Strain susceptibility to active induction and passive transfer of experimental autoimmune glomerulonephritis in the rat

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Abstract
Background. Previous studies have shown that different inbred rat strains vary in their susceptibility to experimental autoimmune glomerulonephritis (EAG). The Wistar Kyoto (WKY) rat is highly susceptible and develops crescentic glomerulonephritis, while the Lewis (LEW) rat is resistant. When immunized with collagenase-solubilized rat glomerular basement membrane (GBM), both strains produce circulating autoantibodies reactive with rat GBM by enzyme-linked immunosorbent assay, but only the WKY rat shows strong linear deposits of IgG on the GBM.

Methods. We investigated the hypothesis that differences in the characteristics of the anti-GBM antibodies produced, or in the inflammatory response to antibody deposition, could account for susceptibility.

Results. We found that circulating anti-GBM antibodies from WKY rats immunized with GBM were present at a higher concentration than those from LEW rats. Antibodies from WKY rats also recognized the rat $\alpha_3$ chain of type IV collagen [$\alpha_3$(IV)NC1], whereas those from LEW rats did not. Antibody eluted from the kidneys of WKY rats with EAG induced by GBM showed a higher affinity for GBM and recombinant rat $\alpha_3$(IV)NC1 than circulating antibody. This eluted antibody bound strongly to normal kidney sections from both WKY and LEW rats. Passive transfer of eluted anti-GBM antibodies from WKY rats with EAG resulted in similar binding of IgG to the GBM of WKY and LEW rats at 24 h. However, only the WKY recipients went on to develop crescentic glomerulonephritis by 28 days.

Conclusions. This study demonstrates that the characteristics of the anti-GBM antibodies induced in WKY rats contribute to their susceptibility to EAG. However, the passive transfer experiments reveal that factors related to the inflammatory response to antibody deposition are also important in determining susceptibility. A combination of these genetic influences could explain the variation in severity of human anti-GBM disease.

Keywords: anti-GBM antibodies; experimental autoimmune glomerulonephritis (EAG); genetic susceptibility; glomerular basement membrane (GBM); Lewis (LEW) rat; Wistar Kyoto (WKY) rat

Introduction
Goodpasture’s, or anti-glomerular basement membrane (GBM), disease is an autoimmune disorder characterized by rapidly progressive glomerulonephritis and lung haemorrhage [1]. The disease is caused by autoantibodies to basement membranes of glomeruli and alveoli [2], and the pathogenicity of these antibodies has been demonstrated in passive transfer studies [3]. The autoantigen has been identified as the non-collagenous domain of the $\alpha_3$ chain of type IV collagen [$\alpha_3$(IV)NC1] [4,5], and the major epitope involved has been localized to the amino terminal of the $\alpha_3$(IV)NC1 molecule [6,7]. Goodpasture’s disease is associated with certain major histocompatibility complex (MHC) class II alleles; in particular, a positive association has been shown with DR15 and DR4 and a negative association with DR7 and DR1 [8,9]. T cells from patients with Goodpasture’s disease proliferate in response to the Goodpasture antigen [10] and, more recently, it has been shown that the precursor frequency of autoreactive T cells specific for $\alpha_3$(IV)NC1 is higher in patients with active disease than in controls and declines following treatment [11]. The disease relapses extremely rarely, perhaps due to the influence of CD4+CD25+ regulatory T cells [12].

Experimental autoimmune glomerulonephritis (EAG), an animal model of Goodpasture’s disease, can be induced in Wistar Kyoto (WKY) rats by immunization with heterologous or homologous
GBM in adjuvant [13–17]. The model of EAG in this rat strain is characterized by anti-GBM antibody production, accompanied by focal necrotizing glomerulonephritis with crescent formation. In contrast, Lewis (LEW) rats, with the same MHC background (Rt1-l) and immunized with the same antigen, develop circulating anti-GBM antibodies but no histological evidence of nephritis [18]. In recent studies examining the genetic basis of susceptibility to EAG, we have demonstrated that first generation crosses (F1; WKY × LEW) were completely resistant to the development of EAG, while WKY backcross animals (BC1; WKY × F1) showed a range of responses, from severe crescentic glomerulonephritis to no histological evidence of the disease [18]. These results indicate that EAG is inherited as a complex trait, with a role for WKY genes not linked to the MHC.

