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# Acceleration of onset of collagen-induced arthritis by intra-articular injection of tumour necrosis factor or transforming growth factor-beta

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## SUMMARY

We examined whether tumour necrosis factor (TNF) or transforming growth factor-beta 1 (TGF-$\beta$1) could alter the course of collagen-induced arthritis (CIA). Injection of 100 ng TNF or 500 ng TGF-$\beta$1 into ankle joints of normal rats induced a very limited inflammatory response, observable only upon histological analysis. However, when injected into ankle joints of rats 9 days after immunization with bovine type II collagen (CII), identical doses of TNF or TGF-$\beta$1 induced a sustained, clinically obvious inflammation and oedema that began within 8 h on average, as compared to 90 h in CII-immunized control rats given no injections or intra-articular injections of buffer. The incidence of arthritis at 2 weeks post-immunization was 100% for TNF-injected hindpaws, compared with 55% for the control groups, a statistically significant difference. In rats passively immunized with a subarthritic dose of affinity purified antibody to rat-CII, intra-articular injection of 100 ng TNF or 500 ng of TGF-$\beta$1 also induced intense, though transient arthritis. The rapid proinflammatory effects in CIA described in this study and the synergy demonstrated between anti-CII IgG and either cytokine, suggest that these cytokines can participate locally in the pathogenesis of arthritis.

**Keywords** tumour necrosis factor transforming growth factor-beta 1 collagen-induced arthritis rats intra-articular injection

## INTRODUCTION

Several cytokines, including tumour necrosis factor (TNF), transforming growth factor-beta 1 (TGF-$\beta$1), IL-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been detected in the synovial effusions of rheumatoid arthritis patients [1–6]. Immunolocalization of both TNF and TGF-$\beta$1 to synovial lining cells and at the cartilage/pannus junction [7,8] indicates that either may act locally in rheumatoid arthritis. Recent studies have shown that exogenous TGF-$\beta$1 can induce synovial hyperplasia [9,10] and that unregulated production of TNF in an interesting new transgenic mouse line results in spontaneous erosive arthritis [11]. Here we focused on local effects of exogenous TNF and TGF-$\beta$1, on the early stages of collagen-induced arthritis (CIA).

CIA in rats is an erosive autoimmune polyarthritis involving both humoral and cell-mediated immune responses that resembles human rheumatoid arthritis [12–16]. We demonstrate here that intra-articular injection of either TNF or TGF-$\beta$1 accelerates and intensifies arthritis locally in type II collagen (CII)-immunized rats, and that each cytokine also elicits rapid onset of clinical arthritis in passively immunized rats.

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## MATERIALS AND METHODS

### *Animals*

Six week old female Wistar Outbred rats (175 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals were housed at the Nashville Veterans Administration Medical Centre and all protocols used in the study were approved by a joint Vanderbilt University/ Department of Veterans Affairs animal use committee.

### *Cytokines*

Recombinant human TNF and TGF-$\beta$1 obtained from two sources gave identical results. TNF was purchased from Research and Diagnostic Systems (Minneapolis, MN) or donated by Knoll Pharmaceuticals (Whippany, NJ). Endotoxin contamination was not detectable when assayed with an E-Toxate Kit (Sigma Chemical, St Louis, MO). Aliquots containing 1 mg/ml TNF in PBS with 0·1% bovine serum albumin (PBS/BSA) were stored at −70°C, and never refrozen. Endotoxin-free recombinant human TGF-$\beta$1 was donated by Genentech Inc. (San Francisco, CA) or Oncogen (Seattle, WA). Controls utilizing contralateral joints that received injections of cytokine-free PBS/BSA ensured that buffer components did not contribute to our results.

To inactivate TNF, an aliquot of 10 μg/ml TNF in PBS/BSA was heated at 65°C for 3 h and tested in a mouse L-cell cytotoxicity assay [17]. Heating reduced its biological activity by five orders of magnitude compared with an unheated control. SDS–PAGE showed heating caused no loss of protein.

*Collagens*

Bovine and rat type II collagen were prepared as described [18,19]. Collagen was stored lyophilized and was dissolved overnight in 0·1 N acetic acid at 4°C before use as an immunogen or in ELISA.

