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Paradoxical Effects of Adenovirus-Mediated Blockade of TNF Activity in Murine Collagen-Induced Arthritis

Emilia Quattrocchi,* Marita Walmsley,2* Kylie Browne,3* Richard O. Williams,* Lilia Marinova-Mutafchieva,* Wim Buurman,† Debra M. Butler,* and Marc Feldmann4*

Collagen-induced arthritis (CIA) is an experimental model of arthritis widely used to dissect the pathogenesis of human rheumatoid arthritis and to identify potential therapeutic targets. Among these, TNF-α has been recognized to play an important role. Here we investigate the feasibility and therapeutic efficacy of prolonged blockade of TNF-α activity through the adenovirus-mediated gene delivery of a dimeric chimeric human p55 TNFR-IgG fusion protein and compare it to protein therapy in established CIA. A single i.v. administration of the replication-deficient adenovirus yielded microgram serum levels of the chimeric fusion protein and ameliorated CIA for 10 days. Subsequently, benefit was lost and a rebound to greater inflammatory activity was observed despite the continual presence of bioactive TNF fusion protein. A similar trend was also observed in mice injected directly with comparable amounts of a human TNFR-IgG fusion protein, whereas the administration of a control adenovirus-encoding β-galactosidase or of a control human IgG1 protein did not significantly affect the disease course. The mechanisms of the rebound of CIA were investigated, and augmented Ab response to collagen type II and TNFR were identified as potential causes. Our results confirm the feasibility of adenovirus-mediated gene delivery of cytokine inhibitors in animal models of autoimmune diseases for investigational purposes and highlight the importance of prolonged studies. Further investigations are needed to optimize ways of exploiting the potential of adenoviral gene therapy in RA. *The Journal of Immunology, 1999, 163: 1000–1009.

Rheumatoid arthritis (RA) is a common (about 1% prevalence) chronic autoimmune and inflammatory disease in which there is destruction of the synovial joints, leading to progressive disability with loss of joint function. Despite a lack of knowledge about its etiology, there has been recent significant progress in understanding its pathogenetic mechanisms. Analysis of cytokine expression and regulation in rheumatoid synovial cultures has led to the hypothesis that TNF-α regulates the production of other proinflammatory cytokines such as IL-1, IL-6, IL-8, and GM-CSF (1, 2) and therefore could be a potential target for therapy. This concept has been supported by studies in an animal model of RA, collagen-induced arthritis (CIA), where anti-TNF-α Abs given after onset of clinical symptoms significantly inhibit disease progression (3, 4). The important role of TNF-α in RA was finally established in clinical trials where anti-TNF-α mAbs administered to patients with long-standing active RA were found to be beneficial (5–8). More recently, the p55 TNFR Ig fusion protein was found to be effective in RA (9) and, to a lesser extent, also the p55 TNFR Ig fusion protein (10, 11).

The definition of TNF-α as a therapeutic target has led to a search for other efficient means of blocking its activity. Low-molecular mass chemicals that specifically block TNF-α activity have not been identified; however, drugs such as SB 203580, which blocks p38 mitogen-activated protein kinase (12), and phosphodiesterase type IV inhibitors such as rolipram (13) have been shown to reduce the production of TNF-α and other cytokines. Another possible approach is gene therapy, which has the potential to become an effective therapeutic option if certain problems can be overcome, chief among them being the availability of effective vectors for gene delivery. Two viral systems have dominated the literature in this field. Retroviruses were the first to be used, which, as they only infect dividing cells, are used in ex vivo procedures, where proliferating cells are cultured, infected, and selected in vitro, then reimplanted containing the gene of interest (14, 15). Adenoviruses, a more recent entrant to the field, are efficient at transferring the gene of interest, can infect a wide range of dividing and nondividing cells, and can generate large amounts of proteins over extended periods of time (16). The adenoviruses used in gene therapy are replication deficient due to deletion of the E1 early transcriptional region and can be produced and purified in large quantities. There is no insertion into host DNA, therefore no risk of insertional mutagenesis (16). However, there are problems with the use of adenoviruses for gene therapy. As several studies have shown, adenoviruses are immunogenic and also the cells expressing the gene product can become immunogenic, leading to an immune response against the producing cells and cessation of gene expression (17, 18). Nevertheless, immunosuppressive therapy with a variety of agents can reduce the immune response to adenoviruses, thus prolonging gene expression (19, 20).

CIA is induced in DBA/1 mice by intradermal injection of native collagen type II (CII) in CFA and has some important similarities to human arthritis. For example, in both RA and CIA strong

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*Kennedy Institute of Rheumatology, London, United Kingdom; and 1Department of Surgery, University of Limburg, Maastricht, The Netherlands

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2 Current address: National Institute Medical Research, London, U.K.

3 Current address: Austin Hospital Institute for Medical Research, Melbourne, Australia.

4 Address correspondence and reprint requests to Dr. Marc Feldmann, Kennedy Institute of Rheumatology, 1 Aspenlea Road, Hammersmith, London W6 8LH, U.K.