There is still controversy as to whether the development of EAG is associated mainly with humoral or cell-mediated immunity. There is now mounting evidence for the involvement of both limbs of the immune response in the pathogenesis of disease. The main target antigen of the autoantibodies has been shown to be the same in EAG as in Goodpasture’s disease [19,20]: the NC1 domain of the α3 chain of type IV collagen [α3(IV)NC1]. The role of anti-GBM antibodies in the pathogenesis of EAG has been demonstrated in passive transfer studies. This was first demonstrated by Steblay using passive transfer of blood from nephritic sheep [21], and has been confirmed more recently in mice and rats. Passive transfer of EAG has been demonstrated in SJL mice by anti-α3(IV)NC1 antibodies pooled from the serum of nephritic mice [22], and in WKY rats by antibodies purified from the urine of nephritic rats [23]. In addition, induction of disease has been demonstrated using monoclonal antibodies to GBM generated from WKY rats with EAG [24,25], confirming the nephritogenicity of these specific anti-GBM antibodies. More recently, transfer of sera from C57/BL6 mice with EAG to RAG-1 knockout mice (that lack adaptive immunity) induced linear deposits of IgG on the GBM and proteinuria, demonstrating that humoral immunity alone was sufficient to induce EAG [26].

However, there is also increasing evidence to support a role for autoreactive T lymphocytes in the pathogenesis of EAG. T cells have been shown to be present in the glomeruli of animals with EAG [17,22], and splenic T cells from these animals proliferate in response to α3(IV)NC1 [27,28] and can be used to transfer disease to naive recipients [22,29]. Glomerular T cells from rats with EAG show restricted T-cell receptor CDR3 spectratypes, demonstrating that they are an oligoclonal antigen-driven population [30]. More recently, it has been reported that an immunodominant B- and T-cell epitope from the N-terminus of rat α3(IV)NC1 is capable of inducing crescentic nephritis [31–34]. Several studies have shown that anti-T-cell immunotherapy can prevent or ameliorate disease [35–38]. Anti-CD4 mAb therapy is effective in the prevention of EAG [35], and anti-CD8 mAb therapy is effective in both prevention and treatment of established disease [36]. Inhibition of T-cell activation by blockade of the CD28-B7 co-stimulatory pathway [37], or the CD154-CD40 co-stimulatory pathway [38], has also been shown to ameliorate EAG. In addition, oral administration of GBM antigen [39], or nasal administration of recombinant α3(IV)NC1 [40], induces mucosal tolerance and reduces the severity of crescentic nephritis in EAG. Recently, it has been shown that B-cell-deficient mice immunized with α3(IV)NC1 develop proliferative glomerulonephritis with a glomerular infiltrate of T cells and macrophages, demonstrating that cell mediated immunity is sufficient to induce EAG [26]. Thus, there is now compelling evidence for the role of both humoral and cell-mediated immunity in the pathogenesis of experimental models of anti-GBM disease, which is also likely to be true for the human condition.

Apart from a clear role for the MHC, other genetic influences on the development and severity of anti-GBM disease have not been well-defined. In this study, we examine the differences in the characteristics of the anti-GBM antibodies between WKY (responder) and LEW (non-responder) rats, to determine if this could account for the difference in susceptibility to EAG. We demonstrate that anti-GBM antibodies in WKY rats are present in a higher concentration and show greater specificity for recombinant α3(IV)NC1, when compared with those in LEW rats. In addition, we demonstrate that passive transfer of eluted anti-GBM antibodies from kidneys of WKY rats with EAG leads to similar deposition of IgG on the GBM of both WKY and LEW rats, but results in the development of crescentic glomerulonephritis only in WKY rats. These findings illustrate the importance of both the autoimmune response, and also the inflammatory response to deposited antibody, in the development of glomerulonephritis.

### Subjects and methods

#### Experimental animals

Male WKY rats were purchased from Charles River, Margate, UK, and male LEW rats were purchased from Harlan UK Ltd, Bicester, UK. All animals were housed in standard conditions and had free access to normal laboratory diet and water. All experimental procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act.

