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Oral Administration of Glomerular Basement Membrane Prevents the Development of Experimental Autoimmune Glomerulonephritis in the WKY Rat

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Abstract. Experimental autoimmune glomerulonephritis (EAG), an animal model of Goodpasture's disease, can be induced in Wistar Kyoto (WKY) rats by a single injection of collagenase-solubilized rat glomerular basement membrane (GBM) in adjuvant. EAG is characterized by circulating and deposited anti-GBM antibodies, accompanied by focal necrotizing glomerulonephritis with crescent formation. The inhibitory effect of orally administered antigens has been reported in various animal models of autoimmunity but not in EAG in the rat. The effects of feeding rat GBM by gavage, at total doses of 0.5, 2.5, or 5 mg, before immunization were examined. A dose-dependent effect was observed on the development of EAG. A dose of 0.5 mg of GBM had no effect on disease, 2.5 mg resulted in a moderate reduction in the severity of nephritis but no change in anti-GBM antibody production, and 5 mg resulted in a marked reduction in circulating and deposited anti-GBM antibodies, albuminuria, deposits of fibrin in the glomeruli, severity of glomerular abnormalities, and numbers of infiltrating T cells and macrophages. Animals that were fed 5 mg of GBM showed a significant reduction in IgG2a but not IgG1, anti-GBM antibody levels, suggesting downregulation of Th1 responses. There was also a dose-dependent reduction in the proliferative responses of splenic T cells from treated animals to GBM antigen *in vitro*. These results clearly demonstrate that mucosal tolerance can be induced by oral administration of GBM antigen and that this approach is effective in preventing EAG.

The inhibitory effect of orally administered autoantigens has been widely reported in studies using known autoantigens in experimental models of autoimmune disease in rodents, including encephalomyelitis, uveitis, collagen-induced arthritis, thyroiditis, diabetes, and myasthenia gravis (1–6). Recently, similar findings have been reported in glomerulonephritis in SJL/J mice (7) and in tubulointerstitial nephritis in Brown Norway (BN) rats (8). Nasal administration of lower doses of antigen or of immunodominant synthetic peptides has also been shown to induce mucosal tolerance in various models of autoimmunity, such as encephalomyelitis (9,10), collagen-induced arthritis (11,12), and myasthenia gravis (13). The last is of particular relevance to our work, because autoantibodies are clearly pathogenic in myasthenia gravis, as in Goodpasture's disease. In addition, coupling the antigen to the cholera toxin B subunit (CTB) has been shown to enhance development of mucosal tolerance (14), possibly because of more efficient induction of regulatory cells as a result of the binding properties of CTB. There is still controversy as to the mechanisms involved in the induction of mucosal tolerance. There is evidence that high doses of antigen lead to clonal deletion or anergy, whereas lower doses favor the development of suppressor mechanisms (1–6). In some models, this suppression may be due to the stimulation of Th2 cells, which downregulate the inflammatory response involving Th1 cells (15). The balance between Th1 and Th2 cells may be controlled by a regulatory subset of T cells, which has been shown to suppress immune responses *in vitro* and *in vivo* by release of the immunosuppressive cytokine transforming growth factor-β (TGF-β) (16). This subset of T cells has been termed Th3 by some authors (17). There is also evidence that T cells that bear the $\gamma\delta$ T-cell receptor are involved (18).

Experimental autoimmune glomerulonephritis (EAG) is an experimental model of Goodpasture's disease that can be induced in genetically susceptible strains of rats by immunization with heterologous or homologous preparations of glomerular basement membrane (GBM) in adjuvant (19–24). The development of nephritis is associated with both cell-mediated and humoral immunity to the noncollagenous (NC1) domain of the α3 chain of type IV collagen [α3(IV)NC1] (25–29), which is also the autoantigen in Goodpasture's disease (30,31). In the model used in this study, Wistar Kyoto (WKY) rats that were given a single injection of collagenase-solubilized rat GBM in Freund's complete adjuvant (FCA) develop sustained anti-GBM antibody synthesis, linear deposition of IgG on the GBM, deposits of fibrin in the glomeruli, albuminuria, focal necrotizing glomerulonephritis with crescent formation, and variable lung hemorrhage (24). Anti-GBM antibodies from rats

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and mice with EAG have been shown to be pathogenic in passive transfer experiments (7,32). However, there is also increasing evidence for the role of T lymphocytes in the pathogenesis of EAG (33–41). In the present model, EAG is characterized by an influx of T cells that precedes macrophage infiltration and glomerular injury (33). T cells from animals with EAG proliferate in response to cationic monomer and dimer NC1 domains of rat GBM (34). Transfer of Th lymphocytes purified from the spleens of rats with EAG can prime naive recipients for the disease (35), and *in vitro* stimulation of these cells by GBM, before transfer, confers the ability to induce anti-GBM antibody production in recipients (Reynolds *et al.*, unpublished observation). We have previously shown that cyclosporin A (36) and anti-CD4 monoclonal antibody (mAb) (37) can prevent EAG in the BN rat, and have recently found that anti-CD8 mAb (38) and blockade of the CD28-B7 co-stimulatory pathway (39,40) are effective in EAG in the WKY rat. It has also been demonstrated that antibodies to intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1 are effective in both the prevention and the treatment of EAG in the WKY rat (41).

The present model of EAG is highly appropriate for studying mechanisms of oral tolerance because of the close similarities to human anti-GBM disease in both the immune response and the glomerular pathology. We therefore examined the effects of feeding different doses of collagenase-solubilized rat GBM on the development of EAG. The results obtained demonstrate for the first time that mucosal tolerance can be induced in EAG in the WKY rat in a dose-dependent manner by oral administration of GBM antigen.

Materials and Methods

Experimental Animals

Male WKY rats, ages 8 to 10 wk and weighing 120 to 150 g, were purchased from Charles River (Margate, 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 GBM Antigen

Collagenase-solubilized GBM was prepared from Sprague Dawley (SD) rat kidneys, as described previously (21,24). Briefly, the kidneys were decapsulated, the medulla was partly removed, and the cortex was forced through sieves to isolate the glomeruli. After examination by light microscopy, the glomeruli were disrupted ultrasonically and the resulting material was lyophilized and digested with purified type I collagenase (Sigma-Aldrich Company Ltd., Poole, UK) for 1 h at 37°C.

Induction of EAG

EAG was induced in WKY rats by a single intramuscular injection of collagenase-solubilized rat GBM in an equal volume of FCA, at a dose of 5 mg/kg body wt (24). Serial blood samples were taken by tail artery puncture under light anesthesia (Isoflurane), and 24-h urine specimens were obtained by placing animals in metabolic cages.

Experimental Protocol

Collagenase-solubilized rat GBM was administered orally to groups of WKY rats ($n = 5$ to 6) by gavage, at total doses of 0.5, 2.5, or 5 mg, before immunization. In addition, positive control groups, immunized with GBM in FCA, were fed bovine serum albumin (5 mg), and negative control groups, injected with FCA alone, were fed rat GBM (5 mg). All groups of animals were fed five times at intervals of 2 to 3 d with the last feeding 2 d before immunization.

