Nasal Administration of Recombinant Rat α3(IV)NC1 Prevents the Development of Experimental Autoimmune Glomerulonephritis in the WKY Rat

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Experimental autoimmune glomerulonephritis (EAG), an animal model of Goodpasture’s disease, can be induced in Wistar Kyoto (WKY) rats by immunization with either collagenase-solubilized rat glomerular basement membrane (GBM) or the recombinant NC1 domain of the α3 chain of type IV collagen [α3(IV)NC1]. EAG is characterized by circulating and deposited anti–glomerular basement membrane antibodies, focal necrotizing glomerulonephritis with crescent formation, and glomerular infiltration by T cells and macrophages. Previous studies have demonstrated that oral administration of collagenase-solubilized GBM to WKY rats prevented the development of EAG. Nasal administration of specific autoantigens has been reported to be more effective than oral administration in other models of autoimmune disease. The main aim of this study was to investigate further the concept of mucosal tolerance in EAG by examining the effect of nasal administration of recombinant rat α3(IV)NC1. Groups of WKY rats with EAG, induced by immunization with recombinant rat α3(IV)NC1, were given α3(IV)NC1 nasally on 3 consecutive days before immunization, at total cumulative doses of 25, 100, or 250 μg per rat. A dose-dependent effect was observed on the development of EAG. A dose of 25 μg had no effect on disease; 100 μg resulted in a moderate reduction in the severity of nephritis; and 250 μg led to a marked reduction in circulating and deposited antibodies, albuminuria, severity of glomerular abnormalities, and numbers of glomerular CD8+ T cells and macrophages. In addition, there was a reduction in the proliferative response of splenocytes from rats in the high dose group (250 μg) to α3(IV)NC1 in vitro. The results from this study clearly demonstrate for the first time that mucosal tolerance in EAG can be induced by nasal administration of recombinant rat α3(IV)NC1 and that this approach is effective in the prevention of crescentic glomerulonephritis. Further work using new antigen-specific treatment strategies may provide a novel approach to the treatment of patients with anti–glomerular basement membrane disease.

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lomerulonephritis is one of the most common causes of established renal failure worldwide. The exact cause of most types of glomerulonephritis is not known, but immunologic mechanisms are clearly involved. Goodpasture’s, or anti–glomerular basement membrane (GBM), disease is uncommon but is well understood and so provides a good model of autoimmune renal disease. It presents with rapidly progressive glomerulonephritis caused by autoimmunity to a component of the GBM, the α3 chain of type IV collagen [α3(IV)NC1] (1,2). For investigating mechanisms of autoimmunity and inflammation in glomerulonephritis and to test approaches to specific immune intervention, animal models of Goodpasture’s disease known as experimental autoimmune glomerulonephritis (EAG) have been induced in various species by immunization with preparations of GBM (3–9) or more recently with recombinant α3(IV)NC1 (10–13). The disease is characterized by the development of circulating and deposited anti-GBM antibodies, with focal necrotizing crescentic glomerulonephritis and lung hemorrhage. Recent studies have demonstrated that EAG shares many features in common with the human disease, in that the renal pathology is very similar and that the autoimmune response involves anti-GBM antibodies with the same specificity for the main target antigen, α3(IV)NC1 (12). As in Goodpasture’s disease, there seems to be a role for both humoral and cell-mediated immunity to α3(IV)NC1 in the development of EAG (14).

