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ANTICYTOKINE TREATMENT OF ESTABLISHED TYPE II COLLAGEN-INDUCED ARTHRITIS IN DBA/1 MICE

A Comparative Study using Anti-TNF α , Anti-IL-1 α/β , and IL-1Ra

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Objective. To examine the role of tumor necrosis factor α (TNF α), interleukin-1 α (IL-1 α), and IL-1 β in collagen-induced arthritis (CIA), immediately after onset and during the phase of established arthritis.

Methods. Male DBA/1 mice with collagen-induced arthritis were treated with antibodies against murine TNF α and IL-1 α/β at different time points of the disease. IL-1 receptor antagonist (IL-1Ra) was administered using Alzet osmotic minipumps. The effect of anticytokine treatment was monitored by visual scoring. Histology and cytokine reverse transcription polymerase chain reaction (RT-PCR) analyses were performed at the end of the treatment period.

Results. Anti-TNF α treatment showed efficacy shortly after onset of the disease, but had little effect on fully established CIA. Histologic analysis after early treatment revealed that anti-TNF α significantly reduced joint pathology, as determined by infiltration of inflammatory cells and cartilage damage. Anti-IL-1 α/β treatment ameliorated both early and full-blown CIA. This clear suppression of established arthritis was confirmed by administration of high doses of IL-1Ra. Dose-response experiments showed that a continuous supply of 1 mg/day was needed for optimal suppression. Histologic analysis showed markedly reduced cartilage destruction both in the knee and the ankle joints. Autoradiography demonstrated full recovery of chondrocyte synthetic function of articular cartilage. In

addition, we found that the IL-1 β isoform plays a dominant role in established CIA. Profound suppression of CIA was observed with anti-IL-1 β , although elimination of both IL-1 α and IL-1 β still gave better protection. Analysis of messenger RNA with RT-PCR revealed that IL-1 β was highly upregulated in synovium and cartilage at late stages of CIA, whereas anti-IL-1 β treatment markedly reduced IL-1 β message in the synovium.

Conclusion. The present study identified different TNF α /IL-1 dependencies in various stages of CIA and revealed that blocking of TNF α does not necessarily eliminate IL-1. Continuous, high doses of IL-1Ra are needed to block CIA.

Rheumatoid arthritis (RA) is characterized by chronic inflammation in joints and concomitant destruction of cartilage and bone. Although the disease is generally considered an autoimmune process, the autoantigen is still unknown, thus hampering specific immunomodulation as a straightforward therapeutic approach. In fact, detailed analyses of mediator production in inflamed synovial tissue have revealed a relative lack of T cell factors and an abundance of cytokines and growth factors, which are produced by macrophages and synovial fibroblasts (1,2). Fortunately, considerable hierarchy seems to exist in this plethora of factors, and tumor necrosis factor α (TNF α) and interleukin-1 (IL-1) seem of pivotal importance in arthritis (3-5). It has been claimed that TNF α is driving most of the IL-1 production in the inflamed synovia of RA patients (6), making it a prime target for therapy. This has been further substantiated by the demonstration of arthritis in TNF α -transgenic mice and of the efficacy of anti-TNF α treatment in collagen-induced arthritis (CIA) in the mouse (7-9).

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The first clinical trials with neutralizing antibodies against TNF α have demonstrated efficacy in humans with RA (10–12).

CIA is a widely used experimental model of polyarthritis. It can be induced in susceptible strains of mice and rats by immunization with type II collagen, the major component of articular cartilage, and has histopathologic features in common with RA (13–16). Earlier studies revealed that the expression of this autoimmune arthritis can be enhanced with passive addition of cytokines like TNF α , IL-1, and transforming growth factor β (TGF β), whereas TGF β and antibodies to TNF α prevented the onset of disease (17–21). Subsequent studies using antibodies and soluble TNF α receptor fusion protein analyzed the effect of TNF α neutralization after the onset of CIA. Significant amelioration was found both macroscopically and histologically (8,9,22). However, recent studies, using neutralizing antibodies to IL-1 in CIA, suggest that elimination of this cytokine was at least as effective (23,24). Moreover, studies in other arthritis models have provided evidence that IL-1 is perhaps not always the dominant cytokine in the inflammatory process, but is certainly of pivotal importance in cartilage destruction (25,26).

In the present study, we compared the relative efficacy of anti-TNF α and anti-IL-1 α/β treatment in murine CIA, using concomitant treatment protocols in large groups of arthritic mice. Moreover, treatment was started at different time points after the onset of arthritis and in addition to macroscopic scores for arthritis, we investigated the histopathology in sections of knees and ankle joints. It was found that anti-TNF α was effective at the onset of arthritis but less so at later stages, whereas anti-IL-1 α/β was also highly effective in established disease, with a major emphasis on reduction of cartilage destruction. We confirmed the importance of IL-1, using IL-1 receptor antagonist (IL-1Ra) treatment. A critical observation in the latter experiments was the need of continuous, high concentrations of IL-1Ra, supplied by osmotic minipumps. Finally, we examined the relative role of IL-1 β and IL-1 α and analyzed message expression in synovium and cartilage for TNF α , IL-1 β , and IL-1Ra. It is clear from observations in this animal model that IL-1 is a pivotal mediator in both early and late disease, whereas blocking of TNF α does not necessarily eliminate IL-1. Given the cartilage-destroying character of IL-1, this argues at least for a

consideration of both TNF α and IL-1 as therapeutic targets in RA.

MATERIALS AND METHODS

Animals. Male DBA/1 Lac/J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Male C57Bl/6 mice, used for induction of streptococcal cell wall (SCW) arthritis, were bred in our own facilities. Mice were housed in filter top cages, and water and food were provided ad libitum. DBA/1 mice were immunized at the age of 9–10 weeks and C57Bl/6 mice were used at the age of 10–12 weeks.

Materials. LPS (*Escherichia coli* O111:84), ethidium bromide, hamster Ig, rat Ig, rabbit Ig, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). *Taq* DNA polymerase, 100-bp DNA marker, TRIzol reagent, and agarose were obtained from Life Technologies (Breda, The Netherlands). GAPDH, β_2 -microglobulin (β_2 -m), IL-1 β , IL-1Ra, TNF α , and tissue inhibitor of metalloproteinases (TIMP) primers were purchased from Pharmacia Biotech (Roosendaal, The Netherlands). Internal DNA construct (Pmus) was a kind gift of Dr. D. Shire (Sanofi Recherche, Labège, France) (27). Freund's complete adjuvant (FCA) and *Mycobacterium tuberculosis* (MT; strain H37Ra) were obtained from Difco (Detroit, MI). Osmotic minipumps (model 1007D) were purchased from Alza (Palo Alto, CA).

Cells producing rat anti-murine TNF α IgG1 (V1q) were a kind gift of P. H. Krammer (German Cancer Research Centre, Heidelberg, Germany) (28). Rabbit anti-murine IL-1 α/β polyclonal antibodies were prepared in our own laboratory by one of us (AAJVDL) (25,26). Polyclonal rabbit anti-murine IL-1 α and monoclonal hamster anti-murine IL-1 β were generously provided by Robert Schreiber (Washington University Medical School, St Louis, MO), in conjunction with Merck Research Laboratories (Rahway, NJ). Human recombinant IL-1Ra was kindly provided by Synergen (Boulder, CO).

Collagen preparation. Articular cartilage was obtained from the knee joints of 1–2-year-old cows. Bovine type II collagen was prepared according to the method of Miller and Rhodes (29). Collagen was dissolved in 0.05M acetic acid (10 mg/ml) and stored at -70°C .

Induction of CIA. Bovine type II collagen was diluted with 0.05M acetic acid to a concentration of 2 mg/ml and was emulsified in an equal volume of FCA (2 mg/ml of MT H37Ra). The mice were immunized intradermally at the base of the tail with 100 μl of emulsion (100 μg of collagen). On day 21, the animals were given booster injections intraperitoneally with 100 μg of type II collagen dissolved in phosphate buffered saline (PBS). This resulted in the onset of arthritis around day 28 in 20–40% of the mice. Mice with arthritis were selected at this time point to be used in studies of "classic" CIA.

Acceleration of CIA. Mice without any macroscopic signs of arthritis on day 28 were used for LPS-accelerated CIA. Arthritis onset was initiated by a single intraperitoneal injection of 40 μg of LPS (30). This resulted in the onset of CIA within 3 days, and on day 35, full-blown arthritis was

noted in the paws of more than 95% of the animals. Thus, anticytokine treatments were performed in 2 CIA models: classic and accelerated. The histopathologic changes in the knee and ankle joints were comparable in accelerated CIA and classic CIA. As described previously, administration of 40 μg of LPS to nonimmunized DBA/1 mice did not result in any macroscopic or histologic abnormalities (31).

Assessment of CIA. Mice were examined visually for the appearance of arthritis in the peripheral joints, and scores for arthritis severity were given (macroscopic score) as previously described (24,31). Mice were considered to have arthritis when significant changes in redness and/or swelling were noted in the digits or in other parts of at least 2 paws. At later time points, ankylosis was also included in the macroscopic scoring.

The clinical severity of arthritis was graded on a scale of 0–2 for each paw, according to changes in redness and swelling (0 = no change, 0.5 = significant, 1.0 = moderate, 1.5 = marked, and 2.0 = maximal swelling and redness and later, ankylosis). The macroscopic score was the cumulative value for all paws (mean \pm SD), with a maximum of 8. The macroscopic score was assessed by 2 independent, blinded observers (LABJ, MMAH).

