Blockade of the CD154-CD40 costimulatory pathway prevents the development of experimental autoimmune glomerulonephritis

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Blockade of the CD154-CD40 costimulatory pathway prevents the development of experimental autoimmune glomerulonephritis.

Background. Experimental autoimmune glomerulonephritis (EAG) was induced in Wistar-Kyoto (WKY) rats by immunization with rat glomerular basement membrane (GBM) in adjuvant. This model is characterized by anti-GBM antibody production, accompanied by focal necrotizing glomerulonephritis with crescent formation. There is also glomerular infiltration by T cells and macrophages. Our hypothesis was that blocking the interaction between CD154 (CD40L) on Th cells and CD40 on antigen-presenting cells should inhibit T-cell activation, and thus the development of EAG.

Methods. The in vivo effects of a hamster anti-rat monoclonal antibody to CD154 (AH.F5) were examined in EAG starting at day −1 prior to immunization, day +7 after immunization, or day +14 after immunization.

Results. When administered from day −1 at a dose of 10 mg/kg intraperitoneally three times per week for the duration of the study (4 weeks), AH.F5 resulted in a marked reduction in circulating anti-α3(IV)NC1 antibodies, deposits of IgG on the GBM, albuminuria, deposits of fibrin in the glomeruli, severity of glomerular abnormalities, and numbers of glomerular T cells and macrophages. When administered from day +7 at the same dose, AH.F5 resulted in a moderate reduction in the severity of disease, while administration from day +14 had no significant effect.

Conclusion. These studies demonstrate for the first time that early blockade of the CD154-CD40 T-cell costimulatory pathway can prevent the development of crescentic nephritis, and that delayed treatment can reduce the severity of disease. This confirms the importance of T cell mediated immunity in the pathogenesis of EAG, and suggests that strategies targeting T-cell costimulation may provide a novel approach in the treatment of human glomerulonephritis.

Keywords: experimental autoimmune glomerulonephritis, costimulatory molecules, glomerular basement membrane, T lymphocytes, Wistar-Kyoto rats.

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Antigen-specific T-cell activation is regulated by a two-signal pathway. The first signal is provided by engagement of the T-cell receptor with the antigenic peptide-major histocompatibility complex (MHC) molecule on antigen-presenting cells (APC), and is an antigen-specific response. However, this interaction alone is insufficient to induce optimal T-cell activation without secondary costimulatory signals, which are provided by binding of specific receptors on T cells with their ligands on APC. Two of the best characterized costimulatory pathways are (1) the interaction between CD40 ligand (CD154) on T cells and CD40 on antigen-presenting cells should inhibit T-cell activation, and thus the development of EAG.

Methods. The in vivo effects of a hamster anti-rat monoclonal antibody to CD154 (AH.F5) were examined in EAG starting at day −1 prior to immunization, day +7 after immunization, or day +14 after immunization.

Results. When administered from day −1 at a dose of 10 mg/kg intraperitoneally three times per week for the duration of the study (4 weeks), AH.F5 resulted in a marked reduction in circulating anti-α3(IV)NC1 antibodies, deposits of IgG on the GBM, albuminuria, deposits of fibrin in the glomeruli, severity of glomerular abnormalities, and numbers of glomerular T cells and macrophages. When administered from day +7 at the same dose, AH.F5 resulted in a moderate reduction in the severity of disease, while administration from day +14 had no significant effect.

Conclusion. These studies demonstrate for the first time that early blockade of the CD154-CD40 T-cell costimulatory pathway can prevent the development of crescentic nephritis, and that delayed treatment can reduce the severity of disease. This confirms the importance of T cell mediated immunity in the pathogenesis of EAG, and suggests that strategies targeting T-cell costimulation may provide a novel approach in the treatment of human glomerulonephritis.
humoral as well as cell-mediated immunity. More recently, it has been shown that anti-CD154 monoclonal antibody given during the induction and chronic stages of experimental autoimmune myasthenia gravis in the Lewis rat suppressed progression of disease by down-regulation of Th1 responses and up-regulation of CTLA4 [14], demonstrating that blocking the CD154-CD40 T-cell costimulatory pathway can be effective in both the prevention and treatment of autoantibody-mediated disease.

