Therapeutic Effect of Anti–TNF-α Antibodies in an Experimental Model of Anti-Neutrophil Cytoplasm Antibody–Associated Systemic Vasculitis

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The therapeutic options for anti-neutrophil cytoplasm antibody (ANCA)-associated systemic vasculitis (AASV) remain limited and hampered by adverse effects. One potential novel therapeutic avenue involves inhibition of TNF-α, with encouraging uncontrolled data in humans with one agent (infliximab) but disappointing controlled data from another (etanercept). For investigating the potential role of TNF-α as a therapeutic target in AASV, the effect of an anti-rat TNF-α mAb (CNTO 1081) in a rat model of AASV was investigated. For testing the effect of TNF-α blockade in this model, starting on day 28 after immunization (a point when glomerulonephritis is established), animals were randomized to treatment with CNTO 1081 or control mouse IgG. Treatment with CNTO 1081 significantly reduced albuminuria (mean 1.1 ± 0.3 mg/24 h CNTO 1081 *versus* 8.0 ± 1.9 controls; $P < 0.05$) and crescent formation (0% CNTO 1081 *versus* 60% controls; $P < 0.05$). Lung hemorrhage was also reduced (CNTO 1081: median score 0, range 0 to 2; controls: 2, range 1 to 3; $P < 0.05$). When analyzed by intravital microscopy, there was a 43% inhibition of leukocyte transmigration in mesenteric venules in response to topical CXCL1 (a neutrophil chemoattractant) in the CNTO 1081 group compared with controls ($P < 0.001$). Anti-myeloperoxidase antibody titers were similar in both groups throughout the study. In conclusion, these findings indicate that TNF-α plays an important role in the pathogenesis of experimental autoimmune vasculitis and suggest that blockade of this cytokine with an mAb is effective in treating established vasculitis. The therapeutic action of anti–TNF-α reagents may be mediated, in part, by suppression of the enhanced leukocyte-endothelial interactions in this disorder.

J Am Soc Nephrol 17: 160–169, 2006. doi: 10.1681/ASN.2005060616

Anti-neutrophil cytoplasm antibody (ANCA)-associated systemic vasculitis (AASV) is characterized by rapidly progressive glomerulonephritis and lung hemorrhage, in addition to dysfunction in many other organs (1). Despite the extensive interest in the pathogenesis of this chronic inflammatory condition, the therapeutic options for AASV remain limited (2). Before the widespread introduction of cyclophosphamide treatment, this was a disease that was usually fatal and resulted in a high incidence of irreversible renal failure (3). With increased survival, AASV has become a chronic illness of the elderly (4) that has a propensity to relapse and necessitates long-term immunosuppression. Consequently, the cumulative burden of treatment-related side effects, *e.g.*, bone marrow suppression, infection, and malignancy, may result in more injury than the disease process itself (5). There thus is a need for more specific, less toxic therapeutic agents.

In recent years, increased understanding of chronic inflammatory diseases has led to the development of a range of novel biologic therapies for use in conditions such as rheumatoid arthritis and Crohn's disease. These include mAb and fusion proteins designed to block the effects of TNF-α (6), IL-6 (7), T cells (8,9), B cells (10), and adhesion molecules (11). These are proving attractive forms of therapy, as they seem to be efficacious and to be associated with relatively few adverse effects. In addition, in AASV, several uncontrolled studies of TNF-α blockade with infliximab (a chimeric mAb to TNF-α) have been performed (12–14). These have found anti–TNF-α mAb to be of potential benefit in inducing remission in acute disease, in treating refractory vasculitis, and in controlling chronic "grumbling" disease without escalation of immunosuppression. However, in the recently published Wegener's Granulomatosis Etanerept Trial study, a large, prospective, randomized study of etanercept (a soluble inhibitor of TNF-α and lymphotoxin), there was no benefit from anti–TNF-α treatment in inducing and maintaining remission in Wegener's granulomatosis (15). In addition, this agent has been found to be ineffective in Crohn's disease (16). These investigations raise some uncertainty over the potential role of TNF-α as a therapeutic target in AASV and indicate the critical importance of performing controlled studies before the widespread introduction of infliximab.

Received June 14, 2005. Accepted October 5, 2005.

Published online ahead of print. Publication date available at www.jasn.org.

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ISSN: 1046-6673/1701-0160

The association of small-vessel vasculitis with autoantibodies against constituents of the neutrophil granule was described in the mid-1980s (17). The autoantigen in the majority of patients with microscopic polyangiitis was subsequently found to be myeloperoxidase (MPO) (18), an enzyme that is found in azurophilic granules and accounts for approximately 5% of total neutrophil protein (19). In addition to being an aid in diagnosis of vasculitis, ANCA titers tend to mirror disease activity (20). Although AASV may relapse in the absence of a rise in ANCA titer and clinical disease may occasionally persist despite a fall in titer, the association of ANCA titer with vasculitis severity is tight enough to prompt many clinicians to use serial ANCA estimation in the ongoing care of patients with AASV (21,22). In addition, certain drugs (*e.g.*, propylthiouracil, hydralazine, phenytoin) are capable of inducing MPO-ANCA, resulting in some patients in focal necrotizing glomerulonephritis and systemic vasculitis (23). These clinical findings, along with recent studies indicating a pathogenic role for anti-MPO antibodies in a murine model (24), support the concept of an autoimmune response against neutrophil proteins, such as MPO, as the central driving force in AASV.

