

*Original Article***Experimental autoimmune glomerulonephritis (EAG) induced by homologous and heterologous glomerular basement membrane in two substrains of Wistar–Kyoto rat**John Reynolds¹, Kostas Mavromatidis¹, Stephen J. Cashman¹, David J. Evans² and Charles D. Pusey¹¹Renal Unit, Department of Medicine, Royal Postgraduate Medical School; and ²Department of Histopathology, St Mary's Hospital Medical School, London**Abstract**

Background. Goodpasture's, or antiglomerular basement membrane (GBM), disease presents with rapidly progressive glomerulonephritis, and is caused by autoimmunity to the NC1 domain of the $\alpha 3$ chain of type IV collagen. In order to investigate mechanisms involved in the induction and regulation of glomerulonephritis, experimental models of Goodpasture's disease have been developed in the rat which share many characteristics with the human disease. Induction of experimental autoimmune glomerulonephritis (EAG) involves immunization of susceptible strains with either heterologous or homologous GBM in FCA. However, pathological changes have tended to be mild and/or variable, except in certain protocols using Wistar–Kyoto (WKY) rats.

Methods. We studied the susceptibility of inbred WKY rats from two different suppliers to the development of EAG. These substrains of rat had different MHC haplotypes (WKY/CR, RT1-l; WKY/Olac, RT1-k), so we proposed that they might show differences in their immune response to GBM antigens. Both substrains were immunized with sheep GBM, pH 7, or rat GBM buffered to pH 3, pH 5 or pH 7.

Results. All immunized rats developed circulating anti-GBM antibodies detectable at 14 days and rising until 28 days, at which time there was linear deposition of IgG on the GBM. WKY/CR rats developed severe focal segmental proliferative and necrotizing glomerulonephritis, with heavy albuminuria, following immunization with rat GBM, pH 7, but only moderate disease following sheep GBM. WKY/Olac rats showed a more variable response, with moderate disease following both rat and sheep GBM. Immunization of either substrain with rat GBM, pH 5, produced a response similar to that with rat GBM, pH 7, but disease was mild following rat GBM, pH 3.

Conclusion. EAG in the WKY rat varies in severity

according to the substrain of animal and preparation of GBM used for immunization. The model with the most severe and consistent changes was that induced in the WKY/CR rat by rat GBM at pH 7. This model of EAG will be of value for investigating mechanisms of autoimmunity and inflammation in glomerulonephritis, and for attempting novel forms of immunotherapy prior to trials in man.

Key words: autoimmunity; experimental autoimmune glomerulonephritis (EAG); glomerular basement membrane (GBM); Goodpasture's disease; Wistar–Kyoto (WKY) rat

Introduction

Goodpasture's disease presents with rapidly progressive glomerulonephritis, often accompanied by lung haemorrhage, and is caused by autoimmunity to components of the glomerular (GBM) and alveolar basement membranes [1]. It is characterized by the presence of circulating and deposited anti-GBM antibodies, the pathogenicity of which has been demonstrated by passive transfer experiments [2], and is supported by the observation that control of antibody levels by plasma exchange and immunosuppressive drugs is accompanied by clinical improvement [3]. The main target of anti-GBM antibodies has been identified as the NC1 domain of the $\alpha 3$ chain of type IV collagen [4,5]. However, there is increasing evidence for the role of T cells in the pathogenesis of this disorder. They are likely to be involved in providing T cell help for autoreactive B cells, and also perhaps in direct cell-mediated immune injury [6]. Both T cells and macrophages have been observed infiltrating the glomeruli of patients with Goodpasture's disease, and in one study T cells were present at an early stage of the disease before macrophage infiltration [7]. In recent work from our laboratory, T cells from patients with Goodpasture's disease have been shown to proliferate

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in response to affinity-purified Goodpasture antigen [8]. Further indirect evidence for the role of T cells in Goodpasture's disease comes from the close association with class II MHC genes, notably HLA DRB1*1501 (DR2) [9].

Stebly [10] first demonstrated the development of experimental autoimmune glomerulonephritis (EAG) in sheep immunized with human GBM in FCA. He also examined the response of several other species, including the rat, to various preparations of GBM. The sheep was found to be most susceptible, particularly to heterologous (human) GBM [11]. Subsequently, other groups investigated EAG in the rat, and generally reported only mild nephritis [12,13]. More recently, Sado *et al.* [14] demonstrated a strain specific response of inbred rats to induction of EAG. They showed that Wistar–Kyoto (WKY) and SHR rats developed severe glomerulonephritis with marked pulmonary haemorrhage, while F344 and Lewis rats developed mild glomerulonephritis with only petechial pulmonary haemorrhage. This suggested that genetic differences in experimental animals influenced the severity of renal damage.

In the WKY rat, Sado *et al.* found that immunization with trypsin-digested affinity-purified heterologous (bovine) GBM [15], or homologous (rat) GBM [16] resulted in the development of circulating and deposited anti-GBM antibodies, proteinuria and glomerulonephritis with crescent formation. Anti-GBM antibodies purified from the urine of nephritic rats were shown to transfer disease to syngeneic recipients [17]. In contrast, Bolton *et al.* [18] reported that reduction in the pH of collagenase-digested bovine GBM, and the use of Bordetella pertussis in addition to FCA, were necessary to induce crescentic nephritis in the WKY rat. In their model of EAG, rats had positive tests for delayed type hypersensitivity, T cells underwent antigen specific transformation, and T cells and macrophages were present on renal histology. Nishikawa *et al.* [19] used a similar model of EAG in the WKY rat, induced by collagenase-digested bovine GBM, to examine crescent formation. They showed that hyaluronate was an abundant extracellular component of crescents, and played an important role in their formation by its effect on migration and activation of lymphocytes, monocytes/macrophages, fibroblasts, and epithelial cells.

