of acute anti-glomerular basement membrane-antibody disease. It is a biphasic disease, the first phase being caused by binding of the heterologous antibody to antigens in the GBM [56] and the second autologous phase due to the host's reaction to the this foreign serum protein [146, 147]. We have investigated the effect of PGE on this model. PGE_1 was used since it has been shown to be more resistant to catabolism by 15-hydroxy-PG dehydrogenase [148] and can therefore be used in much lower therapeutic

doses [149].

Materials and Methods

Animals

Inbred male JC-Lewis rats of 150-250 g were supplied by IMVS, Gilles Plains. Normal laboratory diet and water were freely available during the experiments.

15(s)-15-methyl PGE_1

15(s)-15-methyl PGE_1 was donated by Upjohn (Kalamazoo, Michigan, USA). It was dissolved in absolute ethanol (5 mg/ml) and stored at -20°. Immediately before injection it was diluted 1:10 with sterile normal saline (final concentration 500 μg PGE/ml in 10% ethanol).

Nephrotoxic Serum

The heterologous antiserum was prepared by immunising a rabbit (IMVS, Gilles Plains) with sonicated rat glomeruli (prepared by the method of Krakowar and Greenspon [56], as modifyed by Spiro [80]). Five mg of sonicated glomeruli emulsified with Freund's complete adjuvant was injected intramuscularly. Two booster doses of 2 mg sonicated glomeruli with incomplete Freund's adjuvant were given subcutaneously. The antibody response was monitored by indirect immunofluorescence. When a strong positive reaction had developed, blood was collected and allowed to clot. The serum was separated from the cells and ammonium sulphate precipitated twice (50% SAS). The IgG fraction was then purified by DE-52

column chromatography, dialysed against PBS and concentrated to 10 mg/ml.

Experimental Protocol - Heterologous Phase

In order to block uptake of iodine by the thyroid, all rats were given 0.1% potassium iodide in their drinking water for 24 hours prior to the experiment.

2.5 Hour Experiment.

Five rats were given 0.5 ml ethanol subcutaneously. These were designated the "untreated" animals. Five PGE_1 treated rats were given 500 µg PGE_1 subcutaneously. One hour later, 1 ml nephrotoxic serum was given intravenously to all rats. The rats were anaesthetised 2.5 hours later. The abdomens were opened and urine was withdrawn from the bladder. The animals were exanguinated by removal of blood from the inferior vena cava and the kidneys removed. Tissue blocks were collected for immunofluorescence (snap frozen in Tissue Tek using isopentane and liquid nitrogen and stored at -70°), light microscopy (stored in formol saline) and electron microscopy (stored in gluteraldehyde). Serum and urine were stored frozen at -20° until analysed.

1 Day Experiment.

Five rats were injected daily with 250 µg PGE_1 subcutaneously. Five rats were injected with an equal volume of 10% ethanol. On day 3 all rats received 5 ml of nephrotoxic serum intraperitoneally. The animals were killed one day later and specimens collected as before.

Experimental Protocol - Autologous Phase

Ten rats were used. Five were injected daily from day 1 to day 16 with 250 µg PGE_1 subcutaneously, five were given 10% ethanol each day. On day 3, all rats were given a 5 ml intraperitoneal dose of nephrotoxic serum. On the 17^{th} day all animals were killed and specimens were collected as set out in the heterologous protocol.

Microscopic Methods

Light.

The tissue blocks were embedded in the wax mixture of Meadows [150]. Five µm sections were cut and stained with haematoxylin and eosin. The number of PMNs per glomerular cross section was counted in two or more glomeruli from each rat kidney.

E.M.

The tissue blocks were post-fixed in osmium tetroxide and embedded in Spurr's resin. Half µm survey sections were cut and stained with toluidine

blue. Two or more glomeruli per animal were then thin-sectioned and stained with uranyl acetate and lead citrate. Mesangial size was visually assessed and graded either normal or enlarged.

Immunofluorescence.

Two µm frozen sections were cut and stained either directly (FITC swine anti-rabbit IgG) or indirectly (1st layer goat anti-rat C3 or goat anti-rat IgG; 2nd layer FITC rabbit anti-goat immunoglobulin).

Biochemical Methods

Urea.

Serum urea was measured by the urease reaction using an ASTRA (Beckman Instruments) automatic chemistry analyser.

Creatinine.

Serum creatinine was estimated on the same instrument using a rate Jaffé reaction.

Protein.