E-mail address: m.feldmann@cxwms.ac.uk

5 Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; AdTNFR, p55 TNFR adenovirus; Adβgal, β-galactosidase adenovirus; CII, collagen type II.

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In the light of the clinical success of TNF-α blockade, by means of Abs or soluble TNFR, we have investigated the feasibility and efficacy of adenoviral gene delivery of the 55-kDa TNFR Ig fusion protein as a therapeutic strategy against CIA.

Materials and Methods

Recombinant adenovirus vectors

The recombinant adenovirus vectors used in this study, based on human adenovirus type 5, have been rendered replication deficient by deleting the E1a and E1b regions of the viral genome; each vector contains an expression cassette with the CMV early promoter, the pUC 19 polylinker, SV40 splice, and poly(A) signal sequences. The adenovirus encoding the human 55-kDa TNF-sensitive Ig fusion protein (AdTNFR, kindly donated by Drs. B. Beutler and J. Kolls, University of Texas, Southwestern Medical Center, Dallas, Texas), was constructed as previously described (25, 26). A control adenovirus encoding the Escherichia coli β-galactosidase (Adβgal) was generously donated by Dr. M. Wood (University of Oxford, Oxford, U.K.).

Viruses were propagated in the 293 human embryonic kidney carcinoma cell line routinely cultivated in DMEM (BioWhittaker, Verviers, Belgium) containing 5% FCS (Life Technologies, Paisley, U.K.), 2 mM L-glutamine (Flow Laboratories, Irvine, U.K.), and 100 U/ml penicillin/streptomycin (Life Technologies) in 5% CO2 at 37°C. When ~80% confluent, culture medium was replaced with DMEM 2% FCS plus live virus at a multiplicity of 10. After 48 h, most of the cells rounded up and confluent, culture medium was replaced with DMEM 2% FCS plus live protein as a therapeutic strategy against CIA.

Animal studies

The induction and assessment of CIA was performed as previously described (27). Briefly, male BALB/c mice, 8–10 wk old, were immunized intradermally with 100 μg of bovine CII emulsified in CFA (Difco Laboratories, West Molesey, U.K.). The onset of arthritis was considered to be the day that erythema and swelling were first observed, and arthritic mice were given a daily clinical score per limb from 0 to 3, with 0 = normal, 1 = slight erythema and swelling, 2 = pronounced edematous swelling, and 3 = joint deformity with ankylosis, resulting in a maximum score of 12 per animal. Paw swelling was assessed using calipers (Krogh, London, U.K.). Paw swelling was assessed by calipers (Krogh, London, U.K.).

Treatment protocol

Adenoviral vectors (2 × 10⁹ and 2 × 10⁸ pfu in 100 μl of isotonic saline) were injected into the tail vein i.v. once at the disease onset (day 1), and mice were monitored for disease activity up to day 21. Unimmunized DBA/1 mice were also injected i.v. with the higher dose of the virus as controls, monitored for 21 days, and showed no signs of illness. All animals that received recombinant adenovirus survived until sacrifice. Postmortem examination of the gross morphology of their liver, which is the target organ of adenovirus i.v. inoculation, showed no signs of pathology.

Human 55-kDa TNFR-human IgG1 fusion protein (p55-S12) was produced at Centocor (a gift of Dr. B. Scallon and J. Ghayeb, Malvern, PA). It is a dimer of the extracellular domain of the human 55-TNFFR where each TNF is fused to a partial J segment that is attached to the whole of the constant region of the human IgG1 H chain (28). Two hundred microliters were administered i.p. in 200 μl of saline on days 1, 3, 5, 7, 11, 13, and 15 after the onset of disease. As control, an IgG1 with the same human IgG1 backbone used for the fusion construct but without the TNFR moieties, also produced at Centocor, was given i.p. following the same regimen.

A murine chimeric rat anti-mouse TNF Ab (murine IgG1 clone cE1q) (provided by Dr. B. Scallon and J. Ghayeb, Centocor), was also injected i.p. at a dose of 500 μg, three times per week, for 3 wk.

Murine CTLA4-Ig, (a gift from Dr. Glenn Larsen, Genetics Institute, Boston, MA) was administered (50 μg/i.p.) at the onset of arthritis, which was 24 h before the AdTNFR i.v. injection. Controls received the same dose of CTLA4-Ig and isotonic saline alone i.v. 24 h later. Blood samples were obtained from all treated and control mice on day 3, 10, and 21, and the serum was stored at −80°C until use.

Soluble TNFR assay

Soluble p55 human TNFR was assayed in diluted serum samples by ELISA as previously described (29). Monoclonal anti-human p55 TNFR was used as capture Ab, and polyclonal rabbit IgG anti-p55 TNFR Ab conjugated to biotin was used as the detection Ab. Streptavidin-HRP conjugates were used to detect the biotinylated Abs followed by a chromogenic substrate of tetramethylbenzidine dihydrochloride (Kirkegaard and Perry, Gaithersburg, MD). Absorption was measured at 450 nm, and results were expressed as the mean of triplicate samples.

