

Anti-CD8 Monoclonal Antibody Therapy Is Effective in the Prevention and Treatment of Experimental Autoimmune Glomerulonephritis

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Abstract. Experimental autoimmune glomerulonephritis (EAG), which is an animal model of Goodpasture's disease, can be induced in Wistar Kyoto rats by a single injection of rat glomerular basement membrane (GBM) in adjuvant. EAG is characterized by circulating and deposited anti-GBM antibodies, focal necrotizing glomerulonephritis with crescent formation, and glomerular infiltration by T cells and macrophages. Our hypothesis was that T cell-mediated immunity, in addition to humoral immunity, was necessary for the development of crescentic nephritis in this model. To investigate the role of CD8⁺ T cells in the pathogenesis of EAG, the *in vivo* effects of an anti-CD8 monoclonal antibody (OX8) were examined, with administration starting at the time of immunization (prevention) or 2 wk after immunization, when glomerular abnormalities were first detected (treatment). When administered intraperitoneally at 5 mg/kg, three times per week, from week

0 to week 4 (prevention), OX8 completely inhibited the development of albuminuria, deposits of fibrin in the glomeruli, glomerular and interstitial abnormalities, the influx of CD8⁺ T cells and macrophages, and glomerular expression of granzyme B and inducible nitric oxide synthase. Circulating anti-GBM antibody levels were not reduced, but there was a reduction in the intensity of antibody deposition on the GBM. When administered at the same dose from week 2 to week 4 (treatment), OX8 greatly reduced the severity of EAG; in particular, the formation of crescents was prevented. These studies demonstrate that anti-CD8 monoclonal antibody therapy is effective in both the prevention and treatment of EAG. They confirm the importance of T cell-mediated immunity in the pathogenesis of this model of Goodpasture's disease. Similar therapeutic approaches may be worth investigating in human crescentic glomerulonephritis.

Goodpasture's disease, or anti-glomerular basement membrane (GBM) disease, presents with rapidly progressive glomerulonephritis, often accompanied by lung hemorrhage (1). It is characterized by the presence of circulating and deposited anti-GBM antibodies, the pathogenicity of which has been demonstrated in passive-transfer experiments (2). Treatment with plasma exchange and immunosuppressive drug therapy is generally effective among patients treated sufficiently early but carries a high risk of serious adverse effects (3,4). Anti-GBM antibodies from all patients recognize the same component of GBM, known as the Goodpasture antigen (5,6). This has been identified as the noncollagenous domain of the $\alpha 3$ chain of type IV collagen [$\alpha 3(\text{IV})\text{NC1}$] (7–11). Certain class II MHC genes are associated with the development of disease; HLA DRB1*1501–02 and DRB1*04 alleles confer susceptibility, and DRB1*01 and DRB1*07 alleles confer protection (12).

These strong MHC associations suggest a T cell-dependent immune response, and we have demonstrated that peripheral blood T cells from patients with Goodpasture's disease proliferate *in vitro* in response to affinity-purified Goodpasture antigen (13). In addition to providing help for the production of anti-GBM antibodies, such T cells may play a role in cell-mediated immune damage in glomeruli (14). Previous studies demonstrated the presence of both CD4⁺ and CD8⁺ T cells in the glomeruli of patients with anti-GBM disease (15).

Experimental autoimmune glomerulonephritis (EAG) is an experimental model of Goodpasture's disease that can be induced in genetically susceptible rat strains by immunization with heterologous or homologous preparations of GBM in adjuvant (16–20). In the model used in this study, Wistar Kyoto (WKY) rats 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, and focal necrotizing glomerulonephritis with crescent formation (20). This model of EAG shares many characteristics with the human disease and involves anti-GBM antibodies with specificity similar to that of human autoantibodies (21). Work from other groups, in related models, supports the idea that the main target antigen, $\alpha 3(\text{IV})\text{NC1}$, is

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generally the same in EAG as in Goodpasture's disease (22–24).

The role of anti-GBM antibodies in the induction of EAG has been demonstrated in passive-transfer experiments, in which the transfer of anti-GBM antibodies from rats and mice with EAG induces disease in the recipients (25,26). There is also increasing evidence to support a role for T lymphocytes in the induction and pathogenesis of EAG. We recently demonstrated that a transient glomerular influx of CD4⁺ T cells precedes an infiltration of CD8⁺ T cells and macrophages and glomerular injury (27). Transfer of CD4⁺ T lymphocytes purified from the spleens of rats with EAG can prime naive recipients for the disease (28), and *in vitro* stimulation of these cells by GBM, before transfer, confers the ability to induce anti-GBM antibody production in the recipients. Furthermore, T cells from animals with EAG exhibit specific proliferation in response to cationic monomer and dimer NC1 domains of rat GBM enriched in α 3(IV)NC1 (J. Reynolds and C.D. Pusey, unpublished observations). We previously demonstrated that cyclosporine A (29) and anti-CD4 monoclonal antibody (mAb) (W3/25) (30) were effective in preventing EAG in Brown Norway rats. More recently, we demonstrated that anti-CD40L mAb (31), blockade of the CD28-B7 costimulatory pathway (32), and oral administration of GBM antigen (33) were effective in preventing the development of crescentic nephritis in WKY rats. Therefore, there seems to be a role for both humoral and cell-mediated immunity to α 3(IV)NC1 in the development of EAG.

The *in vivo* administration of anti-CD8 mAb has proved to be effective in preventing the induction of disease and/or decreasing the severity of disease in various rat models of nephritis (34–38) or autoimmune disease (39–43). In nephrotoxic nephritis, depletion of CD8⁺ cells with anti-CD8 mAb (OX8) prevented the development of crescentic nephritis in both WKY (34,35) and Sprague-Dawley (36) rats. Similar results were demonstrated for Heymann nephritis, in which CD8⁺ T cell depletion prevented the development of proteinuria and glomerular injury in Lewis rats (37,38). In addition, anti-CD8 mAb therapy has been demonstrated to reduce the severity of inflammatory arthritis in HgCl₂-induced autoimmunity in Brown Norway rats (39), to reduce tissue injury and anti-thyroglobulin antibody levels in experimental autoimmune thyroiditis in Buffalo rats (40), to prevent diabetes mellitus in Biobreeding/Worcester rats (41), and to reduce the severity of disease and anti-acetylcholine receptor (AChR) antibody levels in experimental autoimmune myasthenia gravis in Lewis rats (42,43).