The role of anti-GBM antibodies in the pathogenesis of EAG has been demonstrated in passive transfer studies. This was first demonstrated by Steblay (15), using cross circulation of blood from nephritic sheep to normal recipients. More recently, the successful passive transfer of EAG was demonstrated in SJL mice by anti-α3(IV)NC1 antibodies pooled from the serum of nephritic mice (8), in WKY rats by antibodies purified from the
urine of nephritic rats (16), and by antibody eluted from the kidneys of rats with EAG (Reynolds et al., unpublished observation). In addition, Sado’s group has demonstrated transfer of disease by mAb generated from WKY rats with EAG (17,18). However, there is also increasing evidence to support a role for T lymphocytes in the pathogenesis of EAG. T cells and macrophages have been shown to be present in the glomeruli of mice and rats with EAG (8,14); T cells from nephritic animals proliferate in response to α3(IV)NC1 (8,19,20) and can transfer EAG into naive recipients (8,20); an immunodominant T cell epitope from the N-terminus of rat α3(IV)NC1 is capable of inducing severe crescentic nephritis (21–24); glomerular T cells in EAG show restricted T cell receptor CDR3 spectratypes (25); and anti–T cell immunotherapy can prevent or ameliorate disease (26–30).

Previous studies have shown that both cyclosporin A (26) and anti-CD4 mAb (27) are effective in reducing the level of the autoimmune response in EAG in the Brown Norway (BN) rat, and more recently it was shown that anti-CD8 mAb (28) is effective in reducing the severity of crescentic nephritis in EAG in the WKY rat. Selective blockade of T cell co-stimulatory pathways has also proved to be beneficial in both the prevention and the treatment of EAG. Blockade of the CD28-B7 co-stimulatory pathway by the fusion protein CTLA4-Ig (29) and blockade of the CD154-CD40 co-stimulatory pathway with a mAb directed against CD154 (30) both have been shown to be effective in EAG. In addition, oral administration of GBM antigen is effective in preventing the development of crescentic nephritis in EAG (8,19). Thus, there is now compelling evidence for the role of both humoral and cell-mediated immunity in the induction and pathogenesis of experimental models of anti-GBM disease.

Mucosal tolerance is a phenomenon whereby peripheral immunologic tolerance may be induced by the mucosal administration of autoantigens (31–34). The inhibitory effect of orally or nasally administered autoantigens or immunodominant peptides has been widely reported in several experimental models of autoimmune disease in rodents, including enccephalomyelitis, arthritis, myasthenia gravis, and interstitial nephritis (35–43). In several of these studies, it was shown that nasal administration of lower doses of antigen than those given orally has been effective in inducing mucosal tolerance (35,37,39) and in treating established disease (36,40,43). In addition, coupling the antigen to the cholera toxin B subunit (CTB) has been shown to enhance development of mucosal tolerance (44).

Despite considerable research in this area, there is still controversy as to the mechanisms involved in the induction of mucosal tolerance. There is evidence to suggest that mucosal tolerance can be mediated by a number of mechanisms depending on the dose of antigen administered. High doses of antigen may lead to clonal deletion or anergy, whereas lower doses favor the development of suppressor mechanisms (31–34). This suppression seems to be due to a distinct subset of T regulatory cells that secrete anti-inflammatory cytokines, resulting in a switch from a pathogenic Th1-like response (IFN-γ mediated) to a less pathogenic or protective Th2-like response (IL-4 mediated) (45,46). The balance between Th1 and Th2 cells has been shown to be controlled, at least in part, by a regulatory subset of T cells, known as Th3 cells, which can suppress immune responses in vitro and in vivo by release of the immunosuppressive cytokine TGF-β (47,48). More recently, it was shown that CD4+CD25+ T regulatory cells may also be involved in the induction of mucosal tolerance (49,50). In addition, there is mounting evidence that mucosal dendritic cells play a pivotal role in controlling the mucosal immune response (51,52).

In this study, we investigated further the concept of mucosal tolerance in a rat model of autoimmune glomerulonephritis (EAG) by examining the effect of nasal administration of recombinant rat α3(IV)NC1. We demonstrate for the first time that mucosal tolerance can be induced in EAG in a dose-dependent manner by recombinant antigen given nasally. This work may eventually lead to the development of new antigen-specific treatment strategies in patients with anti-GBM disease or other forms of glomerulonephritis.

