

## RAPID ONSET SYNOVIAL INFLAMMATION AND HYPERPLASIA INDUCED BY TRANSFORMING GROWTH FACTOR $\beta$

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Transforming growth factor  $\beta$  (TGF- $\beta$ ),<sup>1</sup> originally isolated from platelets as a 25-kD peptide (1, 2), has been shown to be a product of other inflammatory cells, including lymphocytes (3, 4) and macrophages (5, 6), and to have potent immunomodulatory effects (7). Although five different forms of TGF- $\beta$  have now been characterized (1), only TGF- $\beta$ 1 and its homologue, TGF- $\beta$ 2 (8, 9), have thus far been identified in hemopoietic cells (6). Each of these peptides has been cloned, demonstrating that TGF- $\beta$ 1 is encoded as a 390 amino acid precursor (10), TGF- $\beta$ 2 as a 412 amino acid precursor (11, 12), and that both have a signal peptide of 20–23 amino acids at the NH<sub>2</sub> terminus. The processed 112 amino acid chains of the two peptides share 72% sequence homology and appear to bind equally well to TGF- $\beta$  class III receptors (13), although TGF- $\beta$ 1 binds with greater affinity than TGF- $\beta$ 2 to class I and II receptors (14, 15). Interestingly, lymphoid cells appear to possess only class I and II TGF- $\beta$  receptors (16), although in vitro, lymphocyte and monocyte responses to TGF- $\beta$ 1 and  $\beta$ 2 appear comparable (6, 17, 18). The secretion of TGF- $\beta$  peptides by lymphocytes and monocytes only after activation (3–6) implicates these polypeptides in the evolution of immunologic processes, and in recent studies TGF- $\beta$  has been identified in chronic inflammatory tissues (19–22), including the synovium of rheumatoid arthritis patients (7, 22) and in experimentally induced arthritis in rodents (22). To define the role of TGF- $\beta$  in such lesions, we injected natural or recombinant TGF- $\beta$  directly into the joint cavity of Lewis rats and monitored its effect on cellular recruitment and activation, and on its potential modulation of pathogenic effects. TGF- $\beta$ 1 and TGF- $\beta$ 2 were both found to induce synovial erythema, swelling, and leukocyte infiltration. The infiltrating mononuclear cells, activated to express growth factors, likely regulated the pronounced synovial hyperplasia that was apparent within 2–3 d. Based on these observations, it appears that TGF- $\beta$ , released by platelets and inflammatory cells in the arthritic synovium, may directly contribute to the events associated with inflammatory arthropathies.

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<sup>1</sup> *Abbreviations used in this paper:* AI, articular index; i.a., intraarticular; NRS, normal rat serum; PEC, peritoneal macrophages; SCW, streptococcal cell wall; TGF- $\beta$ , transforming growth factor  $\beta$ .

### Materials and Methods

**Reagents.** Lyophilized, carrier-free, sterile TGF- $\beta$ 1 purified to homogeneity from bovine bone (23) was reconstituted in 4 mM HCl and diluted in PBS (1  $\mu$ g/25  $\mu$ l) just before injection. TGF- $\beta$ 2, also purified to homogeneity from bovine bone (9), was lyophilized in the presence of rat serum albumin (RSA; 25  $\mu$ g RSA/1  $\mu$ g TGF- $\beta$ 2), reconstituted in 10 mM HCl (25  $\mu$ g/100  $\mu$ l), and diluted to 1  $\mu$ g/25  $\mu$ l in PBS for injection. Recombinant TGF- $\beta$ 1 (rTGF- $\beta$ 1) (Genentech, So. San Francisco, CA) (109  $\mu$ g/ml in 4 mM HCl) was also diluted in PBS immediately before use. Corresponding vehicles for control injections in the contralateral joints were 0.6 mM HCl in PBS for TGF- $\beta$ 1, 0.1% RSA in 1.6 mM HCl for TGF- $\beta$ 2, and 1.5 mM HCl in PBS for rTGF- $\beta$ 1. All of these preparations had <0.2 ng/ml (limit of detection) of endotoxin as determined by the Limulus amoebocyte lysate assay (24).