#### Preparation of rat GBM

Collagenase-solubilized rat GBM (csGBM) was prepared from Sprague Dawley (SD) rat kidneys, as previously described [16,17]. Briefly, the kidneys were decapsulated, the medulla partly removed and the cortex passed through a series of sieves in order to isolate the glomeruli. After examination by light microscopy, the glomeruli were disrupted ultrasonically, and the resulting material lyophiolized and digested with purified type I collagenase (Sigma-Aldrich Company Ltd, Poole, UK) for 1 h at 37°C.
Preparation of recombinant rat \( \alpha_3(IV)NC1 \)

Recombinant rat \( \alpha_3(IV)NC1 \) was produced in a mammalian expression system and purified by FLAG affinity chromatography, as previously described [20]. Briefly, a cDNA fragment encoding rat \( \alpha_3(IV)NC1 \) was subcloned into a pFLAG CMV-1 expression vector, and the resultant plasmid was transfected into COS-7 cells. Recombinant rat \( \alpha_3(IV)NC1 \), secreted into the COS-7 cell supernatant, was then purified on an anti-M2 FLAG affinity column.

Active induction of EAG

Groups of male WKY and LEW rats (\( n = 5 \)), aged 8–12 weeks and weighing 120–150 g, were given a single intramuscular injection of rat csGBM in Freund's Complete Adjuvant (FCA) at a dose of 5 mg/kg body weight [16,17]. All animals were sacrificed at day 28 after immunization.

Assessment of disease

Enzyme-linked immunosorbent assay. Circulating anti-GBM antibody concentrations were measured in sera of experimental animals by a solid-phase enzyme-linked immunosorbent assay (ELISA), as previously described [16,17]. Briefly, csGBM or recombinant rat \( \alpha_3(IV)NC1 \) was coated on to microtitre plates (Life Technologies, Paisley, UK) at a concentration of 5 \( \mu \)g/ml by overnight incubation at 4°C, and an optimum dilution of test or control sera applied for 1 h at 37°C. Bound anti-GBM antibody was detected by alkaline phosphatase conjugated sheep anti-rat IgG (Sigma-Aldrich Company Ltd), and developed using the substrate \( p \)-nitrophenyl phosphate (NPP, Sigma-Aldrich Company Ltd). The absorbencies for each well were read at 405 nm using an Anthos Multiskan ELISA plate reader (Lab Tech International, Uckfield, UK), and the results calculated as mean optical density for each triplicate sample.

Rocket immunoelectrophoresis. Urinary albumin concentrations were measured in 24 h collections from experimental animals by rocket immunoelectrophoresis (Amersham Bioscience UK Limited), as previously described [16,17]. Briefly, urine samples from experimental animals were subjected to immunoelectrophoresis at 60 V in an electrophoresis tank containing Barbitone buffer (BDH Laboratory Supplies, Poole, Dorset, UK), pH 9.5, for 6 h, using a 1% agarose gel (BDH Laboratory Supplies) 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.

Direct immunofluorescence. Deposits of IgG within the glomeruli were detected by direct immunofluorescence, as previously described [16,17]. Tissue was embedded in OCT II embedding medium (Miles Inc., Elkhart, Indiana, USA) on cork discs, snap frozen in isopentane (BDH Laboratory Supplies) pre-cooled in liquid nitrogen and stored at \(-70^\circ\)C. Cryostat sections were cut 5 \( \mu \)m thick and were incubated with fluorescein isothiocyanate (FITC) labelled rabbit anti-\( \alpha \)-rat IgG (Serotec Ltd). The degree of immunostaining was graded by a blinded observer on a scale from 0 to 3+.

Light microscopy. Kidney tissue was fixed in 10% neutral buffered formalin, processed and embedded in paraffin wax for light microscopy by standard techniques (Histopathology Department, Hammersmith Hospital, Imperial College London). Briefly, 3 \( \mu \)m sections were stained with haematoxylin and eosin and periodic acid-Schiff. Fifty glomeruli per section were assessed by a blinded observer as: normal, abnormal (small areas of hypercellularity and/or focal necrosis), or severe (>50% of the glomerulus affected by segmental necrosis and/or crescent formation), and expressed as a percentage of glomeruli examined [16,17].

Immunohistochemistry. Kidney sections were stained for T cells and macrophages using a standard avidin-biotin complex immunoperoxidase staining technique. Briefly, formalin-fixed, paraffin embedded kidney sections were stained with monoclonal antibodies: W3/13 (T cells) and ED1 (macrophages) (Serotec Ltd). Numbers of glomerular T cells and macrophages were detected using a biotinylated secondary antibody and avidin-biotin complex (Dako Ltd, Cambridge, UK). The cellular infiltrate was assessed by a blinded observer by counting the number of positively stained cells per 50 consecutive glomeruli in cross section [17].