Neutralizing anticytokine antibodies/IL-1Ra. Hybridoma cells (3×10^6) producing rat anti-murine TNF α (V1q) were injected into nude Balb/c mice, and after 3 weeks, ascites fluid was collected. Thereafter Ig were isolated using a protein G column. This antibody (IgG1) showed efficacy in LPS-mediated lethal shock models (28) and in the L929 bioassay. The neutralizing capacity of this antibody was 3.5 ng of TNF α per μg in the latter assay. Polyclonal rabbit antibodies were raised against murine recombinant IL-1 α and IL-1 β (25,26). The isotype-specific antibodies showed no cross-reactivity with a number of other cytokines, such as IL-6, TNF α , or granulocyte-macrophage colony-stimulating factor. The antibodies have been demonstrated to show neutralizing capacity both in vitro and in vivo. One microgram of purified Ig neutralized 50–100 pg of IL-1 in the NOB-1 bioassay. To confirm the anti-IL-1 α/β treatment results, we compared the in vivo efficacy of our anti-IL-1 antibodies with anti-IL-1 antibodies from other sources. To this end, we used rabbit anti-IL-1 α and monoclonal hamster anti-IL-1 β (from Robert Schreiber), which neutralized 35 pg of IL-1 α per μg of antibody and 175 pg of IL-1 β per μg of antibody, respectively.

IL-1Ra was administered using osmotic minipumps. Pumps were implanted into the peritoneal cavity on day 34 after immunization (6 days after acceleration by LPS), giving sustained release of IL-1Ra for the next 7 days. All anticytokine agents used contained no LPS, as measured by the *Limulus* amoebocyte assay.

Anticytokine treatment of CIA. To investigate the effect of anti-IL-1 α/β or anti-TNF α treatment at different phases of classic CIA, a single dose of these antibodies was injected intraperitoneally at different time points after the onset of CIA. We injected 0.6 mg of rabbit anti-IL-1 α or anti-IL-1 β polyclonal antibodies or 75 μg of rat monoclonal anti-TNF α . Dose-response experiments in established CIA were performed with 0.6 mg of polyclonal rabbit anti-IL-1 α

and 0.12 mg of monoclonal hamster anti-IL-1 β (from Robert Schreiber) as the highest doses, which had the same neutralizing capacity as 0.6 mg of rabbit anti-IL-1 α and anti-IL-1 β as prepared by one of us (AAJVDL). Daily administration of anti-TNF α were performed during accelerated CIA, with a 75- μg dose of rat anti-TNF α for a period of 7 days. As controls in the anti-IL-1/TNF α studies, we injected the same amounts of normal rabbit, rat, or hamster Ig.

IL-1Ra treatment was started on day 34 after immunization by implantation of osmotic minipumps, which released 0.12–1.2 mg of IL-1Ra per day. As a control, we implanted osmotic pumps, which released 1.2 mg of BSA per day. Arthritis was assessed every 2 days, starting on day 28 after immunization with collagen. Knee and ankle joints were isolated and processed for light microscopy 7 days after the start of anticytokine treatment, unless stated otherwise. Tissue samples for measurements of messenger RNA (mRNA) were also isolated at this time point.

Induction of endotoxic shock. Mice, C57Bl/6 were injected intravenously into the tail vein with LPS (100 μg) in a 200- μl saline solution and monitored for survival for 7 days. All deaths occurred within 3 days of LPS administration. Rat anti-murine TNF α (75 μg) was injected intraperitoneally 4 hours before the induction of endotoxic shock.

SCW preparation and induction of SCW arthritis. *Streptococcus pyogenes* T12 organisms were cultured overnight in Todd-Hewitt broth. Cell walls were prepared as described previously (32). The resulting 10,000g supernatant was used throughout the experiments. These preparations contained 11% muramic acid. Bacteria were kindly provided by Dr. M. Hazenberg (Erasmus University, Rotterdam, The Netherlands). Unilateral arthritis was induced by intraarticular injection of 25 μg of SCW (dry weight) in 5 μl of PBS into the right knee joint of naive mice. As a control, PBS was injected into the left knee joint.

Measurement of SCW-induced arthritis. SCW arthritis was quantified by the $^{99\text{m}}\text{Tc}$ -uptake method (33). This method measures by external gamma counting the accumulation of a small radioisotope at the site of inflammation due to local increased blood flow and tissue swelling. The severity of inflammation is expressed as the ratio of the $^{99\text{m}}\text{Tc}$ uptake in the right (inflamed) knee joint over that in the left (control) knee joint. All values exceeding 1.10 were considered inflammation.

Anti-TNF α treatment of SCW-induced arthritis. To examine the role of TNF α during the onset of SCW-induced arthritis, a single injection of 75 μg of rat anti-TNF α antibodies was given, 1 hour before induction of SCW. As a control, we used 75 μg of rat Ig. Knee joint swelling was measured on days 1, 2, and 4 of the arthritis.

RNA isolation. Mice were killed by cervical dislocation, immediately followed by dissection of the patella with adjacent synovium (34). Two synovium biopsies (3-mm diameter) were taken with a disposable biopsy punch (Stiefel, Wackersbach, Germany) from this specimen, one from the lateral and one from the medial side. The synovium samples were immediately frozen in liquid nitrogen. Patellae were transferred to a 5% EDTA solution and kept on ice for 4 hours. Thereafter, the cartilage layer was stripped as

previously described (35). This procedure does not affect mRNA expression.

The total RNA of a pool of 10 cartilage samples was extracted with 1 ml of TRIzol reagent, an improved single-step RNA isolation method based on the method described by Chomczynski and Sacchi (36). Five separate synovium samples were ground into powder using a microdismembrator II (B. Braun, Melsungen, Germany). Total RNA was extracted in 1 ml TRIzol reagent in a procedure similar to that used for cartilage samples.

Polymerase chain reaction (PCR) amplification. One microgram of synovial RNA and the total amount of cartilage RNA was used for reverse transcription PCR (RT-PCR). Messenger RNA was reverse-transcribed to complementary DNA (cDNA) using oligo-dT primers and one-twentieth of the cDNA was used in 1 PCR amplification. PCR was performed according to a standard protocol. Message for GAPDH, IL-1 β , IL-1Ra, and TNF α was amplified using the primers described elsewhere (37–40). Primers for TIMP were designed using Oligo 4.0 (National Bioscientist, Plymouth, MN) or Primer (Whitehead Institute, Cambridge, MA). The results are presented as the relative increase in mRNA expression compared with noninflamed control samples from normal DBA/1 mice. The relative increase in mRNA was calculated as follows: 1.9 (amplification factor [41]) to the power of n, where n is the difference in the number of cycles showing identical staining intensity for experimental and noninflamed control tissue. IL-1 β , TNF α , IL-1Ra, and TIMP mRNA levels were corrected for GAPDH message, if needed.

To validate the calculated values for the mRNA levels, we also performed competitive PCR for β_2m and IL-1 β (27). A serial dilution of an internal DNA standard (Pmus) was added to a fixed amount of cDNA (one-twentieth of the cDNA). The IL-1 β mRNA levels were corrected for the amount of β_2m .

Histology. Mice were killed by ether anesthesia; ankle and knee joints were removed and fixed for 4 days in 4% formalin. After decalcification in 5% formic acid, the specimens were processed for paraffin embedding. Tissue sections (7 μ m) were stained with hematoxylin and eosin or Safranin O. Histopathologic changes were scored according to the following parameters. Infiltration of cells was scored on a scale of 0–3, depending on the amount of inflammatory cells in the synovial cavity and synovial tissues. Proteoglycan depletion was determined using Safranin O staining. The loss of proteoglycans was scored on a scale of 0–3, ranging from fully stained cartilage to destained cartilage or complete loss of articular cartilage. A characteristic parameter in CIA is the progressive loss of articular cartilage. This destruction was separately graded on a scale of 0–3, ranging from the appearance of dead chondrocytes (empty lacunae) to a complete loss of articular cartilage. Histopathologic changes in the knee joints were scored in the patellofemoral region on 5 semi-serial sections of the joint. For the ankle joint, we scored the calcaneous region. Scoring was done in a blinded manner by 2 observers (LABJ, MMAH), as described earlier (24).

