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Immunology

Synergy between anti-CD4 and anti-tumor necrosis factor in the amelioration of established collagen-induced arthritis

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ABSTRACT **Anti-CD4 treatment is reported to prevent collagen-induced arthritis if administered before the onset of clinical disease but has relatively little effect on established arthritis. In contrast, we have recently shown that anti-tumor necrosis factor α/β (TNF) treatment reduces the severity of established arthritis. We now study the effect of combined administration of anti-CD4 monoclonal antibody (YTS 191.1.2/YTA 3.1.2) and anti-TNF monoclonal antibody (TN3-19.12) in established arthritis. Anti-CD4 treatment caused some reduction in paw-swelling but did not significantly prevent joint erosion. A suboptimal dose of anti-TNF alone had no significant effect on arthritis. In contrast, anti-CD4 plus suboptimal anti-TNF significantly reduced paw-swelling, limb involvement, and joint erosion. As previously reported, an optimal dose of anti-TNF alone inhibited paw-swelling, limb involvement, and joint erosion. However, optimal anti-TNF combined with anti-CD4 caused significantly greater reductions in paw-swelling and joint erosion than those achieved by optimal anti-TNF alone. Coadministration of anti-CD4 was also effective in preventing an antibody response to the hamster anti-TNF antibody, which may have implications for long-term therapy in human disease. Thus anti-CD4 acts synergistically with anti-TNF in ameliorating established collagen-induced arthritis and this combined therapeutic approach may provide effective long-term control of rheumatoid arthritis.**

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation within the joint associated with synovitis and erosion of cartilage and bone. A strategy for the treatment of this condition is to administer monoclonal antibodies (mAbs) to block the immunological or inflammatory cascades. Such mAbs may recognize either cells involved in the immune or inflammatory response or neutralize soluble mediators released by the cells. A candidate cellular target is the $CD4^+$ T cell because the abundant presence of these cells in the synovium suggests their active involvement in the disease process (1). Tumor necrosis factor α (TNF-α) is an inflammatory mediator that we have targeted by mAb therapy after its identification as a critical cytokine in the pathogenesis of RA (2). Thus, TNF-α is consistently found in the joints of RA patients (3) and possesses properties that are consistent with a pathogenic role. For example, TNF-α stimulates the secretion of prostaglandin E_2 and collagenase (4), encourages inflammatory cell infiltration (5, 6), and induces cartilage and bone resorption (7, 8). Most importantly, neutralization of TNF-α in rheumatoid synovial cell cultures was shown to cause reduced secretion of the proinflammatory cytokines, interleukin 1 (IL-1) (9) and granulocyte/macrophage colony-stimulating factor (GM-CSF) (10).

The two contrasting therapeutic approaches have been tested in murine collagen-induced arthritis (CIA), as a model for RA. For example, anti-CD4 mAb was found to prevent the induction of arthritis in DBA/1 mice if administered around the time of immunization with type II collagen but was ineffective in established disease (11). In another report, anti-CD4 mAb had no effect on established arthritis but was found to halt disease progression when combined with anti-Thy-1 mAb (12). Anti-T-cell receptor (α/β) mAb treatment completely blocked the induction of arthritis when administered around the time of collagen immunization and led to a reduction in disease severity when given after the onset of arthritis (13). More recently, we have shown that anti-tumor necrosis factor α/β (TNF) mAb administered after the onset of clinical arthritis reduces the clinical and histological severity of arthritis (14).

These findings suggest that although $CD4^+$ T cells are unequivocally involved in the induction phase of the disease, their role during the effector phase is less easy to define. In contrast, the influence of TNF-α is most clearly evident during the effector phase. Bearing in mind that human RA generally runs a more protracted course than murine CIA (15), it is likely that both induction and effector mechanisms are operating on a long-term basis. It follows that there may be considerable therapeutic potential for a form of treatment that targets not only induction but also effector mechanisms, thereby interrupting both the immune and inflammatory disease processes.

The aim of this paper was to determine whether anti-CD4 and anti-TNF mAbs act synergistically in the treatment of established CIA. The approach used was to evaluate the effect of anti-CD4 treatment combined with doses of anti-TNF that we knew from earlier studies (14) to be either suboptimal or optimal. Our results reveal that anti-CD4 acts synergistically with both suboptimal and optimal doses of anti-TNF. The fact that both anti-CD4 and anti-TNF mAbs are being evaluated for the treatment of human RA (16, 17) heightens the potential clinical relevance of this finding.