Urinary protein was assessed by Dipstix (AMES) and by the Folin-Lowry method.

Anti-rabbit IgG.

Serum antibody to rabbit IgG was measured using a solid phase RIA. Rabbit IgG (100µl at 10µg/ml) was evaporated to dryness in PVC microtitre plate wells. Rat serum (100 µl 1:100 dilution) was added and incubated for 5 hours at 4°. Radiolabelled goat anti-rat IgG (100-150,000 cpm) which had previously been absorbed against normal rabbit serum was added and incubated overnight at 4°. The plates were washed 3 times, cut up and the individual wells counted.

Results

Injection of PGE caused immediate but short-lived (approximately 1 hour) lethargy in the rats. The long term effect was a loss in weight and fur condition.

Immunofluorescence

Immediate binding of rabbit IgG to the GBM, was demonstrated by IF as shown in Figure 4.11. At 14 days the autologous antibody response caused weaker granular fluorescence (Figure 4.12). The results are shown in Table 4.3. No statistically significant differences were observed in the amount of rabbit IgG bound in the PGE treated and untreated groups at either 2.5 hours, 1 day or 14 days (Mann-Whitney U test). At 14 days the PGE treatment caused a significant decrease in the number of rats with deposits of rat C3 ($p < 0.05$). The difference in the deposits of rat IgG was not significant ($p > 0.1$), but this probably reflects the size of the experimental groups of animals as there was no fluorescence observable in PGE treated animals compared to 3 out of 5 untreated animals having detectable rat IgG.

Mesangial Enlargement

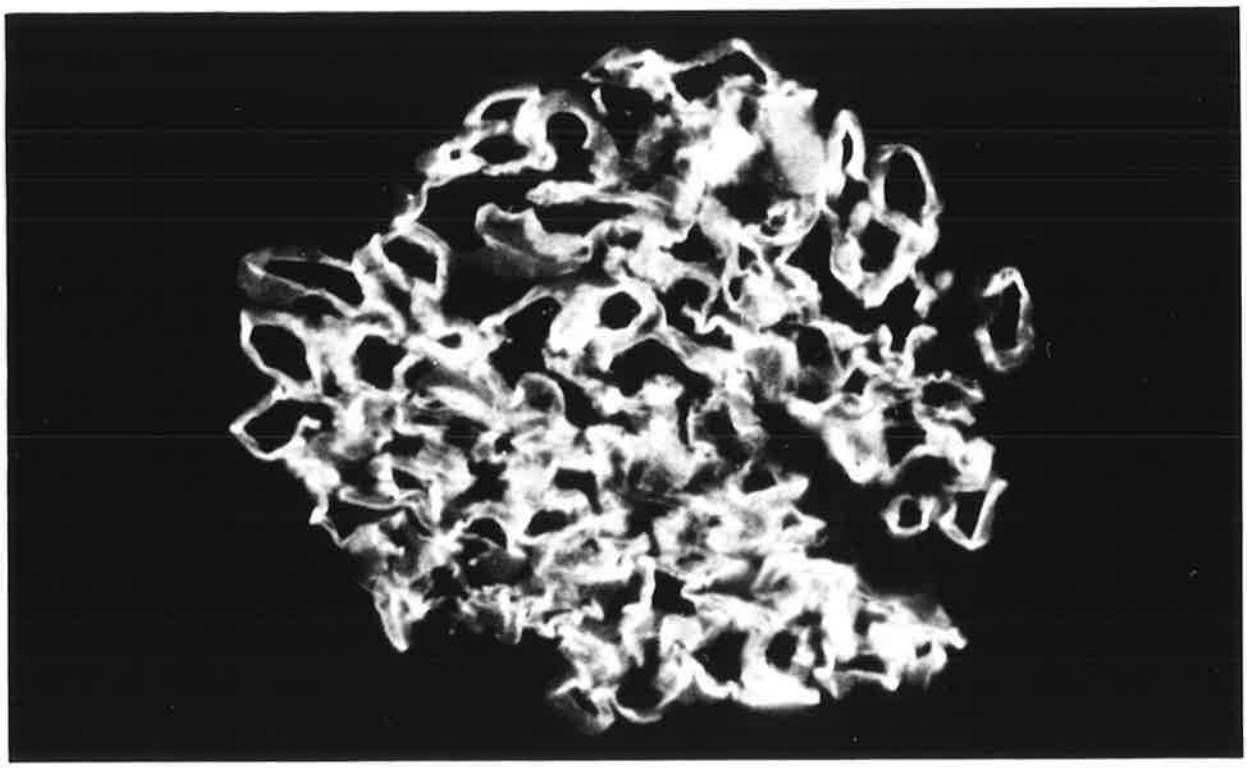
This was assessed at 14 days in both PGE treated and untreated animals. There was no enlargement in either group.