*Immunization with collagen*

Bovine CII (2 mg/ml) in 0·1 N acetic acid was emulsified in an equal volume of Freund's complete adjuvant (FCA; Difco, Detroit, MI) at 4°C. Rats were anaesthetized with Enflurane (Ethrane, Anaquest, Madison, WI) and given intradermal injections of 200 μl of emulsion (200 μg CII) at the base of the tail.

*Isolation by affinity chromatography of anti-bovine type-II collagen IgG cross-reactive with rat type-II collagen*

Sera from immunized rats were pooled and anti-CII IgG was affinity purified by batch absorbtion to rat-CII coupled to Sepharose beads. Serum was filtered through a 0·45 μm filter and then incubated overnight at 4°C with the CII-coupled beads. After extensive washing with PBS, antibody was eluted with 0·2 M glycine buffer (pH 2·8), immediately neutralized, concentrated by pressure ultrafiltration and dialysed against PBS. Aggregated IgG was removed by centrifugation at 100 000 ***g*** for 1 h at 4°C, the IgG was filter sterilized and its anti-rat CII titre determined by ELISA.

*ELISA for CII-reactive antibodies*

Levels of rat CII-reactive antibody were determined by a modification of a previously described method [19]. Each well of a serocluster, U-vinyl microtitre plate (Costar Corporation, Cambridge, MA) was coated overnight at 4°C with 100 μl of 5 μg/ml rat CII and blocked by incubation with 0·5% ovalbumin (Grade V, Sigma) in PBS. After washing, a 100 μl aliquot of each rat serum, diluted in 0·1 M Tris, 0·15% NaCl containing 0·5% Tween 20, was added and the plates were incubated at 25°C for 2 h, washed again, and incubated at 25°C for 1 h with 100 μl of an appropriate dilution of peroxidase conjugated anti-rat IgG (ICN Immunobiologicals, Lisle, IL). The plates were washed and colour development of *o*-phenylenediamine dihydrochloride (Sigma), was monitored at 450 nm in a Model EL310 Microplate Autoreader (Biotek Instruments, Winooski, VT). The anti-CII titre for each sample (1:1000 dilution) was determined from its $OD_{450}$ at the point when a standard anti-CII rat serum (1:5000 dilution) reached an $OD_{450}$ of 1·0 unit. Anti-CII titres were normalized with the same standard anti-CII serum throughout the study.

*Ankle and knee injections with cytokines*

Injections of 10 μl of sterile PBS/BSA either with or without exogenous cytokine were made into ankle joints through the Achilles tendon just above the calcaneous. Knees were injected through the infrapatellar fat pad with the joint slightly flexed. Injections were performed with a 30 gauge needle and a 25 μl Hamilton syringe in a mechanical syringe driver (Hamilton Co., Reno, NV). Animals were anaesthetized with Ethrane for ankle injections and with a mixture of 80 μg Ketamine and 12 μg Xylazine/g of body weight for knee injections. The method of anaesthesia did not influence the results of cytokine injection.

*Evaluation of arthritis*

Clinical severity of arthritis (arthritic or clinical index) was scored on a scale from 0 to 4 (whole integers, with 4 equal to most severe inflammation) based on the enlargement, erythema and oedema of the periarticular tissues [12,20]. Arithmetic means were calculated by dividing the sum of arthritic indices by the number of hindpaws scored.

*Histology of joint tissues*

Rats were sacrificed by $CO_2$ narcosis and the hindpaws or knee joints removed. Specimens were fixed for 24 h in 2% gluteraldehyde in PBS, bisected, decalcified in rapid bone decalcifier (RDO, Dupage, Kinetic Labs, Inc., Plainfield, IL), returned to 2% gluteraldehyde and submitted for routine paraffin embedding. Tissue sections were routinely stained with haematoxylin and eosin and representative tissue sections were also stained for fibrin using Landrum's Acid Picromallory stain [21]. Quantitative analysis of polymorphonuclear leucocyte (PMN) influx was performed exactly as previously described [10,22].