**Intraarticular (i.a.) Administration of TGF- $\beta$ .** Specific pathogen-free 6-wk-old inbred Lewis rats (Harlan-Sprague Dawley, Indianapolis, IN) were anesthetized and i.a. injections (25  $\mu$ l) were given in the ankle joint, to the extensor surface of the talar-navicular joint, using a 25-gauge needle. Rats ( $n = 7-9$ ) received 1  $\mu$ g, unless otherwise indicated, of TGF- $\beta$ 1, TGF- $\beta$ 2, or rTGF- $\beta$ 1 in the right extremity and the appropriate vehicle (25  $\mu$ l) in the left extremity. No evidence of leakage around the joint was apparent. The injection schedules are indicated in the Results. The joints were clinically scored on an articular index (AI) scale of 0 (normal) to 4 (severe inflammation) based on swelling, distortion, and redness of the joints (25).

**Light and Electron Microscopic Analysis.** At various intervals after administration of TGF- $\beta$ , synovial tissues were obtained for histopathologic evaluation. In some experiments, animals were anesthetized (chloralhydrate, 400 mg/kg, i.p.) and perfused with fixative solution containing 2% glutaraldehyde, 2% formaldehyde, and 0.05%  $\text{CaCl}_2$  in a 0.1 M sodium cacodylate buffer, pH 7.4, for 10 min. The joints were then excised and immersed in fresh fixative (22°C) for 2-3 h, washed, and stored at 4°C in 0.1 M cacodylate buffer with 7% sucrose. The joints were demineralized in disodium EDTA at 4°C (26), trimmed into small pieces, and washed again in cacodylate-sucrose buffer. After postfixation with 1%  $\text{OsO}_4$ , the tissues were stained with 0.5% aqueous uranyl acetate, dehydrated in ethanol, and embedded in Spurr epoxy resin. 1- $\mu$ m sections were stained with toluidine blue and examined by light microscopy. Thin sections were stained with uranyl acetate and lead citrate for transmission electron microscopy.

The joint tissues from additional animals were snap frozen in embedding medium (O. C. T. compound; Miles Laboratories, Inc., Naperville, IL), sectioned (8  $\mu$ m) and stained with toluidine blue, hematoxylin and eosin, and Trichrome Masson (25). The slides were coded and analyzed for evidence of an inflammatory reaction and/or other tissue abnormalities. Additional sections were prepared for immunoperoxidase staining.

**Identification of Mononuclear Cell Surface Antigens.** T lymphocytes (OX19), monocytes/macrophages (ED1), and  $\text{Ia}^+$  cells (OX6) were identified using mAbs (Bioproducts for Science, Indianapolis, IN) in combination with an immunoperoxidase-staining technique (ABC Vectastain Kit; Vector Laboratories, Inc., Burlingame, CA) (25). Control sections were incubated without the primary antibody, with an irrelevant mAb or with mouse ascites fluid.

**Peritoneal Macrophage and PBMC Cultures.** Resident peritoneal macrophages (PEC) were obtained from normal Lewis rats by peritoneal lavage with PBS. PBMC were isolated by density gradient (Histopaque 1083; Sigma Chemical Co., St. Louis, MO) centrifugation of heparinized blood obtained by intracardiac puncture.

Supernatants were generated by culturing  $1 \times 10^6$  PBMC or PEC/ml in serum-free DME (Gibco Laboratories, Grand Island, NY) containing 50  $\mu$ g/ml gentamicin, 2 mM glutamine, and  $5 \times 10^{-5}$  M 2-ME in 24-well plates (Costar, Cambridge, MA) in the presence or absence of TGF- $\beta$  for 36 h at 37°C with 5%  $\text{CO}_2$ .

**Chemotaxis Assay.** PBMC were suspended at  $1.1 \times 10^6$  monocytes/ml in Gey's balanced salt solution (GBSS; National Institutes of Health Media Unit) containing 2% BSA, antibiotics, and 0.015 M Hepes. The chemotaxis assay was carried out in 48-well microchamber plates (Neuroprobe, Rockville, MD) with 5- $\mu$ m pore polycarbonate filters (27). TGF- $\beta$ 1, TGF- $\beta$ 2, and rTGF- $\beta$ 1 were diluted and assayed at 0-10 pg/ml. After a 90-min incubation at 37°C, the polycarbonate filters were removed, fixed, and stained with Diff-Quik (American Scientific

Products, Stone Mountain, GA) and quantitated with an Optomax Image Analyzer (Optomax, Hollis, NH). Chemotactic activity is defined as the mean ( $\pm$  SEM) number of cells that migrated through the 5- $\mu$ m pores in three standard fields for each of triplicate filters.