In this study, we investigated the role of CD8⁺ T cells in the pathogenesis of EAG by examining the *in vivo* effects of anti-CD8 mAb therapy in both the prevention and treatment of EAG. We demonstrate for the first time that anti-CD8 mAb is effective in preventing the development of autoimmune crescentic nephritis and in treating established disease after the onset of albuminuria. The results obtained contribute to our knowledge of the mechanisms underlying the immunopathogenesis of anti-GBM disease and should facilitate the design of

more specific and effective treatments for human crescentic glomerulonephritis.

Materials and Methods

Production, Purification, and Characterization of Anti-CD8 mAb

The anti-CD8 antibody-producing murine hybridoma OX8 was obtained from Serotec Ltd. (Oxford, UK). The cells were initially grown by standard tissue culture, as described previously (30), in RPMI 1640 medium supplemented with 5% fetal calf serum, 100 μ g/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine (Life Technologies, Paisley, UK). Cells (5×10^8) were then transferred to a hollow-fiber bioreactor (Harvest Mouse; Serotec) and grown in RPMI 1640 medium supplemented with 5% fetal calf serum, 100 μ g/ml penicillin, 100 μ g/ml streptomycin, 4 mM glutamine, and 2.5 g/L glucose (Life Technologies), which enabled us to produce large quantities of supernatant. IgG-enriched mAb fractions were purified from the supernatants by affinity chromatography using a protein G-Sepharose column (Amersham Pharmacia Biotech, St. Albans, UK). The purified IgG was then dialyzed against phosphate-buffered saline, measured for total IgG content by spectrophotometry at 280 nm, and sterile-filtered before injection. The specificity of the antibody was confirmed by labeling lymphocytes from normal animals and comparing the flow cytometric profile with that for commercially available OX8 supernatant (Serotec).

Preparation of GBM Antigen

Collagenase-solubilized GBM was prepared from normal rat kidneys, as described previously (17,20). Briefly, the kidneys were decapsulated, the medulla was partly removed, and the cortex was passed through sieves (Endecotts, London, UK) for isolation of glomeruli. After light-microscopic examination to ensure purity, the glomeruli were disrupted ultrasonically, and the resulting material was lyophilized (Life Science International, Basingstoke, UK) and digested for 1 h at 37°C with purified type I collagenase (Sigma-Aldrich Co., Poole, UK).

Experimental Animals

Male WKY rats (age, 8 to 12 wk; weight, 120 to 150 g) were purchased from Charles River (Margate, UK). All animals were housed under standard conditions and had free access to normal laboratory diet and water. All experimental procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act.

Induction of EAG

EAG was induced in WKY rats with a single intramuscular injection of collagenase-solubilized rat GBM in an equal volume of FCA, at a dose of 5 mg/kg body wt (20). Serial blood samples were obtained by tail artery puncture under light anesthesia (isoflurane), and 24-h urine specimens were obtained by placement of animals in metabolic cages (RS Biotech, Finedon, UK).

Assessment of EAG

Rocket Immunoelectrophoresis. Urinary albumin concentrations were measured in 24-h collections from animals with EAG by rocket immunoelectrophoresis (Amersham Pharmacia Biotech), as described previously (20). Briefly, urine samples from experimental animals were subjected to immunoelectrophoresis for 6 h at 60 V in an electrophoresis tank containing barbital buffer (pH 9.5; BDH

Laboratory Supplies, Poole, UK), using a 1% agarose gel (BDH Laboratory Supplies) containing rabbit antiserum to rat albumin (raised in our laboratory). Results were calculated by using rat serum albumin standards (which were analyzed at the same time) and were expressed in milligrams per 24 h.

Creatinine Clearance. Creatinine levels in serum and urine samples obtained from animals at week 4 (the time of euthanasia) were measured by standard spectrometric techniques, as described previously (32,33). Creatinine clearance was then calculated for each animal and expressed as milliliters per minute.

Direct Immunofluorescence Assays. Direct immunofluorescence studies to assess deposits of IgG and fibrin within the glomeruli were performed with kidneys obtained at euthanasia, using a method similar to that described previously (20,32,33). Tissue was embedded in OCT II embedding medium (Miles Inc., Elkhart, IN) on cork discs, snap-frozen in isopentane (BDH Laboratory Supplies) that had been precooled in liquid nitrogen, and stored at -80°C . Cryostat (Bright, Huntingdon, UK) sections ($4\ \mu\text{m}$ thick) were cut and incubated with either FITC-labeled rabbit anti-rat IgG (Serotec) or goat anti-rat fibrin (Nordic Immunology, Tilburg, The Netherlands). The degree of immunostaining was assessed and graded (from 0 to 3+) by a blinded observer.

Light Microscopy

Tissue Preparation. Kidney tissue obtained at the time of euthanasia was fixed in 10% neutral buffered formalin (BDH Laboratory Supplies), processed, and embedded in paraffin wax for light microscopy, using standard techniques. Briefly, $3\text{-}\mu\text{m}$ sections were stained with hematoxylin/eosin and periodic acid-Schiff stain and were examined by a blinded observer for the following features.

Glomerular Injury. Fifty consecutive glomeruli per section were assessed. Glomerular appearance was graded as normal, abnormal, or severe injury (>50% of the glomerulus affected by necrosis/crescent formation), and results were expressed as percentages of glomeruli examined (20,32,33).

Interstitial Injury. The degree of tubular injury and interstitial inflammatory infiltration was assessed and graded from 0 to 2+ (44).

Apoptosis Frequency. The number of apoptotic cells per 50 consecutive glomeruli was assessed and expressed as an average number per 50 glomeruli. Cells were considered apoptotic when they exhibited loss of cell volume; chromatin condensation along the nuclear membrane, with intensely basophilic staining; and/or nuclear fragmentation into spherical structures containing condensed chromatin (45).

Immunohistochemical Analyses

Leukocytes. Kidney sections were stained for T cells and macrophages by using standard avidin-biotin complex immunoperoxidase staining techniques. Briefly, formalin-fixed, paraffin-embedded kidney sections were stained with W3/13 (T lymphocytes), OX8 (CD8⁺ lymphocytes), and ED1 (macrophages) (Serotec) mAb. Glomerular T cells and macrophages were detected by using a biotinylated secondary antibody and avidin-biotin complex (Dako, Cambridge, UK). The cellular infiltrate was assessed by a blinded observer, who counted the number of positively stained cells per 50 consecutive glomeruli (32,33,44).