FIGURE 4.11

Direct immunofluorescence of rabbit IgG on rat kidney at 2.5 hours. Linear glomerular wall staining is evident (x 250).

FIGURE 4.12

Direct immunofluorescence of rat IgG on rat kidney at 14 days. Granular staining is evident (x 250).



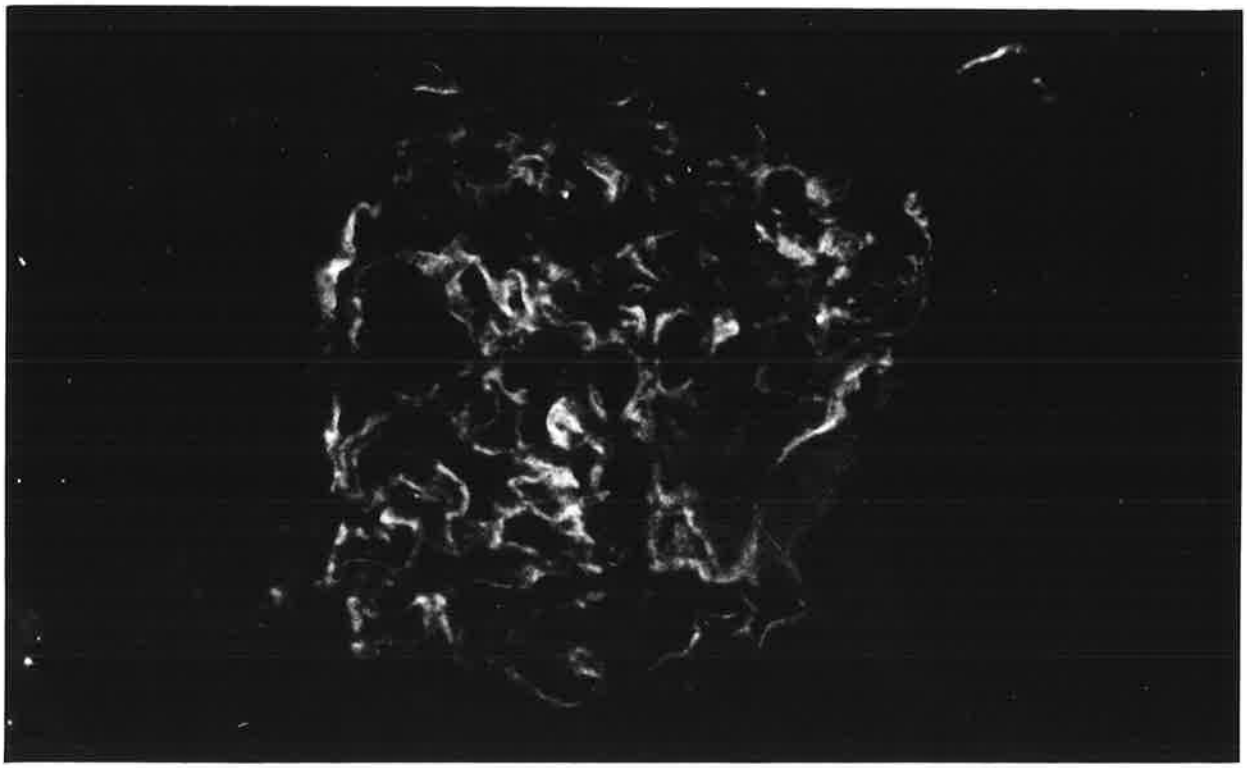


TABLE 4.3

	2.5 hours	1 day	14 days	
Immunofluorescence#				
untreated	5	5	5	}
				}rabbit IgG
PGE_1 treated	5	5	5	}
untreated	0	0	3	}
				}rat IgG
PGE_1 treated	0	0	0	}
untreated	5	ND	5	}
				}rat C3
PGE_1 treated	5	ND	2	}

number of animals showing positive fluorescence (n=5).