Dermal fibroblasts were obtained and subcultured as reported (28). Fibroblast chemotaxis was also determined in 48-well microchamber plates using 8- $\mu$ m PVP-free polycarbonate filters. The fibroblasts ( $2.5 \times 10^5$ /ml) were incubated for 150 min at 37°C, the filters removed, stained, and counted as above.

**IL-1 Assay.** Cell-free supernatants (twofold dilutions) were assayed for IL-1 activity using thymocytes from 6–8-wk-old C3H/HeJ mice as described (25). The cultures were pulsed with 0.5  $\mu$ Ci/well [ $^3$ H]TdR (specific activity 1.9 Ci/mmol; Schwarz-Mann, Orangeburg, NY) for the final 5 h of incubation and the incorporated radioactivity was determined. Data were transformed into units by comparison to an IL-1 standard containing 100 Units/ml (Genzyme Corp., Boston, MA).

**TNF Assay.** Cell-free supernatants were assayed for TNF by the L929 cytotoxicity assay (29). rTNF (gift of Cetus Corp., San Francisco, CA) was used as the standard against which the samples, diluted twofold, were compared.

**Fibroblast Proliferation Assay.** Primary synovial fibroblast cultures were established from the synovium of bacterial cell wall-injected Lewis rats (30) after enzymatic dissociation with collagenase (50 U/ml; Worthington Biochemical Corp., Freehold, NJ). Fibroblasts were cultured at  $5 \times 10^4$  cells/ml/well in 24-well flat-bottomed plates in DME alone (controls) or DME containing 5% normal rat serum (NRS) and TGF- $\beta$ 1 or TGF- $\beta$ 2 (0–20 ng/ml) at 37°C for 48 h. Additionally, fibroblasts were cultured in the presence of diluted cell-free supernatants derived from TGF- $\beta$ -stimulated monocytes. 4 h before harvest, the cultures were pulsed with 1  $\mu$ Ci/ml [ $^3$ H]TdR and proliferation was quantitated by the level of incorporation of [ $^3$ H]TdR in triplicate wells.

**RNA Isolation and Northern Analysis.** Total RNA was isolated from  $15 \times 10^6$  PEC cultured for 4 h with media, TGF- $\beta$ 1, TGF- $\beta$ 2, or rTGF- $\beta$ 1 (1–30 ng/ml) or from excised synovial tissues by the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (31). For Northern blots, 5  $\mu$ g total RNA was fractionated on 1.0% agarose gels containing formaldehyde and transferred to nitrocellulose filters. The blots were prehybridized for 4 h and then hybridized overnight with  $^{32}$ P-labeled cDNA probes. Probes included IL-1 $\beta$  cDNA (32), the cDNA probe for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (33), and the cDNA probe for human TGF- $\beta$ 1 (10). The filters were washed and were exposed to Kodak XAR film with intensifier screens at  $-70^\circ\text{C}$  for 4–48 h.

## Results

**Clinical Manifestations of i.a. TGF- $\beta$  Injection.** Within 12–24 h after the initial injection of 1  $\mu$ g of natural TGF- $\beta$ 1 or its homologue, TGF- $\beta$ 2, significant swelling and erythema became apparent in the treated joints. Two additional injections caused further exacerbation of the response, which reached an AI of  $3.6 \pm 0.5$  for TGF- $\beta$ 1 and a maximum 4.0 for TGF- $\beta$ 2 on the third day (Fig. 1). A similar administration

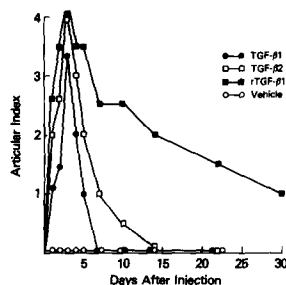


FIGURE 1. Induction of joint swelling and erythema by TGF- $\beta$ . Lewis rats received 3 daily i. a. injections of 1  $\mu$ g of TGF- $\beta$ 1, TGF- $\beta$ 2, rTGF- $\beta$ 1 or vehicle only. AI were determined at 24 h and daily thereafter. Data represent the mean joint scores of seven to nine animals in each group.