To address the role of TNF-α in the pathogenesis of AASV, we investigated the effect of TNF-α blockade in an animal model that was developed recently in our laboratory (experimental autoimmune vasculitis [EAV]) (25). In this model, WKY rats are immunized with purified human MPO (hMPO) in complete Freund's adjuvant (CFA). Anti-hMPO antibodies that cross-react with rat neutrophils are detectable by 2 wk, proliferative glomerulonephritis is present by 4 wk, and pauci-immune crescentic glomerulonephritis is evident by 6 to 8 wk (26). In addition, pulmonary vasculitis with alveolar hemorrhage is a feature of this model. Furthermore, by observing the mesenteric microcirculation of these animals using intravital microscopy, we have shown that the leukocyte–endothelial interactions of firm adhesion and transmigration are exaggerated in EAV, a phenomenon that seems to be due to circulating ANCA (25).

The role of TNF-α in this disease model was investigated through the use of a monoclonal anti-rat TNF-α antibody, CNTO 1081. Collectively, the findings demonstrate the efficacy of CNTO 1081 in treating EAV, even when the disease phenotype is established, and indicate that this therapeutic effect is associated with reversal of the exaggerated leukocyte transmigration response seen in EAV. These data suggest an important role for TNF-α in our animal model of vasculitis and hence support the use of anti–TNF-α therapy in patients with AASV.

Materials and Methods

Animals

WKY/NCrlBR rats were purchased from Charles River (Margate, UK). All animal studies were performed according to the directives of the United Kingdom Home Office Animals (Scientific Procedures) Act (1986).

Immunization Protocol and Characterization of EAV Phenotype

Female rats (135 to 160 g) all were immunized intramuscularly with hMPO (400 μg/kg; Calbiochem, Merck Biosciences, Nottingham, UK) in an equal volume of CFA as described previously (27). Hematuria was assessed with dipstick (Bayer Multistix, Berkshire, UK), and albuminuria was assessed with ELISA (Nephrat ELISA kit; Exocell, Philadelphia, PA). In addition, serum samples were taken for the measurement of anti-hMPO antibodies by ELISA. Briefly, 96-well plates were coated overnight with hMPO (2 μg/ml) in carbonate buffer. Wells then were incubated with dilutions of serum samples in triplicate for 60 min at 37°C, washed, and incubated with anti-rat IgG-alkaline phosphatase conjugate (Sigma, St. Louis, MO) for 45 min at 37°C. Binding was detected with p-NPP (Sigma) and read at 405 nm. Antibody titer was determined by serial log or half-log dilution of serum and defined as the dilution that results in a 50% drop in optical density. This was calculated by regression analysis.

In addition to crescentic glomerulonephritis, rats with EAV develop pulmonary vasculitis and hemorrhage. This was assessed macroscopically by a visual inspection of the lungs at the time of killing and graded according to the amount of lung surface bleeding: 0, no hemorrhage; 1, a single lesion; 2, 2 to 5 lesions; 3, 6 to 12 lesions; and 4, more than 12 lesions/massive macroscopic lung hemorrhage.

Histology and Immunohistochemistry

Kidney and lung tissues were taken from experimental and control groups of rats at the time of killing. Tissues were fixed in 10% buffered formalin and embedded in paraffin wax, and 3-μm sections were cut for light microscopic studies. Sections were stained with hematoxylin and eosin and periodic acid-Schiff for general morphology and to establish the incidence of focal proliferative glomerulonephritis, fibrinoid necrosis, and crescent formation, using standard techniques (28). Accurate quantification of lung histology requires antemortem tracheal intubation and distension of the lungs with mounting medium. This was not performed routinely, so it was not possible to provide quantitative measures of lung inflammation beyond commenting on the presence or absence of perivascular leukocyte cuffing.

Immunoperoxidase staining was performed for the detection of glomerular and lung perivascular cell infiltration, using the anti-rat monocyte/macrophage mAb ED1 (Serotec, Oxford, UK), for monocytes/macrophages, as described previously (29). Briefly, 3-μm-thick paraffin-embedded sections were rehydrated, and antigen retrieval was performed using microwave exposure. The slides then were treated with 1% hydrogen peroxide in PBS to block endogenous peroxidase activity, and nonspecific binding was blocked with 20% goat serum. The slides then were exposed to mouse anti-ED1 (1:200) overnight at 4°C in a humid chamber, followed by incubation with 1:200 biotinylated polyclonal goat anti-mouse antibody (Dakocytomation, Glostrup, Denmark). The sections then were incubated in Streptavidin-Biotin complex (Dakocytomation) and then briefly with 3,3′-diaminobenzidine tetrahydrochloride dehydrate substrate (97%; Sigma-Aldrich, Gillingham, UK). Harris's hematoxylin was used as a counterstain and glomerular cell infiltration was quantified in 50 glomerular cross-sections per slide. The degree of macrophage infiltration in pulmonary tissue was assessed blindly using a semiquantitative analogue score (0 to 3).

Anti–TNF-α Antibody Treatment Protocol

The protocol was designed to treat established EAV, rather than to prevent it, as this mirrors clinical practice. We used CNTO 1081, a recombinant fully mouse IgG2aκ anti-rat TNF-α antibody derived from the parental murine antibody Rt108 (Centocor Inc., Malvern, PA). On the basis of our previous studies with anti-TNF antibodies in a rat model of anti–glomerular basement membrane disease (30), a dose of 16 mg/kg intraperitoneally, three times per week was used. We elected

to begin treatment 28 d after immunization, a point when most rats have developed hematuria, and to continue to day 56, a point when histologic evidence of disease is maximal (31). Thus, in total, each rat received 12 doses of CNTO 1081 or an equal dose of control mouse IgG (Equitech-Bio, Kerrville, TX; $n = 6$ in each group). All therapeutic antibodies were made up in sterile saline for injection (Braun, Melsungen, Germany) and assayed for endotoxin before use by Limulus Amebocyte Lysate testing (Bio-Whittaker, Walkersville, MD). Endotoxin was removed using Detoxigel polymyxin columns (Pierce, Rockford, IL), such that all preparations contained <5 EU/ml.