MATERIALS AND METHODS

Purification of Type II Collagen. Type II collagen was purified from bovine articular cartilage as described (18).

Type II Collagen Immunization. Male DBA/1 mice were immunized intradermally at 8–12 weeks of age with type II collagen (100 μg), emulsified in Freund's complete adjuvant (Difco).

mAbs. *Anti-TNF.* TN3-19.12, a neutralizing hamster IgG1 anti-TNF-α/β mAb (19), and L2, an isotype control, were generously donated by Robert Schreiber (Washington University Medical School, St. Louis), in conjunction with Celltech (Slough, U.K.).

Anti-CD4. Cell-depleting anti-CD4 mAb (rat IgG2b) consisted of a 1:1 mixture of YTS 191.1.2 and YTA 3.1.2 (20–22).

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Abbreviations: TNF-α, tumor necrosis factor α; TNF, tumor necrosis factor α/β; RA, rheumatoid arthritis; mAb, monoclonal antibody; IL-1, interleukin 1; GM-CSF, granulocyte/macrophage colony-stimulating factor; CIA, collagen-induced arthritis; PIP, proximal interphalangeal.

YTA 3.1.2 was a gift from Herman Waldmann (University of Cambridge). An isotype control mAb, HRPN11/12a, was a gift from Stephen Hobbs (Institute of Cancer Research, London).

Assessment of Arthritis. Day 1 of arthritis was considered to be the day that erythema and/or swelling was first observed in one or more limbs. mAb treatment was administered on days 1, 4, and 7. Paw-swelling (measured with calipers) was monitored for 10 days, after which time the mice were killed and joints were processed for histology. From each mouse, the first limb to show clinical arthritis was removed, formalin-fixed, decalcified, and wax-embedded before sectioning and staining with hemotoxylin/eosin. Saggital sections of the proximal interphalangeal (PIP) joint of the middle digit were studied in a blinded fashion for the presence or absence of erosions (defined as demarcated defects in cartilage or bone filled with inflammatory tissue). In this way, comparisons were made between the same joints, and in each case, the arthritis was of identical duration.

Flow Cytometry. To enumerate the proportion of $CD4^+$ cells in spleens or peripheral blood, cells were incubated with phycoerythrin-conjugated anti-CD4 (Becton Dickinson) and then analyzed by flow cytometry (FACScan, Becton Dickinson) with scatter gates set on the lymphocyte fraction.

Immunohistochemistry. Sections were dewaxed, trypsin-digested, and then incubated with anti-CD4 mAb (YTS 191.1.2/YTA 3.1.2). Detection of bound antibody was by alkaline phosphatase–rat anti-alkaline phosphatase complex (APAAP; Dako) and fast red substrate.

Anti-Collagen IgG. Serum anti-collagen IgG levels were measured as described (14).

Anti-TN3-19.12 Response. The IgM response to injected TN3-19.12 was measured by ELISA on day 10 of the treatment period. Briefly, microtiter plates were coated with TN3-19.12 (5 μg/ml), blocked, and then incubated with serially diluted test sera. Bound IgM was detected by goat anti-mouse IgM-alkaline phosphatase conjugate, followed by substrate. It was not possible to measure the IgG anti-TN3-19.12 response because of the close homology between murine and hamster IgG.

Measurement of Unbound TN3-19.12. Microtiter plates were coated with recombinant murine TNF-α (a gift from Genentech), blocked, and then incubated with test sera. Goat anti-hamster IgG-alkaline phosphatase conjugate was then applied, followed by substrate. Quantitation was by reference to a sample of known concentration of TN3-19.12.

RESULTS

Effect of Anti-CD4/Anti-TNF on the Clinical Course of Arthritis. Arthritis became clinically evident around 30 days after immunization. For each mouse, treatment was started on the first day that arthritis was observed and was continued for 10 days. First, a suboptimal dose (50 μg) of anti-TNF alone was compared with the same dose given together with anti-CD4 (200 μg). Neither anti-CD4 alone nor suboptimal anti-TNF alone caused significant reductions in paw-swelling (Fig. 1). However, treatment with anti-TNF plus anti-CD4 resulted in a consistent reduction in paw-swelling relative to the group given control mAb ($P < 0.01$). Furthermore, combined anti-TNF/anti-CD4 treatment caused a reduction in paw-swelling relative to anti-CD4 alone ($P < 0.05$) and anti-TNF alone ($P < 0.05$).