Granzyme B and Inducible Nitric Oxide Synthase. Kidney sections were stained for granzyme B and inducible nitric oxide synthase (iNOS) by using a horseradish peroxidase-streptavidin complex staining system (ImmunoCruz system; Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, formalin-fixed, paraffin-embedded kidney sections were stained with a goat polyclonal antibody raised against granzyme B (N-19) of human origin, which was crossreactive with rat granzyme B (Santa Cruz Biotechnology), and with a mouse anti-rat

mAb against iNOS (B. D. Biosciences, Oxford, UK). Glomerular expression of granzyme B and iNOS was detected by using an appropriate biotinylated secondary antibody and horseradish peroxidase-streptavidin reagent (Santa Cruz Biotechnology). The number of positively stained cells per 50 consecutive glomeruli was assessed by a blinded observer.

Enzyme-Linked Immunosorbent Assay. Circulating anti-GBM antibodies were measured in sera from animals with EAG by using a solid-phase enzyme-linked immunosorbent assay, similar to that described previously (20). Briefly, collagenase-digested rat GBM was coated onto microtiter plates (Life Technologies) by overnight incubation at 4°C , and an optimal dilution of control or test serum (1:16 dilution, as previously determined from a dilution curve) in phosphate-buffered saline/Tween was applied for 1 h at 37°C . Bound anti-GBM antibody was detected by using horseradish peroxidase-conjugated sheep anti-rat IgG (Sigma-Aldrich), with development using the substrate *o*-phenylenediamine dihydrochloride (Sigma-Aldrich). The absorbances of triplicate wells were measured at 492 nm by using an Anthos Multiskan enzyme-linked immunosorbent assay plate reader (Lab Tech International, Uckfield, UK), and the results were expressed as percentages of the binding obtained with a positive reference serum, as described previously (20).

Flow Cytometry. Peripheral blood cells from positive control animals, negative control animals, and animals given OX8 were labeled with mAb to T cell subsets, as described previously (32). Briefly, isolated lymphocytes from peripheral blood were labeled with mAb OX1 (leukocyte common antigen), OX19 (all T cells), W3/25 (CD4⁺ T cells), or OX8 (CD8⁺ T cells) or with isotype-matched negative control mAb (OX23) (Serotec). Labeled cells were detected by incubation with FITC-conjugated sheep anti-mouse IgG (Dako). The cells were then fixed in 2% paraformaldehyde (BDH Laboratory Supplies) and kept in the dark until analysis. Cells were quantified by flow cytometric analysis (EPICS XL; Coulter, Luton, UK). Stained cells were expressed as a percentage of total leukocytes, after subtraction of the negative control mAb values.

Experimental Protocols

Results from our pilot studies examining the effects of anti-CD8 mAb (OX8), administered throughout the disease or only at the time of immunization, on the development of EAG demonstrated that intraperitoneal injection at a dose of 5 mg/kg three times per week for the duration of the study was the most effective dosage. In subsequent experiments examining the prevention and treatment of EAG, groups of animals ($n = 5$ to 8) immunized with rat GBM in FCA were administered OX8 at 5 mg/kg, three times per week, from week 0 to week 4 in the prevention study and from week 2 to week 4 in the treatment study. Positive control groups (GBM/FCA) were given an isotype-matched control mAb (mouse anti-human Thy-1, which did not crossreact with rat cells; kindly donated by Professor M. Ritter, Department of Immunology, Imperial College, London, UK), and a negative control group (FCA alone) was given saline solution.

Statistical Analyses

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

Results

Albuminuria

All positive control animals immunized with rat GBM in FCA exhibited detectable levels of albuminuria by week 2,

which increased by week 3 and peaked at week 4. Animals given OX8 starting at the time of immunization (prevention) developed no albuminuria. Animals given OX8 starting at week 2 after immunization (treatment) demonstrated a significant reduction in albumin excretion, to nearly undetectable levels. Negative control animals given FCA alone did not develop albuminuria. Results are presented in Figure 1.

Creatinine Clearance

All negative control animals exhibited normal creatinine clearance, in the range of 1.0 to 1.2 ml/min. Positive control animals exhibited significant reductions in creatinine clearance. Animals given OX8 starting at the time of immunization (prevention) or starting at week 2 after immunization (treatment) exhibited creatinine clearances similar to those of negative control animals. Results are presented in Figure 2.

Direct Immunofluorescence Assays for Fibrin

All positive control animals exhibited large fibrin deposits within the glomeruli at week 4 after immunization. Animals given OX8 starting at the time of immunization (prevention) or starting at week 2 after immunization (treatment) exhibited no detectable fibrin deposits within the glomeruli. Negative control animals also demonstrated no binding. Results are summarized in Table 1 and illustrated in Figure 3.

Light Microscopy

All positive control animals exhibited severe focal necrotizing glomerulonephritis, with crescent formation, interstitial damage with tubular atrophy, and an increase in the number of apoptotic glomerular cells, at week 4 after immunization. Animals given OX8 starting at the time of immunization (prevention)

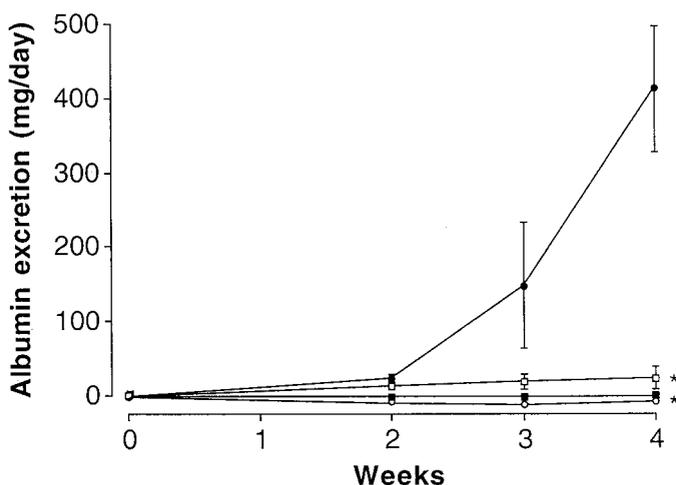


Figure 1. Effect of anti-CD8 monoclonal antibody (mAb) therapy on albuminuria in groups of Wistar Kyoto (WKY) rats (n = 5 to 8) with experimental autoimmune glomerulonephritis (EAG). Results shown represent the mean ± SD for each experimental group, i.e., positive control (●), OX8 therapy from week 0 to week 4 (prevention) (■), OX8 therapy from week 2 to week 4 (treatment) (□), and negative control (○) groups. *P < 0.001, positive control group versus OX8 (prevention) and OX8 (treatment) groups.