Intravital Microscopy

Intravital microscopy on rat mesentery was performed as described previously (32). Briefly, control or EAV rats were anesthetized with intravenous sodium pentobarbitone and maintained at 37°C on a heated microscope stage. After midline abdominal incision, the mesentery adjoining the terminal ileum was carefully arranged over a glass window on the stage and superfused with Tyrode's balanced salt solution. Baseline quantification of leukocyte rolling, firm adhesion, and transmigration was performed using a fixed-stage upright microscope with water immersion objectives (Axioscope; Zeiss, Welwyn Garden City, UK). In experiments that assessed the biologic effect of CNTO 1081 in nonimmunized animals, CNTO 1081 (16 mg/kg) or vehicle (saline) was given intravenously 1 h before laparotomy. On the basis of dose finding experiments, a dose of 1 μg/kg TNF-α (or an equal volume of saline) was injected to inhibit leukocyte rolling and induce firm adhesion after exteriorization of the mesentery and quantification of baseline variables. Leukocyte responses then were quantified at regular intervals for 30 min.

In rats that had EAV and were treated with CNTO 1081 or control IgG, intravital microscopy was performed 57 to 63 d after immunization and 28 to 34 d after commencing antibody treatment, and the rats were killed thereafter. For investigating the effect of recombinant rat CXCL1 (Peprotech, London, UK) in these animals, the chemokine was applied continuously topically to the mesentery in the superfusion buffer (final concentration 3×10^{-9} M) and leukocyte responses were quantified at regular intervals. The experiments also typically involved measurement of baseline and postoperative peripheral differential leukocyte counts, in addition to red blood cell centerline velocity, as previously detailed (32).

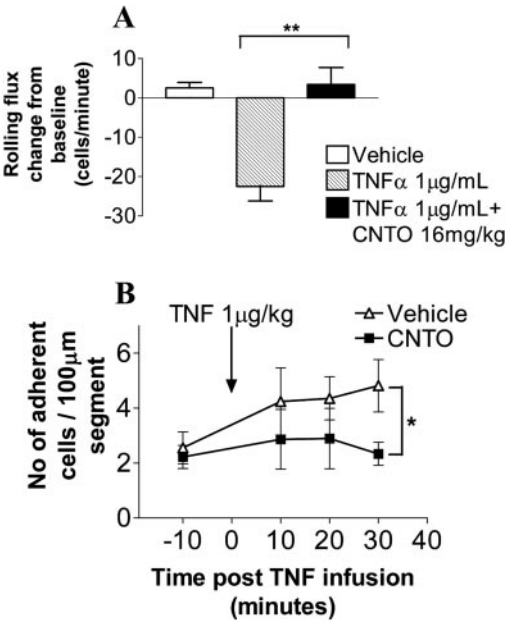

Figure 1. CNTO 1081 blocks the effect of TNF-α on leukocyte rolling (A) and firm adhesion *in vivo* (B). Nonimmunized WKY rats were given TNF-α (1 μg/kg) intravenously or vehicle 1 h after pretreatment with CNTO 1081 (16 mg/kg [■]) or saline (▲), and leukocyte responses within rat mesenteric venules were assessed using intravital microscopy. (A) Absolute change in rolling response from baseline 10 min after administration of saline or TNF-α. The reduction in rolling induced by TNF-α (-22.5 ± 3.7 cells/min) was prevented by CNTO 1081 pretreatment (3.4 ± 4.3). (B) Firm adhesion response over time in the same animals after TNF-α administration. CNTO 1081 prevented the progressive increase in adhesion induced by TNF-α. The data represent the mean ± SEM, $n = 4$ to 12/group. Statistically significant differences between groups of rats are shown by asterisks, *$P < 0.05$, **$P < 0.005$.

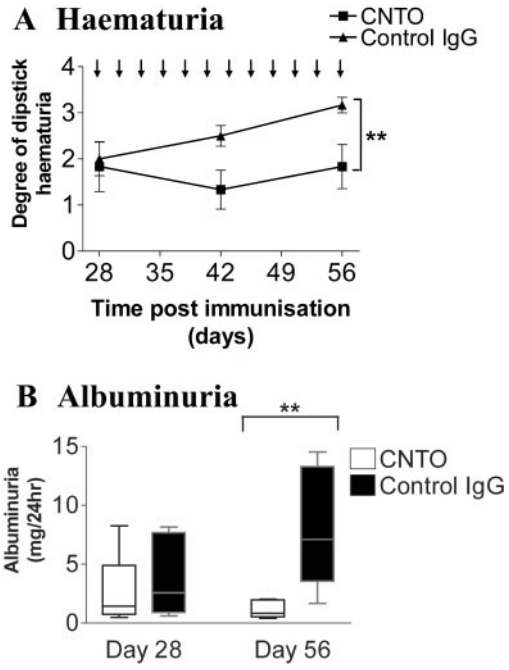

Figure 2. CNTO 1081 treatment ameliorates established renal vasculitis in experimental autoimmune vasculitis (EAV), as quantified by hematuria and urinary albumin excretion rate. CNTO 1081 or control IgG was started at day 28 after immunization with human myeloperoxidase (hMPO), at a point when glomerulonephritis was already established, and continued on alternate days until day 56. In control animals, hematuria (A), as assessed by urine dipstick, increased progressively over time to 3.2 ± 0.2 at day 56, whereas it remained stable in the CNTO 1081 group (mean 1.8 ± 0.4 at day 56). Arrows indicate doses of CNTO 1081/control IgG. Each data point represents the mean ± SEM. (B) Albuminuria was measured by ELISA on urine collected on days 28 and 56 after hMPO immunization. The bars indicate the median ± interquartile range, and the error bars represent the range; $n = 6$ in each group. Statistically significant differences between groups of rats are shown by asterisks, **$P < 0.01$.