Morphology				
untreated	0	ND	0	}
				}mesangial
PGE_1 treated	0	ND	0	}enlargement*
untreated	9.7±1.1	ND	2.7±0.5	}
				}PMNs/
PGE_1 treated	10.8±1.8	ND	2.3±0.5	} glomerulus†

* number of animals showing mesangial enlargement (n=5).
† mean ± SEM.
ND not determined

PMNs/glomerulus

There were increased numbers of PMNs present in the glomeruli of both treated (9.7 ± 1.1) and untreated (10.8 ± 1.8) animals at 2.5 hours following injection of NTS, compared to an expected normal value of about 2 [151]. Fourteen days later, the means were 2.7 ± 0.5 in treated and 2.3 ± 0.5 in untreated animals. No statistically significant differences were demonstrated between PGE treated and untreated groups.

Urinary Protein

The PGE treatment appeared to cause an increase in urine protein at 2.5 hours (Table 4.4). To confirm this observation, a control experiment was performed in which PGE_1 was injected into 5 rats which did not receive NTS. Combined data for all animals 2.5 hours after PGE, both those injected with NTS and those that were not, gave a mean of 17.0 ± 2.4 g/l. The combined untreated animals gave a value of 4.7 ± 0.7 g/l. These figures were significantly different using an unpaired T test ($p < 0.001$). A similar control experiment was performed for the 14 day group. In this case, no difference between the two groups, PGE_1 treated (4.2 ± 0.5) and untreated (3.1 ± 0.4) was demonstrated ($p > 0.1$). The nephrotoxic serum did not cause a significant difference in urine protein concentration either at 2.5 hours ($p > 0.5$) or 14 days ($p > 0.1$, Mann-Whitney U test).

TABLE 4.4

	2.5 hours	1 day	14 days	
untreated	4.8 ± 1.4	5.0 ± 1.3	3.8 ± 0.6	} }urine protein
PGE_1 treated	20.8 ± 3.7	7.4 ± 6.2	3.2 ± 0.4	} (g/l)
untreated	6.5 ± 0.5	4.9 ± 0.1	8.4 ± 0.2	} }serum urea
PGE_1 treated	13.5 ± 0.9	2.3 ± 0.3	7.8 ± 0.2	} (mmol/l)
untreated	0.04 ± 0.003	0.04 ± 0	0.074 ± 0.002	} serum } creatinine
PGE_1 treated	0.04 ± 0.003	0.032 ± 0.002	0.062 ± 0.005	} (mmol/l)
untreated	0.036 ± 0.007	ND	0.62 ± 0.1	} serum rat }anti-rabbit
PGE_1 treated	0.028 ± 0.004	ND	0.78 ± 0.2	} antibody (% binding)

Serum Urea (Table 4.4)

Using the additional control group of rats which were not injected with NTS, combined data for all PGE treated animals compared to all untreated animals at 2.5 hours, showed that prostaglandin caused a significant rise in serum urea at this time (mean 12.9 ± 0.5 mmol/l compared to 6.3 ± 0.3 mmol/l, $p < 0.001$, unpaired T test). By one day after injection, however, the PGE lowered the serum urea (PGE treated rats 2.3 ± 0.3; untreated rats 4.9 ± 0.1, $p < 0.01$, Mann-Whitney U test). The level of serum urea had risen at 14 days in both groups compared to normal ($p < 0.01$).

Serum Creatinine (Table 4.4)

There was no difference in serum creatinine between the PGE treated and untreated rats at 2.5 hours following injection of NTS nor 1 day later. At 14 days, both treated and untreated groups showed statistically significant rises in serum creatinine (from 0.04 to 0.06 mmol/l ($p < 0.05$) and 0.07 mmol/l ($p < 0.01$, Mann-Whitney U test).

Serum Rat Anti-Rabbit Antibody (Table 4.4)

The value at 2.5 hours was 0.032 ± 0.005 % binding (i.e the background level, since this is too early for an antibody response). At l4 days, there was a statistically significant rise in antibody level (0.7 ± 0.15 %, $p<0.005$), but no significant difference between the PGE_1 treated and untreated groups (0.78 compared to 0.62 $p > 0.1$).

Discussion

Whilst the protective effect of PGE_1 in inflammatory reactions has now been established, the mode of action remains unclear. There are several possible mechanisms, or a combination of these may occur. The most likely actions are in the modulation of antibody synthesis, alterations to IC deposition, or changes in the recruitment or activation of inflammatory cells.