Estimates of serum TNFR-Ig concentrations were calculated with reference to a recombinant truncated human p55 TNFR standard with a molecular mass of ~25–30 kDa. The results are expressed as μg/ml of p55 TNFR equivalents, although comparable OD readings were obtained with the recombinant TNFR-Ig fusion protein subsequently provided (Centocor).

Bioassay of TNF inhibitory activity

To verify that the TNF-IgG1 present in serum was bioactive, TNF-α inhibitory levels were tested in serum samples of treated mice. Inhibition of the TNF-sensitive murine fibrosarcoma WEHI 164 was assessed (30). Briefly, 2 × 10⁵ WEHI cells/well with 100 μl DMEM supplemented with 5% FCS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin and were adhered overnight in 5% CO2 at 37°C. Serum samples were serially diluted in separate wells in 100 μl DMEM and incubated with recombinant murine TNF-α (Dr. A. Meager, National Institute of Biological Standards and Controls, Potters Bar, U.K.) at a concentration of 10 μg/ml, which generates 60% cytoxicity and is within the linear region of the dose-response curve, to allow the soluble TNFR-IgG1 present to bind and neutralize TNF-α. After 1 h, they were added to the WEHI cells cultured with actinomycin D at a final concentration of 0.5 μg/ml. Cells were incubated overnight, then 10 μl of MT at 5 mg/ml in PBS were added, followed by another overnight incubation at 37°C. Viable cells were quantitated by solubilizing formazan crystals with 100 μl of 10% SDS in 0.1 M HCl per well. OD was assessed at 540 nm using a Multiskan (Labsystems, Helsinki, Finland).

Proliferation assay

Inguinal and popliteal lymph nodes were aseptically removed from AdTNFR-treated and control arthritic mice on day 10 or 21 after start of treatment. Then, 5 × 10⁷ cells were cultured in 0.2 ml of DMEM supplemented with 2% heat-inactivated FCS in a 96-well flat-bottom plate containing WEHI 164 was assessed (30). Briefly, 2 × 10⁵ WEHI cells/well with 100 μl DMEM supplemented with 5% FCS, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin and were adhered overnight in 5% CO2 at 37°C. Serum samples were serially diluted in separate wells in 100 μl DMEM and incubated with recombinant murine TNF-α (Dr. A. Meager, National Institute of Biological Standards and Controls, Potters Bar, U.K.) at a concentration of 10 μg/ml, which generates 60% cytoxicity and is within the linear region of the dose-response curve, to allow the soluble TNFR-IgG1 present to bind and neutralize TNF-α. After 1 h, they were added to the WEHI cells cultured with actinomycin D at a final concentration of 0.5 μg/ml. Cells were incubated overnight, then 10 μl of MT at 5 mg/ml in PBS were added, followed by another overnight incubation at 37°C. Viable cells were quantitated by solubilizing formazan crystals with 100 μl of 10% SDS in 0.1 M HCl per well. OD was assessed at 540 nm using a Multiskan (Labsystems, Helsinki, Finland).

Ab determinations

Anti-CII Ab levels were tested in individual sera using a standard ELISA. Microtiter plates were coated with 2 μg/ml of CII dissolved in TBS overnight at 4°C (31). After blocking for 1 h with 1% BSA, dilutions of sera from 1/50 to 1/6400 were applied to the wells. For isotype quantitation, anti-mouse IgG1 and IgG2 linked to alkaline phosphatase (The Binding Site, Birmingham, U.K.) were used at a dilution of 1:5000. The plates were developed using p-nitrophenyl phosphate (Sigma, St. Louis, MO) as substrate, and OD was assessed at 405 nm. Anti-CII IgG2b Abs were quantitated using sheep anti-mouse IgG2b conjugated to peroxidase at a dilution of 1:10,000 (The Binding Site) and tetramethylbenzidine dihydrochloride (Kirkegaard and Perry) was used as substrate, and OD was assessed at 450 nm. Each plate included a standard curve of a positive serum used to define arbitrary units of total IgG, IgG1, IgG2a, and IgG2b anti-CII Abs.
Adenovirus-mediated gene transfer of TNFR is therapeutic in the early phase of CIA, but not later. Three groups of DBA/1 mice were immunized for arthritis with CII. On the day of arthritis onset (day 1), $2 \times 10^3$ particles of AdTNFR in 100 µl of saline ($\bullet$, $n = 10$) or $2 \times 10^7$ particles ($\Delta$, $n = 9$), or saline alone ($\blacksquare$, $n = 10$) were injected i.v. into the tail vein. Mice were examined every other day for 21 days for signs of joint inflammation and scored as previously described. Mean clinical score presented as average clinical score per group $\pm$ SEM. Statistically significant differences between treatments are indicated ($*, p < 0.02$, $**, p < 0.001$). A representative result from three experiments is shown.