**Materials and Methods**

**Cloning, Expression, and Purification of Recombinant Rat α3(IV)NC1**

The expression plasmid pcBFT, which contains the BM40 signal peptide, a Flag tag, and rat α3(IV)NC1 domain, was transfected into human embryonic kidney (HEK) 293 cells (Invitrogen, San Diego, CA) using superfect (Qiagen, Chatsworth, CA) (8). Cell lines that secrete recombinant α3(IV)NC1 were selected by adding 600 μg/ml geneticin (G418; Sigma-Aldrich Company Ltd, Poole, Dorset, UK) to DMEM (Invitrogen), which contained 10% FCS and 1% penicillin/streptomycin (Invitrogen). Purification of recombinant rat α3(IV)NC1 from the supernatant was carried out by affinity chromatography using an anti-FLAG M2 affinity column (Sigma-Aldrich Company Ltd), as described previously (8,12).

**Characterization of Recombinant Rat α3(IV)NC1**

Protein eluted from the anti-FLAG affinity column was analyzed by SDS-PAGE and Western blotting, using the PhastSystem (Amersham Bioscience UK Limited, Chalfont St Giles, Buckinghamshire, UK) (12). Briefly, pooled fractions were run on a 12.5% SDS gel and blotted with anti-FLAG M2 mAb (Sigma-Aldrich Company Ltd) at a concentration of 10 μg/ml, serum from an animal with EAG at a dilution of 1:10, or serum from a patient with Goodpasture’s disease at a dilution of 1:100. Bound antibody was detected by alkaline phosphatase-conjugated rabbit anti-mouse IgG, sheep anti-rat IgG, or goat anti-human IgG (Sigma-Aldrich Company Ltd). Each blot then was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich Company Ltd).

**Experimental Animals**

Male WKY rats that were 8 to 10 wk of age and weighed 120 to 150 g were purchased from Charles River (Margate, Kent, 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.

**Induction of EAG**

EAG was induced in WKY rats by a single intramuscular injection of recombinant rat α3(IV)NC1 in an equal volume of Freund’s complete adjuvant (FCA; Sigma-Aldrich Company Ltd), at a dose of 100 μg/rat (12). 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**

Recombinant rat α3(IV)NC1 in PBS, in a volume of 30 μl, was administered into one nostril of each rat using a micropipette (Anachem; Luton, Bedfordshire, UK) in groups of lightly anesthetized (isoflurane) WKY rats (n = 5 to 8), at total cumulative doses of 50, 100, or 250 μg, over 3 consecutive days before immunization with α3(IV)NC1 in FCA. In addition, positive control groups [immunized with α3(IV)NC1 in FCA] and negative control groups (injected with FCA alone) were given BSA in PBS (Sigma-Aldrich Company Ltd) nasally, at a total dose of 250 μg in the same volume.

**Assay Systems**

ELISA. Circulating anti-α3 antibody concentrations were measured in sera of experimental animals by a solid-phase ELISA, as described previously (12). Briefly, recombinant rat α3(IV)NC1 was coated onto microtiter plates (Life Technologies, Paisley, UK) at a concentration of 5 μg/ml by overnight incubation at 4°C, and an optimum dilution of test or control sera was 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 (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 were calculated as mean optical density for each triplicate sample.

Subclass ELISA. Circulating levels of IgG1 and IgG2a anti-α3 antibodies were measured in sera of experimental animals by an indirect ELISA, similar to that previously described (19,29,30). Briefly, recombinant rat α3(IV)NC1 was coated onto ELISA plates, and an optimum dilution of test or control sera was applied for 1 h at 37°C. The isotypes of circulating anti-α3 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 horseradish peroxidase-conjugated rabbit anti-goat IgG (Sigma-Aldrich Company Ltd) and developed using the substrate ortho-phenylene diamine dihydrochloride (Sigma-Aldrich Company Ltd). The absorbances for each well were read at 492 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 experimental animals by rocket immunoelectrophoresis (Amersham Bioscience UK Limited), as described previously (9,14). Briefly, urine samples from experimental animals were subjected to immunoelectrophoresis at 60 V in an electrophoresis tank that contained Barbitone buffer (pH 9.5; BDH Laboratory Supplies, Poole, Dorset, UK), for 6 h, using a 1% agarose gel (BDH Laboratory Supplies) that contained rabbit anti-serum 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 with EAG at week 4 were measured by spectrometry using standard techniques. Creatinine clearance then was calculated for each animal and expressed as ml/min (14,29,30).