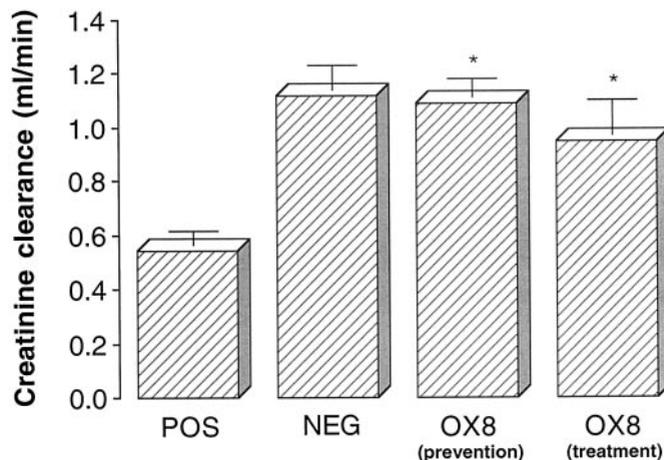


Figure 2. Effect of anti-CD8 mAb therapy on creatinine clearance in groups of WKY rats (n = 5 to 8) with EAG. Results shown represent the mean ± SD for each group at week 4 after immunization. *P < 0.01, positive control group versus OX8 (prevention) and OX8 (treatment) groups. POS, positive control; NEG, negative control.

tion) demonstrated no histologic evidence of disease. Animals given OX8 starting at week 2 after immunization (treatment) exhibited mild glomerular abnormalities in a minority of glomeruli, minimal interstitial injury, and only a small increase in the number of apoptotic cells. Negative control animals exhibited normal renal histologic features. Glomerular injury and apoptotic frequency results are presented in Figure 4, interstitial injury results are summarized in Table 2, and renal histologic findings are illustrated in Figures 5 and 6.

Immunohistochemical Analyses

All positive control animals exhibited a glomerular infiltrate of CD8⁺ T lymphocytes and macrophages, as well as increases

Table 1. Effects of anti-CD8 mAb therapy in WKY rats with EAG on deposition of fibrin in glomeruli and deposition of IgG on the GBM^a

	No. of Animals			
	3+	2+	1+	0
Fibrin				
positive control (n = 6)	4	2	0	0
negative control (n = 5)	0	0	0	5
OX8 (prevention) (n = 8)	0	0	0	8
OX8 (treatment) (n = 6)	0	0	1	5
IgG				
positive control (n = 6)	3	3	0	0
negative control (n = 5)	0	0	0	5
OX8 (prevention) (n = 8)	1	2	3	2
OX8 (treatment) (n = 6)	2	4	0	0

^a WKY, Wistar Kyoto; EAG, experimental autoimmune glomerulonephritis; GBM, glomerular basement membrane; mAb, monoclonal antibody. Results are expressed as numbers of animals with a given intensity of fluorescence at week 4.

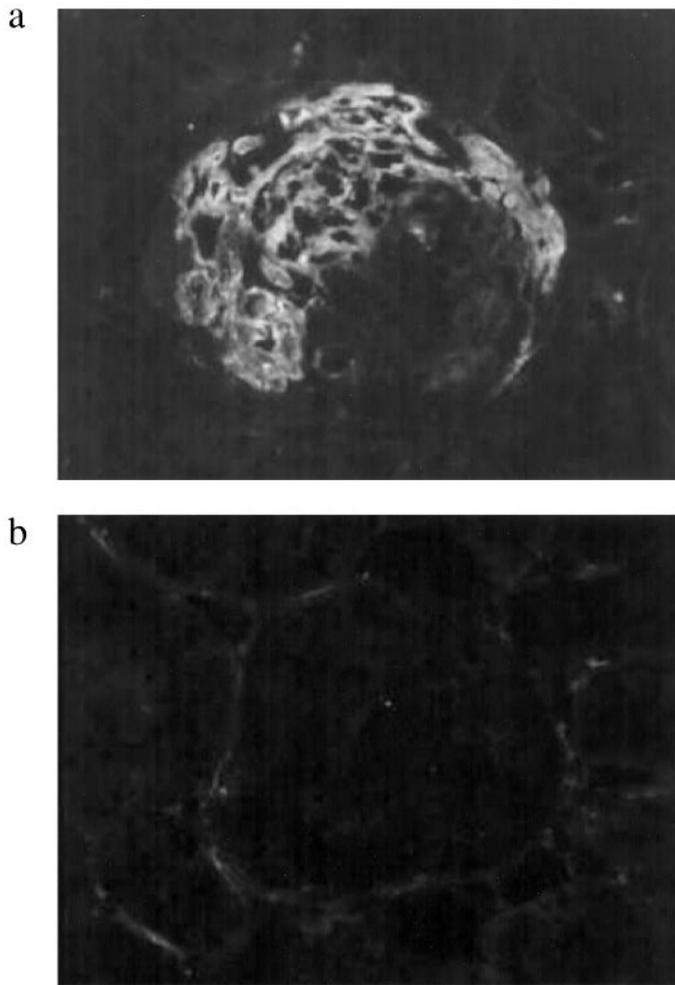


Figure 3. Direct immunofluorescence analysis of kidney tissue from WKY rats with EAG at 4 wk. (a) Large deposits of fibrin within the glomeruli of a positive control animal. Magnification, $\times 300$. (b) Negative findings for an animal given OX8 from week 0 to week 4 (prevention). Magnification, $\times 300$.

in the numbers of granzyme B- and iNOS-positive cells, in the glomeruli at week 4 after immunization. Animals given OX8 starting at the time of immunization (prevention) exhibited no increase in the numbers of glomerular CD8⁺ T cells or macrophages and no evidence of granzyme B- or iNOS-positive cells. Animals given OX8 starting at week 2 after immunization (treatment) exhibited marked reductions in the numbers of infiltrating CD8⁺ T cells and macrophages and in the numbers of granzyme B- and iNOS-positive cells. Results are presented in Figures 7 and 8.

Circulating Anti-GBM Antibody Concentrations

All positive control animals exhibited detectable circulating anti-GBM antibody levels by week 2, which increased to high levels by week 3 and peaked at week 4. Animals given OX8 starting at the time of immunization (prevention) or starting at week 2 after immunization (treatment) demonstrated no significant reduction in circulating anti-GBM antibody levels.