Anti-murine TNFR Abs (total IgG) were also measured by ELISA. Briefly, plates were coated with 1 µg/ml of nonpurified (culture supernatant) murine 55-kDa TNFR overnight at 4°C. After blocking for 2 h with 2% BSA, sera diluted 1:50 were added in duplicates and incubated at room temperature for 1 h. Anti-mouse total IgG linked to alkaline phosphatase (The Binding Site) diluted 1:5000 was then added for 1 h, and plates were developed using p-nitrophenyl phosphate (Sigma). Absorbance was assayed at 450 nm, and results were expressed in OD per individual serum tested.

Histopathology

Arthritic hind paws (the first affected hind paw of each mouse) were removed postmortem on the 21st day after onset of clinical symptoms and of treatment, fixed in 10% buffered formalin, and decalcified in 5.5% EDTA. Microscopic evaluation of arthritic paws was performed by an observer in a blinded fashion. The severity of arthritis in the distal interphalangeal joints, proximal interphalangeal joints, and metatarsophalangeal joints was classified as normal, mild, moderate, or severe based on the following criteria: normal = no damage; mild = minimal synovitis, cartilage loss, and bone erosions limited to discrete foci; moderate = synovitis and erosions present but intact joint architecture; and severe = extensive erosions and disrupted joint architecture.

Statistical analysis

For statistical analysis of macroscopic data, the Mann-Whitney U test of significance was applied using a Minitab statistical software package (Minitab, State College, PA). Differences were considered statistically significant when $p < 0.05$.

Results

Expression and bioactivity of the TNFR fusion protein

Mice injected with $2 \times 10^6$ pfu of AdTNFR and controls were serially bled 3 days, 10 days, and 21 days after the onset of arthritis. Sera were then assayed for the presence of the human
The data showed that while incubation with 10 pg/ml of murine TNF to allow binding to the receptor present, then added to the diluted, preincubated for 1 h with 10 pg/ml of recombinant murine-ized or degraded. For this purpose, serum samples at day 3 and 21 viral construct was still bioactive or whether it had been neutralized or degraded to the development of CIA (31). This observation demonstrated the importance of costimulation and Ag presentation to T cells in the pathogenesis of CIA, even after disease onset. Therefore, to investigate the pathogenetic mechanism leading to the late exacerbation observed in our experiments with the AdTNFR treatment, we first attempted to reduce T lymphocytes responses through the administration of a suboptimal dose of murine CTLA4-Ig. Mice were given one dose of CTLA4-Ig (50 μg) i.p. 24 h before the i.v. injection of 2 × 10^8 pfu of AdTNFR, or saline, and the disease was monitored for 21 days. As shown in Fig. 4, animals that received this low dose of CTLA4-Ig alone (n = 7) showed an improvement in disease progression (p < 0.05) from day 6 to day 12 compared with controls. In contrast, pretreatment on day 1 with CTLA4-Ig and subsequent injection on day 2 with the higher dose of the virus resulted instead in a clinical profile identical with the group treated with AdTNFR alone, with a statistically significant benefit starting on day 5 (p = 0.03) until day 10, followed by an exacerbation of inflammation by day 18 (p = 0.033). Therefore, despite the transient immunosuppression clearly caused in the control mice by the low dose CTLA4-Ig administered, no additive or synergistic effect was observed when both immunosuppressive agents were injected together.

Effect of AdTNFR on lymphocyte proliferation to CII
The development of CIA is associated with high levels of both cell-mediated and humoral immunity to collagen (33). To determine whether AdTNFR treatment was affecting the specific T cell responses to collagen and whether a difference between the early and late phase of AdTNFR treatment could be detected, lymph node T cell proliferation from AdTNFR-treated and arthritic controls, on day 10 and 21 of arthritis, was ascertained. In the same experiment, which is representative of three, the specific proliferative response to the virus in vitro was also monitored, and as an additional control a group of naive DBA/1 mice injected with

55-kDa TNFR fusion protein by ELISA (32). The dimeric protein encoded by the adenovirus was already present at high concentrations on day 3, with the highest level (in the range of 100 μg ml^-1) reached on day 10. Thereafter, levels of TNFR remained high but started to decline on day 21 after disease onset (Fig. 3). Control mice that received either saline or Adβgal virus did not show detectable levels of human TNFR at any time.

Because the TNFR was still present in the sera of AdTNFR-treated mice on day 21, but its effect did not correlate with the disease course after day 10 (Fig. 3), a biological assay on the TNF-sensitive murine fibrosarcoma cell line WEHI 164 was performed to test whether the fusion protein produced by the adenoviral construct was still bioactive or whether it had been neutralized or degraded. For this purpose, serum samples at day 3 and 21 from both the AdTNFR- and the Adβgal-treated mice were serially diluted, preincubated for 1 h with 10 pg/ml of recombinant murine TNF to allow binding to the receptor present, then added to the WEHI cell culture and tested for inhibition of TNF cytotoxicity. The data showed that while incubation with 10 pg/ml of murine TNF-α alone resulted in ~60% cytotoxicity, preincubation with sera from the AdTNFR treated mice reduced the TNF cytotoxic effect to 20% on day 3 and 45% on day 21 (p < 0.05). The reduced degree of TNF inhibition observed on day 21 implied that the virally encoded TNFR was still present, even if at lower concentration, and bioactive at both time points. Thus, a lack of bioactive TNFR was not the mechanism of augmented disease from day 15.