of rTGF- $\beta$ 1 induced a parallel arthritic response achieving an AI of 4 within 2–3 d. Since a maximal AI was obtained after three injections, treatment was stopped and the course of the synovitis monitored for an additional 3 wk. As shown in Fig. 1, the acute swelling and erythema induced by natural TGF- $\beta$ 1 and  $\beta$ 2 declined within 7–10 d, whereas the response to the recombinant peptide persisted for >3 wk, with some clinically apparent swelling evident >6 wk (data not shown). The mechanism for the prolonged response to rTGF- $\beta$ 1 is unclear, since the appropriate vehicle for this preparation induced no clinically apparent inflammatory response when injected into the contralateral joint (Fig. 1), nor did the vehicles for the natural TGF- $\beta$ 1 and  $\beta$ 2.

Since the rTGF- $\beta$ 1 induced a more pronounced clinical response than the natural products, we compared the ability of various dilutions of the recombinant peptide to cause clinically evident synovitis. As shown in Fig. 2, a single injection of 0.01 or 0.1  $\mu$ g rTGF- $\beta$ 1 was ineffective in stimulating synovitis as compared with the 1.0  $\mu$ g injection of TGF- $\beta$ 1. However, after a second injection at these lower concentrations, significant articular indices were evident, even for 0.01  $\mu$ g amounts. After the third and final injection, the AI peaked and then began to decline, reaching baseline by 9 d (0.1 and 0.01  $\mu$ g injections), except for the 1.0  $\mu$ g injection regimen for which the AI persisted after 9 d (see also Fig. 1).

**Histological Manifestations of TGF- $\beta$  Injection.** Because of the pronounced clinical response in the joints to the local administration of TGF- $\beta$ , subsequent studies evaluated the histopathogenesis of these lesions within the synovium. Whereas the synovium of normal joints is represented by a thin layer of synovial lining cells underlaid by loose adipose and connective tissue stroma (Fig. 3 A), within hours after the local administration of TGF- $\beta$ 1 or  $\beta$ 2 the accumulation of inflammatory cells became apparent in the synovial space (Fig. 3 B) and within the synovial tissue (Fig. 3 C). This recruitment of inflammatory cells included some neutrophils with significantly larger numbers of mononuclear cells. Furthermore, many of the mononuclear phagocytes appeared highly activated with large vacuoles and enlarged cytoplasm (Fig. 3 B). Interestingly, the cellular constituents of the TGF- $\beta$ -induced lesions at 24–48 h were similar to what has been observed in synovial tissues 2–3 wk after the onset of antigen-induced arthritis (25).

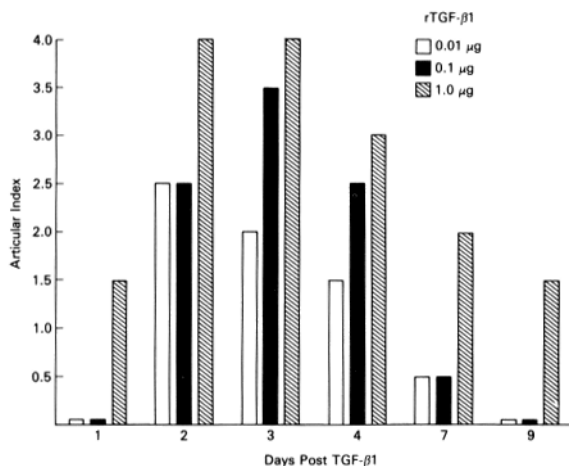


FIGURE 2. Dose-dependent synovitis induced by recombinant TGF- $\beta$ 1. Lewis rats received three daily i.a. injections of 0.01, 0.1, or 1.0  $\mu$ g of rTGF- $\beta$ 1. AI were determined on indicated days.