We have previously demonstrated that EAG can be induced in the Brown Norway (BN) rat, but not in other strains studied, by immunization with either isologous (BN) or homologous (Sprague–Dawley, SD) GBM [20]. This resulted in circulating anti-GBM antibodies, linear deposition of IgG on the GBM, albuminuria and focal segmental glomerulonephritis. The anti-GBM antibodies in this model of EAG had a specificity similar to that of human autoantibodies [21]. We have also found increasing evidence for T cell involvement in the induction of EAG in the BN rat. Transfer of T cells from affected animals resulted in priming for EAG in naive recipients [22], and both cyclosporin A [23] and anti-CD4 monoclonal antibody

[24] were effective in controlling glomerulonephritis. Overall, EAG in the BN rat has been useful for investigating mechanisms of induction and regulation of autoimmunity to GBM. However, because of the variability of the resulting glomerulonephritis, it is not ideal for studies of the development of disease. For this reason we have attempted to develop a model of EAG in the rat with similar immunological characteristics but more consistent and severe glomerulonephritis [25].

In this study we compare the effectiveness of different antigen preparations (sheep or rat GBM) in the induction of glomerulonephritis in two genetically distinct substrains of WKY rat, with the aim of producing a model of EAG similar to Goodpasture's disease in both immunology and pathology. The model with the most severe and consistent glomerulonephritis was that induced by rat GBM at pH7 in the WKY/CR rat. This model should provide the basis for further studies of the genetics of EAG, and the opportunity to study novel approaches to immunotherapy.

Subjects and methods

Experimental animals

Male WKY rats weighing 120–150 g were purchased from Charles River, Margate, UK (WKY/CR, RT1-l) and Olac, Bicester, UK (WKY/Olac, RT1-k). The RT1 types were kindly provided by the suppliers. All animals were housed in standard conditions and had free access to normal laboratory diet and water.

Preparation of glomerular basement membrane antigens

Rat GBM. Rat GBM was prepared by a method similar to that described by Bowman *et al.* [26]. All stages were carried out at 4°C. Kidneys from normal Sprague–Dawley (SD) rats (Sigma, Poole, UK) were decapsulated, the medulla partly removed, and the cortex forced through a 106- μ m stainless steel sieve (Endecotts, London, UK) and washed with ice cold phosphate-buffered saline (PBS), pH 7.4. The separated glomeruli were passed through a 250- μ m sieve to remove large fragments, and collected on a 63- μ m sieve. The isolated glomeruli were washed on the sieve with ice-cold PBS, centrifuged (Jouan, Tring, UK) at 400 g for 5 min, and washed twice with PBS. After examination by light-microscopy (Olympus, London, UK), to exclude tubular contamination, the isolated glomeruli were suspended in PBS and sonicated (Fisons, Loughborough, UK) at 18- μ m amplitude in 30-s bursts, with ice cooling, then examined by light-microscopy for disruption. Sonication was continued until all glomeruli were disrupted (usually 90 s). The disrupted glomeruli were centrifuged at 121 g for 15 min, the supernatant discarded, and the precipitate washed a further two times with PBS at the same speed. The precipitate was then washed twice in ice cold distilled water at 400 g for 5 min and lyophilized (Life Science International, Basingstoke, UK) overnight.

The freeze dried material was cut up finely, suspended in 0.1 M Tris/0.005 M calcium acetate buffer (BDH, Poole, UK), pH 7.4, at 10 mg/ml, and 0.7% w/w collagenase (type I, Sigma) was added together with a mixture of proteinase

inhibitors (1 mM phenylmethane-sulphonyl fluoride, 10 mM N-ethylmaleimide, 10 mM 6-aminocaproic acid, Sigma). The reaction mixture was incubated at 37°C with stirring for 90 min, and the collagenase was then heat inactivated at 60°C for 5 min. The mixture was centrifuged at 700 g for 15 min, and the resulting supernatant of collagenase-solubilized rat GBM was stored at -20°C. Determination of the optical density of the collagenase-digested material on a spectrophotometer (Lightpath Optical, Haroldwood, UK) at 280 nm showed that around 20% of GBM was solubilized from the initial freeze dried material. Acidified preparations of rat GBM for immunization were prepared in 0.2 M glycine-HCl buffer (BDH), and the pH checked immediately before injection.

Sheep GBM. Antigenic material was prepared from normal sheep kidneys (obtained fresh from an abattoir) by a method similar to that described above. However, due to differences in the size of the kidneys and glomeruli, the cortex was first minced in order to allow easier passage through the sieves. The minced cortex was forced through a 150- μ m sieve, followed by a 250- μ m sieve to remove any large fragments, and the glomeruli were collected on a 63- μ m sieve. The other stages of the preparation and digestion were the same as for rat GBM.

Assay systems

Anti-GBM ELISA. Circulating anti-GBM antibodies were measured in sera from WKY rats by a solid-phase enzyme-linked immunosorbent assay (ELISA), similar to that previously described [27,28]. Collagenase-digested rat GBM was coated onto 96-well polystyrene microtitre plates (Gibco, Paisley, UK), at 150 μ l/well of 20 μ g/ml GBM in 0.005 M carbonate buffer (BDH), pH 9.6, by incubation at 37°C for 2 h, followed by further incubation at 4°C overnight. Coated plates were washed three times with PBS/Tween, and either used immediately or stored at 4°C. Coated plates could be stored for up to 2 weeks. The plates were then blocked with 5% bovine serum albumin (BSA, Sigma) at 37°C for 2 h. After further washing in PBS/Tween, 150- μ l aliquots of an optimal dilution of control or test rat serum (1/16 as determined previously by a dilution curve) in PBS/Tween were added to GBM-coated wells in triplicate and incubated at 37°C for 1 h. After this time, the solution was decanted and the plates were washed three times with PBS/Tween and drained. Bound anti-GBM antibody was detected by incubating wells with 150 μ l of horseradish peroxidase conjugated sheep anti-rat IgG (Sigma), at a dilution of 1:1000 for 1 h at 37°C. Excess conjugate was washed off, and 150 μ l of the substrate ortho-phenylenediamine dihydrochloride (OPD, Sigma) in 0.05 M phosphate-citrate buffer (BDH), pH 5, were added for 15 min at room temperature (RT), after which time the reaction was terminated by the addition of 30 μ l of 3M H₂SO₄ (BDH) to each well. The absorbances for each well were read at 492 nm using a Titertek Multiskan ELISA plate reader (Lab Tech International, Uckfield, UK), and the results expressed as an OD for each triplicate sample. In order to compare different experimental groups, results were expressed as percentages of the binding obtained with the positive and negative reference sera, as previously described [26,28].