Next, an optimal dose of anti-TNF (300 μg) alone was compared with the same dose given in combination with anti-CD4. As before, the combined anti-TNF/anti-CD4 treatment resulted in a significant reduction in paw-swelling compared to controls ($P < 0.01$; Fig. 2). In addition, paw-swelling was significantly reduced in the combined anti-CD4/anti-TNF-treated group relative to the groups given anti-CD4 alone ($P < 0.01$) or anti-TNF alone ($P < 0.01$). A reduction in paw-swelling was also observed in the mice given anti-CD4 alone and anti-TNF treatment alone resulted in significantly reduced paw-swelling. The reduction in paw-swelling attributable to anti-TNF treatment was broadly comparable with our previous findings (14).

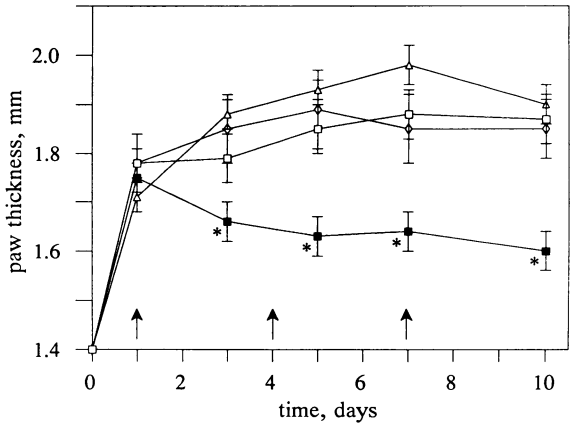

FIG. 1. Paw-swelling. The results are shown of an experiment to compare treatment with a suboptimal dose of anti-TNF (50 μg) administered alone or in combination with anti-CD4 (200 μg). Arrows indicate times of injection. An asterisk indicates a significant reduction compared to the group given control mAb ($P < 0.05$; two-sample t test). There were 18 or 19 mice per group. □, Anti-TNF alone; ■, anti-TNF plus anti-CD4; ◇, anti-CD4 alone; △, control mAb.

In CIA, as in RA, it is unusual for additional limbs to become involved after the initial appearance of disease and new limb involvement is an important indicator of the progression of disease. To determine the effect of anti-CD4/anti-TNF treatment on limb recruitment, the number of limbs with clinically detectable arthritis at the end of the treatment period was compared with the number of arthritic limbs before treatment. In control mice there was an increase in limb involvement over the 10-day period of around 50% (Table 1). There were insignificant reductions in new limb involvement in the groups given anti-CD4 alone or suboptimal anti-TNF alone. However, in the group given optimal anti-TNF, the increase in limb involvement was <10% ($P < 0.05$). Even more striking was the fact that in the groups given

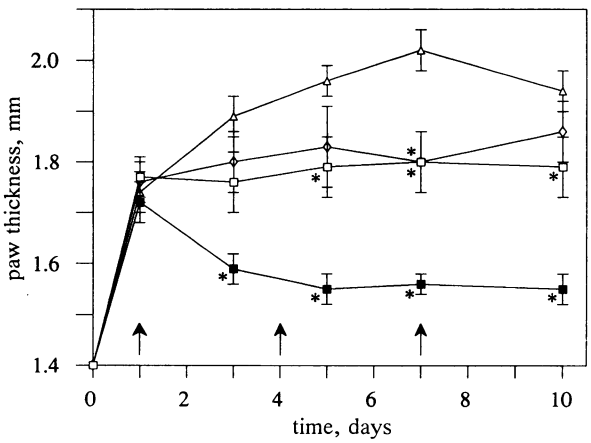

FIG. 2. Paw-swelling. Comparison of an optimal dose of anti-TNF (300 μg) alone vs. the same dose of anti-TNF plus anti-CD4 (200 μg). Arrows indicate times of injection. An asterisk indicates a significant reduction compared to the group given control mAb ($P < 0.05$; two-sample t test). There were 11–13 mice per group. □, Anti-TNF alone; ■, anti-TNF plus anti-CD4; ◇, anti-CD4 alone; △, control mAb.