Assessment of EAG

Enzyme-Linked Immunosorbent Assay. Circulating anti-GBM antibody concentrations were measured in sera from animals with EAG by a solid-phase enzyme-linked immunosorbent assay (ELISA), as described previously (24). Collagenase-digested rat GBM was coated onto microtiter plates (Life Technologies, Paisley, UK) by overnight incubation at 4°C, and test or control sera were applied for 1 h at 37°C. Bound anti-GBM antibody was detected by horseradish peroxidase conjugated sheep anti-rat IgG (Sigma-Aldrich Company Ltd.) and developed using the substrate orthophenylenediamine dihydrochloride (Sigma-Aldrich Company Ltd.). The absorbances for each well were read at 492 nm using an Anthos Multiskan ELISA plate reader (Lab Tech International, Uckfield, UK), and the results initially were calculated as mean optical density for each triplicate sample. To compare different experimental groups, results were finally expressed as a percentage of the binding obtained with a positive reference serum (24).

Subclass ELISA. Circulating levels of IgG1 and IgG2a anti-GBM antibodies were measured in sera from animals with EAG at week 4 after immunization, by an indirect ELISA similar to that previously described (39,42). Briefly, collagenase-digested rat GBM was coated onto microtiter plates and test or control sera were applied, as described above. The isotypes of circulating anti-GBM antibodies were detected by mouse mAb specific for rat IgG1 and IgG2a (Serotec Ltd., Oxford, UK), followed by goat anti-mouse IgG (Serotec Ltd.). Levels of bound IgG1 and IgG2a anti-GBM antibodies were detected by alkaline phosphatase conjugated rabbit anti-goat IgG (Sigma-Aldrich Company Ltd.) and developed using the substrate p-nitrophenyl phosphate (Sigma-Aldrich Company Ltd.). The absorbances for each well were read at 405 nm, and the results were expressed as mean optical density for each triplicate sample.

Rocket Immunoelectrophoresis. Urinary albumin concentrations were measured in 24-h collections from animals with EAG by rocket immunoelectrophoresis (Amersham Pharmacia Biotech, St Albans, UK) as described previously (43). Briefly, urine samples from experimental animals were subjected to immunoelectrophoresis at 60 v in an electrophoresis tank containing Barbitone buffer (BDH Laboratory Supplies, Poole, 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 milligrams per 24 h.

Creatinine Clearance. Creatinine levels in serum and urine from animals at week 4 were measured by spectrometry using standard techniques. Creatinine clearance was then calculated for each animal and expressed as milliliters per minute.

Direct Immunofluorescence. Deposits of IgG and fibrin within the glomeruli were detected by direct immunofluorescence, as described previously (44). Tissue was embedded in OCT II embedding medium (Miles Inc., Elkhart, IN) on cork discs, snap-frozen in isopentane (BDH Laboratory Supplies), precooled in liquid nitrogen, and stored at −70°C. Cryostat sections were cut 4 μm thick and were incubated with FITC-labeled rabbit anti-rat IgG (Serotec Ltd.) or goat anti-rat fibrin (Nordic Immunology, Tilburg, The Netherlands). The extent and the intensity of immunostaining were assessed and graded from 0 to 3+ by a blinded observer.

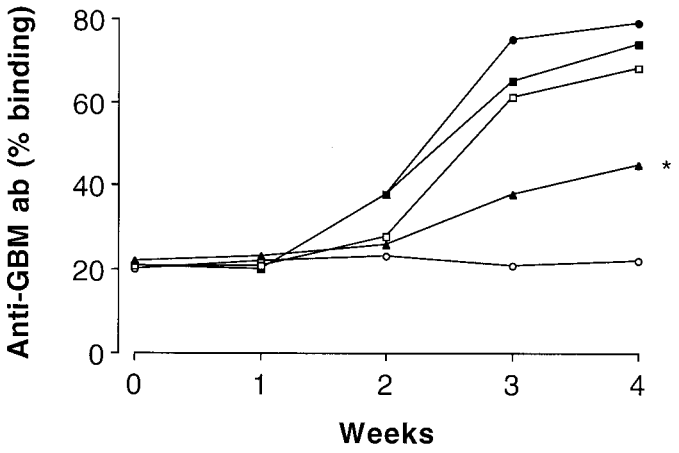

Figure 1. Effect of oral administration of glomerular basement membrane (GBM) on circulating anti-GBM antibody concentrations in groups of WKY rats ($n = 5$ to 6) with experimental autoimmune glomerulonephritis (EAG). Results shown represent the mean of each group: positive control (●), GBM 0.5 mg (■), GBM 2.5 mg (□), or GBM 5 mg (▲), and negative control (○). *, $P < 0.01$ (positive control *versus* GBM 5 mg).

Light Microscopy. Kidney tissue was fixed in 10% neutral buffered formalin, processed, and embedded in paraffin wax for light microscopy by standard techniques. Briefly, 3-μm sections were stained with hematoxylin and eosin and periodic acid-Schiff. Fifty glomeruli per section were assessed and graded by a blinded observer as normal, abnormal, or severe (>50% of the glomerulus affected by necrosis/crescent formation) and expressed as a percentage of glomeruli examined (24).

Immunohistology. Formalin-fixed kidney sections were stained with mAb ED1 (for macrophages) and OX8 (for CD8+ lymphocytes) (Serotec Ltd.) by standard indirect immunoperoxidase staining techniques. The cellular infiltrate was assessed by a blinded observer by counting the number of positively stained cells per 50 consecutive glomeruli in cross section (45).

T-Cell Proliferation Assay

Spleens were dissociated into a single cell suspension (34) and cultured with GBM antigen as described previously (22,35,46). Spleen cells from positive and negative controls or animals fed with GBM were suspended in RPMI 1640 medium (supplemented with 10% fetal calf serum, 100 μg/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine) and cultured in 96-well plates (Life Technologies) at a concentration of 2×10^5 cells/well. Collagenase-solubilized rat GBM (10 μg/ml) was added, and spleen cells were incubated at 37°C in a humidified environment with 5% CO_2 for 7 d. As a control, spleen cells were also cultured with the mitogen Concanavalin A (2 μg/ml; Sigma-Aldrich Company Ltd.) for 3 d. Tritiated thymidine (Amersham International, Amersham, UK) was added at 1 μCi/well 16 h before harvesting, and thymidine incorporation was measured using an automated β counter (Amersham Pharmacia Biotech, St. Albans, UK). Results were expressed as a stimulation index, which was calculated by dividing the cpm in wells cultured with antigen by cpm in wells with no antigen.