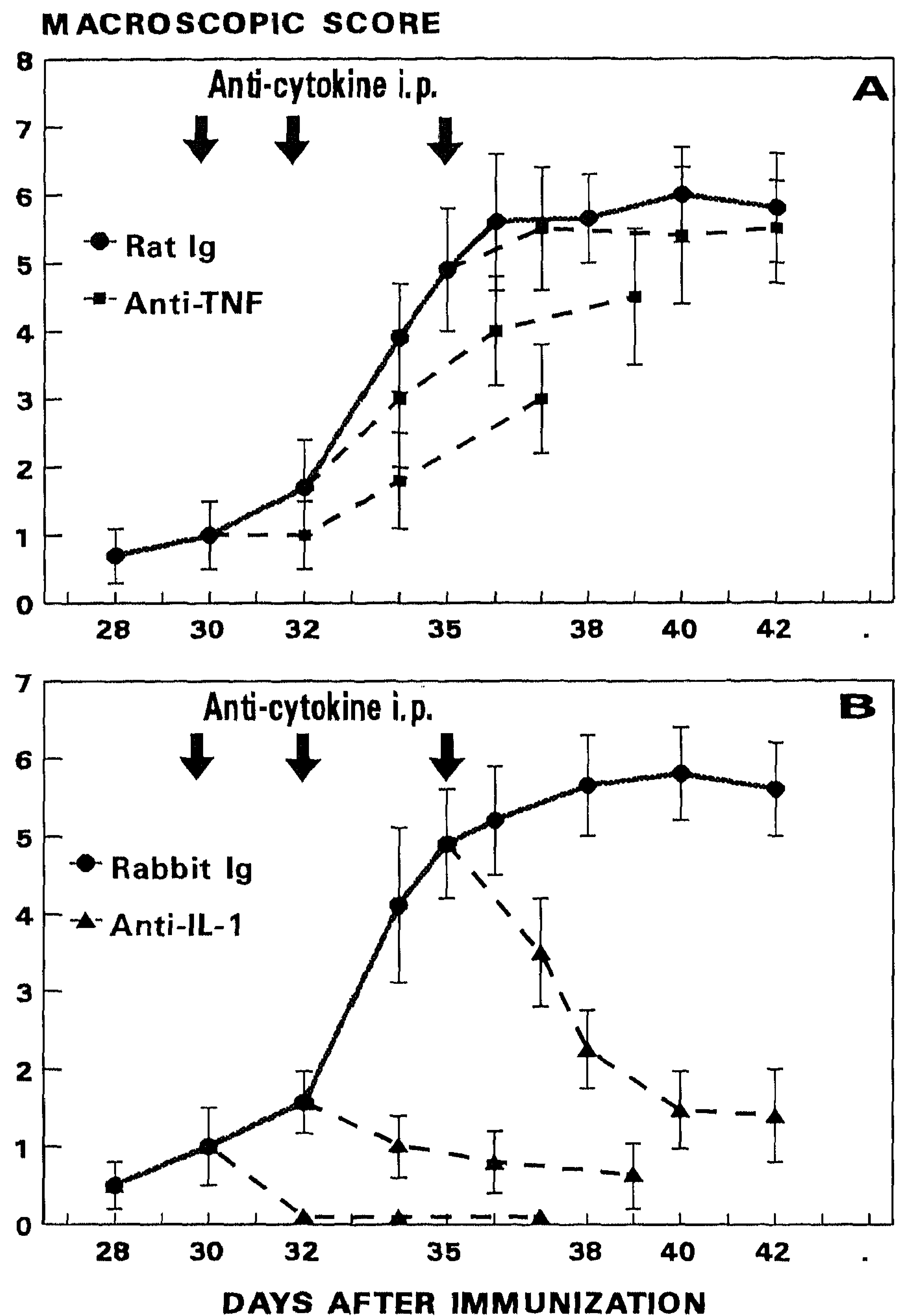


Figure 1. Time-course of tumor necrosis factor α /interleukin-1 (TNF α /IL-1) involvement in classic collagen-induced arthritis (CIA). A single treatment with anti-TNF α or anti-IL-1 α/β was given (A and B). At time points indicated by arrows, separate groups were injected with either control Ig or anticytokine. CIA was scored visually by 2 independent observers (0–8 cumulative value for all paws; see Materials and Methods). This experiment was repeated twice, with similar outcomes. A, ■ = rat anti-TNF α , 75 μ g; ● = control rat Ig, 75 μ g. B, ▲ = rabbit anti-IL-1 α/β , 0.6 mg each; ● = control rabbit Ig, 1.2 mg. Values are the mean \pm SD of at least 10 mice per group (n = 3 experiments). i.p. = intraperitoneal.

Statistical analysis. Differences between experimental groups were tested using the Wilcoxon rank test, unless stated otherwise.

RESULTS

Time course of TNF α /IL-1 involvement in classic CIA. At various time points after the onset of classic CIA, mice were given a single intraperitoneal injection

of antibodies raised against murine $\text{TNF}\alpha$ or murine $\text{IL-1}\alpha$ and $\text{IL-1}\beta$. When $75 \mu\text{g}$ of monoclonal anti- $\text{TNF}\alpha$ was given just after onset (day 30), a marked reduction of arthritis was noted. The effect was less pronounced when treatment was started on day 2 after onset (day 32), whereas no significant relief was seen when treatment was delayed till day 7 after onset (Figure 1A). In contrast, after neutralization of IL-1 with a combination of antibodies against both isoforms, $\text{IL-1}\alpha$ and $\text{IL-1}\beta$, complete prevention of CIA onset was noted upon early treatment. Of interest, a pronounced suppression was still observed when treatment was given on day 2 after onset.

Unlike the effect of anti- $\text{TNF}\alpha$, anti- $\text{IL-1}\alpha/\beta$ treatment was also able to significantly suppress established CIA. Figure 1B shows a macroscopic arthritis score of 5 on day 35, which slowly increased to 5.5 in the control group on day 42, whereas the score was

Table 1. Histology after anticytokine treatment classic of collagen-induced arthritis*

Group	Start of treatment	Infiltrate	Cartilage damage	Proteoglycan depletion	Day of killing
Knee joints					
Rat Ig	Day 30	1.5 ± 0.6	1.0 ± 0.6	1.9 ± 0.8	37
Rabbit Ig	Day 30	1.4 ± 0.7	0.9 ± 0.5	1.8 ± 0.9	37
$\text{TNF}\alpha\text{Ab}$	Day 30	0.9 ± 0.3	0.6 ± 0.5	1.1 ± 0.5	37
Anti- IL-1	Day 30	$0.1 \pm 0.1^\dagger$	$0.1 \pm 0.1^\dagger$	$0.1 \pm 0.1^\dagger$	37
Rat Ig	Day 32	1.4 ± 0.4	1.1 ± 0.3	2.4 ± 0.6	39
Rabbit Ig	Day 32	1.5 ± 0.5	1.0 ± 0.4	2.5 ± 0.3	39
$\text{TNF}\alpha\text{Ab}$	Day 32	1.0 ± 0.5	0.7 ± 0.5	1.8 ± 0.6	39
Anti- IL-1	Day 32	$0.1 \pm 0.1^\dagger$	$0.1 \pm 0.1^\dagger$	$0.1 \pm 0.1^\dagger$	39
Rat Ig	Day 35	1.3 ± 0.4	1.2 ± 0.3	2.2 ± 0.5	42
Rabbit Ig	Day 35	1.4 ± 0.3	1.3 ± 0.4	2.1 ± 0.4	42
$\text{TNF}\alpha\text{Ab}$	Day 35	1.2 ± 0.5	1.0 ± 0.5	2.0 ± 0.6	42
Anti- IL-1	Day 35	$0.6 \pm 0.1^\dagger$	$0.5 \pm 0.3^\dagger$	$0.9 \pm 0.2^\dagger$	42
Ankle joints					
Rat Ig	Day 30	1.5 ± 0.3	0.9 ± 0.4	1.6 ± 0.4	37
Rabbit Ig	Day 30	1.6 ± 0.4	0.8 ± 0.4	1.7 ± 0.5	37
$\text{TNF}\alpha\text{Ab}$	Day 30	0.9 ± 0.5	0.6 ± 0.5	1.1 ± 0.7	37
Anti- IL-1	Day 30	$0.1 \pm 0.1^\dagger$	$0.1 \pm 0.1^\dagger$	$0.2 \pm 0.2^\dagger$	37
Rat Ig	Day 32	2.0 ± 0.5	1.0 ± 0.3	2.4 ± 0.3	39
Rabbit Ig	Day 32	1.9 ± 0.4	0.9 ± 0.2	2.3 ± 0.4	39
$\text{TNF}\alpha\text{Ab}$	Day 32	1.3 ± 0.5	0.7 ± 0.3	1.6 ± 0.8	39
Anti- IL-1	Day 32	$0.2 \pm 0.1^\dagger$	$0.1 \pm 0.1^\dagger$	$0.2 \pm 0.2^\dagger$	39
Rat Ig	Day 35	1.9 ± 0.4	1.1 ± 0.2	2.0 ± 0.5	42
Rabbit Ig	Day 35	1.8 ± 0.2	1.0 ± 0.3	1.9 ± 0.3	42
$\text{TNF}\alpha\text{Ab}$	Day 35	1.7 ± 0.4	1.0 ± 0.3	1.9 ± 0.5	42
Anti- IL-1	Day 35	$0.8 \pm 0.2^\dagger$	$0.5 \pm 0.2^\dagger$	$1.0 \pm 0.2^\dagger$	42

* Start of treatment represents the number of days after immunization of DBA/1 mice with bovine type II collagen. Synovial infiltrates, cartilage damage, and proteoglycan depletion were scored on a scale of 0-3. Values are the mean \pm SD. $\text{TNF}\alpha$ = tumor necrosis factor α ; IL-1 = interleukin-1.

$^\dagger P < 0.01$ versus controls, by Wilcoxon rank test.