Statistical Analyses

Differences between the two treatment groups were compared with the Mann-Whitney test. Comparison of the fraction of animals with crescentic nephritis was performed using Fisher exact contingency test. Changes in rolling from baseline were compared with the Kruskal Wallis test, with Dunn's multiple comparison *post hoc* testing to compare individual groups. Intravital microscopy adhesion and transmigration data and hematuria levels over time were log-transformed and analyzed using two-way ANOVA considering time and treatment group as independent variables. Values at individual time points were compared using Bonferroni *post hoc* tests. For clarity, the nontransformed data are depicted in the graphs, although indications of significance levels refer to the appropriately transformed data.

Results

CNTO 1081 Inhibits TNF-α–Induced Microvascular Responses In Vivo

To confirm that CNTO 1081 can inhibit the effects of TNF-α in the leukocyte migration cascade, we tested the effect of CNTO 1081 on TNF-α–induced leukocyte responses within rat mesenteric venules. For this purpose, nonimmunized WKY rats were pretreated with CNTO 1081 or vehicle 1 h before TNF-α (1 μg/kg), or saline was infused over 5 min. Leukocyte responses of rolling flux and firm adhesion were recorded by intravital microscopy both before and after TNF-α administration. In saline-treated rats, rolling flux remained stable or increased slightly during the period studied, whereas after intravenous TNF-α, rolling flux was reduced dramatically (Figure 1A). This TNF-α–induced reduction in rolling flux was completely blocked in rats that were pretreated with 16 mg/kg CNTO 1081. In addition, infusion of TNF-α induced a progressive increase in leukocyte firm adhesion over time (Figure 1B), a response that was also completely prevented by CNTO 1081. Therefore, in the dose range used later in the therapeutic study, CNTO 1081 blocks the effect of TNF-α in the inflammatory cascade.

Urinary and Pulmonary Manifestations of EAV Are Alleviated by CNTO 1081

Having established a biologic effect of the anti–TNF-α antibody *in vivo*, we treated rats that had established EAV with CNTO 1081 or control mouse IgG. EAV was induced by immunization with 400 μg/kg hMPO in CFA at day 0. As found in previous studies (31), approximately 50% of animals had developed renal vasculitis manifested by dipstick-positive hematuria by day 28 (Figure 2A). Animals were randomly assigned to treatment with either CNTO 1081 or control IgG 16 mg/kg, three doses per week for 4 wk (day 56). They were matched with respect to renal vasculitis severity at the beginning of treatment, as determined by hematuria and albuminuria estimation (Table 1). By day 56, all of those in the control group had developed marked hematuria. In the CNTO 1081–treated group, the level of hematuria remained static over the course of the experiment. Excretory renal function was the same in both groups (creatinine clearance 1.1 ± 0.2 CNTO *versus* 1.2 ± 0.2 control IgG; NS).

With respect to the proteinuric response, rats with EAV in the control group developed moderate albuminuria by day 56 (5 to 15 mg/24 h; Figure 2B). This was completely reversed by treatment with CNTO 1081 (Table 1), with albuminuria declining to levels seen in nonimmunized WKY rats (27) (CNTO 1081 group: median 0.8 mg/24 h, interquartile range 0.5 to 1.9; control group: median 7.1 mg/24 h, interquartile range 3.5 to 13.3; $P < 0.01$).

Pulmonary vasculitis with alveolar hemorrhage is a feature of EAV. We quantified the degree of lung hemorrhage with a visual analogue scoring system (0 to 4). All rats that had EAV and were treated with control IgG developed petechiae of varying severity over the lung surface (Figure 3). In the CNTO 1081 group, two animals had mild lung hemorrhage and the remaining four had none (mean lung hemorrhage score 0.5 ± 0.3 and 2.0 ± 0.4 in CNTO 1081 and control groups, respectively; $P < 0.05$).

Table 1. Hematuria and albuminuria at the time of randomization (day 28) and culling (day 56) in individual rats[a]

Animal	Hematuria		Albuminuria (mg/24 h)	
	Randomization	Culling	Randomization	Culling
CNTO				
1	+++	0	1.5	0.8
2	+++	+++	8.2	2.0
3	+	+++	1.0	1.9
4	+	++	1.5	0.9
5	0	++	0.5	0.4
6	+++	+	1.4	0.6
Control				
1	+++	++++	8.1	14.5
2	+	+++	1.2	1.7
3	++	+++	3.2	7.2
4	++	+++	7.2	12.1
5	+++	+++	1.9	5.4
6	+	+++	0.6	7.0

[a]Hematuria was assessed using dipstick, and albuminuria was measured using ELISA.