Table 1. Combined anti-CD4/anti-TNF inhibits the progression of clinical arthritis

Treatment	Number of limbs affected		Increase, %
	Day 1	Day 10	
Suboptimal anti-TNF (50 μg)			
Anti-CD4 (n = 18)	1.30 ± 0.10	1.90 ± 0.12	46.1
Anti-TNF (n = 19)	1.20 ± 0.09	1.65 ± 0.17	37.5
Anti-CD4/TNF (n = 18)	1.40 ± 0.17	1.45 ± 0.22	3.4*
Control mAb (n = 18)	1.43 ± 0.15	2.24 ± 0.18	56.6
Optimal anti-TNF (300 μg)			
Anti-CD4 (n = 12)	1.27 ± 0.10	1.80 ± 0.14	42.0
Anti-TNF (n = 11)	1.50 ± 0.17	1.64 ± 0.20	9.5†
Anti-CD4/TNF (n = 13)	1.25 ± 0.11	1.25 ± 0.11	0‡
Control mAb (n = 12)	1.53 ± 0.19	2.27 ± 0.25	47.8

Number of limbs showing clinical evidence of arthritis (erythema/oedema) before treatment (day 1 of arthritis) was compared with the number of arthritic limbs after treatment (day 10) (mean ± SEM). The Mann–Whitney test was used for statistical comparisons. *, $P < 0.05$ (anti-CD4/TNF vs. control mAb); †, $P < 0.05$ (anti-TNF vs. control mAb); ‡, $P < 0.005$ (anti-CD4/TNF vs. control mAb).

anti-CD4 plus suboptimal or optimal anti-TNF, the increase in new limb involvement was only 3% ($P < 0.05$) and 0% ($P < 0.005$), respectively.

Histology. After establishing that combined anti-CD4/anti-TNF was remarkably effective in inhibiting the clinical progression of arthritis, we then determined whether this form of treatment could prevent erosion of cartilage and bone. At the end of the treatment period, arthritic limbs were processed for histology. One limb was studied from each mouse and in each case the paw chosen was the first to show clinical evidence of arthritis. To compare the effects of the different treatments, the PIP joint from the middle digit of each paw was examined and classified according to the presence or absence of erosion in either cartilage or bone (Fig. 3). Erosions were observed in almost 100% of the PIP joints from the control groups and in around 70–80% of the joints given either anti-CD4 alone or suboptimal anti-TNF alone (Table 2). However, in the group given an optimal dose of anti-TNF alone, the proportion of joints showing erosive changes was 54% ($P < 0.01$) whereas in the groups given anti-CD4 plus either suboptimal or optimal anti-TNF, only 22% ($P < 0.01$) and 31% ($P < 0.01$) of the joints, respectively, were eroded. Thus, 300 μg of anti-TNF alone gave a degree of protection against joint