**CTLA4-Ig prevents the development of arthritis yet does not prevent its late exacerbation in AdTNFR-treated mice**

In our laboratory, it has previously been shown that the i.p. injection of CTLA4-Ig at the time of collagen immunization prevents the development of CIA (31). This observation demonstrated the importance of costimulation and Ag presentation to T cells in the pathogenesis of CIA, even after disease onset. Therefore, to investigate the pathogenetic mechanism leading to the late exacerbation observed in our experiments with the AdTNFR treatment, we first attempted to reduce T lymphocytes responses through the administration of a suboptimal dose of murine CTLA4-Ig. Mice were given one dose of CTLA4-Ig (50 μg) i.p. 24 h before the i.v. injection of 2 × 10^8 pfu of AdTNFR, or saline, and the disease was monitored for 21 days. As shown in Fig. 4, animals that received this low dose of CTLA4-Ig alone (n = 7) showed an improvement in disease progression (p < 0.05) from day 6 to day 12 compared with controls. In contrast, pretreatment on day 1 with CTLA4-Ig and subsequent injection on day 2 with the higher dose of the virus resulted instead in a clinical profile identical with the group treated with AdTNFR alone, with a statistically significant benefit starting on day 5 (p = 0.03) until day 10, followed by an exacerbation of inflammation by day 18 (p = 0.033). Therefore, despite the transient immunosuppression clearly caused in the control mice by the low dose CTLA4-Ig administered, no additive or synergistic effect was observed when both immunosuppressive agents were injected together.

**FIGURE 3.** Comparison between clinical score and expression of TNFR fusion protein. Gene expression in individual animals was determined by periodic serum quantification of the dimeric TNFR protein. Mice injected with 2 × 10^6 particles of AdTNFR on the day of arthritis onset (day 1) were scored for signs of inflammation and bled 3, 10, and 21 days later for serum TNFR determination by ELISA, as previously described. The results are expressed as μg/ml of p55 TNFR equivalents, although comparable OD readings were obtained with the recombinant TNFR-Ig fusion protein afterwards provided. Data represent mean ± SEM of seven mice.

**FIGURE 4.** Suboptimal administration of CTLA4-Ig controls arthritis progression but does not affect AdTNFR treatment. CIA-immunized mice were injected i.v. on the day of arthritis onset (day 1) with 2 × 10^8 particles of AdTNFR (•, n = 10), with CTLA4-Ig 50 μg i.p. in saline (□, n = 7; and ●, n = 7), or saline alone (□, n = 10). On day 2, one group of mice that received CTLA4-Ig (●, n = 7) was also injected with 2 × 10^6 particles of AdTNFR i.v. Arthritis incidence was then monitored every other day for 21 days and data expressed as mean clinical score per group ± SEM. Statistically significant differences between treatments are indicated (*, p < 0.05; **, p < 0.01).
AdTNFR and lymph node cells was assayed 10 days later. The results showed on day 10 a much higher proliferation to CII in arthritic vs nonimmunized DBA/1 mice (Fig. 5A). The AdTNFR-injected arthritic mice at day 10 also showed a degree of proliferation to the virus at the dose of 60 pfu/ml \( (p = 0.05) \). At both time points, all CIA immunized mice showed a statistically significant proliferation to CII \( (p = 0.03) \), regardless of the AdTNFR treatment (Fig. 5, A and B). Our results suggest that the AdTNFR early clinical amelioration and late exacerbation of CIA are not associated with changes in the specific T cell response to CII.

**Effect of the soluble 55-kDa TNFR fusion protein on CIA**

To rule out the possibility that the late exacerbation of CIA by AdTNFR was due to adenosinoviral infection, the effect of injecting human TNFR fusion protein was compared with the adenoviral delivery of a similar protein (but with a murine IgG tail) on CIA. Because of the high serum levels reached with AdTNFR, a large amount, 200 \( \mu g \) of p55-Sf2 fusion protein, was administered i.p. on alternate days (Fig. 6A). The clinical response was similar to that observed in animals that had received the higher dose of the adenovirus construct, suggesting that the paradoxical late disease-enhancing effect repeatedly observed was attributable to the TNFR fusion protein alone and not to the viral vector. To further investigate whether the human Ig portion present in our fusion protein could somehow affect the late stage of CIA, 200 \( \mu g \) of a control human IgG1 (of the same isotype used to make the fusion protein construct) were injected at the same regimen in a following experiment. As shown in Fig. 6B, the human IgG1 protein did not alter the disease process, thus suggesting that the soluble 55-kDa human TNFR part of the fusion protein, and not the human Ig portion of the molecule, affects the late course of CIA.