Rocket immunoelectrophoresis. Urinary albumin concentrations were measured by rocket immunoelectrophoresis (Pharmacia, St Albans, UK) using a similar method to Laurell [29]. Urine samples from experimental animals were

subjected to immunoelectrophoresis at 60 v in an electrophoresis tank containing barbitone buffer (BDH), pH 9.5, for 6 h, using a 1% agarose gel (BDH) containing rabbit antisera to rat albumin raised in our laboratory. Results were calculated using rat serum albumin standards (which were run at the same time), and expressed in mg per 24 h.

Renal histology

Direct immunofluorescence. Direct immunofluorescence (IF) studies were carried out on kidneys obtained at the time of killing, by a method similar to that previously described [30]. Tissue was embedded in OCT II embedding medium (Bayer, Basingstoke, UK) on cork discs, snap frozen in isopentane (BDH) precooled in liquid nitrogen, and stored at -70°C. Cryostat (Bright, Huntingdon, UK) sections were cut at 5 μ m and air dried for 1 h. Sections were then washed twice in PBS for 15 min, and fixed in acetone (BDH) for 10 min. After further washing in PBS (two changes for 10 min), the sections were blocked with 20% normal rabbit serum for 30 min at RT in a moist chamber. The sections were then overlaid with fluorescein isothiocyanate (FITC) labelled rabbit anti-rat IgG (Dako, High Wycombe, UK) at a dilution of 1:20 for 1 h at RT in a moist chamber. Excess conjugate was washed off with PBS (two changes for 15 min), and the sections were mounted in PBS/glycerol for examination under a fluorescence microscope (Olympus). The degree of immunostaining was assessed 'blind' by two observers (JR, CDP) and graded from 0 to 3+.

Light-microscopy. Renal tissue, taken at the time of killing, was fixed in 10% neutral-buffered formalin, processed and embedded in paraffin wax for light-microscopy by standard techniques. Sections (3 μ m) were stained with H & E, and periodic acid-Schiff. Glomerular damage was graded 'blind' by one experienced pathologist (DJE). Fifty glomeruli per kidney were assessed as being normal, abnormal, or showing necrosis, and results expressed as a percentage of glomeruli examined.

Induction of EAG

EAG was induced in WKY rats by a single i.m. injection of the different preparations of collagenase-solubilized GBM at a dose of 2 mg/kg body weight in an equal volume of Freund's complete adjuvant (FCA, Sigma) [20,25]. The optimum dose of GBM for these studies was determined by pilot experiments. Control rats received an i.m. injection of emulsion containing an equal volume of acetate buffer in FCA. Serial blood samples were taken by tail artery puncture under ether (BDH) anaesthesia, and 24-h urine specimens were obtained by placing animals in metabolic cages (R. S. Biotech, Finedon, UK). Animals were monitored weekly by assessing circulating anti-GBM antibody concentrations by ELISA and albuminuria by rocket immunoelectrophoresis. Kidneys were examined at the time of killing for deposition of IgG along the GBM by immunofluorescence, and glomerular abnormalities by light-microscopy.

Comparison between homologous and heterologous antigen. Groups of male WKY rats ($n=5$) from both suppliers (WKY/CR and WKY/Olac) were given a single i.m. injection of: (a) homologous (rat) GBM in FCA at a dose of 2 mg/kg; (b) heterologous (sheep) GBM in FCA at a dose of 2 mg/kg; (c) FCA alone.

Effect of pH of homologous antigen. Groups of male WKY rats ($n=5$) from both suppliers (WKY/CR and WKY/Olac) were given a single i.m. injection of: (a) homologous (rat) GBM, pH 7, in FCA at a dose of 2 mg/kg; (b) homologous (rat) GBM, pH 5, in FCA at a dose of 2 mg/kg; (c) homologous (rat) GBM, pH 3, in FCA at a dose of 2 mg/kg; (d) FCA alone.

Statistical analysis. Differences between data were determined by the two-sample Student's *t* test. Analysis of variance was used to confirm differences between multiple data.

Results

Comparison between homologous and heterologous antigen in induction of EAG

Anti-GBM antibody. The kinetics of circulating anti-GBM antibodies in all groups of animals are shown in Figure 1. All immunized animals developed circulating antibody detectable by 14 days and rising until day 28. WKY/CR rats given rat GBM produced a higher level of anti-GBM antibodies at all time points than other groups. WKY/Olac rats given rat GBM produced higher levels of antibody than WKY/CR or WKY/Olac rats given sheep GBM. Control animals given FCA alone did not develop detectable circulating antibody.

Albuminuria. The albumin excretion in all groups of animals is shown in Figure 2. There were high levels of albuminuria by day 14 in WKY/CR rats given rat GBM compared with other groups. Albuminuria increased further by day 21, and peaked at day 28. WKY/Olac rats given rat GBM also showed an increase in albuminuria between day 14 and day 21, which fell by day 28. WKY/CR and WKY/Olac rats given sheep GBM showed a slight increase in albuminuria between day 21 and 28. Animals given FCA alone showed no significant albuminuria.