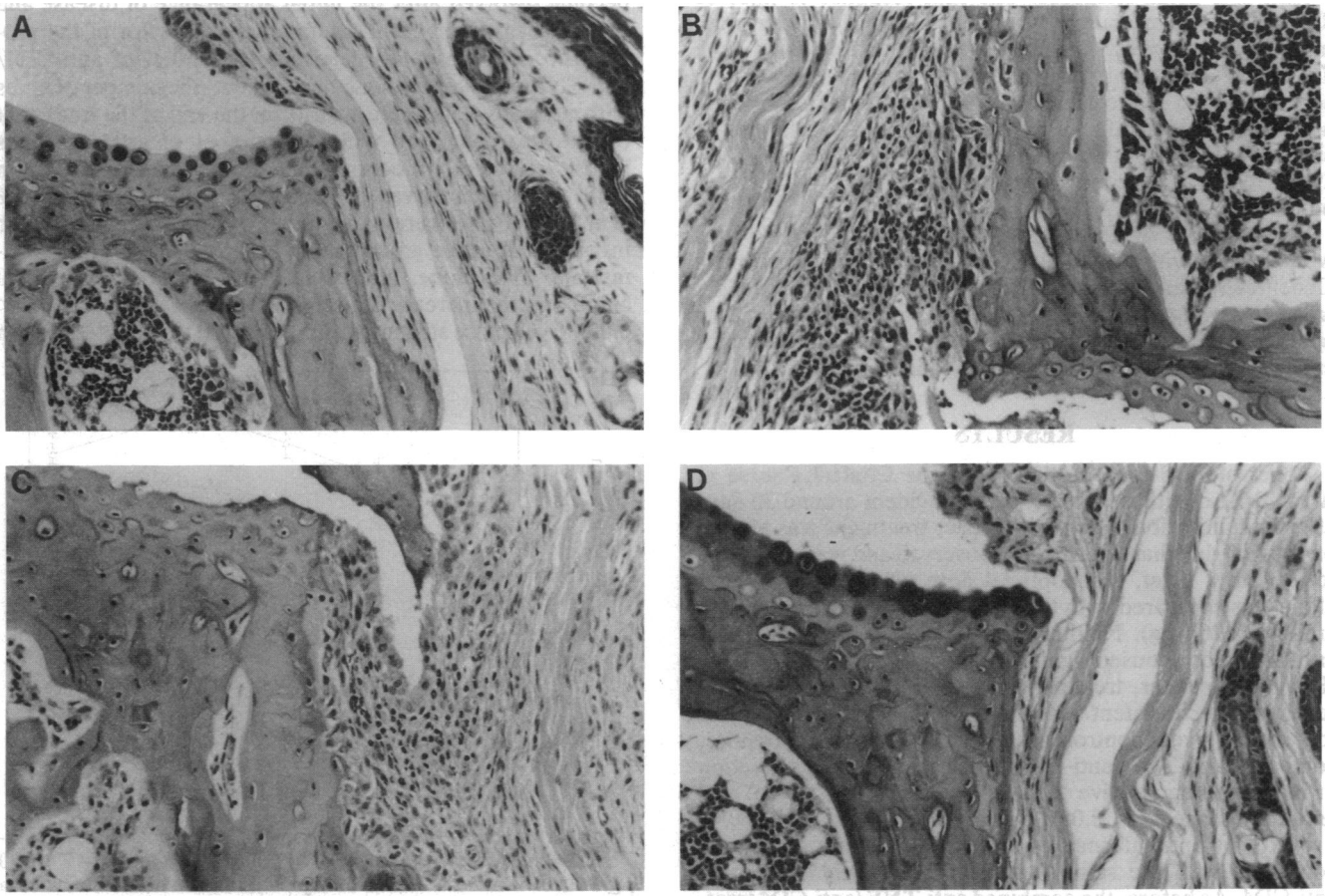

FIG. 3. Saggital histological sections of PIP joints. (*A*) Normal mouse. (*B*) Arthritic mouse treated with control mAb. Note the marginal erosion. (*C*) Mouse treated with anti-CD4 alone. Treatment fails to prevent erosion. (*D*) Mouse treated with anti-CD4 plus anti-TNF. There is no discernible erosion. (Hematoxylin/eosin; ×100.)

Table 2. Histology

Treatment	No. joints with erosions/ no. total joints
Suboptimal anti-TNF (50 μg)	
Anti-CD4	13/18 (72)
Anti-TNF	14/19 (74)
Anti-CD4/TNF	4/18 (22)*
Control mAb	17/18 (94)
Optimal anti-TNF (300 μg)	
Anti-CD4	10/12 (83)
Anti-TNF	6/11 (54)†
Anti-CD4/TNF	4/13 (31)‡
Control mAb	12/12 (100)

Proportions of PIP joints showing significant erosion of cartilage and/or bone. Data were compared by χ^2 analysis. Data in parentheses are percent joints with erosion. *, $P < 0.01$ (anti-CD4/TNF vs. anti-CD4 alone, anti-TNF alone, and control mAb); †, $P < 0.01$ (anti-TNF alone vs. control mAb); ‡, $P < 0.01$ (anti-CD4/TNF vs. anti-CD4 alone and control mAb).

erosion but combined anti-CD4/anti-TNF provided even greater protection.

Depletion of CD4+ T Cells. The extent to which anti-CD4 treatment depleted peripheral CD4+ T cells was determined by flow cytometry. Anti-CD4 treatment resulted in 98% (±1%) depletion of CD4+ T cells in the spleen and 96% (±3%) depletion of CD4+ T cells in the blood. Membrane-bound rat IgG was not detected by flow cytometry on spleen or peripheral blood cells taken from anti-CD4-treated mice, indicating that the lack of CD4+ T cells detected was not due to masking by rat anti-CD4+ mAb.