Experimental autoimmune glomerulonephritis (EAG), an animal model of Goodpasture’s disease [15, 16], can be induced in genetically susceptible strains of rat by immunization with heterologous or homologous preparations of glomerular basement membrane (GBM) in adjuvant [17–21]. In the model used in this study, Wistar-Kyoto (WKY) rats given a single injection of collagenase-solubilized rat GBM in complete Freund’s adjuvant (CFA) 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 [20]. The main target antigen is the same in EAG as in Goodpasture’s disease [22, 23], the noncollagenous (NC1) domain of the α3 chain of type IV collagen (α3(IV)NC1). The role of anti-GBM antibodies in the pathogenesis of EAG has been demonstrated in passive transfer experiments in both rats and mice with EAG. The successful passive transfer of EAG has been demonstrated in WKY rats using antibody purified from the urine of nephritic rats [24], and more recently by antibody eluted from the kidney of WKY rats with EAG [abstract; Reynolds et al, J Am Soc Nephrol 12:659, 2001]. A model of EAG in SJL/J mice can also be transferred using anti-α3(IV)NC1 antibodies pooled from the serum of nephritic mice [25].

There is mounting evidence for the role of T cells in EAG. Glomerular infiltration with T cells precedes an influx of macrophages [26]; transfer of T lymphocytes can prime naive recipients for disease in BN rats [27]; T cells from WKY rats with EAG proliferate in response to enriched α3(IV)NC1 [28] and can transfer crescentic nephritis to recipients [29]; and glomerular T cells in EAG show restricted T-cell receptor CDR3 spectrotypes [abstract; Walters et al, J Am Soc Nephrol 12:644, 2001]. Furthermore, several approaches to anti-T-cell immunotherapy can prevent and ameliorate disease [30]. We have previously shown that cyclosporine A [31] and anti-CD4 monoclonal antibodies [32] are effective in preventing EAG in the BN rat, and more recently have demonstrated that anti-CD8 monoclonal antibody is effective in both the prevention and treatment of EAG in the WKY rat [33]. Antibodies to intercellular adhesion molecule-1 (ICAM-1) and leukocyte function-associated molecule-1 (LFA-1), important in leukocyte migration and activation, are also effective in both the prevention and treatment of EAG [34]. Oral administration of GBM antigen induces mucosal tolerance and prevents the development of crescentic nephritis in this model [28], and similar findings have been reported in EAG in the mouse [25]. Of particular relevance to the present study, we have recently shown that blockade of the CD28-B7 costimulatory pathway, by either the fusion protein CTLA4-Ig or a mutant form of CTLA4-Ig (Y100F) which only blocks B7.1, was effective in preventing the development of crescentic nephritis in EAG in the WKY rat [35].

In this paper, we address the question of whether blocking the interaction between CD154 on T cells and CD40 on B cells or other antigen presenting cells inhibits T-cell activation, and thus the development of EAG. We examined the in vivo effects of blocking the CD154-CD40 pathway with an anti-CD154 monoclonal antibody (AH.F5) at different time points during the course of EAG. We demonstrate for the first time that CD154-CD40 blockade is effective in both the prevention and treatment of crescentic nephritis in the rat. This study confirms the importance of T-cell-mediated immunity in the pathogenesis of EAG, and suggests that strategies targeting T-cell costimulation may provide a novel approach in the treatment of human glomerulonephritis.

METHODS

Experimental animals

Male WKY rats, aged 8 to 10 weeks 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.

Monoclonal antibody

AH.F5, a novel hamster antirat monoclonal antibody to CD154, which blocks CD40-dependent T-cell costimulation [36], was kindly provided by Biogen, Inc. (Boston MA, USA).

Preparation of GBM antigen

Collagenase-solubilized GBM was prepared from Sprague-Dawley rat kidneys, as previously described [19, 20]. Briefly, the kidneys were decapsulated, the medulla partly removed, and the cortex passed through a series of sieves in order to isolate the glomeruli. After examination by light microscopy to check for tubular contamination, the glomeruli were disrupted ultrasonically, and the resulting material lyophilized and digested with purified type XI collagenase (Sigma-Aldrich Company Ltd., Poole, UK) for 1 hour 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 CFA, at a dose of 5 mg/kg body weight [20, 26]. Serial blood samples were taken by tail artery puncture under light anesthesia with isoflurane, and 24-hour urine specimens obtained by placing animals in metabolic cages.