Elution of kidney bound anti-GBM antibodies

Anti-GBM antibody was eluted from the GBM of animals with EAG, as previously described [41,42]. Briefly, kidneys from WKY or LEW rats immunized with csGBM were pooled, and glomeruli isolated and sonicated as described above. The disrupted glomeruli were washed in PBS until the optical density (OD) at 280 nm was less than 0.05. Anti-GBM antibody was eluted from the fragmented GBM with 0.1M Glycine/HCl, pH 2.5, for 30 min at room temperature (RT), followed by centrifugation at 700 \( \times \) g for 5 min and immediately neutralized with 1M Tris. The amount of protein in the eluate was then measured on a spectrophotometer at 280 nm.

Indirect immunofluorescence for eluted antibodies

Binding of eluted anti-GBM antibody from WKY or LEW rats immunized with GBM in FCA was assessed by indirect immunofluorescence, as previously described [41]. Briefly, kidney tissue from normal WKY and LEW rats was embedded in OCT II embedding medium (Miles Inc., Elkhart, Indiana, USA) on cork discs, snap frozen in isopentane (BDH Laboratory Supplies) pre-cooled in liquid nitrogen and stored at \(-70^\circ\)C. Cryostat sections were cut 5 \( \mu \)m thick and were incubated with eluted anti-GBM antibodies for 1 h at RT. After washing, bound anti-GBM antibody was detected with FITC labelled rabbit anti-rat IgG (Serotec Ltd). The degree of immunostaining was graded by a blinded observer on a scale from 0 to 3+.

Concentration of anti-GBM antibodies in WKY and LEW rats

The concentration of circulating and eluted anti-GBM antibodies from WKY or LEW rats immunized with csGBM in FCA was measured by ELISA, as described above. Briefly, csGBM [17] or recombinant \( \alpha_3(IV)NC1 \) [20]...
was coated on to microtitre plates; serum from immunized or control WKY and LEW rats was applied at logarithmic dilutions of 1/30, 1/100, 1/300 or 1/1000, and eluate was applied at doubling dilutions of 1/4, 1/8, 1/16 or 1/32, for 1 h at 37°C. Bound anti-GBM antibody was detected by horseradish peroxidase (HRP) conjugated rabbit anti-rat IgG (Sigma-Aldrich Company Ltd) and developed using the substrate ortho-phenylenediamine dihydrochloride (OPD, Sigma-Aldrich Company Ltd). The absorbances for each well were read at 492 nm, and the results expressed as mean optical density for each triplicate sample.

**Functional affinity of anti-GBM antibodies from WKY rats**

The affinity of circulating and eluted anti-GBM antibodies from WKY rats immunized with csGBM in FCA was measured by ELISA, incorporating the mild chaotropic agent diethylamine (DEA), as previously described [42]. Briefly, plates were coated with csGBM or recombinant \( \alpha_3(IV)NC1 \), and serum was applied at an optimal dilution of 1/30, and eluate applied at an optimal dilution of 1/4, in the presence of DEA at concentrations of 5, 10, 20, 30 or 40 mM. Bound anti-GBM antibody was detected by HRP conjugated rabbit anti-rat IgG, as aforedescribed.

**Passive transfer of EAG to WKY and LEW rats**

Groups of male WKY and LEW rats (n=5), aged 8–12 weeks and weighing 120–150 g, were given an i.v. injection of anti-GBM antibody eluted from the kidneys of WKY rats with EAG at a dose of 500 \( \mu \)g per rat. Groups of animals were sacrificed at 24 h and 28 days after transfer. Deposition of IgG in the glomerulus, extent of glomerular abnormalities, and number of glomerular T cells and macrophages were assessed as described above.

**Statistical analysis**

Differences between data were determined by the Mann–Whitney U-test. ANOVA was used to confirm differences between multiple data.