The possible persistence of CD4+ T cells in the joint despite apparent elimination of peripheral CD4+ T cells was next investigated by immunohistochemical analysis of sections from treated arthritic mice. Small numbers of CD4+ cells were detected in the joints, not only of control mice but also of those treated with anti-CD4 (data not shown), suggesting that anti-CD4 treatment had not eliminated all CD4+ T cells from the joint. Positive staining was not due to the presence of injected antibody on the surface of cells, since negligible staining was seen when an isotype control mAb was substituted for anti-CD4 in the staining protocol or when the primary antibody was omitted.

Anti-Collagen IgG Levels. Serum levels of anti-type II collagen IgG were not significantly altered within the 10-day treatment period by anti-CD4, anti-TNF, or anti-CD4 plus anti-TNF (data not shown).

Anti-Globulin Response. To find out whether anti-CD4 treatment prevented a neutralizing anti-globulin response against the anti-TNF mAb, IgM anti-TN3-19.12 levels on day 10 were compared. The results demonstrated that anti-CD4 was highly effective in preventing the development of an anti-TN3-19.12 antibody response (Table 3). Next, to determine whether anti-CD4 treatment led to increased levels of circulating TNF-α (by reducing the antibody response to the hamster anti-TNF), an ELISA was carried out in which recombinant murine TNF-α was used to detect free TN3-19.12 in the sera of mice on day 10 of the experiment. Levels of TN3-19.12 were slightly elevated in the groups given anti-CD4 plus anti-TNF compared to anti-TNF alone, though the differences were not significantly different (Table 3).

Table 3. IgM anti-TN3 titers and levels of unbound TN3 in arthritic mice on day 10 after initiation of treatment

Treatment	Reciprocal of anti-TN3 titer	Unbound TN3, μg/ml
Suboptimal anti-TNF (50 μg)		
Anti-TNF (n = 12)	242	8.6 ± 2.0
Anti-CD4/TNF (n = 12)	84*	12.1 ± 1.9
Optimal anti-TNF (300 μg)		
Anti-TNF (n = 12)	528	90.7 ± 11.9
Anti-CD4/TNF (n = 12)	91*	102.7 ± 12.5

Intraperitoneal injections of TN3 (with or without anti-CD4) were given on days 1, 4, and 7. Data for the anti-TN3 titer are the mean and for unbound TN3 are the mean ± SEM. *, Significantly reduced anti-TN3 titer ($P < 0.005$; Mann–Whitney test).

DISCUSSION

The experiments reported here show that anti-CD4 acts synergistically with anti-TNF in ameliorating the joint disease of established CIA. The beneficial effects of anti-CD4/anti-TNF treatment were observed in all the parameters tested: in the suppression of paw-swelling, in the prevention of new limb involvement, and above all, in the protection against the erosive changes normally associated with this form of arthritis. Anti-CD4 treatment caused some reduction in paw-swelling but had little effect on the histological outcome of arthritis even at a cumulative dose of 24 mg/kg of body weight. In human RA, anti-CD4 has been shown to provide clinical benefit to some patients (16), though many patients do not respond to treatment despite severe peripheral T-cell depletion (23, 24). This suggests that, as in CIA, depletion of peripheral CD4+ T cells alone is insufficient to consistently modify ongoing disease. In the present study, CD4+ T cells were found in small numbers in the joints of arthritic mice, in keeping with a previous report (25). However, a small number of CD4+ cells were also found in the joints of anti-CD4-treated mice despite the apparent elimination of >95% of peripheral CD4+ T cells. This may be significant in the context of a report that anti-CD4 treatment of mice after immunization with type II collagen does not abrogate collagen-specific T-cell proliferative responsiveness, suggesting that activated collagen-specific T cells are resistant to depletion (26).

In agreement with our previous findings (14), anti-TNF reduced the severity of arthritis (Fig. 2), though anti-CD4 increased the beneficial effects of both optimal and suboptimal doses of anti-TNF. It is likely that the principal ameliorative effect of anti-TNF involves neutralization of TNF-α in the joint, with a consequent reduction in TNF-α-mediated pathology. The available evidence indicates a major role for TNF-α in joint pathology. Thus, anti-TNF antibodies or soluble TNF receptors protect against CIA (14, 27, 28), whereas TNF-α exacerbates disease (27, 29, 30), and the joints of mice expressing human TNF-α transgenes show erosive changes that are similar to those found in CIA and RA (31).