This experiment showed that PGE_1 had no effect on glomerular rabbit antibody deposition, nor an effect on synthesis of rat antibody to the nephrotoxic serum. However a reduction by the PGE in C3 deposition and rat IgG deposition was demonstrated at 14 days. These data contrast with those obtained by Kunkel et al [152], who, in a similar experiment, found no differences between PGE treated and untreated animals in rat IgG or C3 twenty days following administration of NTS. This difference may reflect qualitative or quantitative differences in the antibodies used in the two studies. It is possible that the antibody used in this experiment was less nephrotoxic than that of Kunkel et al, resulting in the induction of milder disease and consequently allowing the observation of subtler differences in antibody deposition.

The administration of prostaglandin did not alter PMN recruitment to the glomerulus, despite the fact that this effect has been shown *in vitro* using rabbit neutrophils [135]. Probably the acute stimulus of NTN prevents

modulation by PGE of these responses. Knowledge of the effect of PGE on monocyte numbers in this disease model would be valuable, since these are responsible for much of the glomerular hypercellularity in NTN [153]. No differences in mesangial enlargement were demonstrable, in contrast to the results obtained by Kunkel who showed decreased glomerular hypercellurlarity and mesangial enlargment in PGE treated rats compared to untreated animals. This too, probably reflects the more severe course of the disease induced by Kunkel's group, since the data obtained here did not show differences in mesangial size between animals which had and had not received nephrotoxic serum. The animals in this study did not suffer from proteinuria. PGE_1 thus did not appear to affect the level of urinary protein over 14 days. However, Kunkel's group showed elevated proteinuria and an effect on this by PGE at 3 days. Whilst it is possible that this is a transient effect, it seems more likely, as before, that the differing severity of the disease is responsible for this difference between the studies. The transient proteinuria caused by PGE treatment demonstrated here may be due to enhanced vasopermeability, leading to leakiness of the GBM. This explanation cannot, however, account for the simultaneous rises in blood urea and creatinine, which must be due either to altered production or clearance of these metabolites, or to interference of PGE_1 in the chemical assays for these products. Over the course of 14 days a slight rise in urea and creatinine was observed in both groups of animals, evidence for

progressive renal damage by the nephrotoxic serum. The PGE treated animals in both cases showed lower values although these were not statistically significant, possibly due to the small number of animals in the experimental groups.

The effects of PGE_1 on two differing models of glomerulonephritis have now been investigated. These are, firstly, the NZB/W mouse model of lupus erythematosus, which is believed to be caused by deposition of circulating immune complexes. PGE reduces proteinuria and enhances survival by reducing the glomerular deposition of complexes in this model. The second model is the acute nephrotoxic nephritis model of *in situ* antibody binding. In this case, the data obtained here, and that of Kunkel et al [152], shows that PGE_1 does not alter deposition of the antibody. It still, however, exerts a protective effect against the effects of the disease in the longer term. This study also shows that PGE does not alter antibody synthesis, and so the protective effect appears to be mediated through the inflammatory cells or the release of their products. The understanding of the manner in which PGE is protective in these experimental models of disease may prove to be of importance in the treatment of human glomerulonephritis.

Chapter 5.

Conclusions and Implications.

In consideration of immunological disease it is important to distinguish between causes and immunopathogenetic mechanisms. Whilst the latter are now comparatively well understood, the aetiology of immune glomerulonephritis remains largely speculative.

Circulating Immune Complex Disease - Importance in Glomerulonephritis

Most complexes are efficiently cleared by the reticuloendothelial (RE) system and do not initiate disease. Models of circulating immune complex disease have been widely studied and many of the factors leading to glomerular accumulation have been elucidated. These include circulating complex size (Ag:Ab ratio) and antibody affinity and class which have all been shown to be of importance in the formation and site of deposition of the complexes [13]. In addition, other factors of importance in the deposition of ICs include saturation of the RE system with CIC which leads to increased glomerular IC deposition [154] and the C3-dependent resolubilisation of deposited immunoglobulin and macrophage ingestion of soluble complexes [155]. After initial complex deposition, free antigen [156] or free antibody or IC [157] can combine with or modify the immune deposits.

Some of the documented immune complexes are DNA-antiDNA [158], rheumatoid factor Ig-anti-IgG complexes [1, 159, 160] and idiotype-anti-idiotype complexes [161, 162].

