

Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis

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ABSTRACT There is considerable evidence implicating tumor necrosis factor α (TNF- α) in the pathogenesis of rheumatoid arthritis. This evidence is based not only on the universal presence of TNF- α in arthritic joints accompanied by the upregulation of TNF- α receptors but also on the effects of neutralizing TNF- α in joint cell cultures. Thus, neutralization of TNF- α *in vitro* results in inhibition of the production of interleukin 1, which like TNF- α , is believed to contribute to joint inflammation and erosion. To determine the validity of this concept *in vivo*, the effect of administering TNF-neutralizing antibodies to mice with collagen-induced arthritis has been studied. This disease model was chosen because of its many immunological and pathological similarities to human rheumatoid arthritis. TN3-19.12, a hamster IgG1 monoclonal antibody to murine TNF- α/β , was injected i.p. into mice either before the onset of arthritis or after the establishment of clinical disease. Anti-TNF administered prior to disease onset significantly reduced paw swelling and histological severity of arthritis without reducing the incidence of arthritis or the level of circulating anti-type II collagen IgG. More relevant to human disease was the capacity of the antibody to reduce the clinical score, paw swelling, and the histological severity of disease even when injected after the onset of clinical arthritis. These results have implications for possible modes of therapy of human arthritis.

There is now abundant evidence to suggest that tumor necrosis factor α (TNF- α) plays an important role in the pathogenesis of inflammatory joint diseases, including rheumatoid arthritis (RA). TNF- α has been identified in the synovial membrane and particularly at the cartilage-pannus junction of patients with RA (1) and is spontaneously produced by cultured synovial cells derived from RA patients (2). The properties of TNF- α are consistent with a pathogenic role. Thus, TNF- α induces cartilage and bone resorption and inhibits the synthesis of proteoglycan and collagen in cartilage (3-5). In addition, TNF- α induces the release of prostaglandin E₂ and collagenase by synovial cells (6), contributes to fibrosis by stimulating fibroblast proliferation (7), and facilitates inflammatory cell infiltration by promoting adhesion of endothelial cells to neutrophils (8) and lymphocytes (9). TNF- α may also lead to the production of other cytokines such as interleukin 1, which is also implicated in joint damage in RA (10), because antibodies to TNF- α have been shown to diminish the production of proinflammatory cytokines such as interleukin 1 and granulocyte-macrophage colony-stimulating factor by rheumatoid synovium-derived mononuclear cells (11, 12). In the light of these observations, it was suggested that TNF- α is important in the pathogenesis of RA not only for its ability to directly promote connective tissue degradation but also for its involvement in the induction of the chronic inflammatory state (13). TNF- α may, therefore, represent an important therapeutic target in RA (11, 13).

In this paper we describe experiments aimed at validating the concept of an *in vivo* role for TNF- α in the pathogenesis of inflammatory joint disease by determining the effect of administering a neutralizing anti-TNF- α/β monoclonal antibody (mAb) to mice with type II collagen-induced arthritis either before the onset of clinical symptoms or after the establishment of full-blown disease. Collagen-induced arthritis is often considered to be a model for human RA because of the involvement of localized major histocompatibility complex class II-restricted T-helper-cell activation in the pathogenesis of both diseases (14) and because of the observed similarities with respect to synovial inflammation and cartilage/bone destruction (15), both of which suggest common pathological mechanisms. Our findings demonstrate that anti-TNF- α/β treatment causes a significant reduction in the clinical and histopathological severity of collagen-induced arthritis whether carried out before or after the onset of clinical disease.

MATERIALS AND METHODS

Purification of Type II Collagen. Type II collagen was purified from bovine articular cartilage by the method of Miller (16). Briefly, powdered cartilage was treated with 4 M guanidine hydrochloride to remove proteoglycans, and then type II collagen was extracted by pepsin digestion in 0.5 M acetic acid and removed from solution by salt precipitation. Residual pepsin activity was removed by stirring the redissolved collagen in a solution of Tris·HCl (0.05 M, pH 7.4) containing NaCl (0.2 M).

mAbs. The anti-TNF antibody used was a hamster IgG1 mAb, TN3-19.12. This antibody has been demonstrated to effectively neutralize murine TNF- α and TNF- β *in vitro* and *in vivo*, the neutralizing titer of TN3-19.12 being 25 ng/unit of TNF- α (17). L2, a nonneutralizing hamster IgG1 mAb raised against interleukin 2, was used as a control. Both antibodies were generously supplied to us by Robert Schreiber (Washington University Medical School, St Louis), in conjunction with Celltech (Slough, U.K.).