Statistical Analyses

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

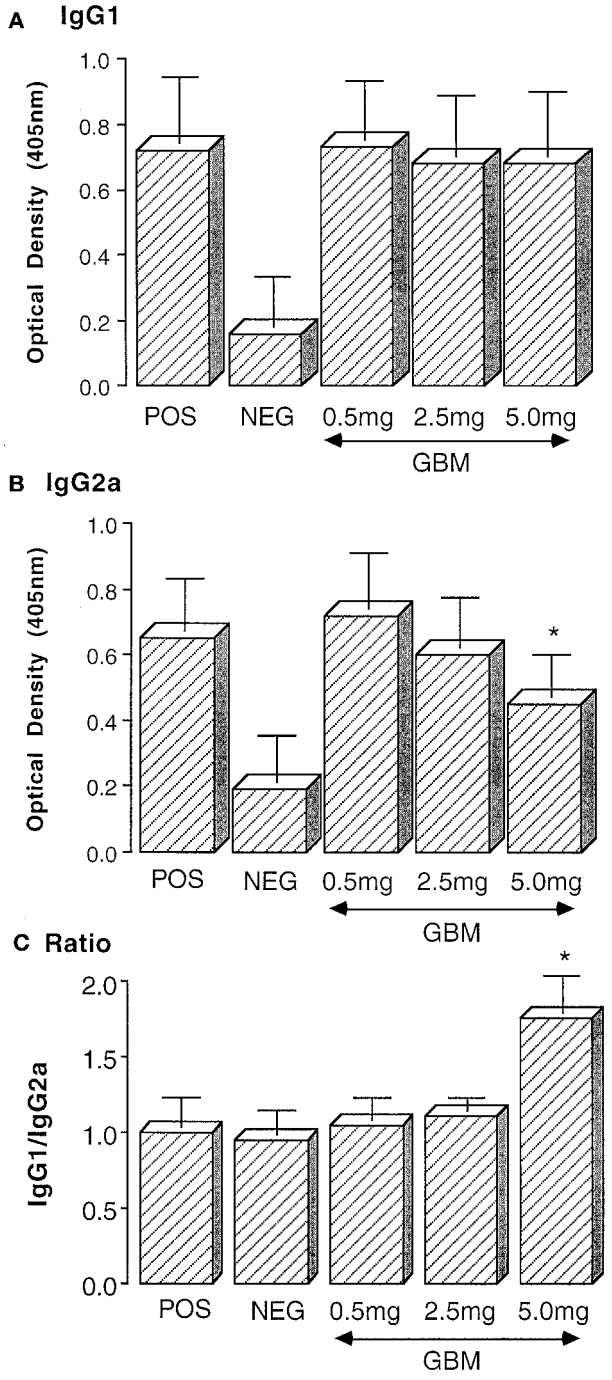

Figure 2. Effect of oral administration of GBM on circulating levels of IgG1 (A), IgG2a (B), and ratio of IgG1/IgG2a anti-GBM antibodies (C) in groups of WKY rats ($n = 5$ to 6) with EAG. Results shown represent the mean ± SD of each group at week 4 after immunization. *, $P < 0.01$ (positive control *versus* GBM 5 mg).

Results

Circulating Anti-GBM Antibody Concentrations

All positive control rats immunized with rat GBM in FCA and fed bovine serum albumin orally at a total dose of 5 mg produced detectable circulating anti-GBM antibody levels by week 2, which increased to high levels by week 3 and peaked

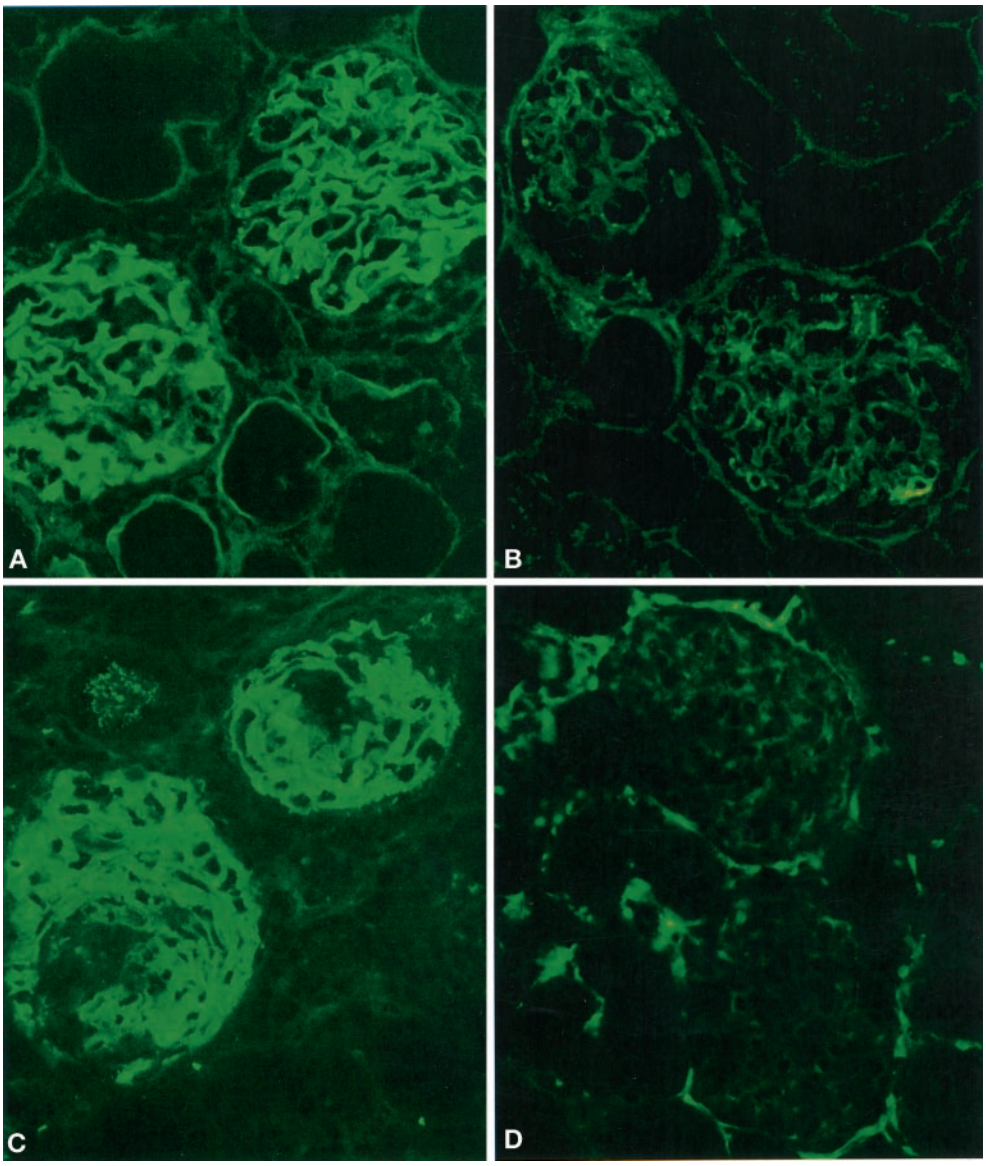

Figure 3. Direct immunofluorescence of kidney tissue at week 4 in WKY rats with EAG showing strong linear deposition of IgG along the GBM in a positive control animal (A), marked reduction in the deposition of IgG in an animal given GBM orally at 5 mg (B), large deposits of fibrin within the glomeruli of a positive control animal (C), and negative findings for fibrin in an animal given GBM orally at 5 mg (D). Magnification, ×300.

at week 4. Oral administration of GBM had a dose-dependent effect on circulating antibody levels. A total dose of 0.5 mg had no effect, whereas 2.5 mg led to a slight, though not significant, reduction and 5 mg resulted in a marked reduction in antibody concentration. Negative control animals injected with FCA alone and fed GBM at a total dose of 5 mg did not develop circulating antibody. Results are shown in Figure 1.