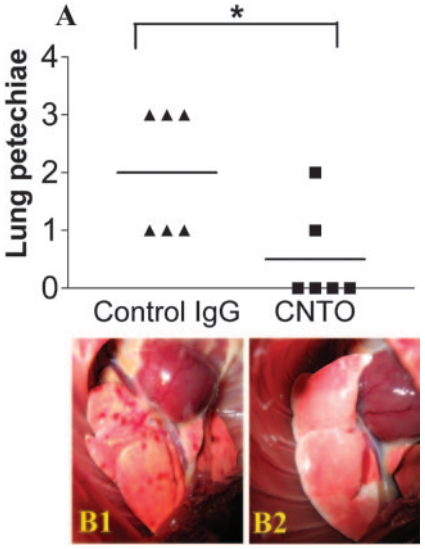

Figure 3. CNTO 1081 treatment ameliorates lung hemorrhage in EAV. Eight weeks after immunization with hMPO and 4 wk after starting treatment with CNTO 1081 or control IgG, WKY rats were killed and the macroscopic appearance of the lungs was scored. In CNTO 1081–treated animals, macroscopic pulmonary hemorrhage was significantly reduced (A). (B) Macroscopic lung appearance in control IgG-treated (B1) and CNTO 1081–treated (B2) animals. Statistically significant differences between groups of rats are shown by asterisks, *$P < 0.05$.

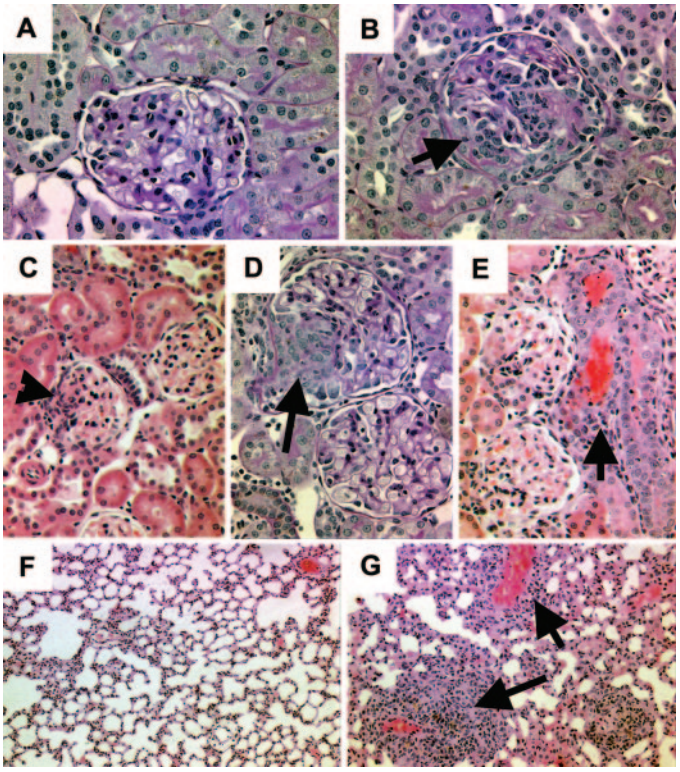

Figure 4. CNTO 1081 treatment reduces glomerular abnormalities, crescent formation, and lung vasculitis in EAV. (A) No glomerular abnormalities in a rat that had EAV and was treated for 4 wk with CNTO 1081. (B) Extracapillary proliferation with crescentic glomerulonephritis in a rat that was treated with control IgG (arrow). (C) Mild focal proliferative glomerulonephritis in a rat that was treated with CNTO 1081 (arrowhead). (D) Focal necrotizing glomerulonephritis (arrow) in a rat that had EAV and was treated with control IgG. (E) Focal proliferative glomerulonephritis with tubulointerstitial nephritis and a red cell cast (arrow) in a rat that was treated with control IgG. (F) Normal lung histology in a rat that was treated with CNTO 1081. G. Lung vasculitis in a rat that was treated with control IgG (arrows). Magnification, ×40 in A, B, and D, periodic acid Schiff; ×20 in C and E, hematoxylin and eosin; ×10 in F and G, hematoxylin and eosin.

CNTO 1081 Had No Effect on Production of ANCA

All rats had developed high levels of anti-hMPO antibodies by 8 wk after immunization (mean 96 ± 1 *versus* 95 ± 2% of reference standard in CNTO 1081– and control IgG–treated animals, respectively). Treatment with CNTO 1081 had no effect on anti-hMPO antibody titers. Median titer was 1:9500 (interquartile range 3700 to 15500) and 1:7500 (interquartile range 4700 to 12000) in the CNTO 1081 and control IgG groups, respectively ($P = 0.6$).

Histologic Evidence of Crescentic Nephritis Is Abolished by Treatment with CNTO 1081

As has been found in previous studies of EAV, 60% of animals that were immunized with 400 μg/kg hMPO developed crescentic nephritis in the control group, with noncrescentic proliferative glomerulonephritis in the remaining 40%. All rats that were treated with CNTO 1081 were free of crescents, with mild proliferative glomerular changes in 50% ($P < 0.05$; Figures 4 and 5). Interstitial nephritis, a feature of EAV in previous studies, was moderately severe in rats with EAV in the control group (Figures 4 and 5). This was significantly reduced in CNTO 1081–treated animals (mean score 1.2 ± 0.2 in CNTO 1081 group *versus* 2.3 ± 0.3 in controls; $P < 0.05$). Consistent with this amelioration in glomerulonephritis, there was a reduction in the degree of macrophage infiltration as determined by immunostaining with ED1 (mean 36.3 ± 13 ED1+ cells/50 glomeruli in CNTO 1081–treated rats *versus* 90.0 ± 19 in controls; $P < 0.05$; Figure 6). In rats with lung hemorrhage, there was evidence of perivascular leukocyte cuffing (Figure 4), with monocyte/macrophage lung infiltration evident on ED1 staining (Figure 7). As preservation of lung architecture mandates specialized lung handling at the time of killing, it was not possible to quantify accurately the degree of lung vasculitis. However, in line with the findings on macroscopic lung hemorrhage, perivascular cuffing seemed more extensive in control-treated animals. The degree of macrophage infiltration, as assessed by blinded scoring of ED1-stained lung sections, was significantly greater in control-treated animals (mean 1.5 ± 0.4 in CNTO 1081–treated rats *versus* 0.3 ± 0.2 in controls; $P < 0.05$).