Despite the success in demonstrating the factors leading to glomerular deposition of ICs, a number of questions remain concerning the role of CICs in human disease. Although CICs are readily demonstrable in

SLE and bacterial endocarditis, in many other diseases of presumed IC mediation (due to granular immunoglobulin deposits shown by immunofluorescence), CICs have not been consistently found. This may be due to failure of methods of detection, to the intermittent presence of the complexes, or to *in situ* or local formation of antigen-antibody complexes. Additionally, not all occurrences of CIC are associated with glomerular disease (e.g. in primary biliary cirrhosis, [163]). The demonstration of antigen or antigen-specific antibody in glomerular deposits (which is necessary for convincing proof of the CIC mediation of disease) has been achieved in disappointingly few cases of presumed IC disease (some cases of lupus have anti-DNA antibodies [164]).

Relevance of the *In Situ* Model of Glomerulonephritis

The previous discussion indicates that the CIC mechanism of disease is unlikely to be responsible for all cases of glomerulonephritis that are not attributable to antibodies directed against structural glomerular antigens. As a consequence, recent interest has focused on the models of *in situ* glomerulonephritis. However, as yet no human disease has been proven to result from this pathogenic route.

Until recently, SLE was considered to be the prototype of circulating immune complex disease. The possibility that the anti-DNA antibodies associated with the disease bind directly to anionic sites in the GBM and then fix complement or rheumatoid factors is now being considered [10]. Certainly it has not proved easy to demonstrate free DNA or DNA-antiDNA complexes

in the circulation or DNA antigen deposits in the kidney (reviewed Eilat [165]). There is also evidence that DNA antibodies can cross-react with cardiolipin so that DNA may not be the primary antigen in the immune complexes [166].

These studies, together with the possibility that complexes containing cationic antibodies may interact with fixed anionic sites within the glomerulus [72] have led to speculation that interactions other than primary antibody-antigen binding may be important mechanisms of immune deposit formation. Certainly, the pI of antibodies in the glomeruli of mice with SLE has been shown to be restricted [167].

In this study it was established that cationic IgG can bind directly to the GBM (with the possibility of leading to subsequent disease). This is in agreement with the data obtained by Vogt [70]. However, such cationic IgG molecules could not be detected in the glomerular eluates studied here from patients with membranous nephropathy. In addition, although bacterial neuraminidase is a likely candidate for the *in vivo* production of cationic subpopulations of IgG, it was shown *in vitro* that treatment of IgG with neuraminidase does not sufficiently alter the charge to cause binding. Thus it would seem that cationic IgG is unlikely to be the primary antigen involved in membranous nephropathy. However, it remains possible that other exogenous antigens of a cationic nature bind to the GBM on the basis of charge, although none of the other likely compounds (gold, mercury and captopril) showed the same affinity for binding to the GBM that DNA does.

Experimental Models of Anti-GBM Disease - General Considerations and Specificity of Antibodies

Despite much clinical interest and experimental work performed in the study of anti-GBM disease, no certain cause for the appearance of anti-GBM antibodies has yet been elucidated. Factors considered to be of importance are-

1. Increased amounts or altered composition of endogenous basement membrane antigens due to exposure to infectious agents and environmental factors or to reduced RE clearance of these antigens.
2. Abnormalities of the immune response (association with HLA DR2).

Some unanswered questions about the disease concern its variable nature, with the recent identification of milder forms of anti-GBM disease [101] and the variable specificity of the antibodies, indicated by the presence and absence of anti-lung cross reactivity.

There is no doubt that different antibodies are involved in the different experimental models of anti-GBM disease. In nephrotoxic antisera there are clearly multiple antibodies due to the crude nature of the immunogen [56] and the primary reason that binding is fairly specific for the GBM appears to be one of accessibility. Immunofluorescence reveals a double line of reactivity for the nephrotoxic antibody [58], whereas, in the case of the autoimmune (Steblay) antibody there is a single line as is the case in human disease [59]. These differences between the experimental models and the human disease mean that caution should be exercised in the interpretation and comparison

of experimental results.

Anti-GBM Antibody Assay

Despite the obvious advantages of RIA over IIF (such as faster throughput and the requirement for less skilled technical operators) the radioimmunoassay for anti-GBM antibody is not widely available. This is due in part to the rarity of the disease and partly to the difficulty of establishing the assay. The comparison described here of the techniques of IIF and solid-phase RIA for the detection of anti-GBM antibody, did not reveal any great advantages in performing the RIA over IIF with regard to sensitivity of detection of antibody. Despite this, there remains a need within a community the size of Australia for reference to be available to such an assay due to the severity of the disease and the occasional difficulties in diagnosis. The number of 'false positives' obtained using the RIA in this study as well as in other published series [13, 27, 92, 93] indicates that there are potentially several antigens which react with circulating antibodies to be found within the glomerulus. Whether these additional antibodies (to the traditional Goodpasture's antibody) have a role in the different manifestations of the disease remains speculative.