**Results**

**Active induction of EAG**

**Assessment of disease following active induction of EAG.**

**Immunization with csGBM.** WKY rats immunized with rat csGBM in FCA developed circulating anti-GBM antibodies, strong linear deposits of IgG on the GBM, high levels of albuminuria, severe focal necrotizing glomerulonephritis with crescent formation, and increased numbers of glomerular T cells and macrophages, at day 28 after immunization. In contrast, LEW rats immunized with the same antigen developed a lower level of circulating anti-GBM antibodies, and no deposits of IgG on the GBM. They did not develop albuminuria, histological evidence of nephritis or glomerular infiltration by T cells and macrophages. Control WKY and LEW rats given FCA alone showed no manifestations of EAG. Results are summarized in Table 1.

**Concentration of circulating antibodies.**

**Directed to csGBM.** Serum anti-GBM antibodies from WKY rats immunized with csGBM in FCA showed a high level of binding to rat GBM by ELISA at serum dilutions of 1/30, 1/100 and 1/300, which titrated out to background levels at a dilution of 1/1000. Serum anti-GBM antibodies from LEW rats immunized with the same antigen showed a moderate level of binding to GBM at a serum dilution of 1/30, which titrated out to background levels at a dilution of 1/1000 (Figure 1A). Normal rat serum alone showed no binding to GBM.

**Directed to recombinant \( \alpha_3(IV)NC1 \).** Serum anti-GBM antibodies from WKY rats immunized with csGBM in FCA showed a high level of binding to recombinant rat \( \alpha_3(IV)NC1 \) at serum dilutions of 1/30, 1/100 and 1/300, which titrated out to background levels at a dilution of 1/1000. Serum anti-GBM antibodies from LEW rats immunized with the

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**Table 1. Summary of results from WKY and LEW rats (n=5) immunized with GBM in FCA or FCA alone. Results are expressed as a mean ± standard deviation of each group at day 28 after immunization**

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>LEW</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>GBM</td>
<td>FCA</td>
</tr>
<tr>
<td>Circulating anti-GBM antibody (OD at 405 nm)</td>
<td>2.1 ± 0.45*</td>
<td>0.28 ± 0.14</td>
</tr>
<tr>
<td>Deposits of IgG on GBM (intensity of fluorescence)</td>
<td>3+,**</td>
<td>0</td>
</tr>
<tr>
<td>Albuminuria (mg/day)</td>
<td>450±90**</td>
<td>0</td>
</tr>
<tr>
<td>Abnormal glomeruli (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) Total</td>
<td>100**</td>
<td>0</td>
</tr>
<tr>
<td>(b) Severe</td>
<td>85 ± 19**</td>
<td>0</td>
</tr>
<tr>
<td>Immunohistochemistry (cells per 50 glomeruli)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) T cells</td>
<td>180 ± 35**</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>(b) Macrophages</td>
<td>276 ± 48**</td>
<td>3 ± 1</td>
</tr>
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*P < 0.01, **P < 0.001; WKY vs LEW.
same antigen showed no binding to recombinant rat α3(IV)NC1 at all four dilutions (Figure 1B). Normal rat serum alone showed no binding to α3(IV)NC1.

**Concentration of deposited antibody.**

**Directed to csGBM.** Eluted anti-GBM antibodies from WKY rats immunized with csGBM in FCA showed a high level of binding to GBM at dilutions of 1/4, 1/8 and 1/16, which titrated out to background levels at a dilution of 1/32. Glomerular eluates from LEW rats immunized with the same antigen showed no binding to GBM at all four dilutions (Figure 2A). Elution buffer alone showed no binding to GBM.

**Directed to recombinant α3(IV)NC1.** Eluted anti-GBM antibodies from WKY rats immunized with csGBM in FCA showed a high level of binding to recombinant rat α3(IV)NC1 at dilutions of 1/4 and 1/8, and moderate binding at a dilution of 1/16, which titrated out to background levels at a dilution of 1/32. The glomerular eluate from LEW rats immunized with the same antigen showed no binding to recombinant rat α3(IV)NC1 at all four dilutions (Figure 2B). Elution buffer alone showed no binding to recombinant rat α3(IV)NC1.

**Functional affinity of circulating and deposited antibody.**

**Directed to csGBM.** Eluted anti-GBM antibodies from WKY rats immunized with csGBM in FCA showed higher functional affinity to csGBM than serum antibodies, in that the addition of DEA at a concentration of 40 mM was needed to inhibit binding of the eluate, while a lower dilution of 20 mM inhibited binding of the circulating antibodies (Figure 3A).