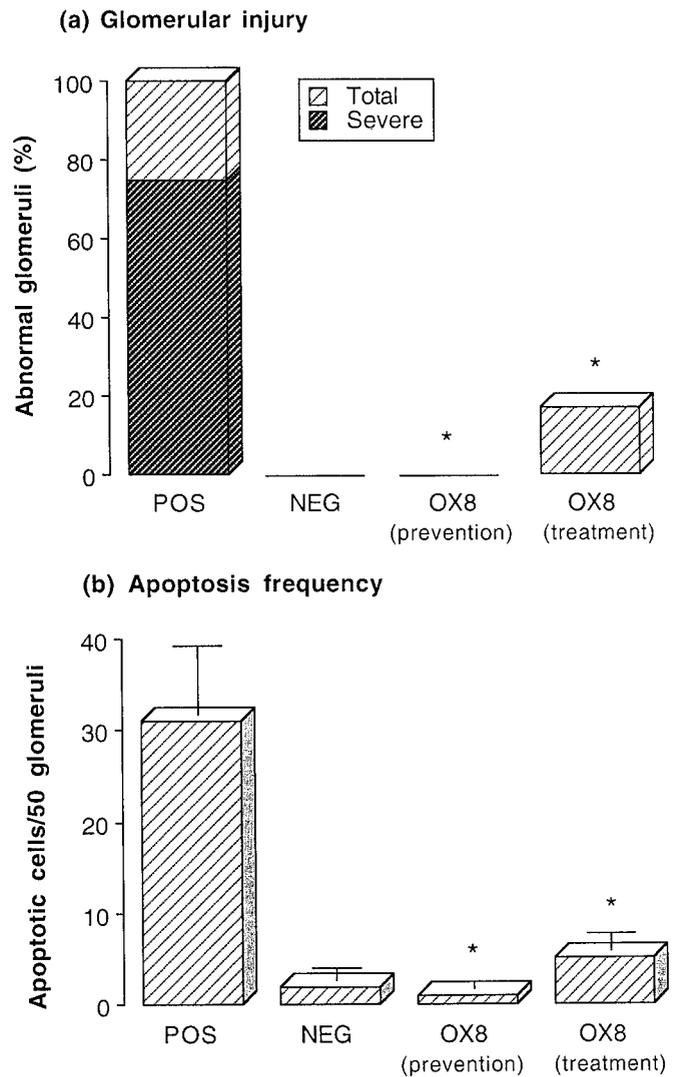


Figure 4. Effects of anti-CD8 mAb therapy on the degree of glomerular injury (a) and apoptosis frequency (b), as assessed by light-microscopic analysis of kidney tissue, in groups of WKY rats ($n = 5$ to 8) with EAG. Results shown represent the mean \pm SD for each group at week 4 after immunization. $*P < 0.001$, positive control group versus OX8 (prevention) and OX8 (treatment) groups. POS, positive control; NEG, negative control.

Negative control animals developed no circulating anti-GBM antibodies. Results are presented in Figure 9.

Direct Immunofluorescence Assays for IgG

All positive control animals demonstrated strong linear deposits of IgG along the GBM, and to lesser extent the tubular basement membrane, at week 4 after immunization. Animals given OX8 starting at the time of immunization (prevention) demonstrated moderate reductions in IgG deposits on the GBM. Animals given OX8 starting at week 2 after immunization (treatment) exhibited no reduction in the intensity of IgG on the GBM. Negative control animals demonstrated no antibody binding. Results are summarized in Table 1 and illustrated in Figure 10.

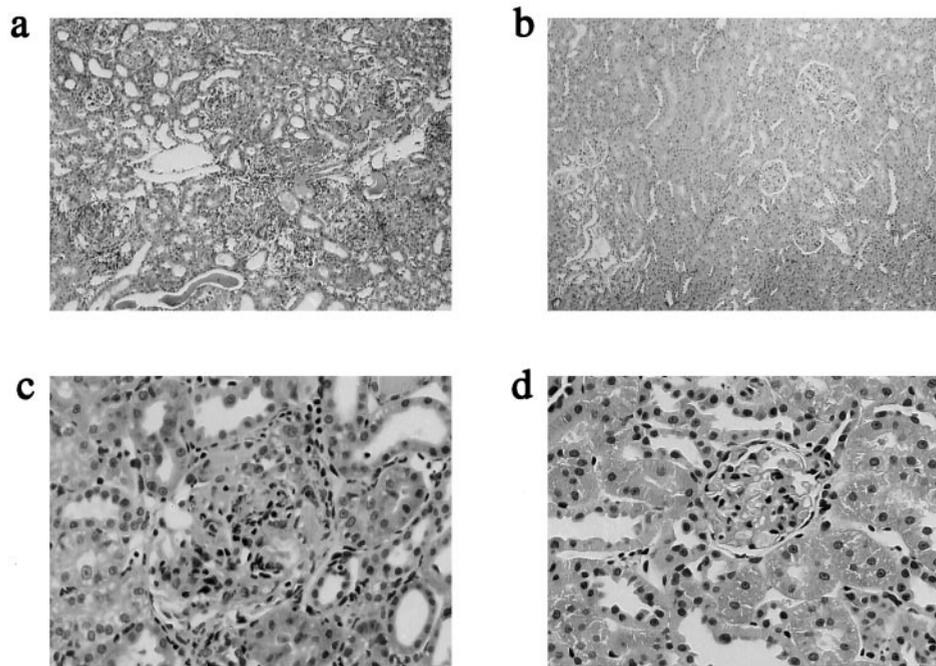


Figure 5. Light-microscopic analysis of kidney tissue from WKY rats with EAG at 4 wk. (a) Marked interstitial infiltrate and severe tubular damage in a positive control animal. Magnification, $\times 150$. (b) Normal interstitial architecture in an animal given OX8 from week 0 to week 4 (prevention). Magnification, $\times 150$. (c) Marked segmental necrosis of the glomerular tuft, with crescent formation, in a positive control animal. Magnification, $\times 300$. (d) Normal glomerular architecture in an animal given OX8 from week 0 to week 4 (prevention). Magnification, $\times 300$.

Flow Cytometric Analyses of Peripheral Blood

All positive and negative control animals exhibited approximately 35% positive T cells in peripheral blood at week 4 after immunization, of which 25% were CD4⁺ and 10% were CD8⁺. Animals given OX8 starting at the time of immunization (prevention) demonstrated marked reductions in the numbers of OX8⁺ cells (<2%) in the peripheral blood, compared with positive and negative control animals. Results are summarized in Table 3.