Direct immunofluorescence. Direct IF of kidney tissue at week 4 revealed that WKY/CR and WKY/Olac rats

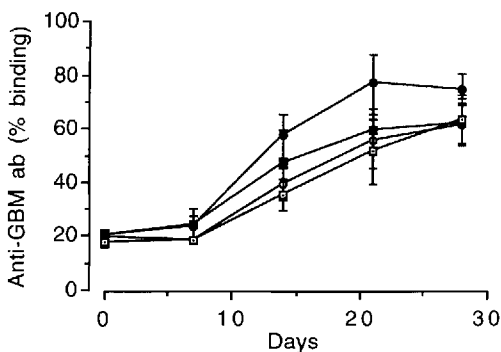


Fig. 1. Circulating anti-GBM antibody concentrations in groups of WKY/CR rats ($n=5$) immunized with rat GBM in FCA (●), or sheep GBM in FCA (○); and WKY/Olac rats ($n=5$) immunized with rat GBM in FCA (■), or sheep GBM in FCA (□). Results shown represent the mean \pm SD of each group. ($P < 0.001$, CR/rat vs Olac/rat, CR/rat vs CR/sheep).

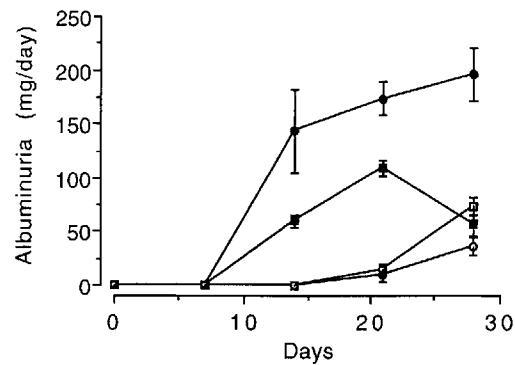


Fig. 2. Albuminuria in groups of WKY/CR rats ($n=5$) immunized with rat GBM in FCA (●), or sheep GBM in FCA (○); and WKY/Olac rats immunized with rat GBM in FCA (■), or sheep GBM in FCA (□). Results shown represent the mean \pm SD of each group. ($P < 0.001$, CR/rat vs Olac/rat, CR/rat vs CR/sheep, Olac/rat vs Olac/sheep).

given rat GBM had strong linear deposition of IgG along the GBM and to a lesser extent the TBM. In contrast, WKY/CR and WKY/Olac rats given sheep GBM showed less intense linear deposits of IgG along the GBM, with some loops exhibiting granular deposits, in addition to weak, intermittent deposits on the TBM. Control animals given FCA alone showed no positive findings on IF. Results are summarized in Table 1 and illustrated in Figure 3.

Light-microscopy. Light-microscopy of kidney tissue at week 4 in WKY/CR rats given rat GBM showed severe focal proliferative glomerulonephritis (FPGN) affecting up to 90% of glomeruli, with segmental necrosis/crescent formation affecting up to 25% of glomeruli, while WKY/CR rats given sheep GBM showed milder FPGN affecting up to 40% of glomeruli, with segmental necrosis/crescent formation affecting up to 15% of glomeruli. In contrast, WKY/Olac rats given rat GBM showed moderate FPGN affecting up to 60% of glomeruli, with segmental necrosis/crescent formation affecting up to 20% of glomeruli, while WKY/Olac rats given sheep GBM showed mild FPGN affecting up to 50% of glomeruli, with segmental necrosis/crescent formation affecting up to 15% of glomeruli. Control animals from both suppliers given FCA alone showed normal histology. Results are shown in Figure 4 and illustrated in Figure 5.

Table 1. Deposition of IgG on the GBM, as judged by intensity of immunofluorescence at week 4, in groups of WKY/CR rats ($n=5$) and WKY/Olac rats ($n=5$) immunized with rat GBM or sheep GBM

	3+	2+	1+	-
CR/rat	5	0	0	0
CR/sheep	0	4	1	0
CR/FCA	0	0	0	5
Olac/rat	4	1	0	0
Olac/sheep	0	2	3	0
Olac/FCA	0	0	0	5