**Splenocyte Proliferation Assay.** Spleens from experimental animals were dissociated into a single-cell suspension and cultured with recombinant rat α3(IV)NC1, as described previously (8,13,19). Briefly, spleen cells were isolated by sieving, and red blood cells were removed by lysis. Cells were suspended in X-vivo 20 medium (BioWhittaker, Walkersville, MD) and plated out in round-bottom 96-well plates (In-vitrogen) at a concentration of 5 × 10^5 cells/well. Recombinant rat α3(IV)NC1 was denatured by boiling for 10 min with immediate cooling on ice. Denatured α3(IV)NC1 has been shown, in previous studies, to generate better T cell proliferative responses than native α3(IV)NC1 (Reynolds et al., unpublished observation). Cells then were cultured with α3(IV)NC1 at a concentration of 1, 3, or 10 μg/ml in a humidified environment with 5% CO2 at 37°C for 72 h. Tritiated thymidine (Amersham Bioscience UK Limited) was added at a concentration of 1 μCi/well at 16 h before harvesting, and thymidine incorporation was measured using an automated β counter (Amersham Bioscience UK Limited). Results were expressed as a stimulation index, which was calculated by dividing the cp/min well cultured with antigen by the cp/min wells with no antigen.

**Renal Histology**

**Direct Immunofluorescence.** Deposits of IgG within the glomeruli were detected by direct immunofluorescence, as described previously (9,14). 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 5 μm thick and were incubated with FITC-labeled rabbit anti-rat IgG (Serotec Ltd). The degree of immunostaining was assessed and graded from 0 to 3+ by a blinded observer.

**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, Imperial College London). 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 (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 (9,14).

**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 mAb OX8 (CD8+ 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 (14,28–30).

**Statistical Analyses**

Differences between multiple groups of data were determined by a nonparametric Kruskal-Wallis test followed by a Dunn post test.

**Results**

**Characterization of Recombinant Rat α3(IV)NC1**

The Western blots shown in Figure 1 show that the anti-FLAG M2 mAb bound strongly to both the dimeric and the two monomeric isoforms of recombinant rat α3(IV)NC1 eluted from the anti-FLAG affinity column. Serum from an animal with EAG and from a patient with Goodpasture’s disease also recognized the same characteristic dimer and monomer components of recombinant rat α3(IV)NC1.
Circulating Anti-α3(IV)NC1 IgG Antibody Levels

All positive control rats that were immunized with recombinant rat α3(IV)NC1 in FCA produced detectable levels of both IgG1 and IgG2a anti-α3(IV)NC1 antibodies by week 4. Animals that were given recombinant α3(IV)NC1 nasally at a total dose of 25 or 100 µg showed no reduction in either IgG1 or IgG2a antibodies, whereas 250 µg resulted in a marked reduction in levels of both IgG1 and IgG2a antibodies. Negative control animals that were given FCA alone showed a similar level of background binding as normal rat serum. Results are shown in Figure 2.

Figure 2. Effect of nasal administration of recombinant α3(IV)NC1 on circulating IgG1 (A) and IgG2a (B) anti-α3(IV)NC1 antibodies in groups of Wistar Kyoto (WKY) rats (n = 5 to 8) with EAG. Results shown represent the mean ± SD of each group at week 4 after immunization. *P < 0.05, positive control versus α3(IV)NC1 250 µg nasally.