*Passive immunization with affinity purified anti-rat CII IgG and cytokine injection*

Rats were anaesthetized with Ethrane, and 4 mg sterile anti-CII IgG in PBS was injected into the tail vein. Preliminary experiments not shown indicated that 4 mg of anti-CII IgG per rat was insufficient to induce arthritis. After 1 h, each rat was anaesthetized and 100 ng TNF or 500 ng of TGF-β1 in PBS/BSA were injected into the left ankle and PBS/BSA into the right. The rats were examined periodically for hindpaw swelling and assigned a clinical index as described above. Repeat injections were performed in the same manner.

*Immunolocalization of rat IgG in articular cartilage*

Whole knee or ankle joints fixed overnight at 4°C in acetone were bisected in the sagittal plane. The joint tissue was embedded in JB-4 glycolmethacrylate and sections immunostained. After hydration in PBS and incubation in 0·3% $H_2O_2$ in methanol, the sections were rehydrated in PBS, incubated with 10% normal rabbit serum, and incubated overnight at 4°C with horseradish peroxidase conjugates of either rabbit anti-rat IgG or anti-human IgG (control) at 1:200 dilution. After washing, the sections were incubated with diaminobenzidine in 0·1 M TRIS, pH 7·5 for 10 min and counter-stained with haematoxylin or toluidine blue.

*Statistical analysis*

The significance of the differences observed in incidence of disease was determined by $\chi^2$ analysis. Continuous variables (clinical index, time of onset, antibody levels) were compared using the paired Student's *t*-test.

**Table 1.** Correlation of time of onset of arthritis with antibody levels measured 9 days after type II collagen (CII) immunization and incidence of arthritis at 4 weeks

| Mean level of antibody* | Average time of onset† | Arthritis incidence |
|---|---|---|
| 1·23 ± 0·03 (range 0·93–1·6) | within 17 h | 5/5 |
| 0·27 ± 0·02 (range 0·12–0·67) | 92 h | 6/10 |
| 0·03 ± 0·03 (range 0·01–0·08) | none at 4 weeks | 0/8 |

* Anti-CII measured by ELISA and compared to a standard rat CII antiserum ($OD_{450} = 1·00$) used throughout the studies. Values for each group are the mean $OD_{450} \pm$ s.e.m.

† Defined as the time at which the first signs of hindpaw swelling were observed, measured from $t_0$, the time serum samples were taken for the selection procedure 9 days post-immunization.

## RESULTS

*Effect of intra-articular injection of cytokines on non-immunized rats.*

Intra-articular injections and histological studies were performed on non-immunized rats, to determine appropriate doses of each cytokine to use in the experiments below. Similar results were observed at doses of 0·1–1 μg of TNF and 0·5–1 μg of TGF-β1, in both knee and ankle (tibiotarsal) joints.

Despite the absence of clinical swelling, histological analysis showed that both cytokines rapidly produced marked cellular inflammation in the synovial membrane and joint space compared with PBS/BSA injection into contralateral joints. PMN influx was observed 2–4 h after intra-articular injection of 1 μg of either cytokine and peaked at approximately 6 h, but only a few PMN were at the articular cartilage surface [22]. An accumulation of plasma exudate containing fibrin was observed in the joint space as well. Later (4 days), evidence of synovial hyperplasia and membrane thickening was apparent after injections of 1 μg TGF-β1 [10,22]. In contrast, inflammation subsided rapidly with only minimal changes present in joints 24 h after TNF injections. With neither cytokine was there evidence of cartilage degradation or bone erosion.

Since in non-immunized animals no swelling occurred after injection of ankle joints with 100 ng TNF or 500 ng TGF-β1, these doses were used in the experiments described below.

*Selection of immunized rats for evaluation of intra-articular cytokine injection on the development of CIA*

The onset time of arthritis for individual CII-immunized rats varies considerably. Therefore, a selection procedure was developed to obtain animals with an average onset time appropriate for testing the effects of cytokine injection on CIA. Previous studies reported that onset of arthritis correlated with anti-CII titre [14,23]. Daily measurements of anti-CII by ELISA (7–12 days post-immunization) indicated that antibody levels measured on day 9 were useful in predicting the time of onset of arthritis. Results of such measurements in a group of 23 CII-immunized rats are presented in Table 1.