Circulating Anti-GBM Antibody Isotypes

All positive control rats immunized with rat GBM in FCA produced detectable levels of both IgG1 and IgG2a anti-GBM antibodies by week 4. Animals that were given GBM orally at a dose of 0.5 mg showed no reduction in either IgG1 or IgG2a antibodies, whereas 2.5 mg showed a slight reduction in IgG2a antibodies and 5 mg led to a marked reduction in the levels of IgG2a but not IgG1 antibodies. Negative control animals that were given FCA alone did not develop detectable levels of IgG1 or IgG2a antibodies. The ratio of IgG1/IgG2a anti-GBM antibodies was significantly increased in the 5 mg group compared with positive controls. Results are shown in Figure 2.

Direct Immunofluorescence for IgG

Direct immunofluorescence for IgG on kidney tissue at 4 wk revealed that positive control animals that were given rat GBM in FCA showed strong linear deposits of IgG along the GBM and, to lesser extent, the TBM. Animals that were given GBM orally at 0.5 mg showed no reduction in IgG deposits, whereas

Table 1. Effects of oral administration of GBM to WKY rats with EAG on deposition of IgG on the GBM and deposition of fibrin in the glomeruli. Results are expressed as intensity of fluorescence at week 4 in individual animals

	Intensity of Immunostaining			
	3+	2+	1+	–
IgG				
positive control ($n = 6$)	3	3	0	0
negative control ($n = 5$)	0	0	0	5
GBM 0.5 mg ($n = 6$)	4	2	0	0
GBM 2.5 mg ($n = 5$)	0	3	2	0
GBM 5 mg ($n = 6$)	0	1	2	3
Fibrin				
positive control ($n = 6$)	4	2	0	0
negative control ($n = 5$)	0	0	0	5
GBM 0.5 mg ($n = 6$)	3	3	0	0
GBM 2.5 mg ($n = 5$)	1	2	2	0
GBM 5 mg ($n = 6$)	0	0	1	5

those that were given 2.5 mg showed a moderate reduction and those that were given 5 mg showed a marked reduction in intensity of staining. Negative control animals that were given FCA alone showed no IgG deposition. Results are summarized in Table 1 and illustrated in Figure 3.

Albuminuria

All positive control rats that were immunized with rat GBM in FCA produced detectable levels of albuminuria by week 2, which increased further by week 3 and peaked at week 4. Animals that were given GBM orally at 0.5 mg showed no reduction in albuminuria, whereas those that were given 2.5 mg showed a moderate reduction and those that were given 5 mg showed a marked reduction in levels at all time points. Negative control animals that were given FCA alone did not develop albuminuria. Results are shown in Figure 4.

Creatinine Clearance

Negative control animals that were given FCA alone showed a normal creatinine clearance in the range of 1.2 to 1.5 ml/min. Positive control rats that were immunized with rat GBM in FCA showed a marked reduction in creatine clearance. Animals that were given GBM orally at 0.5 mg or 2.5 mg showed a moderate reduction in creatinine clearance, whereas those that were given 5 mg had clearances similar to negative control animals. Results are shown in Figure 5.

Direct Immunofluorescence for Fibrin

Direct immunofluorescence for fibrin on kidney tissue at 4 wk revealed that positive control animals that were given rat GBM in FCA showed large areas of fibrin deposits within the glomeruli. Animals that were given GBM orally at 0.5 mg showed no reduction in fibrin deposits, whereas those that were given 2.5 mg showed a moderate reduction and those that were given 5 mg showed a marked reduction in extent and intensity of staining. Negative control animals that were given FCA alone showed no antibody binding. Results are summarized in Table 1 and illustrated in Figure 3.

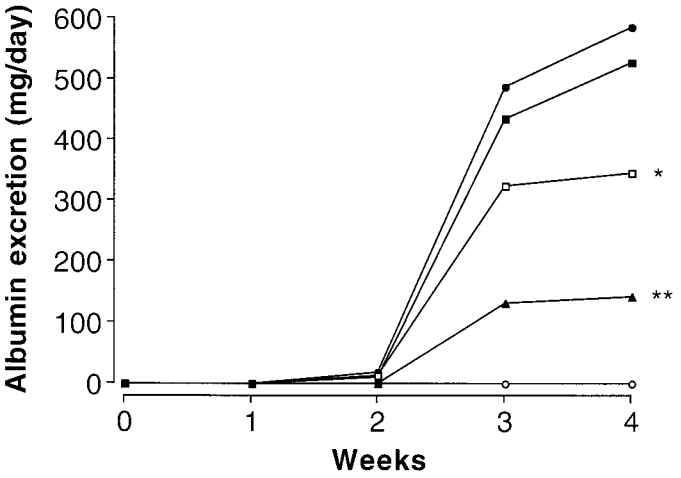

Figure 4. Effect of oral administration of GBM on albuminuria in groups of WKY rats ($n = 5$ to 6) with EAG. Results shown represent the mean of each group: positive control (●), GBM 0.5 mg (■), GBM 2.5 mg (□), or GBM 5 mg (▲), and negative control (○). *, $P < 0.01$ (positive control *versus* GBM 2.5 mg); **, $P < 0.001$ (positive control *versus* GBM 5 mg).

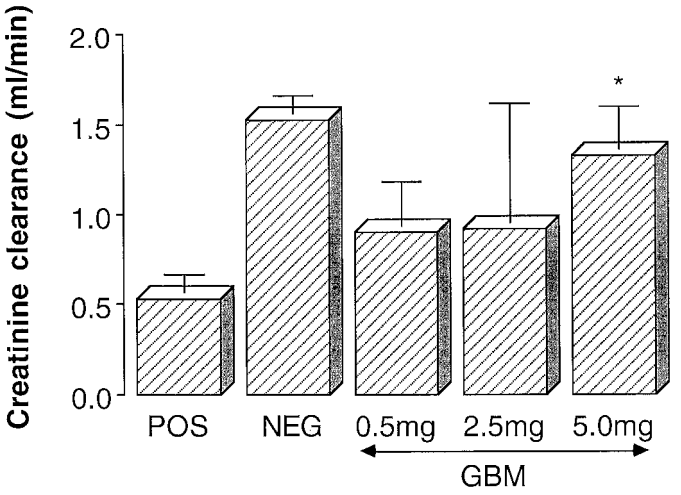

Figure 5. Effect of oral administration of GBM on creatinine clearance in groups of WKY rats ($n = 5$ to 6) with EAG. Results shown represent the mean ± SD of each group at week 4 after immunization. *, $P < 0.01$ (positive control *versus* GBM 5 mg).

Light Microscopy

Light microscopy of kidney tissue at 4 wk revealed that positive control animals that were given rat GBM in FCA showed diffuse necrotizing glomerulonephritis affecting 100% of glomeruli, with severe segmental necrosis/crescent formation (>50% of the glomerulus) affecting 65% of glomeruli.