Circulating Anti-α3(IV)NC1 IgG1 and IgG2a Antibody Levels

All positive control rats that were immunized with recombinant rat α3(IV)NC1 in FCA produced high levels of circulating anti-α3(IV)NC1 IgG by week 4 after immunization. Animals that were given recombinant α3(IV)NC1 nasally at a total dose of 25 or 100 µg produced similar levels of antibody to positive controls, whereas 250 µg resulted in a marked reduction in levels of both IgG1 and IgG2a antibodies. Negative control animals that were given FCA alone showed a similar level of background binding as normal rat serum. Results are shown in Figure 3.

Figure 3. Effect of nasal administration of recombinant α3(IV)NC1 on circulating levels of IgG1 (A) and IgG2a (B) antibodies in groups of WKY rats (n = 5 to 8) with EAG. Results shown represent the mean ± SD of each group at week 4 after immunization. *P < 0.05, positive control versus α3(IV)NC1 250 µg nasally.

Albuminuria

All positive control rats that were immunized with recombinant rat α3(IV)NC1 in FCA produced high levels of albuminuria by week 4 after immunization. Nasal administration of recombinant α3(IV)NC1 had a dose-dependent effect on albuminuria. Animals that were given α3(IV)NC1 nasally at 25 µg showed no reduction in albuminuria, whereas those that were
given 100 µg showed a moderate reduction and those that were given 250 µg showed a marked reduction in the level of albuminuria. Negative control animals that were given FCA alone did not develop albuminuria. Results are shown in Figure 4A.

**Creatinine Clearance**

All negative control rats that were given FCA alone showed normal creatinine clearance, in the range of 1.0 to 1.2 ml/min. Positive control rats that were immunized with recombinant rat α3(IV)NC1 in FCA showed a marked reduction in creatinine clearance. Animals that were given α3(IV)NC1 nasally at 25 and 100 µg also showed a reduction in creatinine clearance, whereas those that were given 250 µg showed a significant increase in creatinine clearance when compared with positive controls. Results are shown in Figure 4B.

**Splenocyte Proliferation**

Spleen cells from positive control rats that were immunized with recombinant rat α3(IV)NC1 in FCA showed an increased proliferative response when cultured with α3(IV)NC1 in vitro for 72 h at a concentration of 10 µg/ml. Spleen cells from animals that were given α3(IV)NC1 nasally at 250 µg showed a marked reduction in proliferation, which was similar to that of negative control animals. Results are shown in Figure 5.

**Direct Immunofluorescence for IgG**

Direct immunofluorescence for IgG on kidney tissue at 4 wk revealed that positive control rats that were immunized with recombinant rat α3(IV)NC1 in FCA showed strong linear deposits of IgG along the GBM. Animals that were given α3(IV)NC1 nasally at a total dose of 25 µg showed binding similar to positive controls, whereas 100 µg led to a slight reduction and 250 µg a marked reduction in the deposits of IgG on the GBM. Negative control animals that were given FCA alone showed no IgG deposition. Results are shown in Figure 6 and illustrated in Figure 7.

**Light Microscopy**

Light microscopy of kidney tissue at 4 wk revealed that all positive control rats that were immunized with recombinant rat α3(IV)NC1 in FCA showed focal proliferative glomerulonephritis (FPGN) affecting 100% of glomeruli, with segmental necrosis/crescent formation affecting 85% of glomeruli. Animals that were given α3(IV)NC1 nasally at a total dose of 25 µg also showed severe FPGN affecting 100% of glomeruli, with segmental necrosis/crescent formation affecting 80% of glomeruli; those that were given 100 µg showed moderate FPGN affecting 70% of glomeruli, with segmental necrosis/crescent formation affecting 40% of glomeruli; and those that were given 250 µg showed mild FPGN affecting 20% 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 8 and illustrated in Figure 9.