All rats with high anti-CII titres ($OD_{450} > 0·9$) developed hindpaw swelling within 17 h of serum sampling, too short a time interval to be useful in this study. Among rats with midrange anti-CII titres ($OD_{450}$ from 0·1 to 0·7), approximately 60% developed hindpaw swelling an average of 92 h after serum sampling. Rats with very low titres ($OD_{450} < 0·1$) never exhibited hindpaw swelling (30 days of observation).

In view of these results we subsequently selected rats with mid-range titres 9 days post-immunization for the studies described below. Histologic examination of tissue from such rats revealed that no inflammatory changes were yet present in ankle joints (data not shown).

*Effects of intra-articular injection of cytokines on the onset, incidence and severity of CIA in selected rats*

Rats selected as described above were divided into (i) cytokine-injected; (ii) PBS/BSA-injected; and (iii) non-injected groups as shown in Table 2, such that the mean levels of anti-CII for each group were similar. Intra-articular injections of either 100 ng TNF, 500 ng TGF-β1 or PBS/BSA were given into ankle joints approximately 6 h after serum was collected, the time required for analysis by ELISA.

Introduction of either cytokine into ankle joints dramatically accelerated the onset of arthritis relative to controls. As summarized in Table 2, injection of either TGF-β1 or TNF resulted in obvious swelling of the injected hindpaw within an average of 7–8 h, respectively. In contrast, swelling did not occur until an average of 86 and 95 h later, respectively, for non-injected or PBS/BSA-injected hindpaws. Biologically active TNF was required for this effect, as rats given intra-articular injections of heat-inactivated TNF exhibited a similar disease course to control groups. It was not possible to inactivate TGF-β1, therefore this control was not performed.

The kinetics of onset and the overall incidence of arthritis in each group of rats is illustrated in Fig. 1. By 8 h post-injection, the incidence of hindpaw swelling was 100% for TNF-injected and 77% for TGF-β1-injected rats, compared with only 20% for PBS/BSA-injected and 30% for non-injected rats, even at 96 h. Arthritis in cytokine-injected hindpaws persisted in all TNF-injected rats and in all but one TGF-β1-injected rat, shown at 96 h in Fig. 2 and at 7 days in Table 2. The incidence of arthritis remained the same for 2 weeks for the cytokine-injected rats, while it rose in control groups to 55% (data not shown). However, the increased incidence relative to controls at 7 and 14 days was statistically significant only for TNF.

Intra-articular injection of either cytokine also increased the extent of hindpaw swelling. Figure 2 illustrates the kinetics and severity of the swelling induced by each cytokine, expressed by a clinical index score. Rats injected with TNF or TGF-β1 displayed severe swelling within 6–8 hours, unlike rats in the control groups which showed significantly less severe swelling that began 2–3 days afterwards.

The local proinflammatory effects just described could not be duplicated by systemic administration of 10 μg TNF or 2 μg TGF-β1 injected either into the peritoneal cavity or subcutaneously, remote from the joints. Also, neither the time of onset nor the severity of arthritis was significantly different from controls in contralateral hindpaws of rats that received cytokine injections in the other paw.

Whether intra-articular injections of 100 ng of TNF could induce persistent arthritis in animals with low day 9 titres

**Table 2.** Effects of intra-articular injection of tumour necrosis factor (TNF) or transforming growth factor-beta 1 (TGF-β1) on incidence and time of onset of collagen-induced arthritis

| Group | Incidence at 48 h | Incidence at 7 days | Average time of onset* | Mean level antibody† |
|---|---|---|---|---|
| TNF‡ ($n=10$) | 10/10 | 10/10 | 7·8±2·8 h (range 6·0–12·0 h) | 0·343±0·017 |
| TGF-β1§ ($n=13$) | 9/13 | 8/13 | 6·9±2·2 h (range 6·0–21·0 h) | 0·324±0·127 |
| PBS/BSA ($n=10$) | 1/10 | 5/10 | 94·5±49·5 h (range 60·0–168·0 h) | 0·356±0·025 |
| No injection ($n=10$) | 1/10 | 6/10 | 86·4±21·5 h (range 72·0–120·0 h) | 0·268±0·018 |
| Heated TNF | 0/3 | 2/3 | >96 h¶ | 0·314±0·050 |

* Values are the mean ±s.e.m. for each experimental group.