Animals that were given GBM orally at a total dose of 0.5 mg also showed glomerulonephritis in 100% of glomeruli, with severe changes in 50% of glomeruli, whereas those that were given 2.5 mg showed glomerulonephritis in 75% of glomeruli, with severe changes in 30% of glomeruli, and those that were given 5 mg showed glomerulonephritis only in 30% of glomeruli, with no histologic evidence of segmental necrosis/crescent formation. Negative control animals that were given FCA alone showed normal renal histology. Results are shown in Figure 6 and illustrated in Figure 8.

Immunohistology

Immunohistology of kidney tissue at 4 wk showed that positive control animals that were given rat GBM in FCA had glomerular infiltration with CD8+ T lymphocytes and macrophages. Animals that were given GBM orally at 0.5 mg showed no reduction in the numbers of T cells and macrophages infiltrating the glomeruli, whereas those that were given 2.5 mg showed a moderate reduction and those that were given 5 mg showed a marked reduction. Negative control animals that were given FCA alone showed no cellular infiltrate. Results are shown in Figure 7 and illustrated in Figure 8.

T-Cell Proliferation

Spleen cells from positive control animals that were given rat GBM in FCA showed a significant T-cell proliferative response (stimulation index = 9.5) when cultured with GBM *in vitro* for 7 d at a concentration of 10 μg/ml. Spleen cells from animals that were given GBM orally at 0.5 or 2.5 mg showed a proliferative response similar to that of positive control animals, whereas splenocytes from animals that were fed 5 mg of GBM showed a marked reduction in T-cell proliferation, which was similar to that of negative control animals. Results are shown in Figure 9.

Discussion

The inhibitory effect of orally (1–8) and nasally (9–13) administered antigens in various animal models of autoimmunity has been widely reported, but there is still much controversy as to the mechanisms involved. Furthermore, there are no reported studies in EAG in the rat. To investigate this, we examined the effects of feeding different doses of collagenase-solubilized rat GBM on the development of EAG in the WKY rat. This resulted in a dose-dependent effect on the development of EAG. A total dose of 0.5 mg of GBM had no effect on disease, 2.5 mg resulted in a moderate reduction in the severity of nephritis but no significant change in anti-GBM antibody production, and 5 mg resulted in a marked reduction in circulating and deposited anti-GBM antibodies and in the severity of glomerular abnormalities. In addition, animals that were fed 5 mg of GBM showed a significant reduction in IgG2a (but not IgG1) anti-GBM antibody levels and a reduced T-cell proliferative response to GBM antigen *in vitro*. Our results clearly

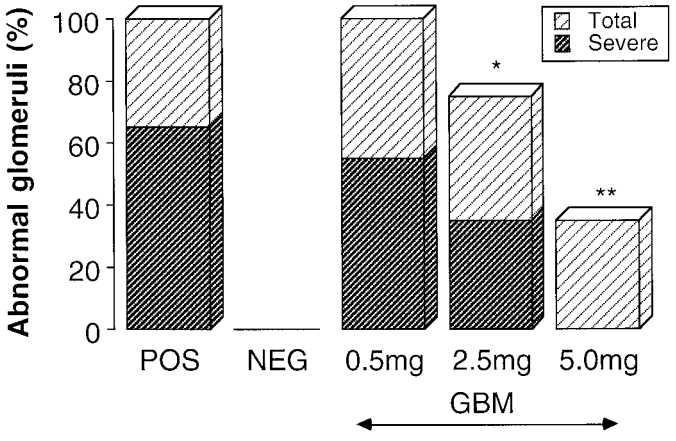

Figure 6. Effect of oral administration of GBM on renal histology at week 4 in groups of WKY rats (n = 5 to 6) with EAG. Results shown represent the severity of glomerular abnormalities, which were graded as normal, abnormal, or severe (>50% of the glomerulus affected by necrosis/crescent formation). *, $P < 0.01$ (positive control *versus* GBM 2.5 mg; **, $P < 0.001$ (positive control *versus* GBM 5 mg).

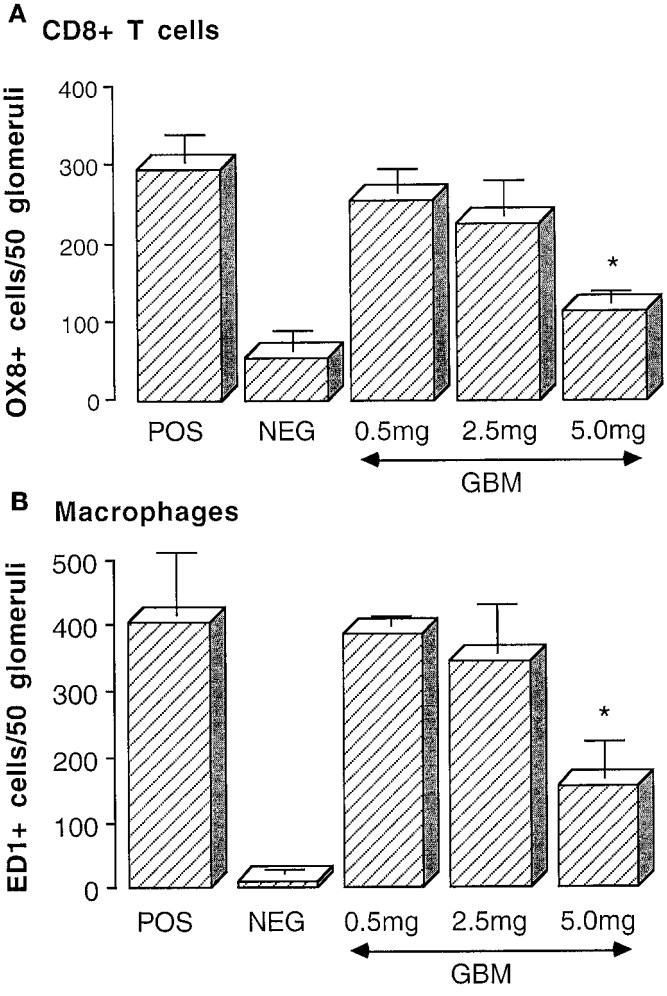

Figure 7. Effect of oral administration of GBM on the numbers of CD8+ T cells (A) and macrophages (B) detected by immunoperoxidase staining of kidney tissue in groups of WKY rats (n = 5 to 6) with EAG. Results shown represent the mean ± SD of each group at week 4 after immunization. *, $P < 0.01$ (positive control *versus* GBM 5 mg).