Rats with EAV Treated with CNTO 1081 Exhibit a Reduced Leukocyte Transmigration Response

To investigate potential mechanisms underpinning the therapeutic effect of anti–TNF-α antibody treatment in EAV, we

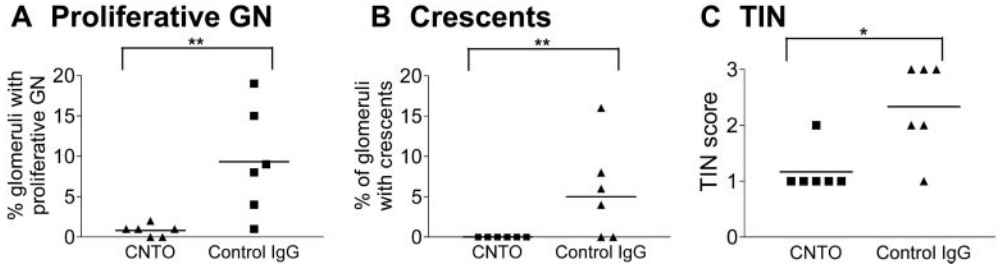

Figure 5. CNTO 1081 treatment reduces glomerular abnormalities and crescent formation in EAV. Histologic sections were scored blindly to quantify the degree of focal proliferative glomerulonephritis (GN; A), crescentic glomerulonephritis (B), and tubulointerstitial nephritis (TIN; C). Each data point represents an individual animal, and asterisks illustrate statistically significant differences: $*P < 0.05$, $**P < 0.01$.

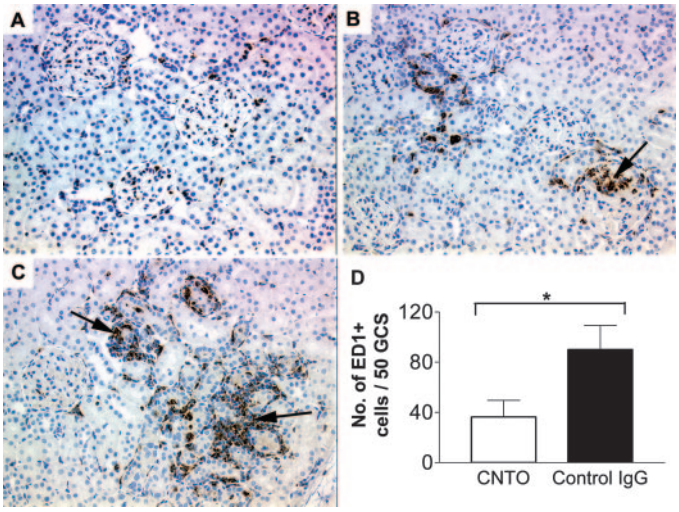

Figure 6. Effect of CNTO 1081 on renal macrophage infiltration. Kidney sections were probed with anti-CD68 (ED1) mAb, and binding was detected with horseradish peroxidase–3,3′-diaminobenzidine tetrahydrochloride dehydrate (HRP-DAB). (A) Occasional peri- and intraglomerular macrophages in a rat that had EAV and was treated with CNTO 1081. There was virtually no tubulointerstitial macrophage infiltration. (B) Focal macrophage infiltration in a glomerular crescent (arrow), with mild peritubular macrophage infiltration in a rat that had EAV and was treated with control IgG. (C) Dense peri- and intratubular macrophage infiltrate (arrows) in a rat that had EAV and was treated with control IgG. (D) Macrophage infiltration was counted in 50 randomly chosen glomeruli/CD68-stained tissue section. GCS, glomerular cross-section; $n = 6$ animals in each group. Bars represent the mean ± SEM. Statistically significant difference between treatment groups is shown by an asterisk, $*P < 0.05$. Magnification, ×10 in A through C.

analyzed leukocyte adhesion and transmigration responses in treated animals using intravital microscopy. In previous studies (33), we showed that, in response to the physiologic inflammatory stimulus CXCL1, a rat homolog of IL-8 (CXCL8), adhesion and transmigration are exaggerated in EAV. This effect is due to circulating ANCA, as demonstrated after passive transfer of ANCA from rats with EAV to naïve rats.

Using a similar approach, the mesentery of rats that had EAV and were treated with CNTO 1081 or control IgG was superfused with CXCL1 (3×10^{-9} M, $n = 4$ in each group). Baseline adhesion and transmigration values before administration of CXCL1 were similar in the two groups (Figure 8A), as was basal rolling flux (15.5 ± 3.2 and 19.1 ± 2.5 cells/min in CNTO 1081 and control groups, respectively). There was a trend toward a reduced total leukocyte count in the control group ($P = 0.1$; Table 2). After the addition of topical CXCL1, there was a rapid increase in firm adhesion (9.0 ± 0.7 *versus* 7.8 ± 1.4 cells/100-μm segment at 20 min in CNTO 1081 and control groups, respectively). Whereas this declined over time in the control group, it remained at the same level for the duration of the experiment in the CNTO 1081 group and, considering all time points, was significantly higher than in the control animals ($P < 0.05$).