† Anti-type II collagen (anti-CII) measured by ELISA on day 9 after immunization and compared to a standard rat CII antiserum ($OD_{450}=1·000$) used throughout the studies. Values for each group are the mean $OD_{450}$ ±s.e.m.

‡ Differences in incidence for TNF-injected animals compared with PBS/BSA- and non-injected controls, $P<0·01$ at 48 h and $P<0·02$ at 7 days after injection.

§ Differences in incidence for TGF-β1-injected animals compared with PBS/BSA- and non-injected controls, $P<0·02$ at 48 h and $P<0·25$ (NS) at 7 days after injection.

¶ Not examined between 96 h and 7 days.

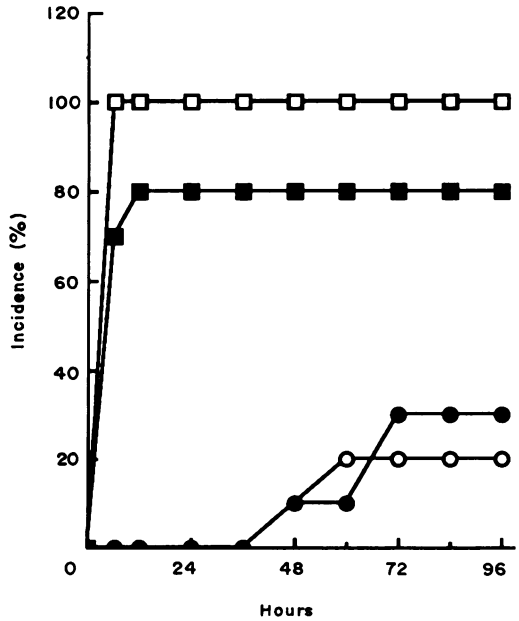


**Fig. 1.** Effect of intra-articular injection of tumour necrosis factor (TNF) or transforming growth factor-beta 1 (TGF-β1) on the incidence of arthritis in type II collagen (CII)-immunized rats. CII-immunized rats selected on the basis of anti-CII titre 9 days post-immunization (see Results) received one intra-articular injection of: TNF (0·1 μg in 10 μl PBS/BSA) (□); TGF-β1 (0·5 μg in 10 μl PBS/BSA) (■); PBS/BSA (10 μl) (○); or no injection (●). Incidence is expressed as percentage of animals in each group developing clinically obvious swelling in the injected paw. $t_0$ is defined as the time of intra-articular injection.

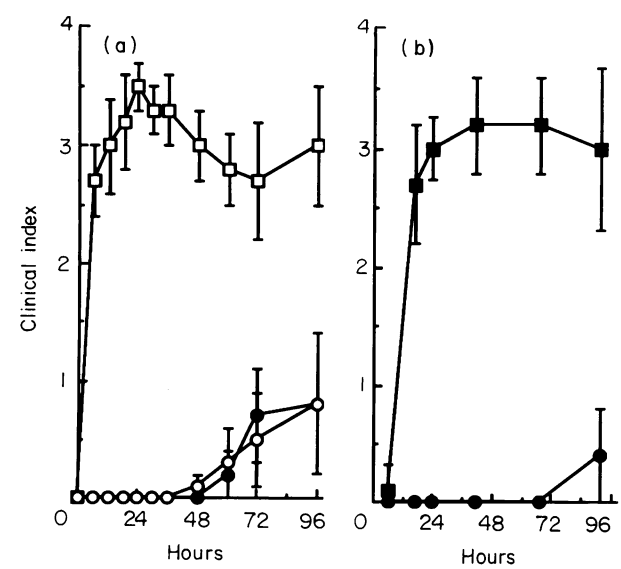


**Fig. 2.** Effect of intra-articular injection of tumour necrosis factor (TNF) or transforming growth factor-beta 1 (TGF-β1) on severity of type II collagen (CII)-induced arthritis in rats. CII-immunized rats selected as described in Results received intra-articular injection of one ankle at $t_0$ with: (a) TNF (0·1 μg in 10 μl PBS/BSA) (□); PBS/BSA (10 μl) (○); or no injection (●). (b) TGF-β1 (0·5 μg in 10 μl PBS/BSA) (■); PBS/BSA (10 μl) (●). Hindpaws were monitored three times daily and swelling scored on a scale of 0–4 (4 equal to severe swelling). Data shown are for the injected paw only. Differences in the clinical index at 48 h compared with controls were statistically significant for both TNF- and TGF-β1-injected animals ($P<0·001$).