Discussion

There is still controversy regarding the respective roles of humoral and cell-mediated immunity to $\alpha 3(\text{IV})\text{NC1}$ in the development of anti-GBM disease. Although much attention has been paid to the pathogenicity of anti-GBM antibodies (1,2), it seems likely that T cell-mediated glomerular injury is also important, both in human Goodpasture's disease (12–15) and in animal models of EAG (16–20). There is now compelling evidence for the presence of glomerular CD8⁺ T cells in patients with anti-GBM disease (15) and rats with EAG (27,32,33). To further address this issue, we have investigated the role of CD8⁺ T cells in the pathogenesis of EAG, by examining the *in vivo* effects of anti-CD8 mAb therapy in prevention and in the treatment of established disease. In the prevention study, OX8 completely inhibited the development of glomerulonephritis and, importantly, reduced the frequency of apoptotic cells and prevented the accumulation of T cells and macrophages in the glomeruli. In addition, there were marked reductions in glomerular expression of granzyme B

Table 2. Effects of anti-CD8 mAb therapy in WKY rats with EAG on the severity of interstitial damage, *i.e.*, tubular injury and interstitial infiltrate^a

	No. of Animals		
	2+	1+	0
Tubular injury			
positive control ($n = 6$)	4	2	0
negative control ($n = 5$)	0	0	5
OX8 (prevention) ($n = 8$)	0	0	8
OX8 (treatment) ($n = 6$)	0	1	5
Interstitial infiltrate			
positive control ($n = 6$)	1	5	0
negative control ($n = 5$)	0	0	5
OX8 (prevention) ($n = 8$)	0	0	8
OX8 (treatment) ($n = 6$)	0	0	6

^a Results are expressed as numbers of animals with a given degree of injury at week 4.

and iNOS. Circulating anti-GBM antibody levels were not reduced but, interestingly, there was a reduction in the intensity of IgG deposition on the GBM. In the treatment study, OX8 greatly reduced the severity of EAG, particularly preventing the formation of crescents. Infiltration by T cells and macrophages was reduced, as was glomerular expression of gran-

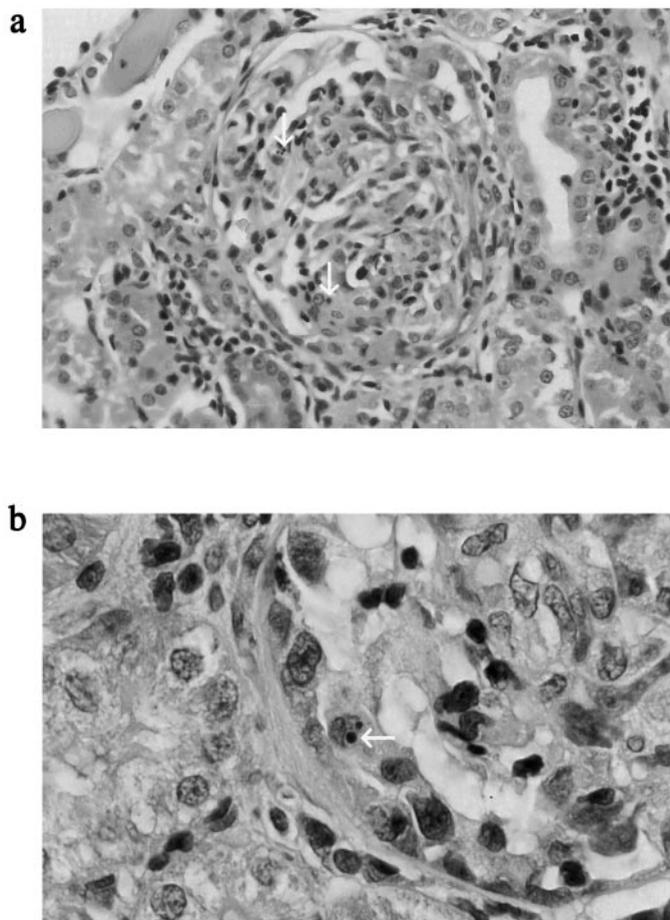


Figure 6. Light-microscopic analysis of a glomerulus from a positive control animal at 4 wk. (a) Severe crescentic glomerulonephritis, with several apoptotic glomerular cells (arrows). Magnification, $\times 300$. (b) Apoptotic bodies being engulfed by a mononuclear cell (arrows). Magnification, $\times 600$.

zyme B and iNOS, but there was no reduction in the deposition of IgG on the GBM.

The mechanism by which CD8⁺ T cells contribute to glomerular injury is unknown. Cytotoxic T lymphocyte-mediated cytotoxicity generally occurs via two independent pathways (46,47). The principle pathway is mediated by secretion of the pore-forming protein perforin, which facilitates the entry of cytotoxic serine proteases (such as granzymes) into target cells by forming transmembrane channels, leading to target cell lysis. An alternative nonsecretory pathway relies on the interaction of Fas ligand, which is upregulated during T cell activation, with the apoptosis-inducing Fas molecule on target cells. Both pathways lead to cell death. However, in addition to functioning as cytotoxic cells, CD8⁺ T cells can perform Th1- and Th2-like activities (48–50). Functionally distinct subsets of CD8⁺ T cells can produce different combinations of cytokines, which determine the functional phenotype of CD4⁺ T cells and the isotype of Ig expressed by B cells. Type 1 CD8⁺ T cells produce interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), and interleukin-2 (IL-2) and are restricted by MHC class I molecules, whereas type 2 CD8⁺ T cells predominantly

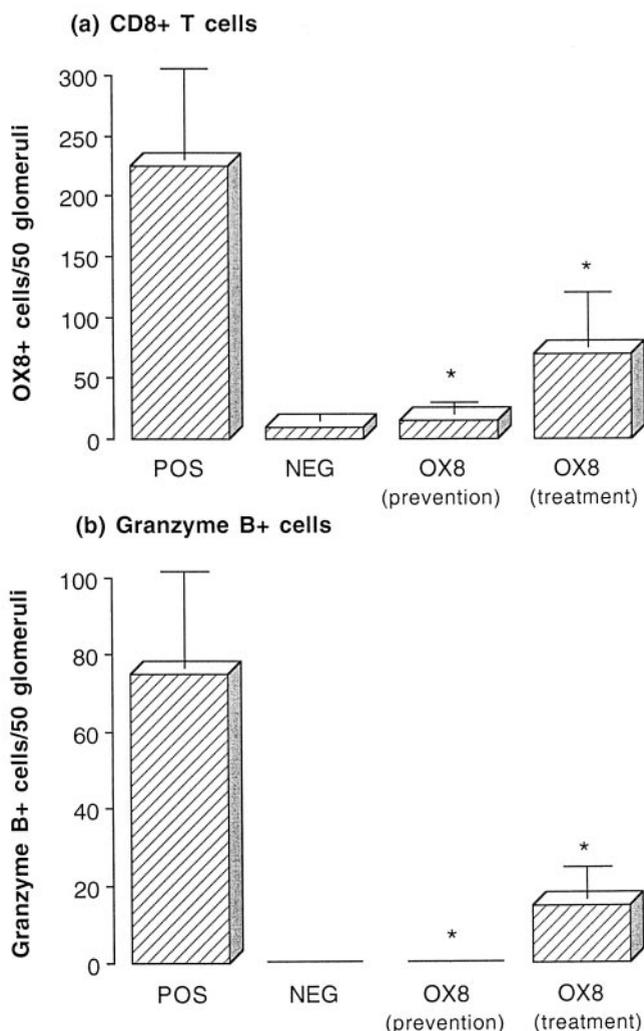


Figure 7. Effects of anti-CD8 mAb therapy on the numbers of CD8⁺ T cells (a) and granzyme B-positive cells (b), as detected by immunohistologic analysis of kidney tissue, in groups of WKY rats ($n = 5$ to 8) with EAG. Results shown represent the mean \pm SD for each group at week 4 after immunization. * $P < 0.001$, positive control group versus OX8 (prevention) and OX8 (treatment) groups. POS, positive positive control; NEG, negative control.