Induction of Arthritis. Male DBA/1 mice (8-12 weeks old) were injected intradermally at the base of the tail with type II collagen (100 μ g) emulsified in Freund's complete adjuvant (Difco). Two regimes were used to assess the effect of anti-TNF treatment. (i) Mice were given i.p. injections of TN3-19.12 [250 μ g per mouse; equivalent to 10 mg/kg (body weight)], L2, or phosphate-buffered saline (PBS) once a week for 4 weeks, starting the day before immunization with type II collagen. (ii) Anti-TNF treatment was initiated immediately after the onset of clinical arthritis. Mice were inspected daily and each mouse that exhibited erythema and/or swelling in one or more limbs was randomly assigned to one of three groups and given twice-weekly intraperitoneal injections of TN3-19.12, L2, or PBS. In one experiment, 300 μ g

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Abbreviations: TNF- α , tumor necrosis factor α ; RA, rheumatoid arthritis; mAb, monoclonal antibody.

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of mAb [equivalent to 12 mg/kg (body weight)] was administered and in a second experiment, doses of 50, 300, or 500 μg were administered [equivalent to 2 mg/kg, 12 mg/kg, and 20 mg/kg (body weight), respectively]. Arthritis was monitored over a 14-day treatment period in terms of the following indices of disease.

Clinical score. A scoring system was used where 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced edematous swelling, and 3 = joint rigidity. Each limb was graded, giving a maximum score of 12 per mouse.

Paw swelling. The thickness of each affected hind paw was monitored using calipers. The results were expressed as the percentage increment in paw width relative to the paw width before the onset of arthritis.

Anti-collagen antibody level. Measurement of anti-type II collagen IgG was by a modification of an ELISA as described in detail for the detection of human IgG (18). Briefly, microtiter plates were coated with native bovine type II collagen (2 $\mu\text{g}/\text{ml}$), blocked, and then incubated with serially diluted test sera. Bound IgG was detected by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG, followed by substrate (dinitrophenyl phosphate). Optical densities were measured at 405 nm.

Histopathology. Arthritic paws (one or two per mouse) were removed post-mortem, fixed in 10% (wt/vol) buffered formalin, and then decalcified in EDTA in buffered formalin (5.5%). The paws were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic evaluation, which was performed in a blinded fashion. The severity of arthritis in each joint was classified as mild, moderate, or severe based on the following criteria: mild, minimal synovitis, cartilage loss, and bone erosions limited to discrete foci; moderate, synovitis and erosions present but normal joint architecture intact; severe, synovitis, extensive erosions, and joint architecture disrupted.

RESULTS

Effect of Anti-TNF Treatment Prior to Onset of Arthritis. Mice were treated with TN3-19.12 ($n = 9$) every 7 days for 4

Table 1. Effect of anti-TNF treatment (250 μg per mouse) before the onset of arthritis

Parameter	Treatment		
	TN3-19.12	L2	PBS
Clinical severity			
Incidence of arthritis	6/9	10/10	8/10
Day of onset	29.0 \pm 1.3	31.2 \pm 1.1	29.6 \pm 1.8
Clinical score	3.7 \pm 1.0	4.6 \pm 0.6	4.4 \pm 0.8
Maximum paw swelling, %	30 \pm 8 [†]	53 \pm 5	51 \pm 6
Histopathology			
Total no. joints assessed	16	14	20
Mild, no.	6 (37)	3 (21)	2 (10)
Moderate, no.	7 (43)	1 (7)	5 (25)
Severe, no.	3 (19)*	10 (71)	13 (65)
Anti-collagen IgG, arbitrary units			
	74.8 \pm 5.4	73.0 \pm 4.6	66.6 \pm 5.8