**Anti-collagen and anti-TNFR Ab responses**

CIA development has been shown to be dependent on humoral immune responses to CII, with the anti-collagen IgG2a isotype playing a major pathogenic role (34, 35). Previous studies from our laboratory on treatment of CIA for 10 days with p55-Sf2 did not show differences in total serum anti-collagen Ab levels between treated groups and controls (36). Therefore, we decided to evaluate and compare the anti-collagen IgG isotype distribution present in the AdTNFR-injected mice in the p55-Sf2-treated mice and in same stage arthritic controls over a longer time period (Fig. 7). Our data showed that in the 21-day CIA control group all anti-collagen IgG subclasses were present, with IgG2a and IgG2b equally represented and higher than the IgG1 subset. Interestingly, both the
FIGURE 7. TNFR-treated mice have increased humoral responses to CII. CIA-immunized DBA/1 mice were administered 2 × 10⁸ particles/mouse of AdTNFR on the day of arthritis onset (AdTNFR group; n = 10) or multiple injections of p55-Sf2 200 µg i.p. in saline as in Fig. 6A (p55-Sf2 group; n = 10) or saline alone (controls; n = 10). Mice were bled on day 21, and levels of anti-CII Abs were measured by ELISA. Open bars, total IgG1; filled bars, IgG2a; striped bars, IgG2b. Mean values ± SEM and IgG2a/IgG1 ratios are reported for each treatment group.

AdTNFR- and the p55-Sf2-treated groups had higher concentrations of all subclasses of anti-collagen Abs, but their IgG2a/IgG1 ratio was comparable to the arthritic controls. Therefore, although the response to collagen was quantitatively increased in the TNFR-treated groups, it seems qualitatively comparable with the late stage of CIA development.

It then seemed possible that the human p55 TNFR was eliciting a response that cross-reacted with the mouse p55 TNFR. Abs to the human p55 TNFR were assayed by ELISA, but, probably because of the very high levels of human TNFR Ig in the mouse serum (see Fig. 3), these were not detected.

Anti-mouse TNF responses were also evaluated: total IgG Abs toward the murine 55-kDa TNFR were detected, but a degree of binding was also observed in all arthritic controls. Nevertheless, the difference between AdTNFR-treated and control groups (n = 7 per group) was statistically significant at day 10 (p = 0.01) in three separate experiments (data not shown). As most mAbs to p55 TNFR are agonistic, we attempted to ascertain if the sera from AdTNFR or control mice were agonistic and able to kill the TNF-sensitive cell line WEHI 164. Mouse serum at concentration >1% interfered with their adherence, and while some lysis was detected with sera from AdTNFR and p55 IgG immunised mice, this did not reach statistical significance.

Effect of prolonged TNF neutralization with anti-TNF-α mAb on CIA

To elucidate whether prolonged neutralization of TNF during the course of CIA was responsible for the late exacerbation of disease observed with the administration of the soluble p55 TNFR, we injected i.p. a group of mice (n = 10) with 500 µg of a rat anti-mouse TNF Ab (cV1q) three times a week for 3 wk. Control mice (n = 10) received PBS. As shown in Fig. 8, blockade of TNF activity, in this experiment, significantly suppressed CIA (p = 0.0007 on day 21), strongly arguing against a direct involvement of prolonged TNF blockade per se in the rebound effect observed with the TNFR fusion protein treatment.

Effect of AdTNFR treatment on joint histopathology

The histological analysis of the interphalangeal joints from arthritic and Adβgal controls and from mice treated with 2 × 10⁸ particles of AdTNFR was performed on day 21 after disease onset. Fig. 9 shows photomicrographs taken from representative AdTNFR-treated and control animals. Arthritic controls and mice that received the control adenovirus Adβgal showed active chronic proliferative synovitis. The synovial lining layer was villous, hyperplastic, and infiltrated by neutrophils. The subintima contained a diffuse infiltrate of neutrophils, lymphocytes, and plasma cells with fibroblasts in the periphery. Pannus formation caused severe erosions of the articular cartilage and the subchondral bone. Prominent neovascularization associated with pannus was also observed. In some joints, pannus was very extensive with expansion into the bone marrow space, and marrow elements were replaced by granulation tissue. The cartilage showed severe degenerative changes with fragmentation of the surface and many necrotic chondrocytes (Fig. 9A). Paws removed from AdTNFR-treated mice showed a delay in the disease progression. Joint pathology was characterized by acute inflammation of the synovium, including oedema and intense infiltration by neutrophils and mononuclear cells. Acute synovitis was accompanied by hyperplasia of synovial intima and accumulation within the joint space of polymorphs, cell debris, and macrophages immersed in a fibrin web. Active ingrowth of granulation tissue from the synovium, leading to the formation of pannus that covered and eroded the articular cartilage and the subchondral bone, was also observed. The surrounding tissue was oedematous with the presence of mast cells.
The distribution and local accumulation of mast cells varied between specimens, but they were mainly increased at sites of pannus invasion and in the fibrous synovial tissue than in areas with active inflammatory cells infiltrate (Fig. 9B).