MACROSCOPIC SCORE

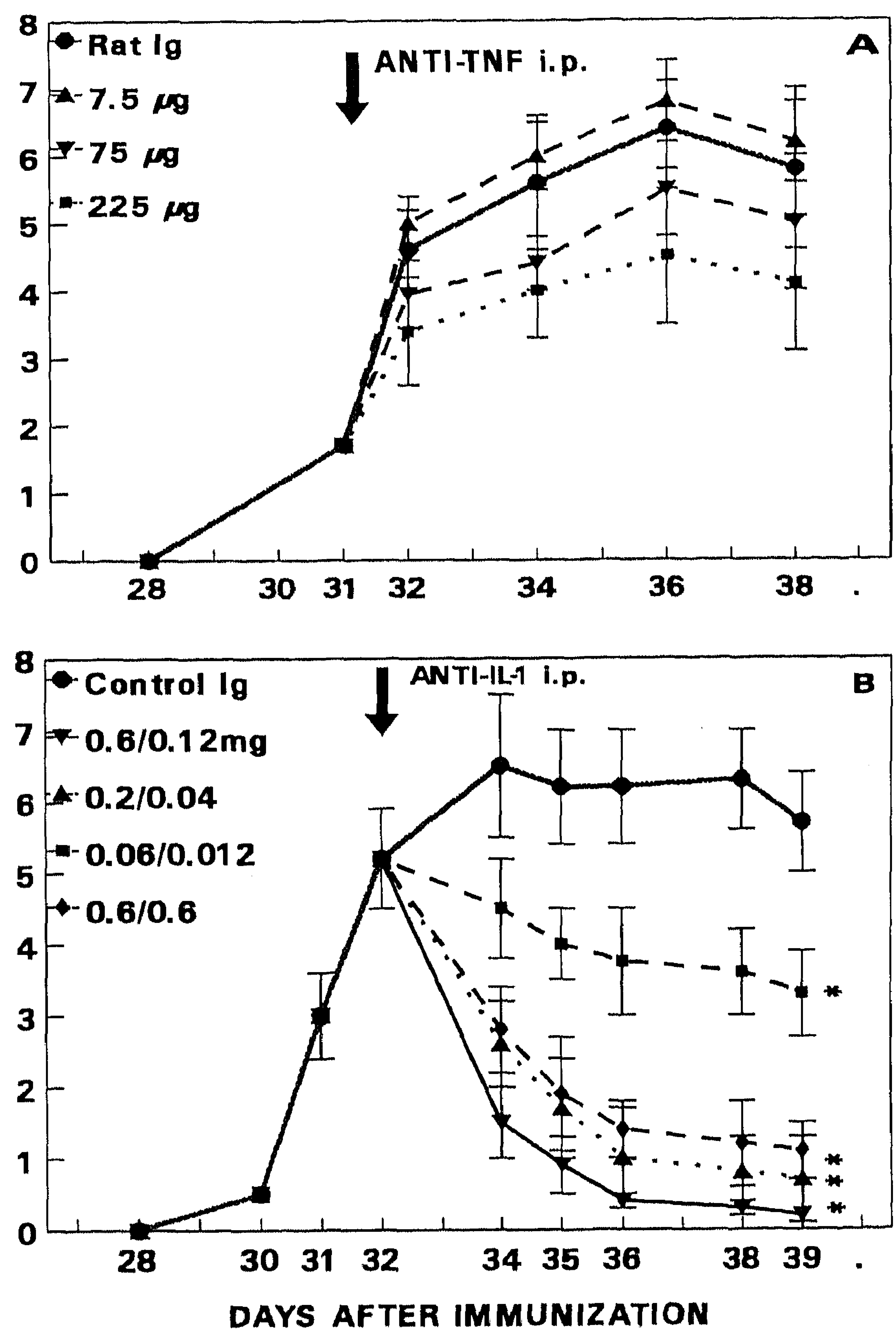


Figure 2. Treatment of accelerated CIA with different doses anti- $\text{TNF}\alpha$ /anti- IL-1 . A, A single injection of rat anti- $\text{TNF}\alpha$ was given just after disease onset (day 31; arrow), at the doses shown. As a control, $75 \mu\text{g}$ of rat Ig was injected. B, An injection of rabbit anti- $\text{IL-1}\alpha$ /monoclonal hamster $\text{IL-1}\beta$ (at the first 3 doses shown) was given on day 32 (arrow), when arthritis was fully developed. As a control, 0.6 mg of rabbit Ig and 0.12 mg of hamster Ig were injected at the same time point. Similar results with rabbit anti- $\text{IL-1}\alpha/\beta$ antibodies ($0.6/0.6 \text{ mg}$ \blacklozenge) were found in accelerated CIA as in classic CIA (see Figure 1B). * = $P < 0.01$ versus controls, by Wilcoxon rank test. Values are the mean \pm SD for at least 10 mice per group. See Figure 1 for definitions.

reduced below 2 by treatment on day 35 with anti- $\text{IL-1}\alpha/\beta$. When anti- $\text{IL-1}\alpha/\beta$ treatment was delayed till day 56, a phase characterized by joint ankylosis, no significant suppression of macroscopic signs was noted (data not shown).

In addition to macroscopic scoring, we ana-

Table 2. Histology after anticytokine treatment of accelerated collagen-induced arthritis*

Group	Start of treatment	Infiltrate	Cartilage damage	Proteoglycan depletion
Knee joints				
Rat Ig	Day 31	1.4 ± 0.6	1.2 ± 0.6	2.0 ± 0.9
Anti-TNF α , 225 μ g	Day 31	0.8 ± 0.7	1.0 ± 0.7	1.7 ± 0.6
Rabbit Ig	Day 32	1.4 ± 0.3	1.1 ± 0.6	2.1 ± 0.4
Anti-IL-1 α/β				
0.06/0.012 mg	Day 32	0.6 ± 0.5	0.8 ± 0.8	1.3 ± 0.7
0.2/0.04 mg	Day 32	0.4 ± 0.2†	0.5 ± 0.5†	0.6 ± 0.5†
0.6/0.12 mg	Day 32	0.2 ± 0.2†	0.4 ± 0.1†	0.4 ± 0.5†
Control	Day 34	1.5 ± 0.7	1.7 ± 0.8	2.2 ± 0.8
IL-1Ra, 1.2 mg/day	Day 34	0.4 ± 0.2†	0.6 ± 0.2†	0.6 ± 0.4†
Ankle joints				
Rat Ig	Day 31	1.7 ± 0.5	0.9 ± 0.3	1.8 ± 0.6
Anti-TNF α , 225 μ g	Day 31	1.0 ± 0.7	0.6 ± 0.5	1.2 ± 0.6
Rabbit Ig	Day 32	1.9 ± 0.5	0.9 ± 0.2	2.2 ± 0.4
Anti-IL-1 α/β				
0.06/0.012 mg	Day 32	1.0 ± 0.3†	0.5 ± 0.2†	0.8 ± 0.2†
0.2/0.04 mg	Day 32	0.8 ± 0.4†	0.4 ± 0.4†	0.7 ± 0.5†
0.6./0.12 mg	Day 32	0.4 ± 0.2†	0.3 ± 0.2†	0.5 ± 0.5†
Control	Day 34	1.9 ± 0.4	1.4 ± 0.3	2.0 ± 0.8
IL-1Ra, 1.2 mg/day	Day 34	0.7 ± 0.1†	0.5 ± 0.2†	0.5 ± 0.2†

* For details, see Table 1. Histology was performed 7 days after the start of anticytokine treatment. Control was 1.2 mg of bovine serum albumin. Values are the mean \pm SD. TNF α = tumor necrosis factor α ; IL-1 α/β = interleukin-1 α/β ; IL-1Ra = IL-1 receptor antagonist.

† $P < 0.01$ versus controls, by Wilcoxon rank test.

lyzed the histologic changes in both knee and ankle joints on day 7 after anticytokine treatment. Anti-TNF α treatment had only a slight effect on this parameter. Cellular infiltration was marginally reduced when treatment was given early. No effect was seen after late treatment (Table 1). In contrast, anti-IL-1 α/β treatment highly reduced cellular infiltration, even when treatment was given on day 35. Of importance, cartilage proteoglycan depletion and damage to the articular surface were also highly reduced by anti-IL-1 (Table 1). This set of data indicates that TNF α is involved in CIA onset, but plays a minor role at later stages, whereas IL-1 is a dominant mediator in both the onset and perpetuation of CIA.

Anti-IL-1 and anti-TNF α treatment of accelerated CIA. Experiments were also performed in mice with LPS-accelerated CIA. Selected mice, showing no clear signs of CIA on day 28, received a single intraperitoneal injection of 40 μ g LPS. Anticytokine treatment was given on day 31, when the animals showed definite arthritis. When accelerated CIA was treated on day 31 with a single dose of 75 μ g of anti-TNF α , some suppression of the arthritis was noted. A low

dose of 7.5 μ g was without effect. A higher dose (225 μ g) did not show greater efficacy compared with 75 μ g (Figure 2A).

As found in the classic CIA, treatment of accelerated CIA with anti-IL-1 α/β resulted in a complete suppression of the arthritis (Figure 2B). To confirm these findings, similar studies were done with yet another source of anti-IL-1 antibodies. Rabbit anti-IL-1 α combined with monoclonal hamster anti-IL-1 β showed a dose-dependent suppression of CIA (Table 2). These findings were confirmed histologically (Figure 3).

Efficacy of anti-TNF α treatment. The studies described above were performed with monoclonal rat anti-TNF α antibodies (V1q). To exclude the possibility that the apparent inefficacy of anti-TNF α treatment was related to inferior pharmacokinetics or inadequate neutralization, a number of control experiments were performed. First, we tested the efficacy of rat monoclonal anti-murine TNF α (V1q) in LPS-induced shock. A single intraperitoneal injection of 75 μ g completely prevented lethality when 100 μ g of LPS was injected intravenously 4 hours later. Second, we performed daily dosing for 7 days during CIA. As can be seen in