Paw thickness was monitored throughout the period of arthritis and the maximum paw swelling for each affected hindlimb was significantly reduced in the TN3-19.12-treated group compared to the control groups. Joints from mice (four mice per group) that had demonstrated clinical arthritis for the same time were examined microscopically. Significantly fewer joints had severe pathology in the TN3-19.12-treated group compared to the control groups. However, treatment did not prevent or delay the onset of arthritis nor did it affect the level of anti-collagen IgG. Data for day of onset, clinical score, and maximum paw swelling are the mean \pm SEM. Data in parentheses are percent. *, $P < 0.01$ (two-sample t test; TN3-19.12-treated vs. L2-treated animals); [†], $P < 0.05$ (χ^2 test; TN3-19.12-treated vs. L2-treated animals).

weeks starting on the day before immunization with type II collagen. Controls were given L2 ($n = 10$) or PBS ($n = 10$). The first signs of arthritis were observed \approx 4 weeks after immunization and consisted of erythema and swelling of the feet and ankles. No significant differences could be detected between the TN3-19.12-treated group and the control groups with respect to the proportion of mice that developed arthritis, the time of onset of disease, mean clinical scores, or serum levels of anti-type II collagen IgG (Table 1). However, paw swelling (which was monitored throughout the experiment) was found to be significantly reduced in the TN3-19.12-treated group compared to the control groups ($P < 0.05$). Similarly, joints that were examined microscopically demonstrated reduced histopathological severity of arthritis in anti-TNF-treated mice compared to control mice. Thus, 19% of joints from mice given TN3-19.12 were classified as severe compared to 71% for L2-treated mice ($P < 0.05$). Serum TNF was undetectable by immunoassay in either normal mice or arthritic mice (data not presented).

Effect of Anti-TNF on Established Arthritis. After establishing that anti-TNF treatment was beneficial when administered before the onset of disease, a second experiment more relevant to human arthritis was carried out that determined the effect of anti-TNF treatment on already established arthritis. Mice ($n = 10$) were given TN3-19.12 twice a week for 2 weeks starting on the day that clinical arthritis was first observed. Controls received L2 ($n = 10$) or PBS ($n = 10$).

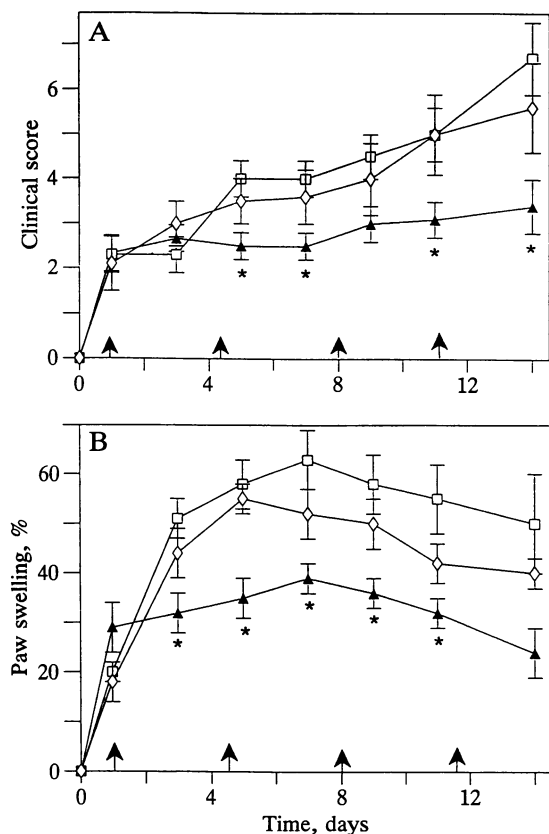


FIG. 1. Effect of anti-TNF on the clinical progression of established collagen arthritis. \blacktriangle , TN3-19.12 (300 μg); \square , L2 (300 μg); \diamond , PBS. Arrows indicate time of injection. (A) Clinical score. The scoring system was based on the following criteria: 0 = normal; 1 = slight swelling and/or erythema; 2 = pronounced edematous swelling; 3 = joint rigidity. Each limb was graded, giving a maximum score of 12 per mouse. *, $P < 0.05$ (Mann-Whitney U test; TN3-19.12-treated vs. L2-treated animals). (B) Paw swelling. Data are expressed as the percentage increment in paw width relative to the paw width before the onset of arthritis. *, $P < 0.05$ (two-sample t test; TN3-19.12-treated vs. L2-treated animals).