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**Figure 4.** Effect of nasal administration of recombinant α3(IV)NC1 on albuminuria (A) and creatinine clearance (B) in groups of WKY rats (n = 5 to 8) with EAG. Results shown represent the mean ± SD of each group at week 4 after immunization. (A) *P < 0.01, positive control versus α3(IV)NC1 100 µg nasally; **P < 0.005, positive control versus α3(IV)NC1 250 µg nasally. (B) *P < 0.01, positive control versus α3(IV)NC1 250 µg nasally.

**Figure 5.** Effect of nasal administration of recombinant α3(IV)NC1 on the in vitro proliferative responses of splenocytes cultured with α3(IV)NC1 at 10 µg/ml in groups of WKY rats (n = 5 to 8) with EAG. Results shown represent the mean ± SD of the stimulation index of each group at week 4 after immunization. *P < 0.01, positive control versus α3(IV)NC1 250 µg nasally.
Immunohistochemistry

Immunohistochemistry of kidney tissue at 4 wk showed that positive control rats that were immunized with recombinant rat α3(IV)NC1 in FCA had glomerular infiltration with T cells and macrophages. Animals that were given α3(IV)NC1 nasally at a total dose of 25 μg showed no reduction in the numbers of CD8+ T cells and macrophages infiltrating the glomeruli, whereas those that were given 100 μg showed a moderate reduction and those that were given 250 μg showed a marked reduction. Negative control animals that were given FCA alone showed no cellular infiltrate. Results are shown in Figure 10.

Discussion

The inhibitory effect of nasally administered antigens in various animal models of autoimmunity has been widely reported, but there is still much controversy as to the mechanisms involved (31–34). To address this issue in autoimmune renal disease, we examined the effect of various nasally administered doses of recombinant rat α3(IV)NC1 on the development of EAG in the WKY rat. Although our previous studies demonstrated that recombinant rat α3(IV)NC1 could be transiently expressed in COS-7 cells (12), this cell line was not stable, making it difficult to produce large quantities of recombinant α3(IV)NC1. For this study, we therefore expressed recombinant rat α3(IV)NC1 in stably transfected HEK 293 cells. To our knowledge, this is the first report of this technique to produce recombinant α3(IV)NC1. The addition of the FLAG epitope to recombinant α3(IV)NC1 allowed us to purify large quantities of antigen on an anti-FLAG affinity column. Recombinant rat α3(IV)NC1 was produced from the HEK 293 cells at approximately 5 mg/L. To ensure that our purified protein was α3(IV)NC1, we characterized the eluted fractions from the affinity column by Western blotting, using both an anti-FLAG mAb to confirm that the protein expressed the FLAG epitope, and sera from WKY rats that were immunized with α3(IV)NC1 and sera from patients with Goodpasture’s disease to confirm the presence of α3(IV)NC1. Recombinant rat α3(IV)NC1 was detected as two monomeric isoforms at the predicted molecular weight of approximately 30 kD, which may be due to posttranslational modification or degradation of the recombinant protein, and one dimeric band, suggesting the spontaneous formation of dimers, possibly via interactions between the short collagenous segments of recombinant monomers (12).

In our study, nasal administration of recombinant α3(IV)NC1 resulted in a dose-dependent effect on the development of EAG. A total cumulative dose of 25 μg had no effect on disease; 100 μg resulted in a moderate reduction in the severity of nephritis; and 250 μg led to a marked reduction in circulating and deposited antibodies, albuminuria, severity of glomerular abnormalities, numbers of glomerular CD8+ T cells and macrophages, and the in vitro proliferative response of splenocytes to α3(IV)NC1. Our results clearly demonstrate for the first time that mucosal tolerance can be induced by nasal administration.
of recombinant rat α3(IV)NC1 and that this approach is effective in the prevention of the development of crescentic glomerulonephritis.