**Directed to recombinant α3(IV)NC1.** Eluted anti-GBM antibodies from WKY rats immunized with csGBM in FCA showed a higher functional affinity to α3(IV)NC1 than serum antibodies, in that the addition of DEA at a concentration of 40 mM was needed to inhibit binding of the eluate, while a lower dilution of 10 mM inhibited binding of the circulating antibodies (Figure 3B).

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**Fig. 1.** ELISA showing the concentration of circulating anti-GBM antibodies from WKY and LEW rats immunized with GBM in FCA to: (A) collagenase-solubilized GBM and (B) recombinant rat α3(IV)NC1. Results shown represent the mean ±SD of each group at week 4 after immunization. *P < 0.01, **P < 0.001; WKY vs LEW. NRS, normal rat serum.

**Fig. 2.** ELISA showing the concentration of deposited anti-GBM antibodies from WKY and LEW rats immunized with GBM in FCA to: (A) collagenase-solubilized GBM and (B) recombinant rat α3(IV)NC1. Results shown represent the mean ±SD of each group at week 4 after immunization. *P < 0.001; WKY vs LEW.
In vitro binding of eluted anti-GBM antibody to normal rat kidney. Eluted anti-GBM antibodies from WKY rats immunized with csGBM in FCA showed positive linear binding to the GBM, of equal intensity, in kidney sections from normal WKY and LEW rats. Glomerular eluates from LEW rats immunized with csGBM in FCA, prepared in the same way, showed no binding to WKY or LEW kidney. Results are illustrated in Figure 4.

Passive transfer of EAG.
In vitro binding of eluted anti-GBM antibody following passive transfer. Normal recipient WKY and LEW rats that received an i.v. injection of anti-GBM antibodies eluted from the glomeruli of WKY donor rats with EAG showed similar linear deposits of IgG on the GBM 24 h after transfer. However, by day 28 after transfer, only recipient WKY rats showed sustained linear deposits of IgG on the GBM. Results are illustrated in Figure 5 and shown in Figure 6A.

Assessment of disease following passive transfer. Recipient WKY rats showed high levels of albuminuria, severe focal necrotizing glomerulonephritis with crescent formation, and increased numbers of glomerular T cells and macrophages, at day 28 after passive transfer. In contrast, recipient LEW rats showed no increase in albuminuria, glomerular abnormalities or glomerular T cells and macrophages, at day 28 after transfer. Results are illustrated in Figure 7, and shown in Figure 6B–D.

Discussion
In this study, we demonstrate that susceptibility to EAG in different rat strains is determined both by differences in the concentration and affinity of anti-GBM antibodies produced, and also by differences in the inflammatory response to antibody deposition. Previous work has shown that WKY and LEW rats vary in their susceptibility to EAG, despite sharing the same MHC genes (Rt1-l); the WKY rat is susceptible
and develops crescentic nephritis, while the LEW rat is resistant [18]. In the present study, all WKY rats immunized with rat GBM in FCA developed circulating and deposited anti-GBM antibodies, and severe focal necrotizing glomerulonephritis with crescent formation, at day 28 after immunization. In contrast, LEW rats immunized with the same antigen developed a lower level of circulating anti-GBM antibodies, but no deposits of IgG on the GBM or histological evidence of nephritis.

Circulating anti-GBM antibodies from WKY rats with EAG were present at higher concentration than those from LEW rats, and also showed a higher affinity to the GBM (Reynolds J et al., unpublished observation). This difference in affinity could be due to the fact that circulating antibodies from WKY rats also bound strongly to rat α3(IV)NC1, whereas those from LEW rats showed no detectable binding. Antibody eluted from the kidney of WKY rats with EAG bound strongly to both GBM and α3(IV)NC1 by ELISA, while eluates from LEW rats showed only background binding. This was not surprising, since LEW rats immunized with csGBM did not show significant deposits of IgG on the GBM by direct immunofluorescence. Interestingly, the affinity of the eluted antibody from WKY rats to both GBM and α3(IV)NC1 was higher than that of the circulating antibody, confirming that the most nephritogenic antibodies are bound in the kidney rather than appearing in the circulation [42]. Taken together, these results serve to confirm that antibodies reactive with α3(IV)NC1, rather than with other components of the GBM, are important in the pathogenesis of EAG. However, Sado et al. [19] have shown that other components of the NC1 domain of type IV collagen, notably α4(IV)NC1, are capable of causing crescentic nephritis in the WKY rat. The characteristics of the actively induced autoantibody response are, therefore, at least partly responsible for genetic susceptibility to EAG in the rat.