Avidity and Relevance of Circulating Antibody

There has been little previous questioning of the pathogenic role of circulating antibody in anti-GBM disease. Evidence for the pathogenic nature of the circulating antibody is based on transfer of experimental disease to disease-free animals with serum antibody [17] and following the

establishment of cross-circulation between sick and well animals [16]. However in the former report, transfer of nephritogenic homologous serum led to transient disease whilst in the latter, persistent disease resulted. Some differences between the studies were the pre-injection of complete Freund's adjuvant (which makes animals 15-20 times more susceptible to nephrotoxic antibody [168]) and the age of the recipient animals (adult recipients may have increased susceptibility to spontaneous nephritis). Large volumes of serum are required for disease to be transferred in the Steblay model, although in the nephrotoxic model of anti-GBM disease, only small quantities of nephrotoxic antibody are required.

An important difference between tne sheep model and the human disease is that increased antibody levels follow nephrectomy in the sheep model [17] but not in humans [13].

Consideration of this evidence suggests that the circulating antibody may be of limited pathogenicity. The study of the avidity of the circulating human anti-GBM antibody presented here establishes that the circulating antibody is of low avidity. This finding, considered with the questions raised earlier concerning the experimental evidence for the pathogenicity of the circulating antibody raises important doubts about the role of the circulating antibody in the continuing disease. This may be similar in both the sheep model and human disease with much or all of the antibody circulating of low avidity and poorly cross-reactive with native GBM.

Treatment of Anti-GBM Disease

In the treatment of anti-GBM disease a combination of prednisolone, immunosuppression and plasma exchange has been found to be effective. This regime has reduced the mortality from 80% [110] to 25% [13]. Although the results presented earlier question the role of circulating antibody in the on-going pathogenesis of the disease, there is no doubt both on clinical and experimental grounds that immunosuppression is effective in treatment. However, the implication of these data is that intervention with the immunological mediators of damage may be the more important factor in the treatment. Thus the current steroid and chemotherapeutic regimes may reduce the importance of plasma exchange in therapy.

Final Remarks

Improvements in the diagnosis and treatment of immunological diseases are being made rapidly. The discovery of the causes for the development of autoantibodies remains one of the outstanding problems in the study of autoimmune disease, and with it disease prevention as well as therapy. Recent approaches to these problems such as the study of anti-idiotypic antibodies [169] and the role of T-cells [170, 171] in the regulation of anti-antibody levels offer promise in these areas.

Appendix 1.

Published work arising from this study.

Wootton, A. M., Smith, P. S., Aaron, I. & Woodroffe, A. J. (1982). Effect of prostaglandin (PGE_1) on experimental anti-glomerular basement membrane antibody disease (nephrotoxic nephritis). *Australian and NewZealand Journal of Medicine, 12*(4), 231–363.

Kidney Int 1984; 26: 243.