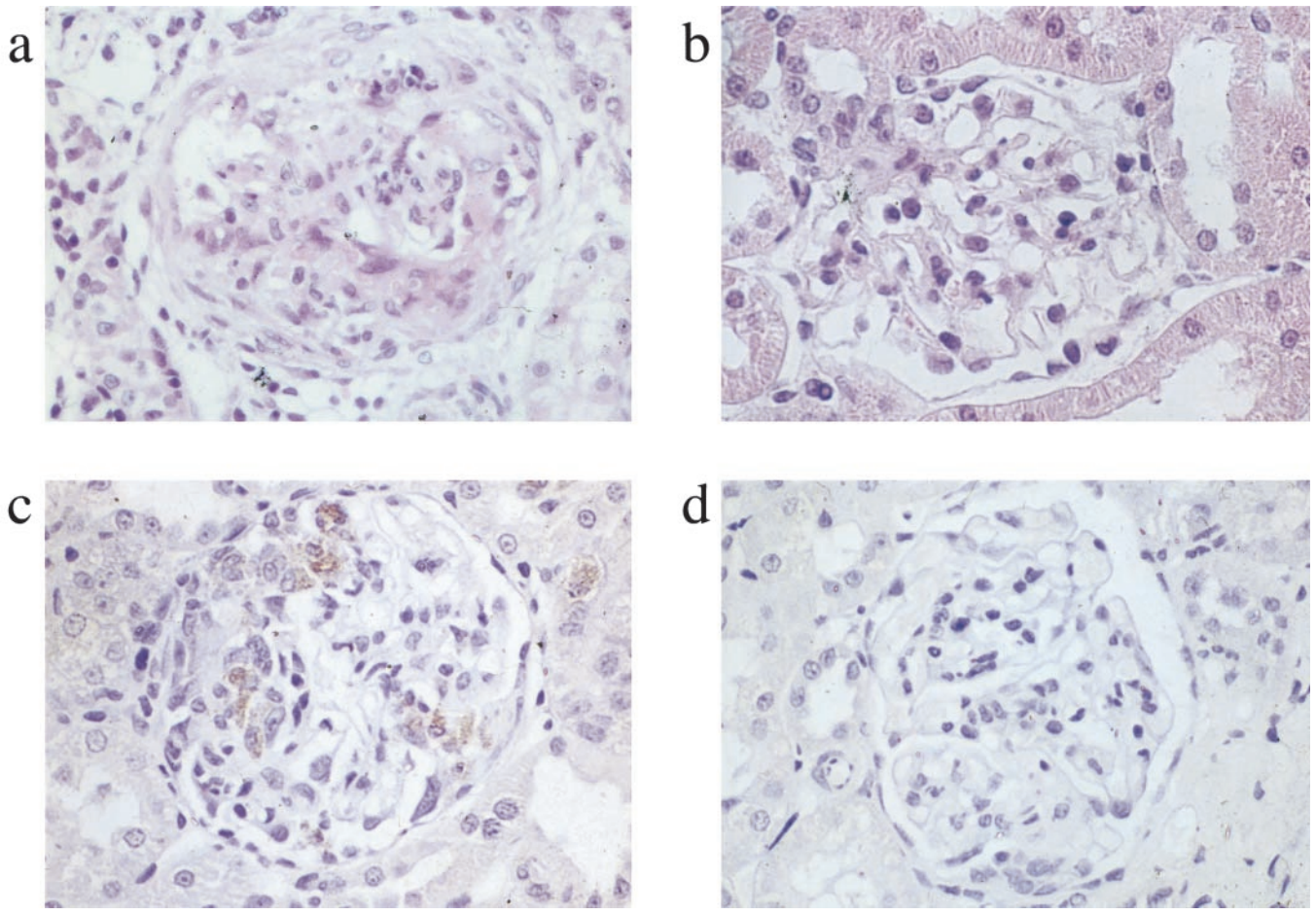

Figure 8. Light microscopy of kidney tissue at week 4 in WKY rats with EAG showing marked segmental necrosis of the glomerular tuft with crescent formation in a positive control animal (A; hematoxylin and eosin), mild segmental proliferation in an animal given GBM orally at 5 mg (B; hematoxylin and eosin), large numbers of macrophages infiltrating the glomeruli and interstitium in a positive control animal (C; immunoperoxidase), and reduced numbers of macrophages in an animal given GBM orally at 5 mg (D; immunoperoxidase). Magnification, ×300.

demonstrate that oral administration of GBM antigen at a total dose of 5 mg before immunization reduces the autoimmune response and completely prevents the development of crescents in this model of EAG.

Previous studies in other animal models of nephritis, although limited, have demonstrated that oral administration of renal antigens can be successful in prevention or amelioration of disease (7,8). In a mouse model of anti-GBM disease, induced in SJL mice by immunization with bovine α3(IV)NC1, oral administration of α3(IV)NC1 dimers, before immunization, resulted in a substantial decrease in numbers of crescents and prevention of interstitial inflammation (7). In addition, a decrease in serum titers of IgG2a anti-GBM antibodies and a reduction of IL-12 expression in the kidney was observed in mice that were fed α3(IV)NC1. In autoimmune interstitial nephritis, induced in BN rats by immunization with renal tubular antigen, oral feeding of renal tubular antigen reduced the severity of interstitial nephritis and renal function, but this was not found to be associated with the suppression of delayed-type hypersensitivity responses or antigen-specific IgG titers (8).

The primary mechanism by which mucosal tolerance to autoantigens is mediated is still unclear, but the determining factor seems to be the dose of the antigen fed. High-dose antigen seems to induce systemic anergy or deletion, whereas a regimen of multiple feedings with low-dose antigen favors the induction of regulatory T cells, whose suppressive activities are mediated through the production of anti-inflammatory cytokines such as interleukin-4 (IL-4), IL-10, and TGF-β (1–6). TGF-β–secreting cells seem to form a unique subset that has been termed Th3 cells (17). It is now widely believed that cytokines associated with the Th1 phenotype (interferon-γ, tumor necrosis factor-α, and IL-12) promote inflammation in the target organ, whereas cytokines associated with Th2 (IL-4, IL-10) and Th3 (TGF-β) responses have a role in suppressing disease (15,16).

The finding that Th1 and Th2 subsets of CD4+ T cells can cross regulate each other, via production of distinct cytokine profiles, supports the idea that oral tolerance induced by low doses of antigen reflects the downregulation of Th1 cells by Th2 cells. In the present study, we examined only the effect of low-dose tolerance, *i.e.*, oral administration of up to 5 mg of GBM, as compared with an immunizing dose of 5 mg/kg. On

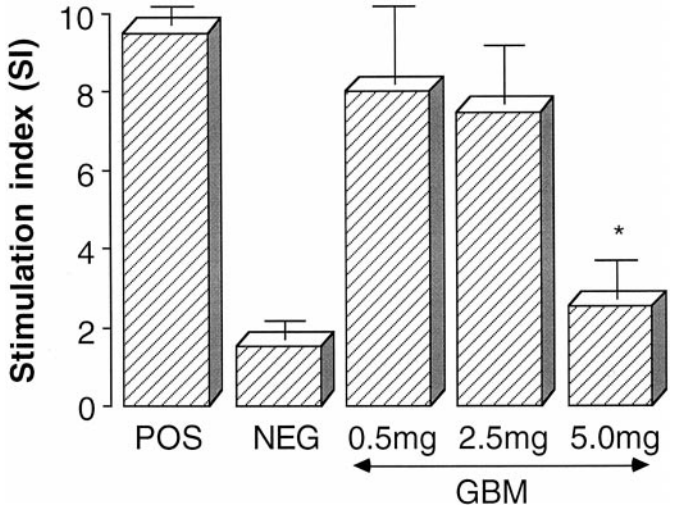

Figure 9. Effect of oral administration of GBM on *in vitro* proliferation of splenic T cells pooled from different groups of WKY rats with EAG. Results shown represent the mean ± SD of each group at week 4 after immunization. *, $P < 0.01$ (positive control *versus* GBM 5 mg).

the basis of the literature (7,47), we would need to give at least 10 times more GBM antigen to examine the effect of high-dose tolerance. The results from our study are consistent with the hypothesis of low-dose tolerance, because we found a decrease in IgG2a anti-GBM antibodies, suggesting downregulation of Th1 cells. In addition, we showed a marked decrease in the deposits of fibrin within the glomeruli and a decrease in the number of infiltrating glomerular T cells and macrophages, which are regarded as markers of a Th1-like response. Furthermore, we demonstrated a reduced proliferative response of splenic lymphocytes to the GBM antigen *in vitro*. This confirms that tolerance has been induced at the level of autoreactive T cells against the GBM antigen. Further work is required to determine whether this is due to production of anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β.