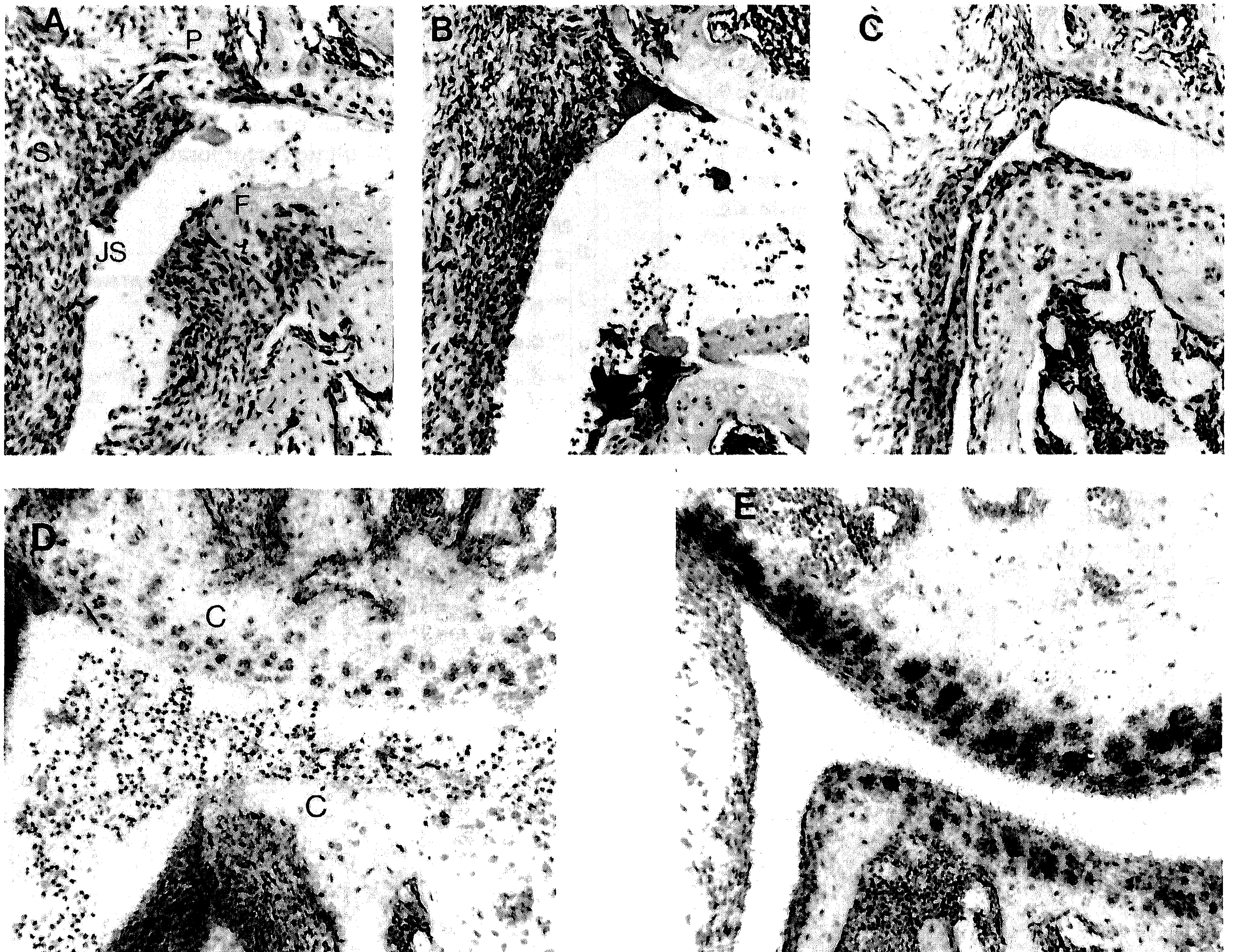


Figure 3. Hematoxylin and eosin-stained frontal sections of knee joints on day 42 of CIA. **A**, Arthritic knee joint of a mouse treated with control IgG on day 30. **B**, Knee joint of a mouse treated with rat anti-TNF α (225 μ g) on day 30. **C**, Knee joint of a mouse treated with rabbit anti-IL-1 α/β (0.6 mg of each antibody) on day 30. **D**, Autoradiograph showing 35 S-sulfate incorporation in an arthritic joint on day 42. **E**, Autoradiograph of a similar region after treatment with IL-1 receptor antagonist (1.2 mg/day for 7 days, starting on day 34). Note the restoration of chondrocyte synthetic function, visualized by distinct labelling. P = patella; F = femur; JS = joint space; C = cartilage; S = synovium. See Figure 1 for other definitions. Original magnification $\times 200$.

Figure 4A, this did not result in more marked suppression of the macroscopic score as compared with the findings in Figure 2A. Finally, we treated SCW-induced arthritis with a single injection of 75 μ g of anti-TNF α . As can be seen in Figure 4B, clear suppression of knee joint swelling was found on days 1, 2, and 4 in this bacterial arthritis model.

Confirmation with IL-1Ra. To further substantiate the important role of IL-1, arthritic mice were

continuously infused with IL-1Ra from day 34, using osmotic minipumps. This experiment was repeated 3 times at the high dosage (1.2 mg of IL-1Ra per day), showing a marked and consistent decline of the arthritis within a few days after the start of treatment. Moreover, there was always a slight suppressive effect when control minipumps were implanted, probably related to the effects of stress. In control arthritic mice without minipumps, the arthritis score increased

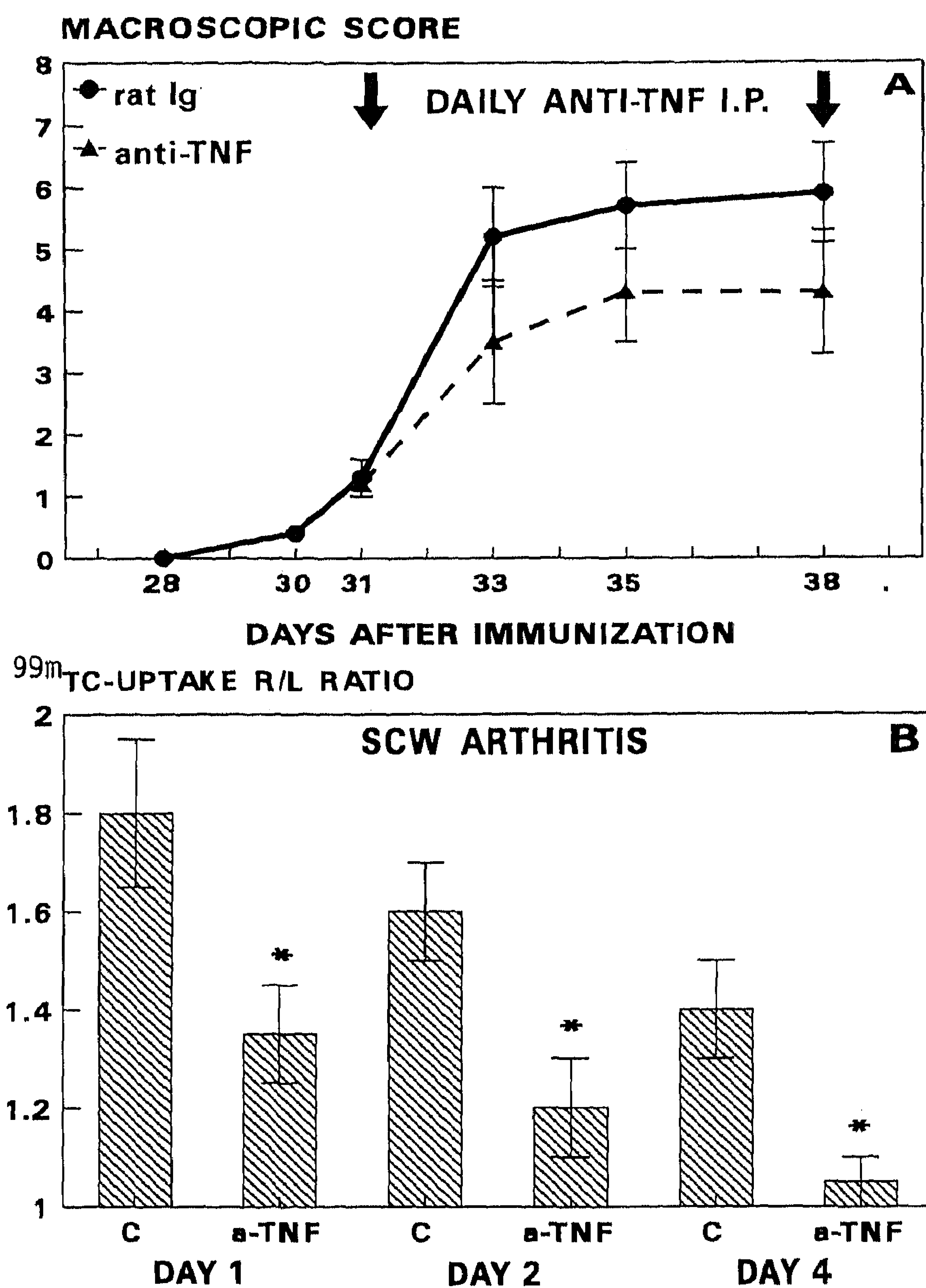


Figure 4. Anti-TNF α treatment of CIA and streptococcal cell wall (SCW)-induced arthritis. **A**, Daily doses of 75 μ g were given for 7 days in mice with accelerated CIA; arthritis was scored macroscopically in the paws. **B**, Unilateral SCW-induced arthritis was treated with a single dose of anti-TNF α (75 μ g), given intraperitoneally (I.P.) 1 hour before arthritis induction. Knee joint swelling was determined by the 99m Tc-uptake method. A right/left (R/L) ratio of 1.1 or more indicates knee joint inflammation. * = $P < 0.01$ versus control (rat Ig at 75 μ g), by Wilcoxon rank test. Values are the mean \pm SD of at least 10 mice per group. C = control; a-TNF = anti-TNF α . See Figure 1 for other definitions.

slightly or remained constant from day 34, whereas in mice with control minipumps, some suppression was consistently seen. Figure 5A shows a typical example of a dose-response experiment. It is clear that relatively high doses of IL-1Ra are needed to obtain pronounced suppression. An intermediate dose of 0.4 mg/day still showed significant suppression, but 0.12 mg/day was ineffective. Similar observations were noted in mice with classic CIA (Figure 5B). Histology performed in the highest dose group confirmed the pronounced suppression of cellular infiltration and

cartilage damage (Table 2), as noted with neutralizing anti-IL-1 antibodies.