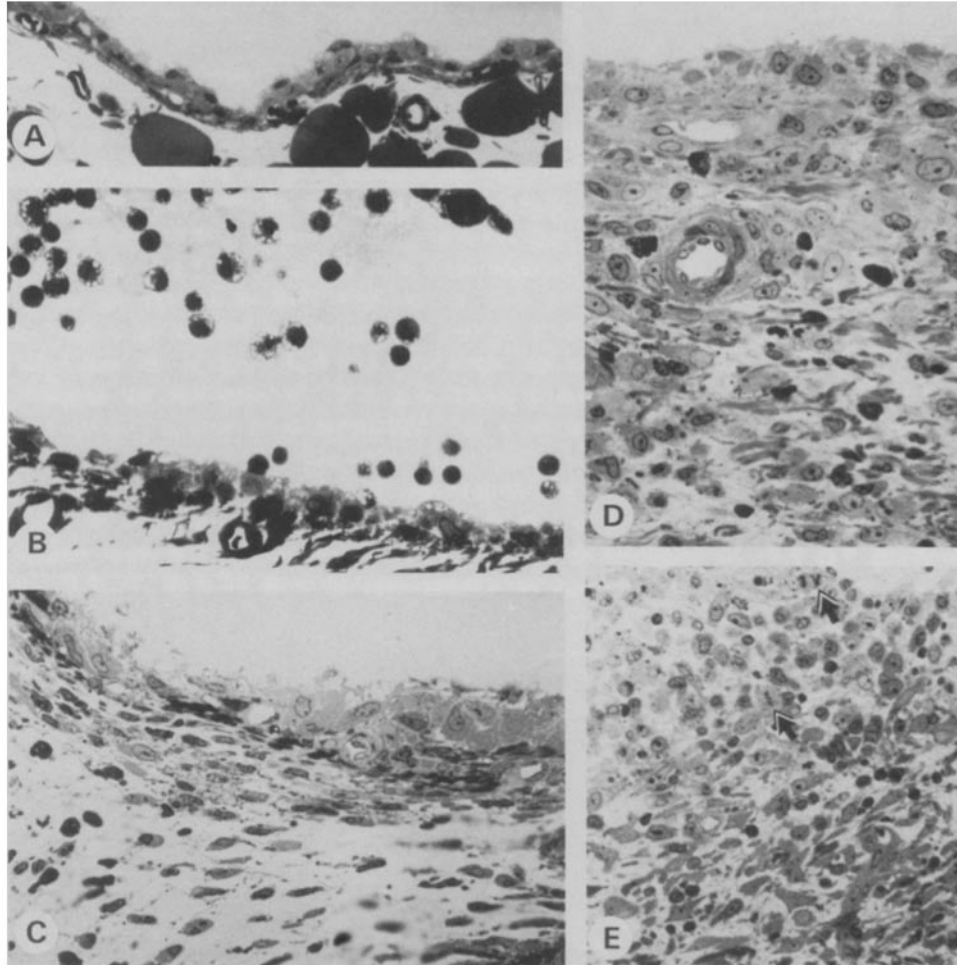


FIGURE 3. Cellular infiltrate and synovial hyperplasia induced by TGF- $\beta$ . Synovial tissues were obtained from vehicle injected control joints (A) (original magnification,  $\times 25$ ) or from TGF- $\beta$ -injected joints at 24 h (B, C), 48 h (D), or 72 h (E). Arrows indicate mitotic figures (original magnification,  $\times 40$ ).

Associated with the accumulating mononuclear infiltrate was an increase in synovial fibroblast-like cells and an apparent angiogenic response by day 2-3 after initiation of TGF- $\beta$  treatment (Fig. 3, D, E). Synovial fibroblast accumulation was likely due to recruitment because of the rapidity of the response, but was also associated with a proliferative response of local cells as evidenced by the presence of mitotic figures (Fig. 3 E). After cessation of TGF- $\beta$  administration, the numbers of mononuclear cells gradually declined in parallel with the clinically apparent decrease in swelling and distortion of the joints. Fibroplasia however, persisted, although at reduced levels, and in the rTGF- $\beta$ -treated joints, enhanced cellularity persisted for 3-4 wk. The nature of the prolonged effect of rTGF- $\beta$ 1 as compared with the natural peptides was not readily apparent. The appropriate vehicle-injected contralateral

joints were not characterized by a cellular infiltrate, although occasional hemorrhage due to injection trauma was seen.