**Assessment of EAG**

*Enzyme-linked immunosorbent assay (ELISA).* Circulating anti-GBM antibody concentrations were measured in sera from animals with EAG by a solid-phase ELISA, as previously described [20, 23]. Briefly, collagenase-digested rat GBM (10 μg/mL), or recombinant human α3(IV)NC1 (10 μg/mL) was coated on to microtiter plates (Life Technologies, Paisley, UK) by overnight incubation at 4°C, and an optimum dilution of test or control sera were applied for 1 hour at 37°C. Bound anti-GBM antibody was detected by alkaline phosphatase-conjugated sheep anti-rat IgG (Sigma-Aldrich Company Ltd.), and developed using the substrate p-nitrophenyl phosphate (NPP) (Sigma-Aldrich Company Ltd.). The absorbencies for each well were read at 405 nm using an Anthos Multiskan ELISA plate reader (Lab Tech International, Uckfield, UK), and the results initially calculated as mean optical density for each triplicate sample. In order to compare different experimental groups, results were finally expressed as a percentage of the binding obtained with a positive reference serum.

*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 [28, 35]. Briefly, recombinant human α3(IV)NC1 (10 μg/mL) was coated on to microtiter plates by overnight incubation at 4°C, and an optimum dilution of test or control sera applied, and expressed as a percentage of glomeruli examined [20, 26].

*Direct immunofluorescence.* Deposits of IgG and fibrin within the glomeruli were detected by direct immunofluorescence, as previously described [20, 26]. Tissue was embedded in 22-oxacalcitriol (OCT II) embedding medium (Miles Inc., Elkhart, IN, USA) on cork discs, snap-frozen in isopentane (BDH Laboratory Supplies) precooled in liquid nitrogen, and stored at −70°C. Cryostat sections were cut at 5 μm and were incubated with fluorescein isothiocyanate (FITC)-labeled rabbit antirat IgG (Serotec Ltd.), or goat antirat fibrin (Nordic Immunology, Tilburg, The Netherlands). The degree of immunostaining, as judged by intensity of fluorescence, was 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. Briefly, 3 μm sections were stained with hemotoxylin and eosin and periodic acid-Schiff. Fifty glomeruli per section were graded by a blinded observer as normal, abnormal (small areas of glomerular hypercellularity and/or focal necrosis), or severe (>50% of the glomerulus affected by necrosis and/or crescent formation), and expressed as a percentage of glomeruli examined [20, 26].

*Immunohistology.* Kidney sections were stained for T cells and macrophages using a standard avidin-biotin complex immunoperoxidase staining technique. Briefly, formalin-fixed, paraffin embedded kidney sections were stained with monoclonal antibodies W3/13 (T lymphocytes), OX8 (CD8+ lymphocytes), 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 [26].

**Experimental protocol**

Results from our pilot studies examining the in vivo effects of different doses of antirat CD154 monoclonal
antibody (AH.F5) from day −1 on the development of EAG showed that administration of AH.F5 at a dose of 10 mg/kg intraperitoneally three times per week for the duration of the study (4 weeks) was the most effective dose. In subsequent experiments, groups of animals (N = 5 to 8) immunized with rat GBM in CFA were therefore given AH.F5 at a dose of 10 mg/kg intraperitoneally three times per week from (1) day −1 prior to immunization; (2) day +7 after immunization; or (3) day +14 after immunization. Positive control groups (GBM/CFA) were given an isotyped-matched control monoclonal antibody (Ha 4/8) (Biogen, Inc.), and a negative control group (CFA alone) was given saline.

**Statistical analysis**

Differences between data were determined by the Mann-Whitney U test (two-tailed). Analysis of variance (ANOVA) was used to confirm differences between multiple data.