In addition to histology, we performed autoradiography to get an impression of the metabolic activity of the articular chondrocytes. Pronounced suppression of 35 S-sulfate incorporation, reflecting

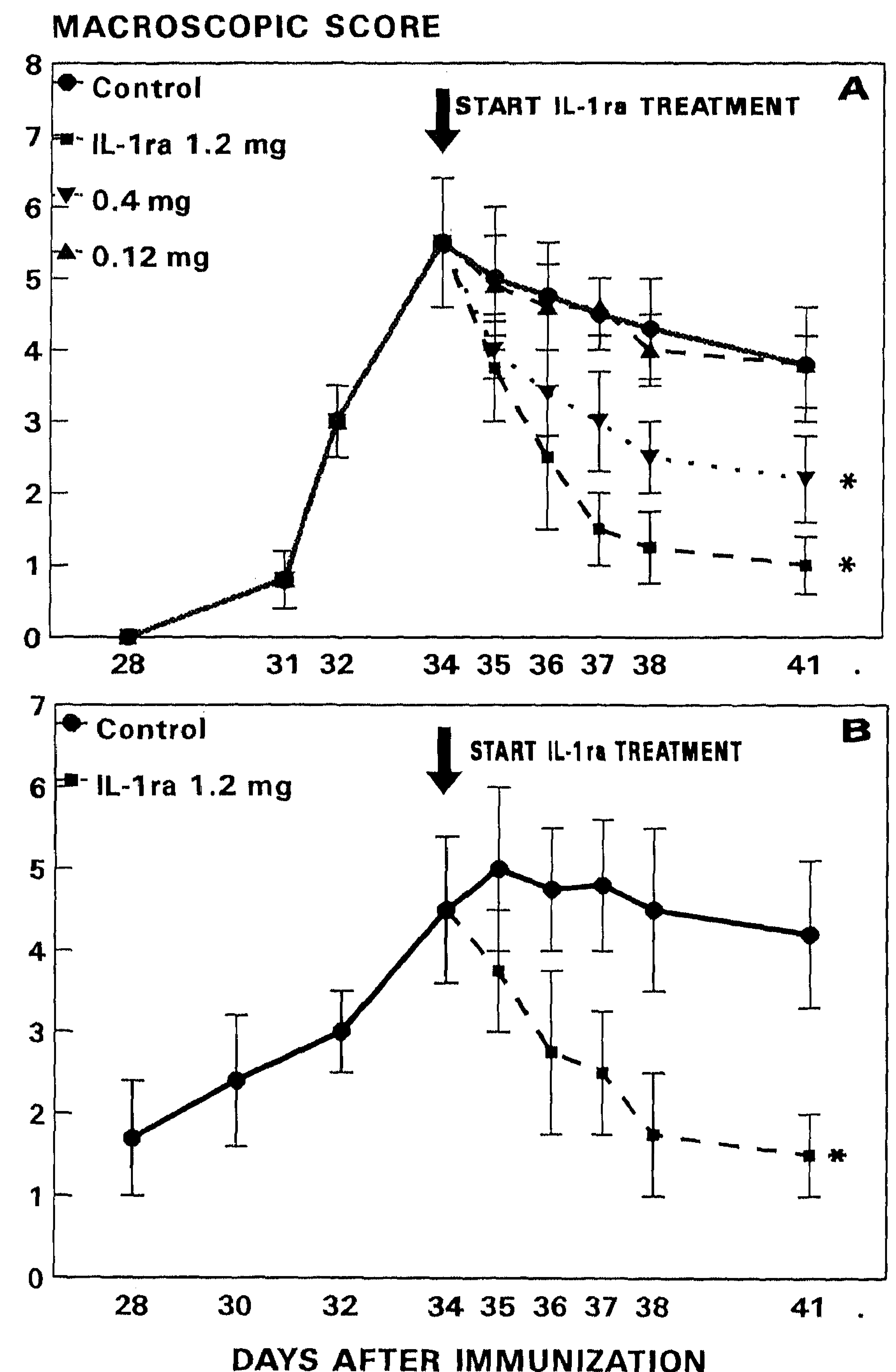


Figure 5. Interleukin-1 receptor antagonist (IL-1ra) treatment of accelerated and classic CIA. **A**, On day 6 after acceleration of CIA, mice were selected based on visual score, and grouped into 4 groups with equal scores for arthritis. Osmotic minipumps (100 μ l) were loaded with 100 (1.2 mg/day), 30 (0.4 mg/day), or 10 (0.12 mg/day) mg/ml of recombinant human IL-1ra and implanted intraperitoneally. Bovine serum albumin (100 mg) was used as a control. Mice were scored daily for arthritis severity, by 2 independent observers. **B**, IL-1ra treatment (1.2 mg/day) of classic CIA was started on day 34 after immunization by intraperitoneal implantation of osmotic pumps. Values are the mean \pm SD of 10 mice per group. * = $P < 0.01$ versus controls, by Wilcoxon rank test. See Figure 1 for other definitions.

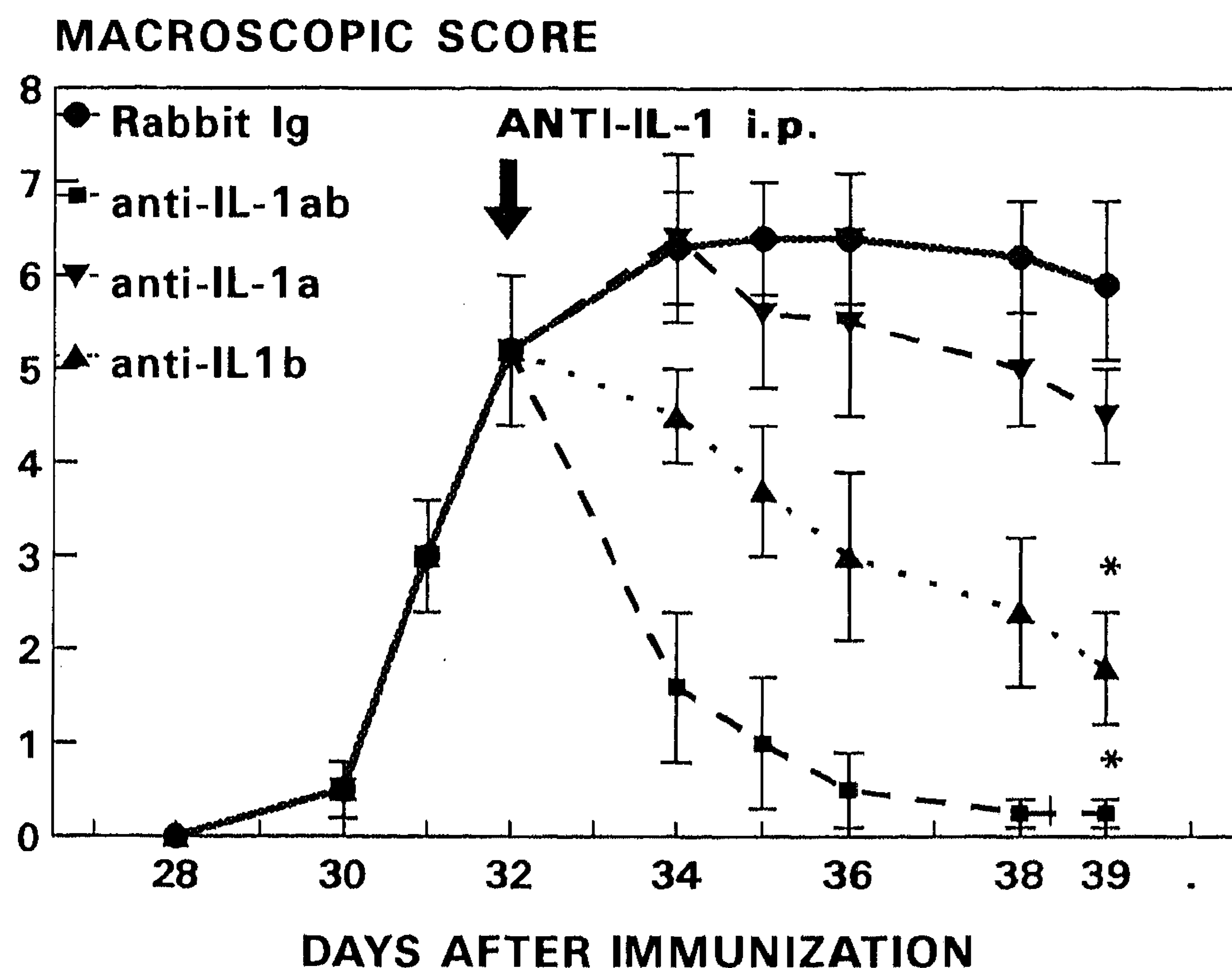
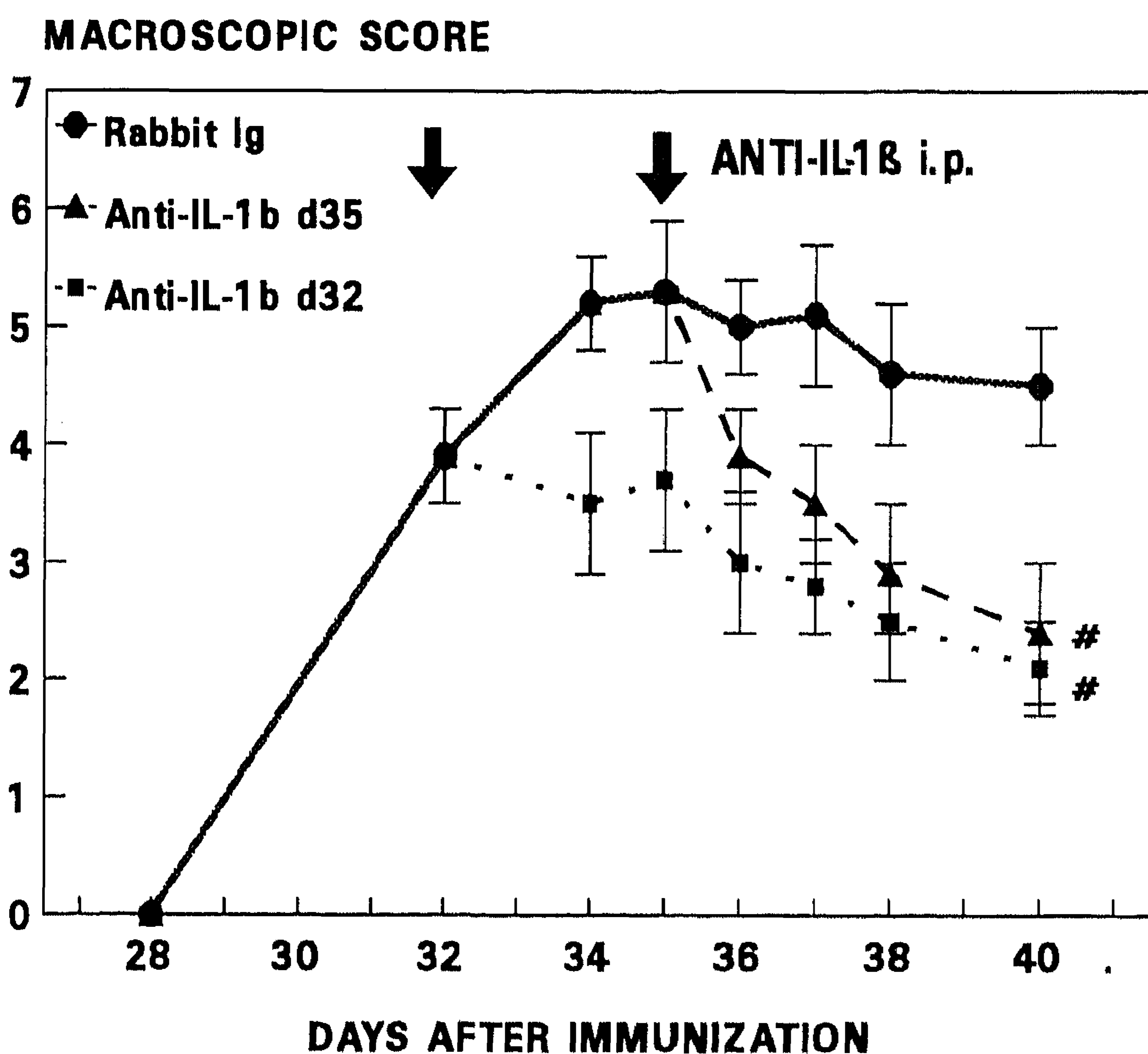