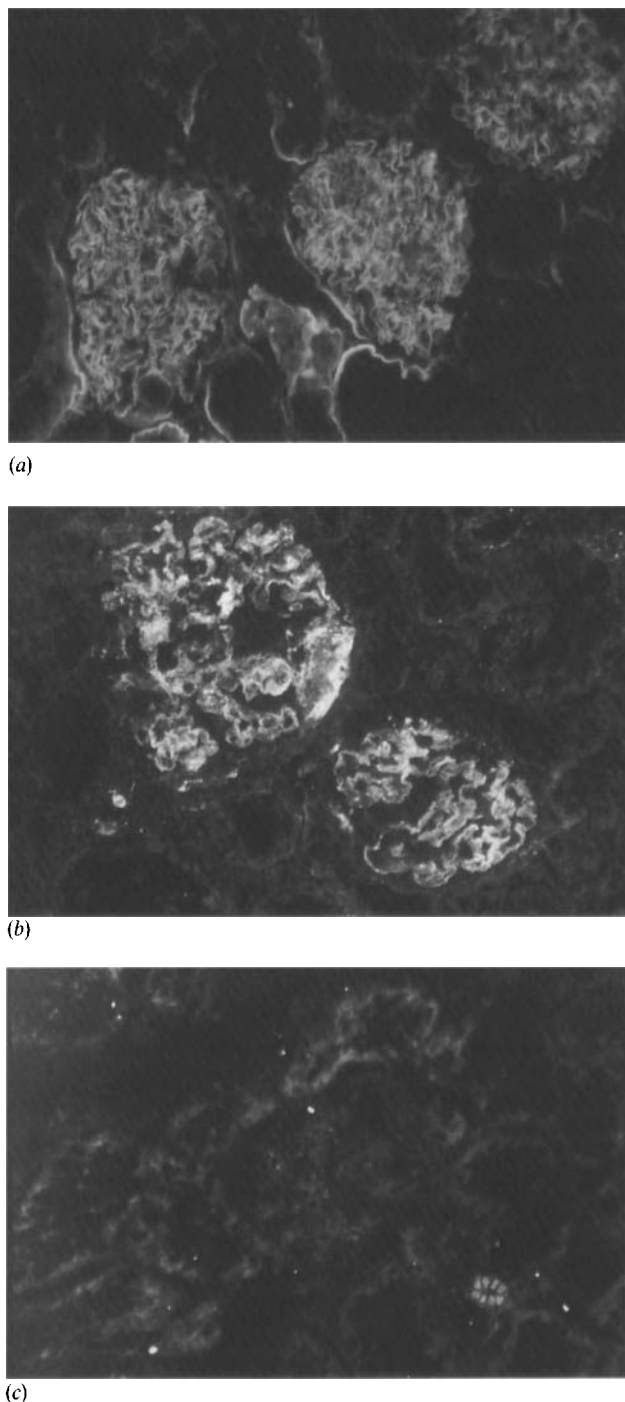


Fig. 3. Direct immunofluorescence of kidney tissue at 4 weeks from WKY/CR rats immunized with (a) rat GBM in FCA showing strong linear deposition for IgG along the GBM; (b) sheep GBM in FCA showing moderate linear and granular deposits of IgG on the GBM; and (c) FCA alone showing background binding only.

Effect of pH of homologous antigen in induction of EAG

Anti-GBM antibody. The kinetics of circulating anti-GBM antibodies in WKY/CR and WKY/Olac rats are shown in Figure 6 (the pH 7 group is the same group of animals described in the first part of this study). All immunized animals developed circulating anti-GBM

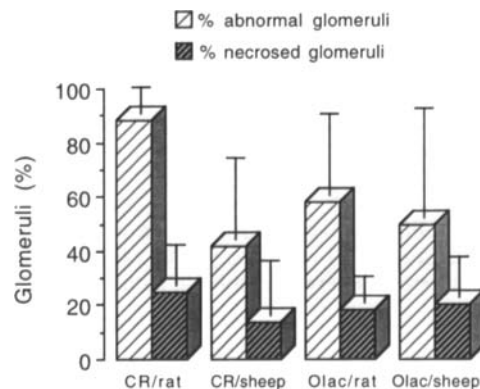


Fig. 4. Percentage of abnormal and necrosed glomeruli from WKY/CR ($n=5$) and WKY/Olac ($n=5$) rats immunized with either rat GBM in FCA or sheep GBM in FCA. Results shown represent the mean \pm SD of each group at week 4 after immunization. ($P < 0.015$, CR/rat vs Olac/rat; $P < 0.002$, CR/rat vs CR/sheep).

antibodies detectable by 14 days and rising until day 28. WKY/CR rats given rat GBM, pH 7, produced a higher level of antibody until day 21 than rats given rat GBM, pH 5, which in turn produced a higher level of antibody until day 21 than animals given rat GBM, pH 3, (Figure 6a). Similarly, circulating anti-GBM antibody levels were lower in WKY/Olac rats given rat GBM at the lower pH (Figure 6b). All groups of immunized animals produced similar levels of antibody by day 28. Control animals given FCA alone produced no detectable circulating anti-GBM antibody.

Albuminuria. The albumin excretion in WKY/CR and WKY/Olac rats is shown in Figure 7. There were high levels of albuminuria by day 14 in WKY/CR rats given rat GBM, pH 7, which increased further by day 21, and peaked at day 28. WKY/CR rats given rat GBM, pH 5, showed an increase in albuminuria between day 14 and day 21, which was maintained at day 28, while those given rat GBM, pH 3, only showed a slight increase above normal (Figure 7a). WKY/Olac rats given rat GBM, pH 7, showed an increase in albuminuria between day 7 and day 14, which peaked between day 21 and day 28. WKY/Olac rats given rat GBM, pH 5, showed increased levels between day 14 and day 21 (lower than with GBM pH 7), which were maintained at day 28, while those given rat GBM, pH 3, only showed a slight increase by day 21 (Figure 7b).

Direct immunofluorescence. Direct IF of kidney tissue at week 4 revealed that WKY/CR and WKY/Olac rats given rat GBM, pH 7, had strong linear deposition of IgG along the GBM, while WKY/CR and WKY/Olac rats given rat GBM, pH 5, showed moderate fluorescence along the GBM, and WKY/CR and WKY/Olac rats given rat GBM, pH 3, showed only weak fluorescence along the GBM. Control animals given FCA alone showed no positive findings on IF. Results are summarized in Table 2.