**Discussion**

The clinical successes of blocking TNF-α, in both RA and Crohn’s disease, are some of the major achievements based on the understanding of the roles of cytokines in disease pathogenesis. Specific down-regulation of TNF-α activity in both RA and CIA has relied predominantly on two approaches using biological agents: injection of neutralizing anti-TNF-α mAbs (3–8) and administration of soluble TNFR fusion proteins (9, 37). These reports have demonstrated consistent therapeutic efficacy, with differences in experimental design, specific pharmacokinetics, and TNF binding properties of the TNF neutralizing compounds presumably accounting for the observed variations on clinical benefit.

Effective control of autoimmune arthritis requires flexible, prolonged neutralization of proinflammatory mediators, and gene therapy offers several potentially unique advantages over previous protein therapies (38), thus we and others have taken an interest in this field. Replication-deficient adenoviruses have proven to be suitable vectors for delivering in vivo high amounts of a specific protein over extended periods of time (16), although limitations related to the host immune responses have been reported (38, 39).

The main goal of the present study was to assess whether blockade of TNF-α activity through adenovirus-mediated gene transfer of a soluble TNFR is feasible and effective compared with protein therapy in a chronic inflammatory disease model such as CIA. The TNF inhibitor we used was an adenovirally encoded chimeric fusion protein consisting of a dimeric mouse IgG1 H chain coupled to two human 55-kDa TNFR extracellular domain (26). This particular adenovirus construct was chosen for its demonstrated efficacy in blocking murine TNF-α activity in vivo (26, 40, 41) and because previous studies performed in our laboratory (36) and by others (42, 43) using a similar human fusion protein showed amelioration of arthritis in the short-term experiments performed. Because the viral transgene expression lasts for weeks (16) as opposed to a serum half-life of hours when the fusion protein is injected (44), an evaluation of its effects on CIA for a prolonged time appeared clinically relevant. The results obtained highlight some interesting and unexpected findings. Firstly, the i.v. administration of AdTNFR yielded high serum levels of the recombinant protein (in the range of 100 μg ml⁻¹) for the entire 21-day period of arthritis. Clinical assessment of the mice revealed a dose-dependent decrease of CIA severity for 10 days. This paralleled previous work with repeated injections of the soluble TNFR fusion protein alone (36). However, the clinical benefit was lost after day 10, despite the continual presence of high levels of bioactive soluble TNFR, with a subsequent inflammatory flare that persisted for the remaining period of observation. The mechanisms of this paradoxical response was analyzed in vivo through the administration of a control adenovirus or repeated injections of a related TNFR fusion protein (human TNFR/human IgG1) and of the control human IgG1 over a 21-day period. Our studies showed absence of systemic inflammatory response to the Adβgal virus or to the control human IgG1 protein, whereas a biphasic response was observed again in the TNFR fusion protein-treated animals (see Fig. 6A). Thus, under the experimental conditions used, therapy with the 55-kDa TNFR ameliorates the early acute phase of established CIA but, if prolonged, causes exacerbation of the disease. There are several possible mechanisms that could account for the exacerbation of arthritis after day 10. One possibility is that TNF blockade is harmful in the late stage of CIA or that prolonged blockade of TNF is harmful. The first hypothesis seems unlikely in the context of arthritis as previous studies performed in patients with longstanding RA using either anti-TNF-α mAbs or the 75-kDa TNFR fusion protein were beneficial (5, 6, 9) even in the long-term (7, 45). However, because of potential differences between human RA and murine CIA, direct comparison between the results of therapy in the two diseases may not always be applicable. Furthermore, it has recently been shown that mice deficient in TNF (TNF⁻/⁻) fail to regulate and limit in vivo an inflammatory response (46). A late florid inflammatory response developed in TNF⁻/⁻ mice injected with Corynebacterium parvum at a time when the inflammatory lesions in the TNF⁺/⁺ control mice had resolved. Indeed, in our study, the histology of arthritic paws from AdTNFR-treated mice showed on day 21 the massive leukocyte infiltration and synovitis...
usually present in the early acute phase of CIA, whereas control animals showed evidence of cartilage destruction and bone erosions with remodeling and fibrosis. Thus, the prolonged blockade of TNF-α achieved in our experiments could be possibly interfering with the up-regulation of homeostatic mechanisms such as the production of IL-10, TGFβ, or other mediators that are likely to be involved in limiting and eventually reversing inflammation (24).

To test this hypothesis we injected mice with a chimeric mouse IgG1 rat Fab anti-TNF-α mAb, at plateau doses, 500 μg three times per week, for 3 wk. This resulted in clinical benefit for the entire duration of the experiment, and argues strongly against TNF-α blockade, at the incomplete level achievable with neutralizing mAbs or fusion proteins, being intrinsically proinflammatory (see Table 1). The results shown in Fig. 8 mimic those obtained clinically, where long-term treatment showed a stable or augmented benefit (45).