Figure 6. Relative role of IL-1 α and IL-1 β in CIA. Into separate groups of mice with established CIA (day 32), we injected anti-IL-1 α (anti-IL-1a; 0.6 mg), anti-IL-1 β (anti-IL-1b; 0.6 mg), or the combination of both (anti-IL-1ab; 1.2 mg). Values are the mean \pm SD of at least 10 mice per group. * = $P < 0.01$ versus controls, by Wilcoxon rank test. See Figure 1 for other definitions.

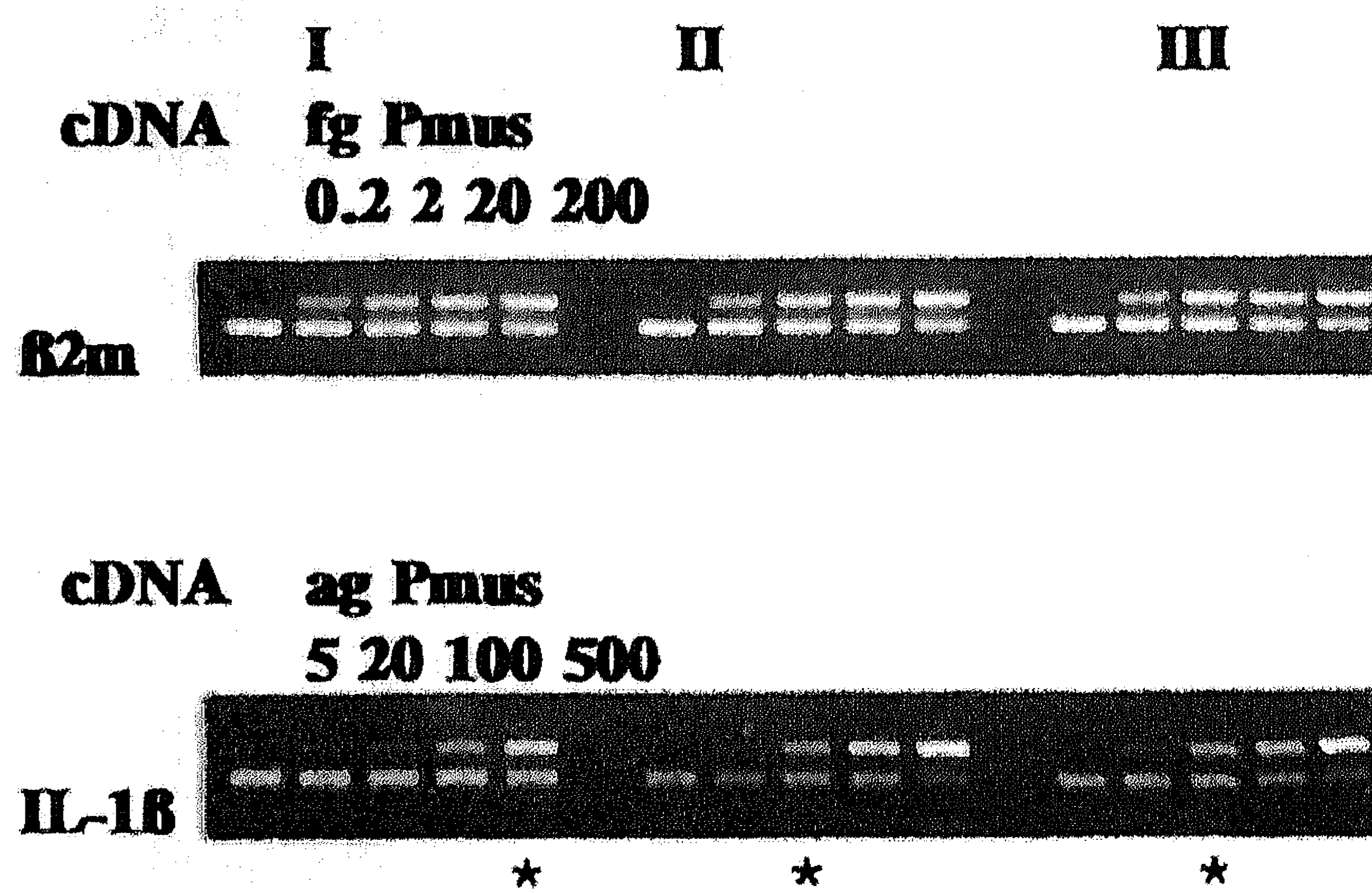
inhibited proteoglycan synthesis, was noted in the control arthritic mice on day 42. In contrast, the IL-1Ra-treated animals showed marked ^{35}S -sulfate incorporation, which was not significantly different from the metabolic activity found in nonarthritic DBA/1 mice (Figure 3). This indicates that IL-1 is a pivotal mediator in this suppression.

Relative roles of IL-1 α and IL-1 β . To investigate the relative contributions of IL-1 α and IL-1 β , we injected mice with either rabbit polyclonal anti-IL-1 α or anti-IL-1 β , on day 32. Selective neutralization of IL-1 β was sufficient to cause marked suppression of arthritis (Figure 6). In a repeat experiment (not shown) we used hamster monoclonal anti-IL-1 β antibodies, which showed roughly similar efficacy. In contrast to the pronounced effect of anti-IL-1 β , we did not observe a major suppression with selective anti-IL-1 α treatment. However, it is clear that the most optimal suppression was reached with the combination (Figure 6).

Cytokine mRNA levels in the arthritic joint. In a repeat experiment, groups of mice were treated with



A



B

Figure 7. Measurement of messenger RNA (mRNA) levels in synovium of control and anti-IL-1 β -treated mice. A, Treatment of accelerated CIA with anti-IL-1 β (Anti-IL-1b) was started at 2 different time points, day 32 (d32) and day 35 (d35) (arrows). In separate groups of 10 arthritic mice, we administered 0.6 mg of antibody raised against murine IL-1 β . The control group received 0.6 mg of control Ig on days 32 and 35. On day 40, mice were killed and samples of synovium and cartilage were isolated for analysis by polymerase chain reaction (PCR). Values are the mean \pm SD of at least 10 mice. # = $P < 0.05$ versus controls, by Wilcoxon rank test. B, Synovium samples from 6 mice per group were pooled, and RNA was extracted. Quantitative PCR was performed as described in Materials and Methods. I = arthritic control group; II = mice treated on day 32; III = mice treated on day 35. The levels for β_2 -microglobulin ($\beta_2\text{m}$; housekeeping gene) were 20 femtograms (fg) in each group. Roughly, a 25-fold reduction (from 500 attograms to 20 ag) in the IL-1 β mRNA level was found after treatment with rabbit anti-murine IL-1 β . * = Equal levels of DNA construct and complementary DNA (cDNA). See Figure 1 for other definitions.

anti-IL-1 β on day 32 or on day 35, and mRNA was extracted from the synovial tissue and patellar cartilage on day 40. The time course of the arthritis is shown in Figure 7A, again proving the efficacy of anti-IL-1 β . Cytokine mRNA levels, as approached by PCR analysis are shown in Table 3. In inflamed synovium, a marked increase compared with normal synovium was found for IL-1 β and IL-1Ra, whereas the enhancement of TNF α and TIMP message was less impressive. Treatment with anti-IL-1 β markedly reduced IL-1 message. This is further illustrated with competitive PCR and an internal standard (Figure 7B). Of interest, IL-1Ra message was also markedly reduced upon anti-IL-1 β treatment, suggesting tight coupling with IL-1. In contrast, the TIMP levels remained constant.