Previous studies in other animal models of nephritis have demonstrated that oral administration of renal antigens can be successful in the prevention of disease. In autoimmune interstitial nephritis in the BN rat, oral feeding of renal tubular antigen reduced the severity of interstitial nephritis and improved renal function (41). In a mouse model of anti-GBM disease, oral administration of purified bovine α3(IV)NC1 dimers to SJL mice reduced the numbers of crescents and interstitial inflammation (8). Similarly, in EAG in the WKY rat, we demonstrated that oral administration of rat GBM reduced the autoimmune response and prevented the development of crescentic nephritis by downregulating Th1-like responses (19). However, to our knowledge, no studies that have examined the effect of nasally administered antigens have been published in experimental glomerulonephritis.

It has been well documented that antigen administration via the nasal mucosa is more effective than oral administration in inducing T cell tolerance and suppressing autoimmune disease in other animal models (35,37,39). Nasal administration means that the antigen is placed in direct contact with the inductive site on the nasal associated lymphoid tissue and is not altered by gastric proteases before presentation within gut associated lymphoid tissue (GALT). As a result, much lower doses of antigen are required to induce a tolerant state via the nasal route, then via oral administration (53,54).

In our previous study, oral administration of GBM at a total cumulative dose of 5 mg was required to prevent the development of EAG, whereas in this study, nasal administration of α3(IV)NC1 at a total cumulative dose of 250 µg was required to prevent the development of disease. Nasal administration of α3(IV)NC1 had an effect on both humoral and cell-mediated immunity. There was a significant reduction in the level of circulating IgG antibodies directed toward α3(IV)NC1 and in the deposition of IgG on the GBM, demonstrating modulation of humoral immunity. In addition, nasal administration of α3(IV)NC1 led to a reduction in both IgG2a and IgG1 autoantibodies, suggesting a downregulation of both Th1- and Th2-like responses. These findings are in contrast to our previous study that examined the effect of oral administration of GBM (19). In that study, we demonstrated a significant reduction in the levels of IgG2a anti-GBM antibodies but not IgG1 antibodies, suggesting selective downregulation of Th1 responses.

Nasal administration of recombinant α3(IV)NC1 greatly reduced the number of infiltrating glomerular CD8+ T cells and macrophages, suggesting that nasal administration of
cells have been reported to be involved in the induction of mucosal tolerance. The balance between Th1 (IFN-γ) and Th2 (IL-4) cytokines has been shown to be important in the induction of mucosal tolerance (45,46). Increased levels of IL-4 production by Th2 cells have been shown to suppress Th1 cell function after nasal administration of antigen. The role of TGF-β in mediating mucosal tolerance has also been well studied. It is now recognized that TGF-β is produced by a unique subset of Th3 cells found within GALT, which are triggered in an antigen-specific manner (47,48). However, more recent evidence also suggests a role for CD4+CD25+ regulatory T cells in mucosal tolerance. There are suggestions that this subset of cells may be involved in the production of both IL-10 and TGF-β (49,50). Finally, it is important to consider the pivotal role for mucosal dendritic cells (DC) in orchestrating the mucosal tolerogenic immune response and in the induction of Tr1 and Th3 cells (51,52). Pulmonary DC that were isolated after respiratory exposure to antigen, via the nasal associated lymphoid tissue, induced Tr1 cells to produce IL-10, whereas DC from the GALT induced Th3 cells to produce TGF-β. Thus, there may be functional links between different regulatory T cells involved in mucosal tolerance, despite that they originate from different compartments and display different functions.

In conclusion, we have demonstrated for the first time that nasal administration of recombinant rat α3(IV)NC1 induces mucosal tolerance in EAG and downregulates both humoral and cell-mediated immunity. The most likely explanation for these findings is that nasal administration of α3(IV)NC1 has resulted in the induction of regulatory cells whose suppressive activities are mediated through the production of anti-inflammatory cytokines, such as IL-4, IL-10, and TGF-β. Further work on mechanisms of mucosal tolerance, including the analysis of cytokine profiles from autoreactive T cells by capture ELISA and ELISpot and analysis of glomerular expression of selected cytokines by real-time PCR, is in progress in our laboratory. It is hoped that this work will lead to the development of new antigen-specific treatment strategies in patients with anti-GBM disease and other autoimmune diseases.

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