Although the precise pathogenetic mechanisms of autoimmune CIA are not completely known, it is generally believed that immune responses to defined cartilage Ags, and to CII in particular, play an important role. However, there is evidence that TNF-α itself, if present chronically, is capable of down-regulating T cell responses (47). Hence, chronic anti-TNF-α therapy could be augmenting T cell responses, contributing to the late exacerbation of arthritis. However, CTLA4-Ig, which is known to block costimulatory signals between T cells and APC ameliorates CIA (31, 48, 49). This suppressed macroscopic inflammation in control animals but did not affect the response to the AdTNFR treatment. This is consistent with the in vitro results that T cell proliferative responses to CII in AdTNFR-treated and control mice were comparable at both early and late stage CIA (see Fig. 5). These results exclude a direct T cell involvement in the exacerbation process observed in the TNFR-treated group. In contrast, it was found that the humoral immune responses to CII were augmented by TNFR treatment, either by adenovirus product or protein (Fig. 7), and thus an alternative hypothesis for the increase in inflammation could involve interference with B lymphocyte regulatory mechanisms, leading to enhanced production of potentially harmful anti-collagen Abs. It has been previously reported that anti-CII Abs, of the IgG2a subclass in particular, exert a pathogenic role in CIA (34, 35), but whether the 2-fold increase detected in our experiments is sufficient to augment inflammation in the manner observed here is not clear.

Another possible mechanism of the inflammatory flare could be the presence of agonistic Abs to the p55 TNFR in mice injected with AdTNFR, which may cross-link the TNFR and hence mimic TNF action. Abs raised against the injected human 55-kDa TNFR may cross-react with its mouse homologue, because both receptors bind human TNF with high efficiency and hence must have similar conformations. We investigated whether Abs to mouse p55 TNFR were raised in the serum of mice treated with TNFR and detectable by ELISA. For the experiment, purified murine 55-kDa TNFR was not available, so we used a nonpurified preparation (culture supernatant) that yielded a degree of nonspecific binding also in control mice. The statistical analysis performed on individual sera from seven AdTNFR-treated mice and seven arthritic controls was consistently significant on samples obtained on day 10 just before relapse, but not significant on day 21 in three different experiments (data not shown). The IgG titer against TNFR is probably high, because we could detect Abs in the presence of high (40–100 μg ml⁻¹) circulating levels of the virally encoded soluble human TNFR. In view of the very high serum levels of human TNFR encoded by the adenovirus, it did not make sense to attempt to detect Abs to the human TNFR. As it has been reported that Abs to the 55-kDa TNFR possess TNF-like activity in vitro due to their ability to cross-link the receptor molecules (50), we set up several assays to test this hypothesis in our system. The most direct was to evaluate whether sera from the AdTNFR mice were capable of lysing murine TNF-sensitive cell lines such as WEHI 164. Increased cytotoxicity was indeed observed, although the results were not statistically significant compared with controls (data not shown), probably due to the deleterious effects of mouse serum on WEHI 164 cells and the consequent large variations. However, in small compartments from which clearance is slow, as for example the mouse joint, concentrated levels of anti TNFR Abs could be highly cytotoxic or stimulatory. It is of interest that, whereas in mice, in acute studies as during sepsis, the human TNFR p55 Fc (lenercept Hoffmann-La Roche, Nutley, NJ) fusion protein is highly effective, more so than the TNFR p75 Fc fusion protein (51), the same is apparently not true in longer-term clinical studies in RA patients. There, the p75 TNFR Fc fusion protein (9) has been very effective, whereas the p55 TNFR Fc fusion protein has been less effective (10, 11). The latter protein has been reported to be immunogenic in 40% treated individuals and it is thus conceivable that its relative lack of effect could be due to agonistic Abs to the human cell-bound TNFR. Such agonistic Abs were recently detected in a subset of RA patients treated with a human TNFR55-IgG1 fusion protein (52). Thus, we think that this is the likeliest mechanism for the disease relapse.

It has also been reported that in certain circumstances soluble cytokine receptors (53) and the dimeric form of the 75-kDa TNFR fusion protein in particular (54, 55), while potent inhibitors of TNF action in vitro, may also stabilize the cytokine, thus prolonging its activity in vitro, but whether this also happens in vivo is unclear. However, worsening of certain disease states has indeed been reported for example in sepsis after p75 TNFR fusion protein treatment (56) and in multiple sclerosis with the p55 TNFR fusion protein, but the mechanisms of these clinical effects are not known (57).

In conclusion, the increasing number of biological activities attributed to TNF and its soluble receptors make it difficult to unravel protective or harmful effects in different disease states. Our findings highlight the feasibility of adenovirus-mediated gene transfer of cytokine inhibitors in vivo as a useful approach to study cytokine functions during the course of chronic inflammatory and immune-mediated diseases and point out a peculiar effect of an immunogenic soluble TNFR administration in the follow-up of CIA. These effects need to be understood before therapy with such agents can be contemplated in humans.
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