In the arthritic cartilage a similar trend of cytokine expression was observed, although values seemed less enhanced compared with the synovium. Remarkably, anti-IL-1 β treatment did not reduce message expression in the chondrocytes.

DISCUSSION

TNF α is of importance at the onset of collagen-induced arthritis, but appears to be less dominant at later stages. In contrast, IL-1 seems to be a critical mediator both at the onset and at later stages of this experimental arthritis. IL-1 dependence is no longer apparent when the disease enters a noninflammatory phase, characterized by total cartilage destruction and the onset of ankylosis. Of interest, IL-1 β seems to be the more prominent IL-1 isotype in this type of experimental arthritis.

CIA is an autoimmune process directed against type II collagen (CII) in the articular cartilage, and the inflammatory process is a combination of local immune complex formation at the articular cartilage surface and an effector T cell reaction to CII. The present study confirms earlier studies with neutralizing antibodies or with soluble TNF receptor (8,9,22), showing that elimination of TNF α just after the onset of arthritis clearly ameliorates, but does not fully suppress, joint inflammation. Combination therapy with anti-TNF α and anti-CD4 yields more protection in CIA than either treatment alone (42). Anti-CD5 therapy also causes significant suppression (43). However, one should be very careful with anti-T cell treatment, since at late stages of the disease, regulatory T cells are also involved and blocking of T cells may then exacerbate arthritis (44).

Table 3. Levels of cytokine mRNA in samples of inflamed tissue at day 40*

Cytokine	Synovium			Cartilage		
	Control	Anti-IL-1 β		Control	Anti-IL-1 β	
		Day 32	Day 35		Day 32	Day 35
IL-1 β	322	13	13	13	13	13
IL-1Ra	322	25	25	47	47	47
TNF α	13	4	4	4	4	4
TIMP	7	7	7	4	4	4

* On day 40, synovium and cartilage samples were isolated as described in Materials and Methods. Values are the relative level of messenger RNA (mRNA), compared with control (nonimmunized) synovium or cartilage. A 24-fold decrease in mRNA levels for interleukin-1 β (IL-1 β) was found after treatment with anti-IL-1 β antibodies. No differences were found in mRNA on day 40 when treatment was started either on day 32 or day 35. Values are the mean of 3 experiments. IL-1Ra = IL-1 receptor antagonist; TNF α = tumor necrosis factor α ; TIMP = tissue inhibitor of metalloproteinases.

The relative roles of TNF α and IL-1 in various stages and forms of chronic arthritis are of therapeutic relevance, even more so because the present study clearly shows that anti-IL-1 treatment is superior to anti-TNF α treatment in this particular model, and substantiates that inhibition of TNF α does not necessarily also inhibit IL-1 production. It must be underscored that one should exercise caution in the overall interpretation of data obtained with different antibodies, in view of potential differences in neutralizing capacity and half-lives in vivo. Moreover, antibodies may significantly differ in their effector functions, depending on the isotype. In addition to the data presented in this paper, we performed experiments with polyclonal anti-TNF α (kindly provided by S. Kunkel and G. Grau) and in fact confirmed the slight suppression after treatment of established disease (unpublished observations).

Our current data with anti-TNF α treatment are consistent with earlier findings in CIA (8,9,44). Moreover, our anti-TNF α was sufficiently potent to fully block LPS-mediated shock, whereas anti-TNF α treatment was also highly suppressive in another murine arthritis model, streptococcal cell wall-induced arthritis (Figure 4B) (45). Apparently, bacterial triggers provoke a more TNF-driven process. Anti-TNF α and anti-IL-1 treatment appeared equally effective in the reactivation of SCW-induced arthritis, and the best suppression was obtained with the combination of anti-IL-1 with anti-TNF α (46).

The importance of IL-1 in fully established CIA is now further substantiated, showing identical sup-

pression with anti-IL-1 antibodies and IL-1Ra. Given the limited half-life of the receptor antagonist, its high pharmacokinetics in mice in general, and the need to occupy almost all IL-1 receptors to block the action of IL-1 (47,48), it is conceivable that profound suppression of established CIA could only be achieved with high levels of IL-1Ra, supplied with osmotic minipumps. An earlier study showed the efficacy of repeated, systemic dosing with IL-1Ra before the onset of CIA (49). However, this treatment clearly suppressed the anti-CII antibody levels and, apart from synovial inflammation, which may require different pharmacokinetics, probably mainly affected cellular processes in the lymphoid organs.

Further comparison with other arthritis models reveals that IL-1 is not a dominant inflammatory mediator in all forms of arthritis. Neither TNF α nor IL-1 is of key importance in the initiating inflammatory process of antigen-induced arthritis (26,49,50). Yet, elimination of IL-1 fully normalized chondrocyte proteoglycan synthesis inhibition in the articular cartilage, proving that IL-1 is a key mediator in this characteristic, undesired disturbance of anabolic processes in arthritic cartilage (26). The general validity of the latter role of IL-1 is now established by similar findings in immune complex arthritis and in T cell-driven flares of antigen-induced arthritis (51,52).

IL-1 blockade in CIA also profoundly reduced cartilage destruction, even when treatment was given late. Since joint inflammation was markedly suppressed as well, it cannot be excluded that the protective effect on articular cartilage was indirect, through concomitant reduction of destructive mediators other than IL-1. Early anti-TNF α treatment also reduced cartilage destruction, consistent with its antiinflammatory effect, but at later stages, no protection could be seen. This excludes a selective, direct role of TNF α in cartilage destruction. Studies with anti-IL-1R antibodies in TNF-transgenic mice provided further support for this (53). Although TNF α can be destructive to cartilage *in vitro* (54), relatively high dosages are needed, and such a role could not be substantiated by intraarticular injection into the knee joints of mice and rabbits (25). However, in the presence of low concentrations of IL-1, TNF α may synergize with IL-1 (55).

To provoke the expression of slumbering CIA, additional TNF α appeared to be as powerful as additional IL-1 (17–20). These cytokines can induce chemotactic factors, and the fact that TGF β , which is a powerful immunosuppressive but also a potent chemotactic factor, can also accelerate CIA expression is in

keeping with this reasoning (19,20,56,57). Recently, it was shown that expression can also be enhanced by systemic administration of LPS (30) or by local injection of Zymosan (31). Dependent on the dose, the latter injection caused primary expression of CIA in the injected joint, or spillover occurred in the draining limb. The inflammation in the draining site, in particular, was sensitive to anti-TNF α treatment. However, at both sites, anti-IL-1 treatment was superior in suppressing inflammation (31). Although it cannot be totally excluded that LPS acceleration may shift cytokine balances, our present data show similar efficacy of anti-TNF α and anti-IL-1 treatment in spontaneous (classic) CIA, as compared with LPS-accelerated CIA.

Although the severity of CIA is generally scored in the paws, profound inflammation is also seen in the knee joint. This in contrast to a rather selective paw involvement in classic adjuvant- and SCW-induced arthritis in Lewis rats (58). A great advantage of knee joint analysis is the higher degree of standardization of histologic sections and the allowance for detailed analysis of message expression in defined samples of synovial tissue and cartilage strips.

Message expression for IL-1 β is highly up-regulated in both synovium and cartilage. Anti-IL-1 β treatment profoundly reduced this message in the synovium, but unexpectedly, the message in the cartilage remained high. The process in the articular cartilage probably is autocrine or paracrine regulated in that phase of the arthritis. Anti-IL-1 β antibodies will not enter the cartilage, and further analysis of message expression and functional analysis of cartilage metabolism after IL-1Ra treatment is warranted. TNF α expression seemed less enhanced compared with IL-1 β , but was nevertheless clearly higher than control values in both synovium and cartilage. TNF α message was also reduced in the synovium after anti-IL-1 β treatment, indicating that reduced activation of the cells in the synovium, in general, was achieved with such treatment.

The first clinical trials with anti-TNF α antibodies in RA patients show promising symptomatic relief, and analysis of synovial tissue points to decreased expression of adhesion molecules, consistent with reduced cell influx and/or local cell activation (3,10–12). Efficacy seems to be related to the subclass of antibodies used. Data on protective effects against cartilage destruction are thus far lacking. Whether the process of CIA is close to that of the disease in humans is, of course, hard to tell, but if so, it would be tempting to speculate that the chronic phase of the

disease in humans should in fact be considered as a repetitive flare, given the $\text{TNF}\alpha$ dependence of the onset of CIA and bacterial flares and the relative insensitivity at later stages. It cannot be excluded that anti-IL-1 treatment would still be superior to anti- $\text{TNF}\alpha$ treatment, particularly in terms of protection against cartilage destruction. Unfortunately, good neutralizing humanized anti-IL-1 antibodies are not yet available, and the recent trials with IL-1Ra have not been fully analyzed (3,4). Based on our observations of the need of extremely high IL-1Ra dosages to affect cartilage proteoglycan synthesis disturbance in antigen-induced arthritis (26), or to suppress CIA, underdosing in the present clinical trials may be a serious concern.

Although it is difficult to extrapolate pharmacokinetic findings in mice to demands in humans, it is hoped that substantially higher doses of IL-1Ra are being considered for upcoming trials, since the first results have been mediocre. Meanwhile, it is tempting to further invest in the development of selective IL-1-production inhibitors or inhibitors of IL-1-converting enzyme (ICE). The present data in CIA have identified IL-1 β as a relevant therapeutic target in this form of experimental arthritis. ICE-deficient mice have recently been established (59), and proper analysis of various forms of arthritis in these "knock-out" mice seems warranted, to further identify IL-1 β or ICE as a valid therapeutic target.

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