**RESULTS**

**Circulating anti-GBM antibody concentrations**

Positive control rats immunized with rat GBM in CFA, and given either saline or control monoclonal antibody, produced detectable circulating anti-α3(IV)NC1 antibody levels by week 2, which increased further by week 3 and peaked at week 4. Animals given AH.F5 from day −1 showed a marked reduction in circulating anti-α3(IV)NC1 antibody concentrations at all time points, when compared to positive controls. Animals given AH.F5 from day +7 showed a slight, though not significant, reduction in anti-α3(IV)NC1 antibodies, while those given AH.F5 from day +14 showed no reduction in antibody levels. Negative control animals given CFA alone did not develop anti-α3(IV)NC1 antibodies (Fig. 1). Similar results were obtained using collagenase-solubilized GBM as the ligand (results not shown).

**Circulating anti-GBM antibody isotypes**

Positive control rats immunized with rat GBM in CFA showed high levels of IgG1 and IgG2a anti-α3(IV)NC1 antibodies at week 4 after immunization. Animals given AH.F5 from day −1 showed a significant reduction in the levels of both IgG1 and IgG2a anti-α3(IV)NC1 antibodies, when compared to positive controls. Animals given AH.F5 from day +7 or from day +14 showed a significant reduction in the levels of IgG2a antibodies, but no reduction in the levels of IgG1 antibodies (Fig. 2).

**Direct immunofluorescence for IgG**

Positive control rats immunized with rat GBM in CFA showed strong linear deposits of IgG along the GBM at week 4 after immunization. Animals given AH.F5 from day −1 showed a marked reduction in deposits of IgG on the GBM, while those given AH.F5 from day +7 showed a moderate reduction in deposits of IgG on the GBM. Animals given AH.F5 from day +14 showed no reduction in antibody binding. Negative control animals given CFA alone showed no antibody binding. Results are summarized in Table 1 and illustrated in Figure 3.

**Albuminuria**

Positive control rats immunized with rat GBM in CFA produced detectable levels of albuminuria by week 2, which increased further by week 3 and peaked at week 4. Animals given AH.F5 from day −1 showed a marked reduction in albumin excretion, to nearly undetectable levels at all time points, while those given AH.F5 from day +7 showed a moderate reduction in albumin excretion, to nearly undetectable levels at all time points, while those given AH.F5 from day +14 showed no reduction in albuminuria at week 4. Animals given AH.F5 from day +14 showed no reduction in albuminuria. Negative control animals given CFA alone did not develop albuminuria (Fig. 4).

**Creatinine clearance**

Positive control rats immunized with rat GBM in CFA showed a significant reduction in creatinine clearance at week 4, while those animals given AH.F5 from day −1 maintained a normal creatinine clearance, similar to that of negative control animals given CFA alone. Animals given AH.F5 from day +7 showed a slight, although not significant, reduction in creatinine clearance, while those given AH.F5 from day +14 showed a reduction in
Fig. 2. Effect of AH.F5 monoclonal antibody on circulating levels of (A) IgG, (B) IgG1, and (C) IgG2a anti-a3(IV)NC1 antibodies in groups of Wistar-Kyoto (WKY) rats (N = 5–8) with experimental autoimmune glomerulonephritis (EAG). Results shown represent the mean ± SD of each group at week 4 after immunization. *P < 0.01 positive control vs. AH.F5 (day −1).
while those given AH.F5 from day +7 showed a moderate but significant reduction. Animals given AH.F5 from day +14 showed no reduction in glomerular leukocytes (Fig. 8).

DISCUSSION

Despite considerable research into the role of T-cell costimulatory molecules in the last few years, the role of the CD154-CD40 costimulatory pathway in the induction and maintenance of an ongoing autoimmune response is still only partially understood [1–7]. To address this issue in autoimmune renal disease, we have examined the effect of blocking the CD154-CD40 costimulatory pathway with a monoclonal antibody to rat CD154 at different time points during the development of EAG. Previous studies have demonstrated that blocking the CD154-CD40 costimulatory pathway is effective in inhibiting the development of various experimental models of autoimmune disease, and effects on both humoral and cellular immunity have been reported [8–14]. To our knowledge, the only successful studies of anti-CD154 monoclonal antibody therapy in renal disease were reported in lupus-like nephritis in the NZB/NZW mouse [11] and the SNF1 mouse [12, 13]. In these studies, blockade of CD154-CD40 prevented the development of serologic changes and histologic abnormalities in the kidney [11] and prolonged survival [12] in prenephritic lupus-prone mice, and decreased the incidence of severe nephritis in mice with established disease [13]. However, no
CD154-CD40 blockade studies have been reported in experimental models of crescentic nephritis. Since EAG in the WKY rat is induced by an antigen-specific autoimmune response directed toward the noncollagenous domain of the α3 chain of type IV collagen, with a contribution to renal disease from both autoantibodies and T cells, it provides an excellent model in which to analyze the effect of CD154-CD40 blockade in the development of crescentic glomerulonephritis.