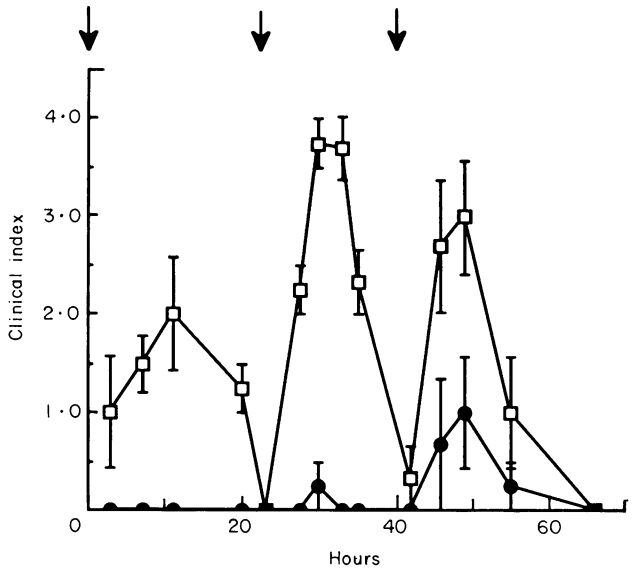


**Fig. 3.** Repeated cycles of clinical arthritis triggered by intra-articular injection of tumour necrosis factor (TNF) into hindpaws of passively immunized rats. Rats ($n=4$) were passively immunized by i.v. injection of 4 mg of affinity purified anti-rat specific IgG isolated from pooled rat sera. At the times indicated by the arrows, TNF (100 ng) in 15 $\mu$l of PBS/BSA was injected into the left hindpaw (□); or 15 $\mu$l of PBS/BSA as a control into the right hindpaw (●). The degree of hindpaw swelling was monitored afterward at the times indicated and scored as described in Materials and Methods (0 = no swelling and 4 = intense swelling and erythema). Each point up to 36 h represents the mean of combined clinical indices of four animals, and since one was sacrificed for histological analysis, three animals thereafter. $t_0$ was 1 h after passive immunization.

($OD_{450}=0{\cdot}059$–$0{\cdot}102$) was also examined. A mild, transient swelling that peaked approximately 14 h after injection, was observed in the injected hindpaws of each of six animals chosen for this experiment. However, only one animal ($OD_{450}=0{\cdot}069$) had sustained arthritis 96 h later (data not shown).

*Long term effects of intra-articular cytokine injections on CII-immunized and non-immunized rats.*

Compared with controls, intra-articular TNF injections did not significantly increase the overall clinical severity of arthritis assessed both at 6 weeks and at 4 months on the basis of a total score for all limbs, although a trend towards increased severity was noted. The histopathology of cytokine-accelerated CIA was no different from controls assessed late in the disease, and included extensive inflammatory changes with erosion of articular cartilage and bone (data not shown).

*Rapid induction of acute clinical inflammation by TNF in rats passively immunized with affinity-purified anti-CII IgG.*

Rats passively immunized with 4 mg of affinity purified anti-CII IgG were given 100 ng TNF by intra-articular injection which rapidly induced intense hindpaw swelling in 6/6 animals. Injection of PBS/BSA into the opposite paw had little or no effect. Fig. 3 shows the mean clinical index for four rats subjected to multiple injections of TNF or PBS/BSA. Note that swelling was virtually undetectable in the PBS/BSA-injected opposite paws, even during maximum TNF-induced swelling. The TNF-induced swelling subsided after approximately 24 h.