During the evolution of the synovial inflammation and hyperplasia of synovial fibroblast-like cells, no apparent evidence of erosion of the connective tissue structures occurred. In contrast, the synovial tissue demonstrated deposition of new connective matrix by the recruited fibroblast population (Fig. 3, *D*, *E*).

*Identification of Mononuclear Cell Subpopulations.* To specifically define the infiltrating mononuclear cells in the TGF- $\beta$ -injected synovium, the tissues were stained with cell-specific mAbs by immunoperoxidase. As shown in Fig. 4, after a single TGF- $\beta$  injection, intense staining occurred with an antibody that recognizes rat monocytes/macrophages (ED1) (34), indicating that the majority of infiltrating leukocytes were of monocytic lineage. In contrast, there were few ED1<sup>+</sup> cells in the thin synovial lining of the vehicle only-treated joints (Fig. 4 *A*). In addition, the majority of these mononuclear phagocytes were Ia<sup>+</sup>, indicative of in situ activation and differentiation (Fig. 4 *E*). Some Ia<sup>+</sup> cells, which were also ED1<sup>-</sup> on sequential sections, were also seen in the normal synovium. A proportion of the infiltrating mononuclear cells appeared to be lymphocytes as indicated by the levels of OX19 staining (Fig. 4 *F*). No apparent differences in the relative numbers of these cellular phenotypes were observed between the tissues injected with TGF- $\beta$ 1, TGF- $\beta$ 2, or rTGF- $\beta$ 1.

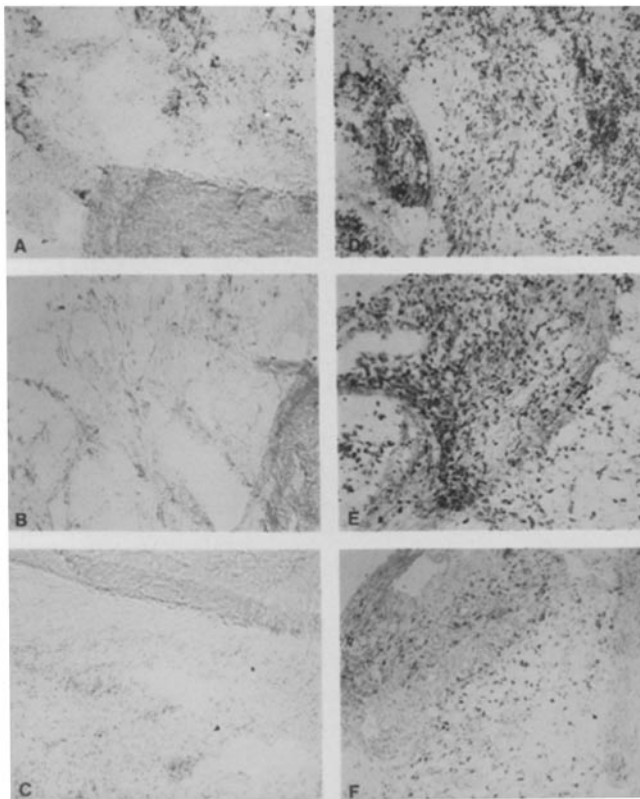


FIGURE 4. Characterization of cellular infiltrates by mAbs. Synovial sections obtained from control (*A*, *B*, *C*) and after a single i. a. injection of TGF- $\beta$ 1 (*D*, *E*, *F*) were stained using an immunoperoxidase technique with mAbs directed at surface antigens on macrophages (ED1) (*A*, *D*), to Ia antigens (OX6) (*B*, *E*), and on T lymphocytes (OX19) (*C*, *F*) (original magnification,  $\times 40$ ).

*Ultrastructural Analysis of TGF- $\beta$ -treated Synovium.* The impressive cellular response to the local administration of TGF- $\beta$  prompted us to further characterize these tissues at the ultrastructural level. The normal synovial lining layer consists of 1 or 2 layers of relatively flattened cells (Fig. 5 A). After i.a. injection of TGF- $\beta$ , the lining showed increased thickness and cellularity, with the cells appearing rounded and more ac-