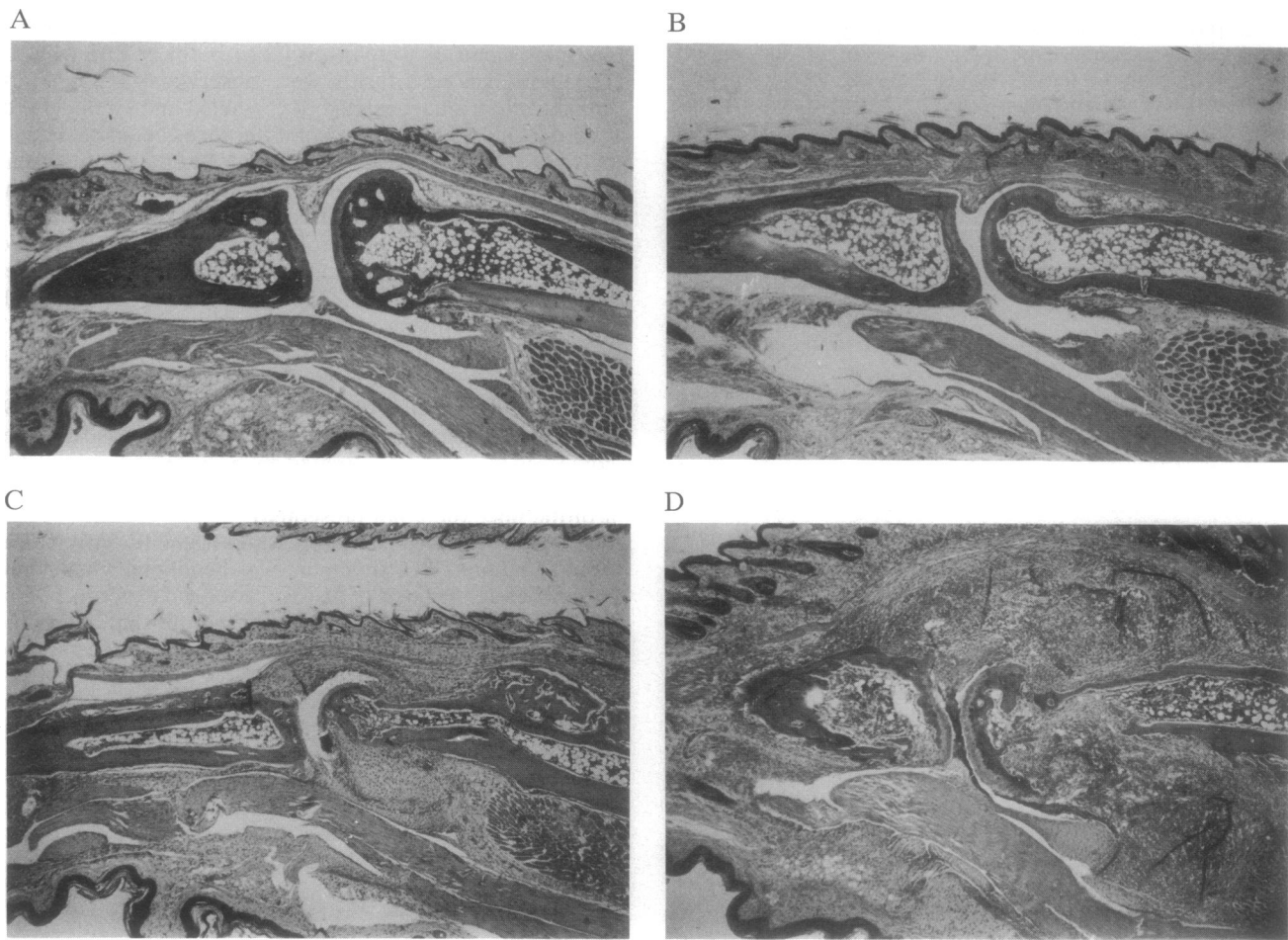


FIG. 2. Histopathological assessment of arthritic joints. (A) Normal interphalangeal joint. (B) Mild arthritis: minimal synovitis without cartilage/bone erosions. Mouse treated with TN3-19.12 (300 μg). (C) Moderate arthritis: synovitis and erosions but joint architecture maintained. Mouse treated with TN3-19.12 (50 μg). (D) Severe arthritis: synovitis, erosions, and loss of joint integrity. Mouse treated with control mAb. (Hematoxylin/eosin. $\times 50$.)