In the present study of EAG, we found that blockade of the CD154-CD40 costimulatory pathway with monoclonal antibodies to CD154 from day −1 before immunization resulted in a marked reduction in the levels of circulating anti-α3(IV)NC1 antibodies, deposits of IgG on the GBM, all features of glomerular injury and numbers of glomerular T cells and macrophages. There was also a significant reduction in both IgG1 and IgG2a anti-α3(IV)NC1 antibodies, suggesting that continued blockade of the CD154-CD40 pathway prior to immunization reduced both Th 1- and Th 2-like responses. These findings are most likely due to the inhibition of activation of antigen-specific T cells reactive with α3(IV)NC1, thus preventing T-cell-dependent autoantibody production and generation of effector T cells. Inhibition of the interaction of CD154 with CD40 on APC may in turn inhibit the expression of CD80/CD86 on the APC and their interaction with CD28 on the T cell. Therefore, continued blockade of CD154 could have the effect of inhibiting two of the major T-cell costimulatory pathways.

In addition, we found that CD154-CD40 blockade was still effective after the induction of the immune response, but before overt glomerular injury. When anti-CD154 monoclonal antibody was administered from day +7 after immunization there was a moderate reduction in the severity of disease. Although there was no significant reduction in the levels of total circulating anti-α3(IV)NC1 IgG, there was a reduction in the levels of IgG2a, but not IgG1. This suggests that anti-CD154 monoclonal antibody given at a later time point during the immune response may preferentially down-regulate Th 1-like responses and thus cell-mediated immune injury. In support of this observation, it has recently been reported that intrinsic renal cell expression of CD40 controls the glomerular infiltration of Th 1 effector cells in murine experimental anti-GBM glomerulonephritis [37]. Our data also supports the suggestion that some degree of T-cell costimulation is still necessary during an ongoing autoimmune response [1–7].

However, when anti-CD154 monoclonal antibody was administered from day +14, after the onset of glomerular injury, no reduction in the severity of disease was observed, even although there was still a significant reduction in levels of circulating IgG2a antibodies. This could be due to that fact that after this time point CD8+ cytotoxic effector cells and macrophages are starting to
infiltrate the glomerulus [26, 33], and the classic T-cell costimulatory molecules are no longer needed. A contributory factor may be that by week 2 after immunization the animals are starting to develop proteinuria, and some monoclonal antibodies may be passing from the serum through the damaged glomeruli into the urine. This suggestion is supported by a drop in the serum levels of anti-CD154 monoclonal antibody on the numbers of cells infiltrating the glomerulus. (A) T cells. (B) Macrophages. Results shown represent the mean ± SD of each group at week 4 after immunization. *P < 0.01 positive control vs. AH.F5 (day +7); **P < 0.001 positive control vs. AH.F5 (day −1).

Fig. 8. Immunoperoxidase staining of kidney tissue in groups of Wistar-Kyoto (WKY) rats (N = 5–8) with experimental autoimmune glomerulonephritis (EAG) showing the effect of AH.F5 monoclonal antibody on the numbers of cells infiltrating the glomerulus. (A) T cells. (B) Macrophages. Results shown represent the mean ± SD of each group at week 4 after immunization. *P < 0.01 positive control vs. AH.F5 (day +7); **P < 0.001 positive control vs. AH.F5 (day −1).

CONCLUSION

We have demonstrated for the first time that blockade of the CD154-CD40 costimulatory pathway with monoclonal antibodies to CD154 can prevent the development of crescentic nephritis in a rat model of Goodpasture’s disease, and can reduce the severity of disease even when given after the induction of the autoimmune response. This study confirms the importance of T-cell-mediated immunity in the pathogenesis of EAG, and suggests that strategies targeting T-cell costimulation may provide a novel approach in the treatment of human glomerulonephritis [38].

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