Light-microscopy. Light-microscopy of kidney tissue at week 4 in WKY/CR rats given rat GBM, pH 7, showed

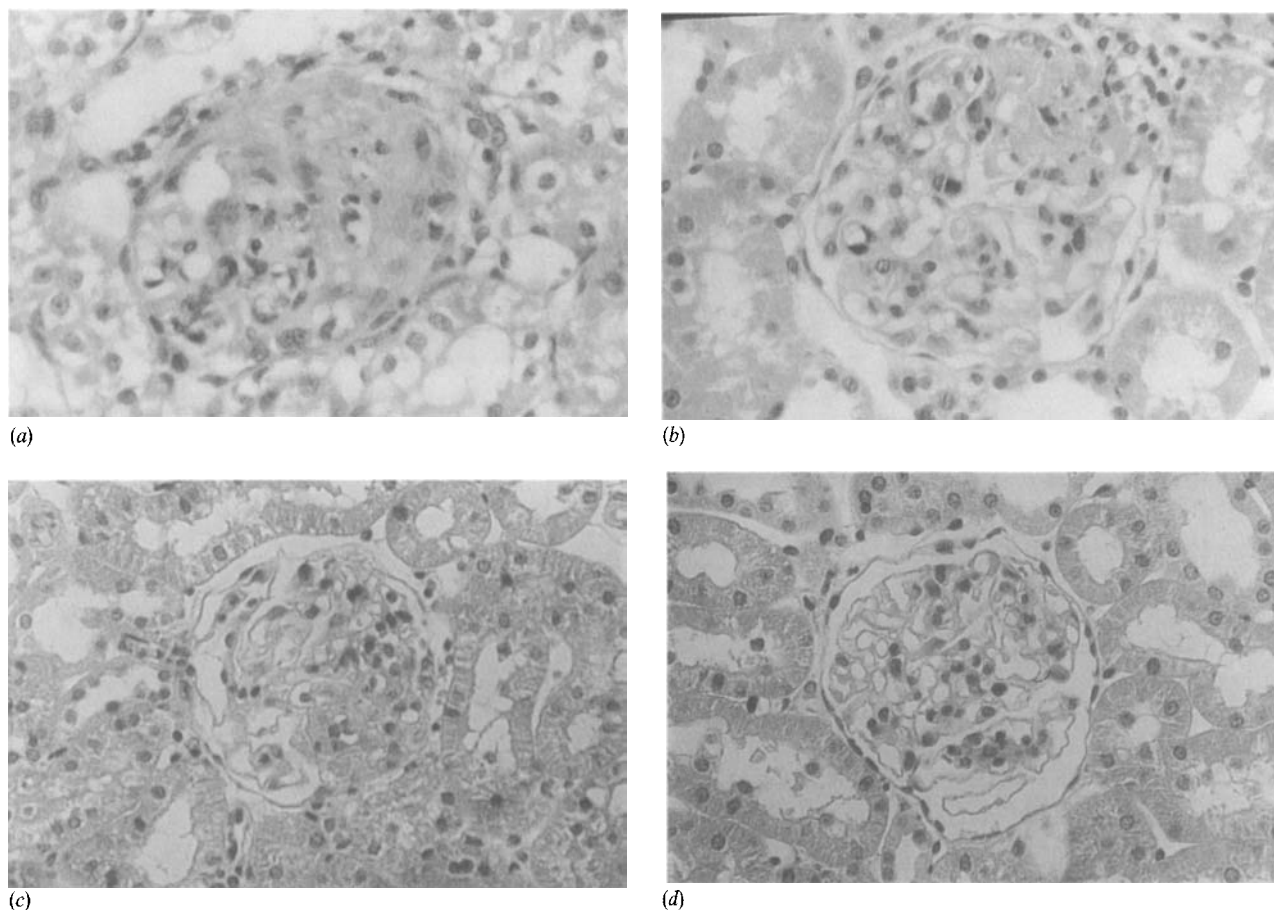


Fig. 5. Light-microscopy of kidney tissue at 4 weeks from WKY/CR rats immunized with (a) rat GBM in FCA showing marked segmental necrosis of the glomerular tuft with crescent formation; (b) rat GBM in FCA showing segmental necrosis; (c) sheep GBM in FCA showing segmental proliferation and necrosis; and (d) FCA alone showing a normal glomerulus.

Table 2. Deposition of IgG on the GBM, as judged by intensity of immunofluorescence at week 4, in groups of WKY/CR rats ($n=5$) and WKY/Olac rats ($n=5$) immunized with rat GBM at a different pH

	3+	2+	1+	-
CR/pH 7	5	0	0	0
CR/pH 5	1	3	1	0
CR/pH 3	0	0	4	1
Olac/pH 7	4	1	0	0
Olac/pH 5	0	3	2	0
Olac/pH 3	0	0	3	2

severe FPGN affecting up to 90% of glomeruli, with segmental necrosis/crescent formation affecting up to 25% of glomeruli. WKY/CR rats given rat GBM, pH 5, showed moderate FPGN affecting up to 65% of glomeruli, with segmental necrosis/crescent formation affecting up to 15% of glomeruli, while those given rat GBM, pH 3, showed mild FPGN affecting up to 20% of glomeruli, with segmental necrosis/crescent formation affecting up to 5% of glomeruli. In contrast, WKY/Olac rats given rat GBM, pH 7, showed moderate FPGN affecting up to 60% of glomeruli, with segmental necrosis/crescent formation affecting up to

20% of glomeruli, while those given rat GBM, pH 5, showed mild FPGN affecting up to 30% of glomeruli, with segmental necrosis/crescent formation affecting up to 5% of glomeruli, and those given rat GBM, pH 3, showed mild FPGN affecting up to 20% of glomeruli, with segmental necrosis/crescent formation affecting up to 10% of glomeruli. Control animals from both suppliers given FCA alone showed normal histology. Results are shown in Figure 8.