Severity of arthritis was monitored by clinical score and paw swelling. Fig. 1 shows the progression of arthritis during the treatment period in terms of clinical score and paw swelling. A significant reduction in clinical score ($P < 0.05$) in the TN3-19.12-treated group was apparent throughout most of the treatment period and a reduction in paw swelling was observed in the TN3-19.12-treated group that reached statistical significance ($P < 0.05$) as early as 2 days after the start of treatment. Mice were killed 2 weeks after the onset of clinical arthritis and affected paws were studied microscopically, after sectioning and staining with hematoxylin and eosin. Examples of arthritic joints classified as mild, moderate, or severe are shown in Fig. 2. Significantly more joints were mildly affected in the TN3-19.12-treated group than in the L2-treated group ($P < 0.001$). In addition, significantly fewer joints from the TN3-19.12-treated mice were severely affected ($P < 0.001$). The serum level of anti-collagen IgG was 82 ± 38 arbitrary units (mean \pm SEM) for the TN3-19.12-treated group, compared with 194 ± 74 arbitrary units for the L2-treated group and 50 ± 18 arbitrary units for the PBS-treated group. These differences were not statistically significant.

Comparison of Different Doses of Anti-TNF on Severity of Established Arthritis. To confirm the earlier findings and to establish a relationship between dose of anti-TNF mAb administered and efficacy of treatment, another experiment was carried out in which multiple doses of TN3-19.12 were administered (50, 300, or 500 μg per mouse; $n = 5$) after the onset of arthritis by using the same protocol as in the

single-dose experiment. Control mice received L2 at 500 μg per mouse ($n = 5$). As in the single-dose experiment, administration of 300 or 500 μg of TN3-19.12 resulted in reduced clinical score and reduced paw swelling (Fig. 3). However, a TN3-19.12 dose of 50 μg per mouse was less effective than a dose of 300 μg in reducing clinical score, and little additional benefit was obtained from a dose of 500 μg compared to 300 μg . Similarly, a dose of 50 μg per mouse was less effective than a TN3-19.12 dose of 300 μg or 500 μg in reducing the extent of paw swelling. In terms of histopathological severity of arthritis, TN3-19.12 administered at 50 μg per mouse did not significantly affect the proportion of joints demonstrating mild or severe lesions (Table 2). On the other hand, a dose of 300 μg per mouse resulted in a significant increase ($P < 0.01$) in the number of mildly affected joints but no additional benefit was seen in the group given 500 μg per mouse. A comparison of TN3-19.12-treated and control joints with similar degrees of arthritis severity failed to reveal any apparent differences in terms of inflammatory infiltrate, pannus formation, osteoclastic activity, or fibrosis. Thus, in lesions of similar severity, arthritis was indistinguishable between anti-TNF-treated and control mice. Serum levels of anti-type II collagen IgG were not found to be significantly different in TN3-19.12-treated mice compared to control mice at any of the doses tested.

DISCUSSION

Attempts to dissect the pathogenesis of human RA at the cellular and molecular level have provided a wealth of