Efficacy of plasma exchange in experimental renal autoimmune disease. *K. Schindhelm, A. J. Pollack, A. M. Wootton, A. J. Woodroffe, B. Pussell, and P. C. Farrell. Centre for Biomedical Engineering, University of New South Wales, Renal Unit Prince Henry Hospital, Sydney, and Renal Unit, Royal Adelaide Hospital, Adelaide, Australia.* Plasma exchange combined with immunosuppressive drug therapy has given encouraging results in the treatment of Goodpasture syndrome (GS). However, the individual roles of plasma exchange and chemotherapy in altering the course of GS have not yet been established. In this study plasmapheresis and immunosuppressive therapy have been separately applied in a sheep model of autoimmune renal disease. Twelve sheep (25 to 35 kg body weight) were induced with Steblay nephritis via multiportal injections of 150 mg human glomerular basement membrane (GBM) in Freund's complete adjuvant given fortnightly until sacrifice. Renal function (^{51}Cr-EDTA clearance), serum biochemistries, whole blood and differential white cell counts, anti-human-GBM-antibody titers and urinalyses were used to monitor disease progression. The sheep were divided into plasma exchange, chemotherapy and control groups. When renal function declined to about 50% of the pre-disease function, treatment was initiated. The plasma exchange group had 1 liter of plasma exchanged with Haemaccel (Behring, Federal Republic of Germany) on six occasions over 8 days using plasma filters (Enka A.G., Federal Republic of Germany). The chemotherapy group received intravenous prednisolone (2 mg/kg) and cyclophosphamide (4 mg/kg) daily until death. The control group was untreated. All data are reported as mean ± SEM (N = 4). Plasma exchange and chemotherapy significantly prolonged survival after disease onset (8.3 ± 1.3 and 6.5 ± 0.7 weeks, respectively, versus 2.8 ± 0.6 weeks for controls). Preterminal renal clearances were 3.2 ± 0.9 ml/min for controls, 5.5 ± 3.1 ml/min for the plasma exchange group and 38 ± 5 ml/min for the chemotherapy group. Plasma exchange resulted in a transient reversal in the rate of decline in renal function, while chemotherapy resulted in a sustained preservation or improvement in renal function. Both control and plasma exchange animals died in renal failure while the other group died as a result of chemotherapy toxicity. Circulating anti-human-GBM-antibody levels were lowered by chemotherapy, but plasma exchange did not cause a long-term decrease in the levels. This study indicates that both plasma exchange and chemotherapy are effective therapeutic measures. Plasma exchange may preserve renal function by removing nonimmunoglobulin inflammatory mediators, while the chemotherapy may reduce renal deposition of immunoglobulin. Plasma exchange with concomitant chemotherapy may be particularly effective by combining the removal of mediators with a decrease in antibody production.

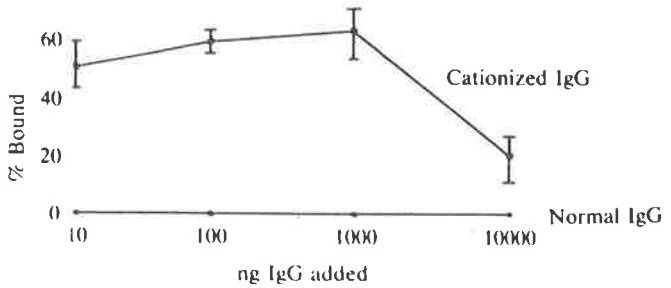
Kidney Int 1984; 26: 245.

Binding studies with isolated glomerular basement membrane (GBM). *A. M. Wooton and A. J. Woodroffe. Clinical Chemistry, Institute of Medical and Veterinary Science and Renal Unit, Royal Adelaide Hospital, Adelaide, Australia.* The binding of various compounds to isolated human GBM was investigated since this may have a role in the etiology of immune deposit renal disease. Lyophilized GBM was sonicated and incubated overnight at 4°C with the compounds listed below. Specifically bound radioactivity was calculated by comparison with tubes containing no GBM. The following results were obtained:

Normal IgG	(−ve)	cationized IgG	(+ve)
Neuraminidase treated IgG	(−ve)*	^{3}H-DNA	(+ve)
^{14}C-Captopril	(−ve)	^{195}Au	(−ve)
^{203}Hg	(+ve)	^{125}I-Clq	(+ve)

* Tested by indirect immunofluorescence.

The binding curves for normal and cationized IgG are shown below. Saturation of the GBM binding sites with cationized IgG occurred at about 8 ng/μg dry wt.



The data obtained using normal IgG (− ve control) and DNA (+ ve control) suggests that this is a suitable system for testing in vitro binding to GBM.

Appendix 2.

Abbreviations.

ALP	Alkaline phosphatase
BM	Basement membrane
CIC	Circulating immune complexes
CSGBM	Collagenase-solubilised glomerular basement membrane
CT	Chemotherapy
DIF	Direct immunofluorescence
FCA	Freund's complete adjuvant
FITC	Fluoroscein isothiocyanate
GBM	Glomerular basement membrane
GN	Glomerulonephritis
IC	Immune complex
IF	Immunofluorescence
IIF	Indirect immunofluorescence
IP	Intraperitoneal
K	Affinity constant (L/mole)
MPGN	Mesangiocapillary glomerulonephritis
NTS	Nephrotoxic serum
NTN	Nephrotoxic nephritis
PBS	Phosphate-buffered saline
PE	Plasma exchange
PIGN	Post-infectious glomerulonephritis
PMNs	Polymorphonuclear leukocytes
RE	Reticuloendothelial
SAS	Saturated ammonium sulphate
SLE	Systemic lupus erythematosus
SSDNA	Single-stranded DNA
TBM	Tubular basement membrane
TBS	Tris-buffered saline

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