Another route to inducing mucosal tolerance in autoimmune disease is by nasal administration of autoantigens or immunodominant synthetic peptides. This approach has been shown to be very effective in both the prevention (9–13) and the treatment (47,48) of various experimental models of disease. Low doses of nasally administered peptides are effective in prevention of disease, whereas high doses seem to be necessary for treatment. In addition, conjugation of the antigen with CTB greatly increases the development of mucosal tolerance (14), allowing considerably lower doses of antigen to be used. If mucosal tolerance is to be used clinically for autoimmune renal disease, then we must first demonstrate it to be effective in treating established disease in an appropriate animal model. The use of nasally administered recombinant rat α3(IV)NC1 (29) or synthetic peptides based on its sequence is likely to be required to achieve high-dose tolerance. These studies are in progress in our laboratory.

In conclusion, we demonstrated for the first time that mucosal tolerance can be induced in EAG in the rat. In particular, oral administration of GBM antigen before immunization can completely prevent the development of crescentic nephritis. In addition, we have demonstrated a significant reduction in IgG2a anti-GBM antibody levels and a reduced T-cell proliferative response to GBM antigen *in vitro*. The most likely explanation for these findings is that oral administration of GBM antigen has resulted in the induction of regulatory T cells whose suppressive activities are mediated through the production of anti-inflammatory cytokines that suppress the Th1-like response in EAG. However, further work, including analysis of cytokine profiles from autoreactive T cells and analysis of glomerular expression of selected cytokines in EAG, is clearly required. The results from this study may have implications for the development of specific immunotherapy for human glomerulonephritis.

Acknowledgments

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References

1. Wraith DC: Antigen-specific immunotherapy of autoimmune disease: A commentary. *Clin Exp Immunol* 103: 509–516, 1996
2. Staines NA, Harper N, Ward FJ, Thompson HS, Bansal S: Arthritis: Animal models of oral tolerance. *Ann N Y Acad Sci* 778: 297–305, 1996
3. Whitacre CC, Gienapp IE, Meyer A, Cox KL, Javed N: Oral tolerance in experimental autoimmune encephalomyelitis. *Ann N Y Acad Sci* 778: 217–227, 1996
4. Xiao BG, Link H: Mucosal tolerance: A two-edged sword to prevent and treat autoimmune disease. *Clin Immunol Immunopathol* 85: 119–128, 1997
5. Weiner HL: Oral tolerance: Immune mechanisms and treatment of autoimmune diseases. *Immunol Today* 18: 335–343, 1997
6. Faria AM, Weiner HL: Oral tolerance: Mechanisms and therapeutic applications. *Adv Immunol* 73: 153–264, 1999
7. Kalluri R, Danoff TM, Okada H, Neilson EG: Susceptibility to anti-glomerular basement membrane disease and Goodpasture syndrome is linked to MHC class II genes and the emergence of T cell-mediated immunity in mice. *J Clin Invest* 100: 2263–2275, 1997
8. Pham K, Smoyer WE, Archer DC, Gabbai F, Kelly CJ: Oral feeding of renal tubular antigen abrogates interstitial nephritis and renal failure in Brown Norway rats. *Kidney Int* 52: 725–732, 1997
9. Liu JQ, Bai XF, Shi FD, Xiao BG, Li HL, Levi M, Mustafa M, Wahren B, Link H: Inhibition of experimental autoimmune encephalomyelitis in Lewis rats by nasal administration of encephalitogenic MBP peptides: Synergistic effects of MBP 68–86 and 87–99. *Int Immunol* 10: 1139–1148, 1998
10. Anderton SM, Wraith DC: Hierarchy in the ability of T cell epitopes to induce peripheral tolerance to antigens from myelin. *Eur J Immunol* 28: 1251–1261, 1998
11. Staines NA, Harper N, Ward FJ, Malmstrom V, Holmdahl R, Bensal S: Mucosal tolerance and suppression of collagen-induced arthritis (CIA) induced by nasal inhalation of synthetic