Discussion

Experimental autoimmune glomerulonephritis (EAG) in the BN rat has proved to be a good model for investigating the immunological mechanisms involved in the induction and control of autoantibody synthesis in anti-GBM disease [20–24]. However, because of the inconsistency of the development of glomerulonephritis, it has proved difficult to investigate the relationship between autoimmunity and resulting tissue injury. For this reason, we decided to develop another model of EAG in the rat with similar immunological characteristics, but more consistent and severe glomerulonephritis. Our early experiments [25] suggested

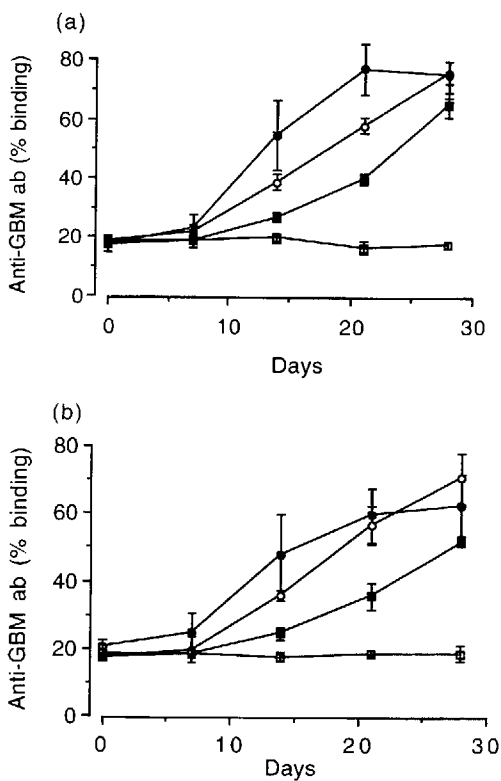


Fig. 6. Circulating anti-GBM antibody concentrations in groups of (a) WKY/CR rats ($n=5$), or (b) WKY/Olac rats ($n=5$) immunized with rat GBM in FCA, pH 7 (●), rat GBM in FCA, pH 5 (○), rat GBM in FCA, pH 3 (■), or given FCA alone (□). Results shown represent the mean \pm SD of each group. ($P < 0.001$, CR or Olac given rat GBM pH 7, pH 5 or pH 3 vs those given FCA).

that the WKY strain was a good candidate, as reported by Sado *et al.* [14–16]. The response of genetically different substrains of WKY rat from two suppliers, Charles River and Olac, to preparations of both homologous (rat) and heterologous (sheep) GBM was therefore examined, to determine which model showed most injury.

The WKY strain of rat has previously been reported by Sado *et al.* to develop glomerulonephritis when immunized with bovine or rat GBM [15,16], and other groups have more recently reported broadly similar results using bovine GBM [18,19]. We chose to examine the use of rat GBM, in order to produce a model with immunological characteristics more similar to that of spontaneous autoimmune disease. In the present study we have shown that WKY/CR rats (RT1-l) immunized with rat GBM are more susceptible to EAG than WKY/Olac (RT1-k) rats. Since CR and Olac WKY rats were derived from the same original colony of Wistar rats, they are likely to be genetically similar. However, they have different RT1 haplotypes, suggesting the involvement of a MHC gene effect in the induction of EAG. In addition, it is possible that there are other important, unknown genetic differences between these substrains. Our previous work showed that Lewis (RT1-l) rats, with the same MHC haplotype as WKY/CR rats, were resistant to the development

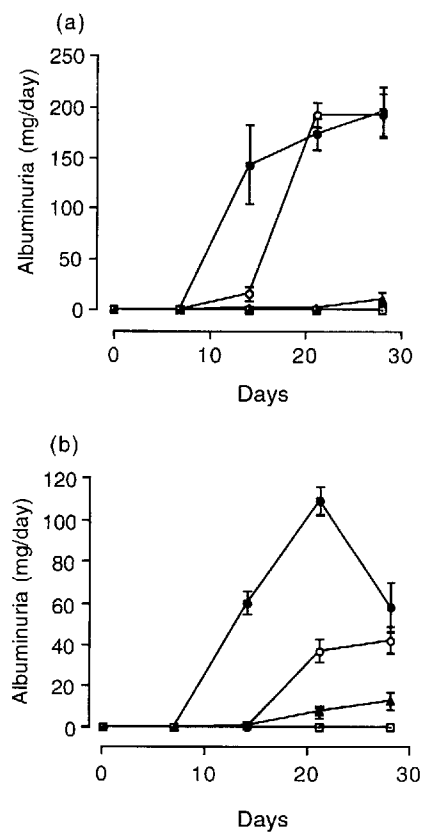


Fig. 7. Albuminuria in groups of (a) WKY/CR rats ($n=5$), or (b) WKY/Olac rats ($n=5$) immunized with rat GBM in FCA, pH 7 (●), rat GBM in FCA, pH 5 (○), rat GBM in FCA, pH 3 (■), or given FCA alone (□). Results shown represent the mean \pm SD of each group. ($P < 0.001$, CR or Olac rats given rat GBM pH 7 or pH 5 vs those given rat GBM pH 3 or FCA).

of EAG when immunized with rat GBM [20]. This has been confirmed by our recent experiments examining genetic susceptibility to EAG [31], and suggests the importance of non-MHC genes in the induction of disease. This genetic association may be analogous to that observed in human anti-GBM disease, which is known to be linked with class II HLA genes [9], but which must also depend on other genetic and environmental factors [6].

The present study shows that both WKY/CR and WKY/Olac rats immunized with collagenase-digested rat GBM develop a more consistent and severe form of EAG than when immunized with collagenase-digested sheep GBM, prepared in exactly the same way. Sheep GBM was chosen as a heterologous antigen to compare with rat GBM because previous work in our laboratory has shown it to have certain characteristics more similar to those of human GBM than bovine GBM [32]. Animals from both suppliers given rat GBM showed severe focal proliferative glomerulonephritis with crescent formation and necrosis. In comparison, animals given sheep GBM developed only a mild proliferative glomerulonephritis. Of interest, the immunofluorescence findings were slightly different in animals immunized with sheep GBM, showing weak