Tolerance induction by anti-CD4 treatment has been described (22, 32) and one way in which anti-CD4 could enhance the neutralization of TNF-α is by preventing a neutralizing anti-TN3-19.12 response, thereby increasing the availability of the injected mAb. Anti-CD4 treatment was indeed found to prevent an anti-TN3-19.12 response, a fact that may be important in terms of maintaining long-term treatment of human disease. However, serum levels of unbound TN3-19.12 were only slightly higher in the anti-CD4-treated mice and it is unlikely that this small difference would be sufficient to account wholly for the reduction in the severity of arthritis. Furthermore, the reduction in arthritis severity was greater in the mice given 50 μg of TN3-19.12 plus anti-CD4 than in those given 300 μg of TN3-19.12 alone, despite the fact that their serum levels of TN3-19.12 were considerably lower (Table 3). It should be pointed out, however, that serum levels of TN3-19.12 do not necessarily reflect the degree of local neutralization of TNF-α within the joints. It is possible, for example, that TN3-19.12 complexed with mouse antibody to hamster IgG retains its ability to bind TNF-α but is unable to penetrate the joints.

Another way in which anti-CD4 could synergize with anti-TNF is by reducing the pathology caused by mediators other than TNF-α that are dependent on CD4$^+$ T cells, such as anti-collagen antibodies (15). Our studies showed, however, that anti-CD4 treatment had no effect on the serum levels of anti-type II collagen IgG, indicating that the beneficial effects of anti-CD4 were not attributable to a reduction in anti-collagen antibody levels. It could also be argued from this finding that anti-collagen antibodies do not alone cause significant pathology, since they were found at high levels in anti-CD4/anti-TNF-treated mice, even in those that were apparently free from pathology.

A number of proinflammatory cytokines, other than TNF-α, have been identified within the inflamed synovial membranes of RA patients, including IL-1 and GM-CSF (33). These cytokines may be important in contributing to the pathology observed in CIA. IL-1, for example, displays properties similar to those of TNF-α (34) and has been shown to trigger the onset of arthritis in collagen-immunized DBA/1 mice (35). It has been proposed that the production of the predominantly macrophage-derived cytokines within the inflamed joint is largely CD4$^+$ T-cell-dependent (1) and this may be an important contributory factor in the synergy observed between anti-CD4 and anti-TNF. However, a pathway of macrophage activation, involving TNF-α, also exists that may be T-cell-independent (36) and studies with rheumatoid synovial cell cultures indicate that TNF-α stimulates the production of both IL-1 (9) and GM-CSF (10).

In the light of these observations, the following hypothesis may account for the synergistic effect of anti-CD4 and anti-TNF in this model. After antigenic stimulation, CD4$^+$ T cells are involved in stimulating the pathological overexpression of cytokines, including TNF-α and IL-1, by macrophage-type cells. Early anti-CD4 treatment interrupts this process and prevents arthritis. Anti-CD4 treatment of established disease, however, has little effect on the disease because of the persistence of small numbers of T cells in the joint and/or because TNF-α (and other mediators), already present within the joints, may perpetuate the disease process. In contrast, anti-TNF treatment reduces TNF-α-mediated pathology but, because of continued CD4$^+$ T-cell involvement, is unable to prevent pathology caused by additional mediators that are dependent on CD4$^+$ T cells. Combined anti-CD4/anti-TNF treatment, on the other hand, reduces the pathology attributable not only to TNF-α but also to the additional CD4$^+$ T-cell-dependent mediators.

In conclusion, we have demonstrated that anti-CD4 acts synergistically with anti-TNF in the amelioration of established CIA. This combined therapeutic approach may provide the basis for an effective long-term treatment for RA, although the potential risks due to diminished immune function would first need to be evaluated. Recently, anti-TNF has been used to treat 20 long-standing and active RA patients, all of whom showed marked clinical improvement, accompanied by a concordant improvement in laboratory indices of disease activity, such as the serum level of C-reactive protein (17). The results presented here argue strongly in favor of a combined approach to the treatment of RA that not only targets TNF-α but also suppresses CD4$^+$ T-cell involvement in the disease process and reduces the risk of sensitization to the therapeutic agents.

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