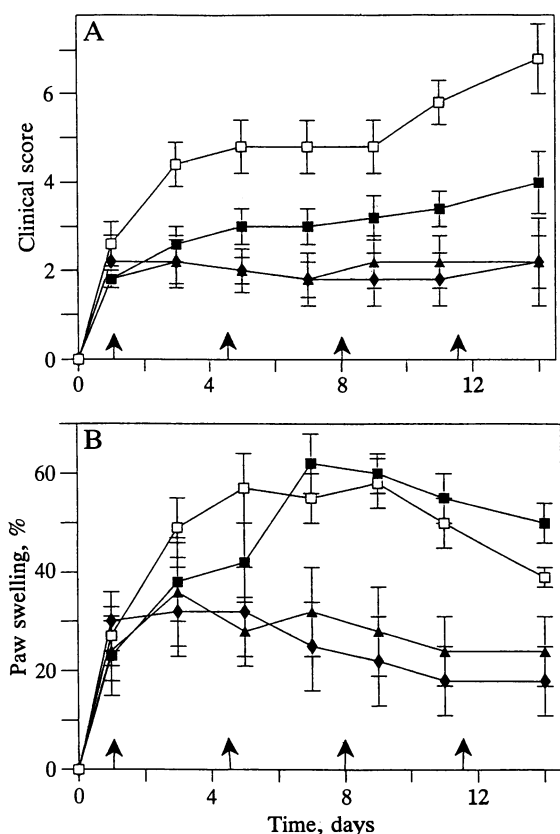


FIG. 3. Comparison of different doses of anti-TNF on clinical progression of established arthritis. ■, TN3-19.12 (50 μ g per mouse); ▲, TN3-19.12 (300 μ g per mouse); ◆, TN3-19.12 (500 μ g per mouse); □, L2 (500 μ g per mouse). Arrows indicate time of injection. (A) Clinical score. (B) Paw swelling.

information concerning the inflammatory processes involved in the disease (for review, see refs. 13 and 19). However, immunological or biochemical data obtained from biopsy specimens or cultured cell populations yield only fragmented information and need to be complemented by *in vivo* studies. Thus, another approach is to administer neutralizing antibodies to cell markers or cell products in experimental models of arthritis, and this may yield important information regarding the pathogenic processes that occur *in vivo* and may, significantly, provide evidence to support a particular therapeutic strategy in human disease. For example, antibodies to the CD4 molecule have been shown to prevent the onset of collagen-induced arthritis if administered around the time of collagen immunization (20, 21), demonstrating the

importance of CD4⁺ cells in this form of arthritis and providing evidence to support the initiation of therapeutic trials of anti-CD4 in human RA (22). It is important to note, however, that although anti-CD4 treatment is effective in preventing the onset of collagen-induced arthritis, it is ineffective in reducing the severity of established disease (21).

There are mounting data suggesting that TNF- α is implicated in RA (for review, see refs. 11 and 13), but the concept of a pivotal role for this cytokine needed to be tested *in vivo*. The simplest approach was to administer a neutralizing antibody to TNF- α in a murine disease resembling RA, and the results of these experiments are described here. We demonstrate that the administration of TN3-19.12, a hamster anti-murine TNF- α/β mAb to mice before or after the onset of collagen-induced arthritis reduces the severity of disease. In mice treated after the onset, the reduction in severity was clinically evident 2 days after the start of therapy. Histological analysis of arthritic joints from TN3-19.12-treated mice revealed a marked shift in the proportion of severely affected vs. mildly affected joints. Thus, treatment with TN3-19.12 resulted in a significant decrease in the proportion of joints with severe arthritis and a significant increase in the number of joints with mild arthritis. The beneficial effects of mAb treatment were specific for TNF-neutralizing antibody since treatment with L2, an isotype-matched nonneutralizing mAb to interleukin 2, did not have a significant effect on the outcome of arthritis. The effects of anti-TNF treatment were dose-dependent, with a dose of 300 μ g providing markedly more clinical and histopathological benefit than a dose of 50 μ g. On the other hand, a dose of 500 μ g was not more effective than the dose of 300 μ g. The precise way in which anti-TNF treatment reduced joint pathology cannot be deduced from the results of this study. Evidence from immunohistochemical studies suggests that the inflamed synovium, and especially the invasive pannus, are major sites of TNF- α production in RA (1). It may be expected, therefore, that in view of the well-established degradative and inflammatory properties of TNF- α , the principal ameliorative effect of anti-TNF therapy would be to neutralize locally produced TNF- α , thereby reducing TNF- α -mediated pathology.