secrete IL-4, IL-5, and IL-10 and are restricted by MHC class II molecules. As a result, depletion of CD8⁺ T cells has the potential to suppress both humoral and cell-mediated immune injury.

Previous studies in other rat models of nephritis demonstrated that anti-CD8 mAb therapy is effective in preventing the development of disease. In nephrotoxic nephritis in both WKY (34) and Sprague-Dawley (36) rats, anti-CD8 therapy prevented proteinuria, crescent formation, and glomerular influx of CD8⁺ T cells and macrophages. However, no reductions in bound anti-rat anti-GBM antibody levels or rat anti-rabbit IgG production were observed. Another study demonstrated that depletion of CD8⁺ T cells in nephrotoxic nephritis in WKY rats resulted in decreases in the accumulation of glomerular macrophages and reductions in the glomer-

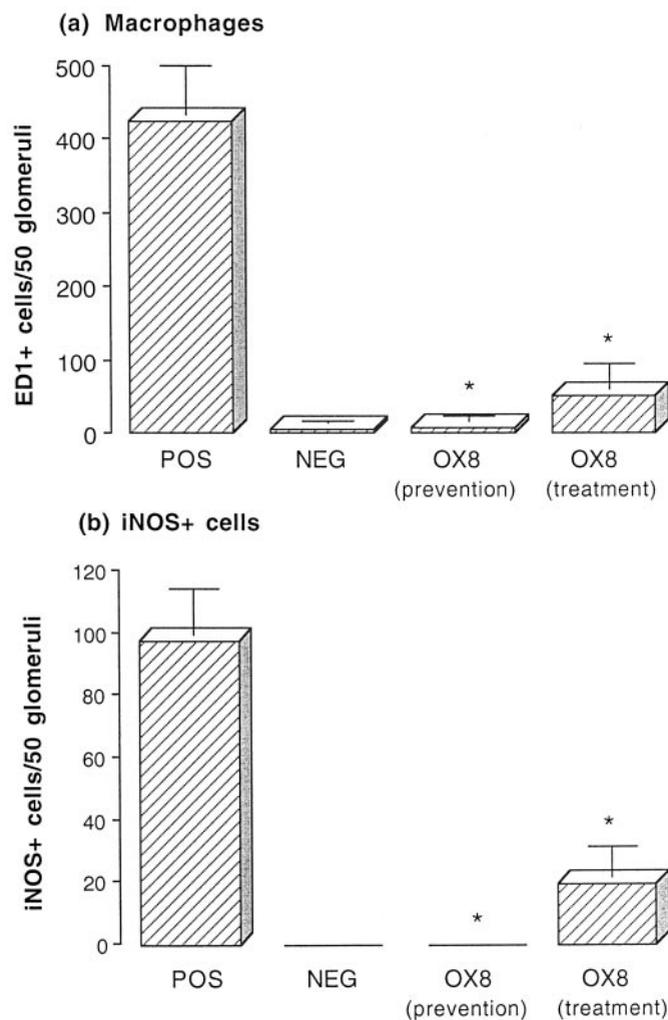


Figure 8. Effects of anti-CD8 mAb therapy on the numbers of macrophages (a) and inducible nitric oxide synthase (iNOS)-positive cells (b), as detected by immunohistologic analysis of kidney tissue, in groups of WKY rats ($n = 5$ to 8) with EAG at 4 wk. Results shown represent the mean \pm SD for each group at week 4 after immunization. * $P < 0.001$, positive control group versus OX8 (prevention) and OX8 (treatment) groups. POS, positive control; NEG, negative control.

ular expression of intercellular adhesion molecule-1 (ICAM-1) mRNA (35). There were also reductions in the expression of mRNA for TNF- α and IFN- γ , which are known to enhance

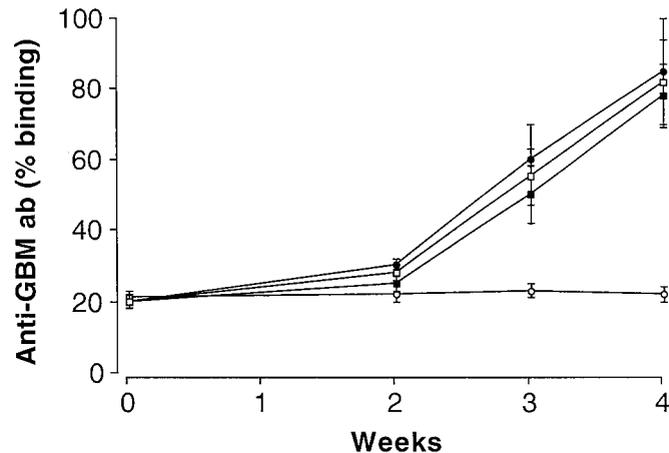


Figure 9. Effects of anti-CD8 mAb therapy on circulating anti-glomerular basement membrane (GBM) antibody concentrations in groups of WKY rats ($n = 5$ to 8) with EAG. Results shown represent the mean \pm SD for each experimental group, *i.e.*, positive control (●), OX8 therapy from week 0 to week 4 (prevention) (■), OX8 therapy from week 2 to week 4 (treatment) (□), and negative control (○) groups.