The failure of adhesion values to fall over time seemed to be due largely to a defect in transmigration in CNTO 1081–treated rats (Figure 8B). In the control group, CXCL1 induced transmigration of 9.5 ± 2.0 cells/100-μm segment after 90 min. This was significantly reduced by 43% (to 5.3 ± 1.1 cells/100-μm segment; $P < 0.001$) in CNTO 1081–treated animals.

Discussion

In a randomized, controlled study in rats with EAV, a model of AASV, we have shown that a monoclonal anti-rat TNF-α antibody reverses established glomerulonephritis and ameliorates pulmonary hemorrhage. This antibody also inhibits the transmigration response to a physiologic inflammatory stimulus, CXCL1, in rats with EAV. We showed previously that rats with EAV exhibit enhanced adhesion and transmigration in response to this chemokine, an effect that is mediated by circulating ANCA (25). Although other mechanisms may be involved, we postulate that the beneficial effect of anti–TNF-α therapy is related at least partly to the interruption of the chain of events that facilitates the interaction of ANCA with the target antigen MPO on the surface of the neutrophil or monocyte, as well as inhibition of endothelial adhesion molecule expression. This may result in the observed reduction in leukocyte transmigration and consequent vascular injury in CNTO 1081–treated animals. These findings provide a cornerstone for the use of these antibodies in patients with AASV.

We elected to begin treatment 4 wk after induction of EAV, at

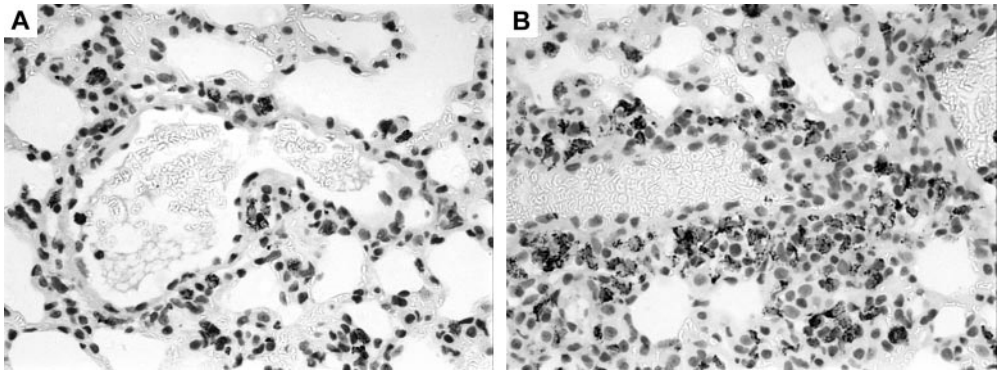

Figure 7. Effect of CNTO 1081 on pulmonary macrophage infiltration. Lung sections were probed with anti-CD68 (ED1) mAb, and binding was detected with HRP-DAB. (A) Occasional alveolar and perivascular macrophages in the lung of a rat that was treated with CNTO 1081. (B) Perivascular infiltrate that contained numerous macrophages in the lung of a rat that was treated with control IgG. Magnification, ×40 in A and B.

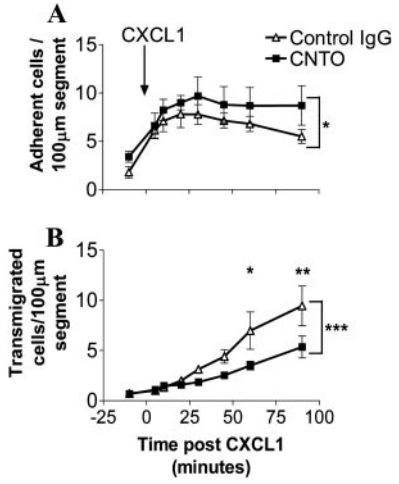

Figure 8. CNTO 1081 treatment inhibits the transmigration response to CXCL1. Eight weeks after immunization with hMPO and 4 wk after treatment was started with CNTO 1081 (■) or control IgG (▲), leukocyte responses in mesenteric venules of WKY rats were assessed using intravital microscopy. After taking baseline firm adhesion and transmigration readings, the mesentery was superfused with CXCL1 (3×10^{-9} M), and responses were quantified for an additional 90 min. (A and B) Firm adhesion (A) and the transmigration responses (B) to CXCL1. The data represent the mean ± SEM; $n = 4$ in each group. Statistically significant differences between groups of rats are shown by asterisks, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$.

a time when most animals are developing histologic and clinical evidence of focal necrotizing glomerulonephritis. This approach mirrors closely the scenario encountered in clinical practice, when patients present with established disease. In addition, we believed that it was critical to use an appropriate mouse IgG as a control, as the administration of IgG in itself has a therapeutic effect in vasculitis (34). Although not identical to human microscopic polyangiitis (rats with EAV develop little excretory renal insufficiency or involvement of organs other than kidney and lung), the EAV model shares many of the features of its human counterpart. It is a model of the autoimmune response to MPO, as observed in AASV, and shares a critical pathologic feature with human vasculitis: The pauci-immune nature of the glomerulonephritis. The fibrinoid necrosis and crescentic glomerular lesions in EAV are identical in appearance to those seen in human AASV (35,36). It therefore is likely that the two conditions share many facets of the effector arm of the vasculitic response. Thus, this model provides an opportunity to test novel biologic agents in an arena where current therapeutic approaches are unfocused and associated with a large burden of adverse effects.