peptide 184–198 of bovine type II collagen (CII) expressing a dominant T cell epitope. *Clin Exp Immunol* 103: 368–375, 1996
12. Garcia G, Komagata Y, Slavin AJ, Maron R, Weiner HL: Suppression of collagen-induced arthritis by oral or nasal administration of type II collagen. *J Autoimmun* 13: 315–324, 1999
13. Barchan D, Souroujon MC, Im SH, Antozzi C, Fuchs S: Antigen-specific modulation of experimental myasthenia gravis: Nasal tolerization with recombinant fragments of the human acetylcholine receptor alpha-subunit. *Proc Natl Acad Sci USA* 96: 8086–8091, 1999
14. Sun JB, Rask C, Olsson T, Holmgren, Czerkinsky C: Treatment of experimental autoimmune encephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit. *Proc Natl Acad Sci USA* 93: 7196–7201, 1996
15. Ma CG, Zhang GX, Xiao BG, Wang ZY, Link J, Olsson T, Link H: Mucosal tolerance to experimental autoimmune myasthenia gravis is associated with down-regulation of AChR-specific IFN-gamma-expressing Th1-like cells and up-regulation of TGF-beta mRNA in mononuclear cells. *Ann N Y Acad Sci* 778: 273–287, 1996
16. Ma CG, Zhang GX, Xiao BG, Link H: Cellular mRNA expression of IFNγ, IL-4, and TGFβ in rats nasally tolerized against experimental autoimmune myasthenia gravis (EAMG). *Clin Exp Immunol* 104: 509–516, 1996
17. Shi FD, Li H, Wang H, Bai X, van der Meide PH, Link H, Ljunggren HG: Mechanisms of nasal tolerance induction in experimental autoimmune myasthenia gravis: Identification of regulatory cells. *J Immunol* 162: 5757–5763, 1999
18. Ke Y, Pearce K, Lake JP, Ziegler HK, Kapp JA: Gamma delta T lymphocytes regulate the induction and maintenance of oral tolerance. *J Immunol* 158: 3610–3618, 1997
19. Sado Y, Naito I, Akita M, Okigaki T: Strain specific responses of inbred rats on the severity of experimental autoimmune glomerulonephritis. *J Clin Lab Immunol* 19: 193–199, 1986
20. Sado Y, Naito I: Experimental autoimmune glomerulonephritis induced in rats by soluble isologous or homologous antigens from glomerular and tubular basement membranes. *Br J Exp Pathol* 68: 695–704, 1987
21. Pusey CD, Holland MJ, Cashman SJ, Sinico RA, Lloveras JJ, Evans DJ, Lockwood CM: Experimental autoimmune glomerulonephritis induced by homologous and isologous glomerular basement membrane in Brown-Norway rats. *Nephrol Dial Transplant* 6: 457–465, 1991
22. Bolton WK, May WJ, Sturgill BC: Proliferative autoimmune glomerulonephritis in rats: A model for autoimmune glomerulonephritis in humans. *Kidney Int* 44: 294–306, 1993
23. Nishikawa K, Andres G, Bhan AK, McCluskey RT, Collins AB, Stow JL, Stamenkovic I: Hyaluronate is a component of crescents in rat autoimmune glomerulonephritis. *Lab Invest* 68: 146–153, 1993
24. Reynolds J, Mavromatidis K, Cashman SJ, Evans DJ, Pusey CD: Experimental autoimmune glomerulonephritis (EAG) induced by homologous and heterologous glomerular basement membrane in two sub-strains of Wistar-Kyoto rat. *Nephrol Dial Transplant* 13: 44–52, 1998
25. Sado Y, Kagawa M, Naito I, Okigaki T: Properties of bovine nephritogenic antigen that induces anti-GBM nephritis in rats and its similarity to the Goodpasture antigen. *Virchows Archiv B Cell Pathol* 60: 345–351, 1991
26. Sado Y, Kagawa M, Kishiro Y, Naito I, Joh K, Ninomiya Y: Purification and characterisation of human nephritogenic antigen that induces anti-GBM nephritis in rats. *J Pathol* 182: 225–232, 1997
27. Abbate M, Kalluri R, Corna D, Yamaguchi N, McCluskey RT, Hudson BG, Andres G, Zoja C, Remuzzi G: Experimental Goodpasture's syndrome in Wistar-Kyoto rats immunized with α3 chain of type IV collagen. *Kidney Int* 54: 1550–1561, 1998
28. Sado Y, Boutaud A, Kagawa M, Naito I, Ninomiya Y, Hudson BG: Induction of anti-GBM nephritis in rats by recombinant α3(IV)NC1 and α4(IV)NC1 of type IV collagen. *Kidney Int* 53: 664–671, 1998
29. Ryan JJ, Reynolds J, Norgan VA, Pusey CD: Expression and characterization of recombinant rat α3(IV)NC1 and its use in induction of experimental autoimmune glomerulonephritis. *Nephrol Dial Tranplant* 2000, in press
30. Saus J, Wieslander J, Langeveld JPM, Quinones S, Hudson BG: Identification of the Goodpasture antigen as the α3 chain of collagen IV. *J Biol Chem* 263: 13374–13380, 1988
31. Turner AN, Mason PJ, Brown R, Fox M, Povey S, Rees AJ, Pusey CD: Molecular cloning of the human Goodpasture antigen demonstrates it to be the α3 chain of type IV collagen. *J Clin Invest* 89: 592–601, 1992
32. Sado Y, Naito I, Okigaki T: Transfer of anti-glomerular basement membrane antibody-induced glomerulonephritis in inbred rats with isologous antibodies from urine of nephritic rats. *J Pathol* 158: 325–332, 1989
33. Reynolds J, Moss J, Smith J, Karkar AM, Shore I, Evans DJ, Woodrow DF, Pusey CD: Ultrastructural abnormalities of kidney and lung in an animal model of Goodpasture's disease [Abstract]. *Kidney Int* 55: 2557, 1999
34. Reynolds J, Sallie BA, Syrganis C, Pusey CD: The role of T-helper lymphocytes in priming for experimental autoimmune glomerulonephritis in the BN rat. *J Autoimmun* 6: 571–585, 1993
35. Reynolds J, Pusey CD: T cell responses to autoantigenic components of GBM in EAG [Abstract]. *J Am Soc Nephrol* 6: 850, 1995
36. Reynolds J, Cashman SJ, Evans DJ, Pusey CD: Cyclosporin A in the prevention and treatment of experimental autoimmune glomerulonephritis in the Brown Norway rat. *Clin Exp Immunol* 85: 28–32, 1991
37. Reynolds J, Pusey CD: In vivo treatment with a monoclonal antibody to T helper cells in experimental autoimmune glomerulonephritis. *Clin Exp Immunol* 95: 122–127, 1994
38. Reynolds J, Norgan VA, Chana U, Pusey CD: Anti-CD8 monoclonal antibody therapy is effective in experimental autoimmune glomerulonephritis [Abstract]. *J Am Soc Nephrol* 10: 519, 1999
39. Reynolds J, Tam FWK, Chandraker A, Smith J, Karkar AM, Cross J, Peach R, Sayegh MH, Pusey CD: CD28-B7 blockade prevents the development of experimental autoimmune glomerulonephritis. *J Clin Invest* 105: 643–651, 2000
40. Nishikawa K, Linsley PS, Collins AB, Stamenkovic I, McCluskey RT, Andres G: Effect of CTLA-4 chimeric protein on rat autoimmune anti-glomerular basement membrane disease. *Eur J Immunol* 24: 1249–1254, 1994
41. Nishikawa K, Guo YJ, Miyasaka M, Tamatani T, Collins AB, Sy MS, McCluskey RT, Andres G: Antibodies to intercellular adhesion molecule 1/lymphocyte function-associated antigen 1 prevent crescent formation in rat autoimmune glomerulonephritis. *J Exp Med* 177: 667–677, 1993

42. Cohen SB, Weetman AP: Serological analysis of experimental autoimmune thyroiditis in the buffalo strain rat. *Int Arch Allergy Appl Immunol* 91: 47–53, 1990
43. Laurell CB: Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal Biochem* 15: 45–52, 1966
44. Cashman SJ, Pusey CD, Evans DJ: Extraglomerular distribution of immunoreactive Goodpasture antigen. *J Pathol* 155: 61–70, 1988
45. Tam FWK, Smith J, Morel D, Karkar AM, Thompson EM, Cook HT, Pusey CD: Development of scarring and renal failure in a rat model of crescentic glomerulonephritis. *Nephrol Dial Transplant* 14: 1658–1666, 1999
46. Derry CJ, Ross CN, Lombardi G, Mason PD, Rees AJ, Lechler RI, Pusey CD: Analysis of T cell responses to the autoantigen in Goodpasture's disease. *Clin Exp Immunol* 100: 262–268, 1995
47. Zou LP, Ma DH, Levi M, Wahren B, Wei L, Mix E, van der Meide PH, Link H, Zhu J: Antigen-specific immunosuppression: Nasal tolerance to PO protein peptides for the prevention and treatment of experimental autoimmune neuritis in Lewis rats. *J Neuroimmunol* 94: 109–121, 1999
48. Metzler B, Wraith DC: Mucosal tolerance in a murine model of experimental autoimmune encephalomyelitis. *Ann N Y Acad Sci* 636: 227–232, 1997