ICAM-1 expression, and mRNA for macrophage inflammatory protein-1 α , macrophage inflammatory protein-1 β , and monocyte chemoattractant protein-1, which are known to activate leukocyte integrins or to act as chemokines. These findings suggested that CD8⁺ lymphocytes play a crucial role in the accumulation of macrophages, through the stimulation of ICAM-1 and the induction of cytokines, and are directly involved in the pathogenesis of nephrotoxic nephritis. In Heymann nephritis (a model of membranous nephropathy), anti-CD8 mAb therapy prevented the development of proteinuria, glomerular injury, and the influx of CD8⁺ T cells and macrophages but had no effect on circulating levels of autoantibodies or deposits of IgG and C3 in glomeruli (37). CD8⁺ cell-depleted animals also demonstrated decreases in the levels of mRNA for IFN- γ , lymphotoxin, and IL-2, which are known to be associated with Th1 cells, mRNA for granzyme A and perforin, which are known to be associated with cytotoxic cells, and mRNA for TNF- α , IL-10, IL-12, and iNOS, which are known to be associated with macrophages (38). These findings suggested that CD8⁺ T cells are essential for the mediation of glomerular injury in Heymann nephritis.

Table 3. Effect of anti-CD8 mAb therapy in WKY rats with EAG on numbers of T cells in peripheral blood, as assessed by flow cytometry^a

	CD8 ⁺ T Cells (%)	CD4 ⁺ T Cells (%)	Pan T Cells (%)
Positive control	10.9	25.3	35.6
Negative control	11.2	24.9	36.2
OX8 (prevention)	1.4 ^b	29.6	30.2

^a Stained cells are expressed as a percentage of total leukocytes, after subtraction of the negative isotype-matched control values. Results are presented as the mean percentage of leukocytes in each group at week 4.

^b $P < 0.01$, positive control versus OX8 (prevention) group.

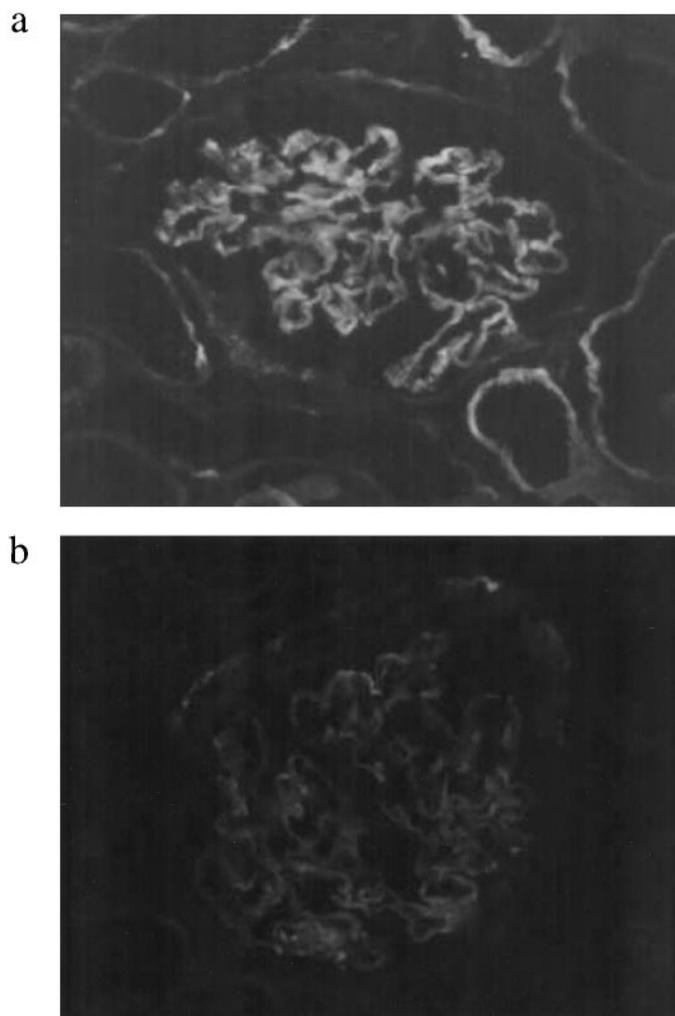


Figure 10. Direct immunofluorescence analysis of kidney tissue from WKY rats with EAG at 4 wk. (a) Strong linear deposition of IgG along the GBM in a positive control animal. Magnification, $\times 300$. (b) Moderate reduction in the deposition of IgG on the GBM in an animal given OX8 from week 0 to week 4 (prevention). Magnification, $\times 300$.

In this study using an autoimmune model of crescentic nephritis, we demonstrated that anti-CD8 mAb therapy prevents the development of albuminuria, crescent formation, interstitial damage, glomerular cell apoptosis, glomerular influx of CD8⁺ T cells and macrophages, and glomerular expression of granzyme B and iNOS. As in the models described above, the effectiveness of this approach may be attributable to inhibition of CD8⁺ cell recruitment into glomeruli and their proinflammatory effects. In support of this suggestion, we demonstrated increased glomerular expression of granzyme B in animals with EAG, which suggests that CD8⁺ T cells may be directly cytotoxic to renal cells, and found that granzyme B expression is reduced by anti-CD8 therapy. In addition, anti-CD8 therapy leads to a decrease in the number of infiltrating macrophages and a decrease in glomerular expression of iNOS (a marker of activated macrophages), suggesting that CD8⁺ cells may also act via macrophage recruitment and activation.

Further work is required to examine the specificity of the infiltrating CD8⁺ T cells and the expression of other cytotoxic molecules and cytokines in the glomeruli of animals with EAG.

The role of anti-CD8 mAb therapy in anti-GBM autoantibody production also requires further investigation. In experimental autoimmune myasthenia gravis, depletion of CD8⁺ T cells led to reductions in anti-AChR antibody levels and in numbers of B and T cells specific for AChR (42,43), demonstrating that this approach can modulate humoral immunity. In our prevention study, there was no effect of treatment on circulating anti-GBM antibody levels but there was a reduction in deposited antibody levels. Whether these findings reflect differences in the specificity or affinity of the autoantibody response, or differences in the deposition or clearance of antibodies in inflamed glomeruli, is not clear. However, the results of our treatment study clearly demonstrate that the effectiveness of anti-CD8 therapy is not dependent solely on changes in anti-GBM antibody production or deposition, because glomerular injury was reduced despite the lack of effect on circulating or deposited antibody levels.

In conclusion, we have demonstrated for the first time that *in vivo* administration of anti-CD8 mAb is effective in preventing the development of autoimmune crescentic nephritis and in treating established disease after the onset of albuminuria. This confirms the importance of CD8⁺ T cell-mediated immunity in the pathogenesis of this model of Goodpasture's disease. Although the precise mechanism of action is not yet known, the prevention of CD8⁺ cell infiltration into glomeruli and the consequent failure of macrophage recruitment are likely to be important. These findings are of particular relevance to the understanding of human disease mechanisms, because of the close similarities between our model and the human disease with respect to both autoimmune responses and renal pathologic features. Similar approaches to treatment may be worth investigating in human crescentic glomerulonephritis.

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