A second injection of TNF into the same ankle joint triggered even more intense swelling, with approximately the same time course as the first inflammatory cycle. A third cycle triggered by TNF injection was less intense, possibly due to depletion of the circulating anti-CII IgG. Mild swelling occurred in one control hindpaw after the third injection of PBS/BSA, suggesting that in the presence of anti-CII IgG, the trauma of repeated injections could occasionally induce swelling.

TGF-$\beta$1 injection (500 ng) was also examined with two rats passively immunized in the same manner (data not shown). Both rats developed arthritis in the injected hindpaw that reached an arthritic index of 4 within 48 h, and that persisted for 3 days with slowly declining severity. As with TNF, a repeat injection of TGF-$\beta$1, once the swelling had subsided, induced a second wave of intense hindpaw swelling. Induction of additional inflammatory cycles was not attempted.

To ascertain whether TNF injection could alter the ability of anti-CII IgG to deposit in/on the articular cartilage, passive immunization experiments were repeated with the TNF injected into knee joints to facilitate histological analysis. Rat IgG was deposited fairly uniformly at the surface and within the articular cartilage, especially in regions that appeared by toluidine blue metachromasia to be depleted of proteoglycan. Some IgG was deposited on the articular cartilage surface in the PBS/BSA-injected knees, but none was found within the cartilage matrix. Similarly, immunoreactivity was seen only at the cartilage surface in rats passively immunized but given no intra-articular injection.

## DISCUSSION

Our results suggest that both TNF and TGF-$\beta$1 can play an important role in initiating the inflammation associated with CIA. A single intra-articular injection of either cytokine produced a dramatic acceleration in onset of arthritis in rats after either active immunization with CII or passive immunization with anti-CII IgG.

Various cytokines have been implicated as positive effectors of arthritic inflammation [24] and the effects of several on the progress of experimental arthritis have been examined, including IL-1 [25,26], IFN [27], and quite recently TGF-$\beta$1 [28]. The observed effects of exogenous cytokines are dependent on the experimental model used, the dose, timing and route of administration. For example, although daily systemic administration of 5 $\mu$g TGF-$\beta$1 had no effect after the onset of CIA, protective effects resulted if TGF-$\beta$1 was given before onset of the disease [28]. The single intra-articular injections of cytokines in the experiments reported here differ from these previous reports.

The fact that TNF [29,30] and TGF-$\beta$1 [31,32] affect the immune response quite differently, the rapidity of the effect of each cytokine on CIA reported here, the inability to duplicate these effects with systemic administration, and the triggering of arthritis in passively immunized animals all suggest that the local proinflammatory effects induced by injection of these cytokines are unrelated to their effects on immune responsiveness.

Both cytokines are likely to affect quite differently other processes relevant to the pathology of arthritis. For example, TGF-$\beta$1's effects on connective tissue tend to enhance produc-

tion and deposition of extracellular matrix components [33]. On the other hand, TNF increases collagenase production by synovial cells [34] and induces chondrocytes both to degrade and to reduce synthesis of proteoglycan [35]. Such proteolytic damage due to direct effects of TNF on chondrocytes and/or via neutral proteases released from neutrophils recruited by either TNF or TGF-β1, could have increased accessibility of cartilage CII to anti-CII IgG. The enhanced penetration of anti-CII IgG into cartilage matrix that we observed in passively immunized rats injected with TNF is consistent with this mechanism. A recent study that demonstrated enhanced reactivity of anti-CII with cartilage after its exposure to neutrophil elastase [36] also supports such a notion.

In rat joints TNF and TGF-β1 did have in common a remarkable effect on PMN recruitment [10,22]. TNF may have recruited PMN through various indirect mechanisms [37–40] whereas TGF-β1 is a very potent direct chemoattractant of PMN [22]. Both cytokines also increase the production of other PMN chemotactic agents such as IL-1 and IL-8 [41–43]. The kinetics of cytokine-induced PMN recruitment into normal rat joints [10,22] correlated well with the onset time of hindpaw swelling induced by cytokine injection in CII-immunized rats, suggesting that recruited PMN in the presence of anti-CII IgG triggered the onset and intensity of clinical arthritis in these models. Further studies are necessary to be certain that this is the case. The results reported here suggest that CIA is an appropriate model for further exploration of these events.

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