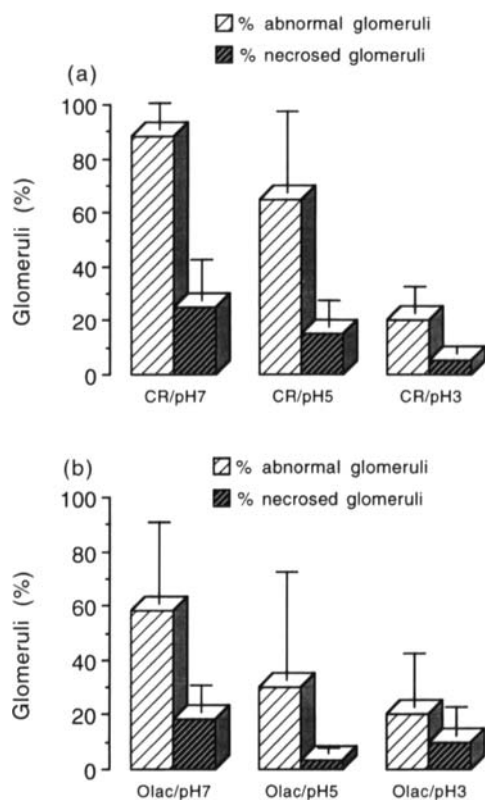


Fig. 8. Percentage of abnormal and necrosed glomeruli in groups of (a) WKY/CR rats ($n=5$), or (b) WKY/Olac rats ($n=5$) immunized with rat GBM in FCA, pH 7, rat GBM in FCA, pH 5, or rat GBM in FCA, pH 3. Results shown represent the mean \pm SD of each group at week 4 after immunization. ((a) $P < 0.001$, CR/pH 7 vs CR/pH 3; $P < 0.005$, CR/pH 5 vs CR/pH 3; (b) $P < 0.008$, Olac/pH 7 vs Olac/pH 3).

granular as well as linear deposits of IgG, which might be due to immune responses to additional components of the heterologous antigen. These results demonstrate that homologous (rat) GBM antigen is more nephritogenic than heterologous (sheep) GBM antigen in the present model of EAG in the WKY rat. This is in contrast to the findings of Sado *et al.*, who reported that heterologous (bovine) GBM was a better immunogen than homologous (rat) GBM [15,16]. This could be due to intrinsic differences between bovine and sheep GBM, or to differences in the preparation of the antigen, such as the method of solubilization.

Previous studies by others [33,34], and more recently in our laboratory [21], using a variety of immunochemical techniques have demonstrated that the target of autoantibodies in EAG in the rat is contained within the NC1 domains of type IV collagen, and is likely to be $\alpha 3(IV)NC1$, as in Goodpasture's disease. To induce EAG, Sado *et al.* used trypsin to digest bovine GBM, whereas we used collagenase for rat and sheep GBM. The different digestion procedures may have resulted in different amounts of the various NC1 domains of type IV collagen being released, although direct comparison between preparations is difficult. In another important study, Kalluri *et al.* showed that $\alpha 3(IV)NC1$ dimers from bovine kidney could induce an organ-specific model of Goodpasture's disease in rabbits,

whereas other $\alpha(IV)$ chains were non-pathogenic [35]. This demonstrates that $\alpha 3(IV)NC1$ contains a nephritogenic epitope which is not shared by the NC1 domains of the other five chains of type IV collagen.

Once it was established which type of GBM gave the more consistent and severe glomerulonephritis, we examined the effect of changing the pH of the GBM on the severity of disease. This has been shown to have the effect of changing the configuration of the antigen [36], and thus may influence nephritogenicity. Bolton *et al.* [18] reported that an acidified preparation of bovine GBM was a better immunogen for EAG, in that rats given an acidic preparation of GBM (pH not stated) developed crescentic nephritis, while those given GBM at pH 7 showed inconsistent and mild glomerulonephritis. This is in contrast to our results using rat GBM, which showed that reducing the pH of the GBM to pH 5 before immunization resulted in a delay in the circulating anti-GBM antibody response, together with a reduction in the severity of glomerulonephritis. Furthermore, animals given GBM at pH 3 showed considerably lower levels of albuminuria and glomerular injury, possibly due to denaturation of the antigen. Similar results were observed in both WKY/CR and WKY/Olac rats in this respect. However, although all groups of animals developed similar levels of circulating anti-GBM antibody by day 28, deposition of antibody on the GBM was less intense as the pH of the antigen decreased. This implies that deposited anti-GBM antibodies on the GBM have a higher specificity for disease than circulating antibodies, perhaps because of their specificity or avidity. In addition, although antibodies appear to be necessary for induction of nephritis, the role of cell-mediated immunity seems likely to be important in determining its severity in this model. It may be relevant that Bolton *et al.* used bovine GBM to induce nephritis, rather than rat GBM, and it is possible that a reduction in pH may have resulted in the unfolding of the bovine antigen differently from that of the rat antigen. Ideally, we should also have examined the effect of reducing the pH of sheep GBM in our model, but we chose to focus on the rat antigen since it produced a better response at neutral pH.

In conclusion, the model of anti-GBM disease produced by the administration of homologous (rat) GBM antigen at pH 7 to WKY/CR rats showed a consistent and severe form of crescentic glomerulonephritis. The use of a homologous antigen is preferred in studies of autoimmunity, provided it is effective in inducing disease, since it avoids the broader immune response which may be induced by foreign antigens. The present model of EAG shares many immunological and pathological features in common with Goodpasture's disease, and therefore provides a suitable experimental model in which to evaluate the mechanisms involved in the pathogenesis of anti-GBM nephritis. In particular it should prove valuable in examining the genetic basis of susceptibility to EAG [31], and in investigating novel immunotherapeutic strategies, including the induction of mucosal tolerance [37] and the use of

CTLA4-Ig to prevent T cell activation [38]. Greater understanding of the principles of autoimmunity to the GBM should lead to the development of more specific forms of immunotherapy for patients with Goodpasture's disease.

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