Before committing to the use of CNTO 1081 in a full therapeutic study, we investigated its ability to block a biologic effect of TNF-α *in vivo*. On the basis of pilot studies in normal rats, the most physiologically relevant and reproducible effect of intravenous TNF-α infusion was a rapid and reversible reduction in leukocyte rolling, coupled with a progressive increase in leukocyte adhesion. The reduction in rolling in this model probably occurs as a result of increased adhesion and leukocyte sequestration in vascular beds such as the lungs, as it is accompanied by reversible peripheral leukopenia (unpublished observations). Pretreatment with CNTO 1081, at the same dose as that used in the therapeutic study, completely prevented these effects. This provided a basis for proceeding with the therapeutic study and for extending our intravital microscopy observations to investigate rats that had EAV and were treated for 4 wk with CNTO 1081 or control IgG. In these experiments, rather than investigate the effect of CNTO 1081 on TNF-α–induced leukocyte endothelial interaction, we studied the effect of CNTO 1081 treatment on the response to the chemokine CXCL1. This was chosen because of our previous observation that rats with EAV exhibit exaggerated leukocyte-endothelial interactions in response to this agent (25). We found that treatment with CNTO 1081, as well as effectively inhibiting macrophage tissue infiltration and reversing crescent formation, inhibited leukocyte transmigration in rats with EAV. The level of transmigration in response to CXCL1 observed in the CNTO 1081–treated group was similar to that observed in previous studies of nonimmunized animals (25). Consistent with this transmigration block, the number of firmly adherent cells re-

Table 2. Baseline leukocyte count, blood flow, and vessel diameter in test rats[a]

	Baseline Leukocyte Count		Mean Blood Flow		Vessel Diameter (μm)
	PMN ($\times 10^6$/ml)	PBMC ($\times 10^6$/ml)	Baseline (mm/s)	30 min (mm/s)	
Nonimmunized					
saline	1.3 ± 0.2	4.1 ± 0.5	1.7 ± 0.1	2.0 ± 0.2	29.8 ± 0.6
TNF-α	0.8 ± 0.1	4.6 ± 0.8	1.6 ± 0.03	1.9 ± 0.1	29.6 ± 0.8
TNF-α + CNTO 1081	1.1 ± 0.3	4.3 ± 0.4	1.9 ± 0.2	2.0 ± 0.1	30.5 ± 0.4
hMPO-immunized					
CNTO 1081	1.5 ± 0.3	4.5 ± 0.2			29.5 ± 0.5
control IgG	0.8 ± 0.2	3.6 ± 0.4			29.2 ± 1.1

[a]Nonimmunized WKY rats were treated with intravenous TNF-α 1 μg/kg or saline 1 h after pretreatment with CNTO 1081 16 mg/kg or vehicle (n = 4 to 12 in each group). WKY rats were immunized with hMPO 400 μg/kg 8 wk previously and subsequently were treated with CNTO 1081 or control IgG (each 16 mg/kg, n = 6 in each group) on alternate days. After baseline readings were taken, they were treated with topical CXCL1 (3×10^{-9} M, n = 4 from each group). No significant differences between the groups within each experiment were observed. Values are mean ± SEM. PMN, polymorphonuclear leukocytes; PBMC, peripheral blood mononuclear cells.

mained static over time in the CNTO 1081–treated group, whereas the number of adherent cells fell (as leukocytes transmigrated) in the control group.

In previous intravital microscopy studies in the presence of circulating ANCA (25), we have shown that transmigration is enhanced. We postulate that this exaggerated transmigration is dysregulated, in that transmigrating leukocytes are activated inappropriately after interaction with ANCA, with consequent potential for microvascular injury. In addition, we showed previously that neutrophils are the dominant glomerular infiltrating leukocytes 2 to 4 wk after immunization of WKY rats with hMPO (37). This fits well with the paradigm built up over the past decade, which proposes a series of mechanisms by which ANCA could induce vasculitis (see ref 38 for review). According to this schema, TNF-α–primed neutrophils and monocytes express autoantigens (MPO and proteinase-3) on their surface, allowing interaction with circulating autoantibodies (ANCA). The sequence of events that follows includes inappropriate degranulation (39), reduced cellular deformability (40), and dysregulated, proinflammatory apoptosis (41,42). Although TNF-α is pleiotropic, with many effects on inflammatory and endothelial cells, it is possible that one of the modes of action of TNF-α blockade involves disruption of this process by interrupting the interaction of cell surface MPO with circulating ANCA.

We have contributed to clinical studies that have tested potential uses for anti–TNF-α antibodies in AASV (12,13) and the rational use of ANCA-depleting strategies such as plasma exchange (43–46). The EAV model that we have now developed should facilitate further dissection of the mechanisms whereby TNF-α blockade exerts a therapeutic effect, in addition to permitting investigation of other biologic agents. There is now an unprecedented opportunity to develop novel therapies for AASV targeted at the pathophysiologic mechanisms involved.

Acknowledgments

This work was funded by a Wellcome Trust Research Training Fellowship to M.A.L., a National Kidney Research Fund Research Training Fellowship to C.L.S., and grants from the British Heart Foundation.

Presented in part at the ANCA workshop; Heidelberg, Germany; 2005.

We acknowledge the technical assistance of Sarah Khan, John Dangerfield, Rashmi Yadav, Jennifer Smith, Karen Larbi, and Fred Tam.

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