In this study we have also demonstrated that cytokine-specific immunotherapy may be a valid alternative to the administration of cell-depleting reagents, such as anti-CD4 mAb, which may have profound immunosuppressive effects. One pharmacological agent that has been shown to inhibit the development of ongoing arthritis is cyclosporin A, and this has also been attributed to the potent immunosuppressive properties of the drug (21). Evidence from the present study indicates that the ameliorative effects of anti-TNF therapy did not arise as a result of generalized immunosuppression since levels of circulating antibody to type II collagen, a

Table 2. Histopathological severity of arthritis in individual joints

Treatment	No. of joints assessed	Severity, no.		
		Mild	Moderate	Severe
Single-dose experiment				
TN3-19.12 (300 μ g)	69	19 (27)*	23 (33)	27 (39)*
L2 (300 μ g)	68	2 (3)	13 (19)	53 (78)
PBS	55	3 (5)	10 (18)	42 (76)
Multiple-dose experiment				
TN3-19.12 (50 μ g)	21	3 (14)	2 (10)	16 (76)
TN3-19.12 (300 μ g)	15	5 (33)*	5 (33)	5 (33)*
TN3-19.12 (500 μ g)	20	6 (30) [†]	4 (20)	10 (50)
L2 (500 μ g)	24	0 (0)	6 (25)	17 (74)

Mild, minimal synovitis, erosions limited to discrete foci, and cartilage surface intact; moderate, synovitis and erosions present but normal joint architecture intact; severe, synovitis, extensive erosions, and joint architecture disrupted. Data in parentheses are percent. *, $P < 0.01$; [†], $P < 0.05$ (χ^2 test; TN3-19.12-treated vs. L2-treated animals).

T-cell-dependent antigen, were not significantly reduced by anti-TNF treatment. It was also of interest that anti-TNF, unlike anti-CD4 (21), exerted a protective effect even after the onset of clinical arthritis.

From these findings it is evident that, as in RA, TNF plays a prominent role in collagen-induced arthritis. The results are consistent with the finding that mice expressing TNF- α transgenes develop chronic inflammatory arthritis that is prevented by treatment with anti-TNF- α antibodies (23). Further evidence for the involvement of TNF- α in murine arthritis comes from the demonstration of accelerated onset and increased incidence of arthritis, without any increase in the humoral immune response to type II collagen, in type II collagen-immunized rats after intraarticular injection of TNF- α (24). Naive rats that were given TNF- α did not show evidence of clinical arthritis, a fact that is also consistent with our results in that anti-TNF treatment did not completely prevent joint pathology even at high doses. This clearly demonstrates that in addition to TNF, other mediators are involved in this form of disease. One of the characteristic features of collagen-induced arthritis is the presence of high levels of circulating anti-type II collagen antibody (20), and passive transfer of hyperimmune serum from type II collagen-immunized mice and rats or affinity-purified anti-type II collagen antibody is capable of inducing significant joint pathology in naive recipients including severe cartilage and bone degradative changes, by a complement-dependent mechanism (25–27). It is likely therefore that cartilage-specific localization of anti-type II collagen antibodies, with the consequent activation of the complement cascade, contributed to the residual pathology observed in the joints of anti-TNF treated mice, although it would be necessary to demonstrate an absence of TNF in such joints to confirm this observation. Support for the role of anti-collagen antibodies in determining pathology is provided by the presence of neutrophil-rich infiltrates in anti-TNF treated and control mice, since neutrophil infiltration is reported to be a dominant pathological feature of passively transferred collagen arthritis (26). It is possible that even greater protection from joint damage could be provided by a therapeutic strategy based on anti-TNF treatment coupled with B- and/or T-lymphocyte down-regulation.

Immunohistochemical techniques have yielded important information concerning the identification of cytokines in tissues affected by autoimmune disease, and studies into the effect of cytokines on different cell types in culture have led to an increase in our knowledge of the *in vitro* properties of these molecules. Consequently, it has become increasingly necessary to study the effects of manipulating cytokine levels or cytokine activities *in vivo*, to determine their physiological importance. In this study, an important *in vivo* role for TNF in murine collagen-induced arthritis has been identified, and the efficacy of anti-TNF therapy in a model of RA has been established. Confirmation of the pathological importance of TNF- α in human RA can only come from the amelioration of this condition by TNF- α -targeted therapy, and results such as the ones provided here reinforce the case for testing the effect of blocking TNF- α in RA.

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