Although this study was designed to examine the response of different rat strains to immunization with a preparation of rat GBM containing whole NC1 domains, it would clearly be of interest to examine the response of these strains to immunization with recombinant α3(IV)NC1. We already know that WKY
rats develop crescentic glomerulonephritis when immunized with this antigen [20], and have recently found that LEW rats do not respond to immunisation with the same dose of α3(IV)NC1 (Reynolds J et al., unpublished observation). The reason for this remains unclear, since both strains possess the same MHC genes (RT1-l), but presumably relates to other genes influencing the immune or inflammatory response.

In order to investigate further the role of anti-GBM antibodies in the pathogenesis of EAG, we went on to examine the inflammatory response to antibody deposition. Before performing passive transfer experiments, we tested the binding of the eluate from WKY rats with EAG to normal kidney sections from WKY and LEW rats. Since the eluate bound strongly to the GBM of both strains in vitro, it seems likely that the autoantigenic components of the GBM in WKY and LEW kidney are similar. This is consistent with our previous results showing that susceptibility to EAG is not linked to the col4a3 gene in this strain combination [18]. We then passively transferred the anti-GBM eluate to WKY or LEW recipients, and sacrificed animals either at 24h after transfer, to examine antibody binding in vivo, or at 28 days after transfer, to assess development of disease. Both WKY and LEW recipients showed strong linear deposits of IgG at 24h after transfer, demonstrating that the transferred antibody had successfully bound to the GBM of both strains in vivo. However, only WKY recipients still showed linear deposits of IgG on the GBM at 28 days after transfer. The reasons for this are unclear: perhaps the eluted anti-GBM antibody had a lower affinity for the GBM of LEW rats in vivo; perhaps the antibody was cleared more efficiently in LEW rats; or perhaps WKY rats made a subsequent autoantibody response to the damaged GBM. The latter explanation is attractive, although we could not detect circulating anti-GBM antibodies in the recipient WKY rats at day 28.

WKY recipients of eluted anti-GBM antibody developed glomerulonephritis similar to that seen in actively immunized rats, although less severe, including: albuminuria, focal segmental glomerulonephritis with crescent formation and a glomerular infiltrate of T cells and macrophages. In contrast, LEW recipients developed no evidence of nephritis. These passive transfer studies demonstrate that other genetic factors, presumably related to the inflammatory response to antibody deposition, are important in determining susceptibility to EAG. Notably, the glomerular lesions observed following passive transfer of eluted anti-GBM antibody in the WKY rat were similar to those reported by Sado and colleagues [24,25], using antibodies purified from the urine of
nephritic rats [23], or monoclonal antibodies generated from WKY rats with EAG. In support of the importance of the inflammatory response to antibody deposition, we have recently shown that administration of heterologous anti-GBM antibody, in the model of nephrotoxic nephritis, induced crescentic nephritis in WKY rats but not in LEW rats [43]. A genome-wide linkage analysis revealed that susceptibility was linked to copy number polymorphisms of the fcgr3 gene. It is therefore possible that Fc receptor polymorphisms, which influence the inflammatory response, also contribute to susceptibility to EAG.

In conclusion, we have demonstrated that anti-GBM antibodies from WKY rats immunized with csGBM in FCA are present in higher concentrations and are more specific for \alpha(IV)NC1, than antibodies from LEW rats. On the other hand, passive transfer of anti-GBM antibody eluted from the kidneys of WKY rats with EAG can induce crescentic nephritis in WKY, but not in LEW recipients. These differences in the characteristics of the anti-GBM antibodies produced, and in the inflammatory response to antibody deposition, suggest that both factors contribute to the susceptibility of rats to EAG. We are currently investigating the genetic basis of susceptibility to EAG in a genome-wide linkage analysis in this strain combination [44]. Similar genetic influences may be important in the immunopathogenesis of human anti-GBM disease, and this possibility should be investigated further in studies of the progression from autoimmunity to organ-specific damage.

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**Conflict of interest statement.** None declared.

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