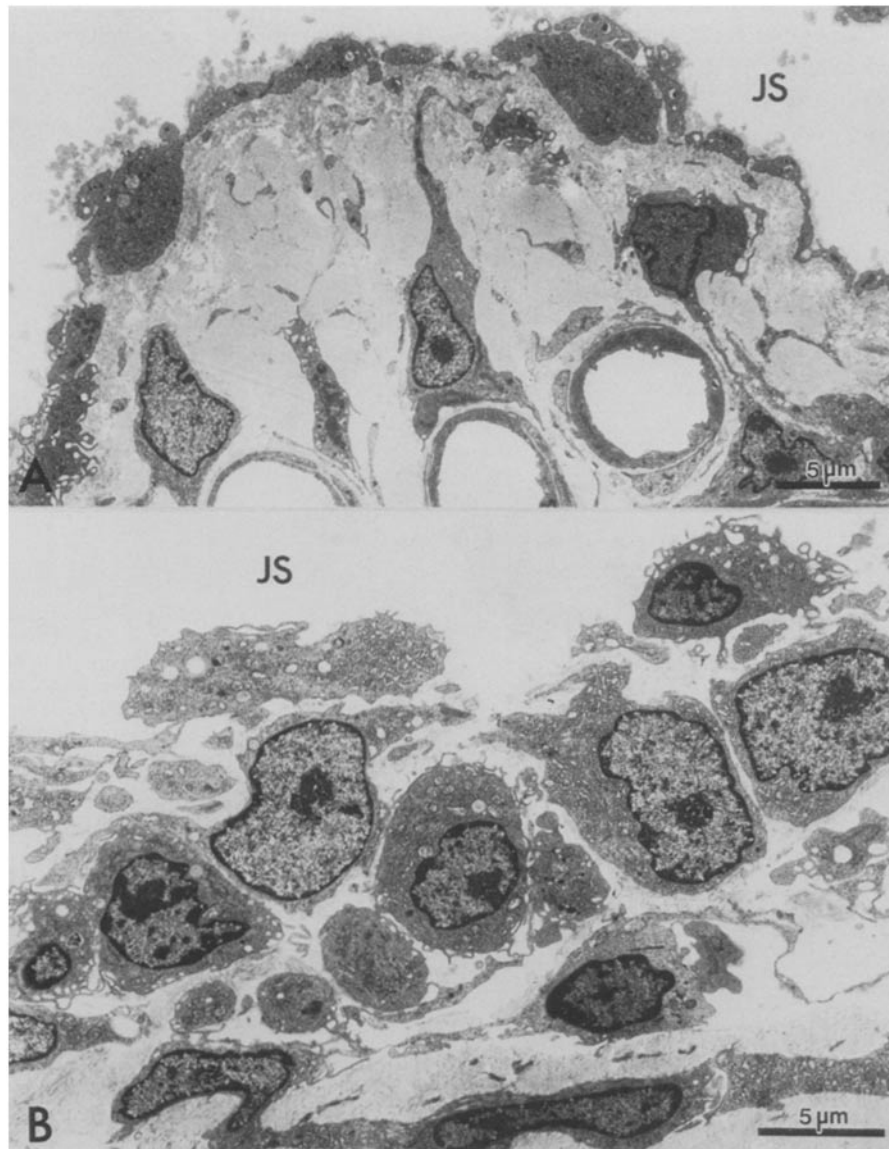


FIGURE 5. Ultrastructural analysis of synovium. (A) Vehicle-injected synovial tissue. The joint space (JS) is lined by a layer of one to two flattened cells. The underlying connective tissue contains abundant collagen, scattered cells, and blood vessels. (B) 3 d after TGF- $\beta$ 1 administration. The synovial lining layer is increased in thickness and cellularity, the cells have rounded up and appear more active. Joint space (JS).

tive (Fig. 5 *B*). Furthermore, the underlying matrix, which was relatively acellular in the controls, became infiltrated with cells and the collagen bundles appeared disrupted (Fig. 5, *A, B*). As defined at the light microscopic level and further by immunocytochemical analysis, the underlying tissue was infiltrated by many cells of monocytic lineage (Fig. 6, *A, B, C*). Numerous examples were found of an apparent intimate relationship between these monocytic cells and fibroblast-like cells (Fig. 6, *A, B*), as well as an association between mononuclear cells and mitotic cells, presumably fibroblasts (Fig. 6 *C*). Interestingly, an increased population of mast cells was observed in the TGF- $\beta$ -treated synovial tissues (Fig. 7).

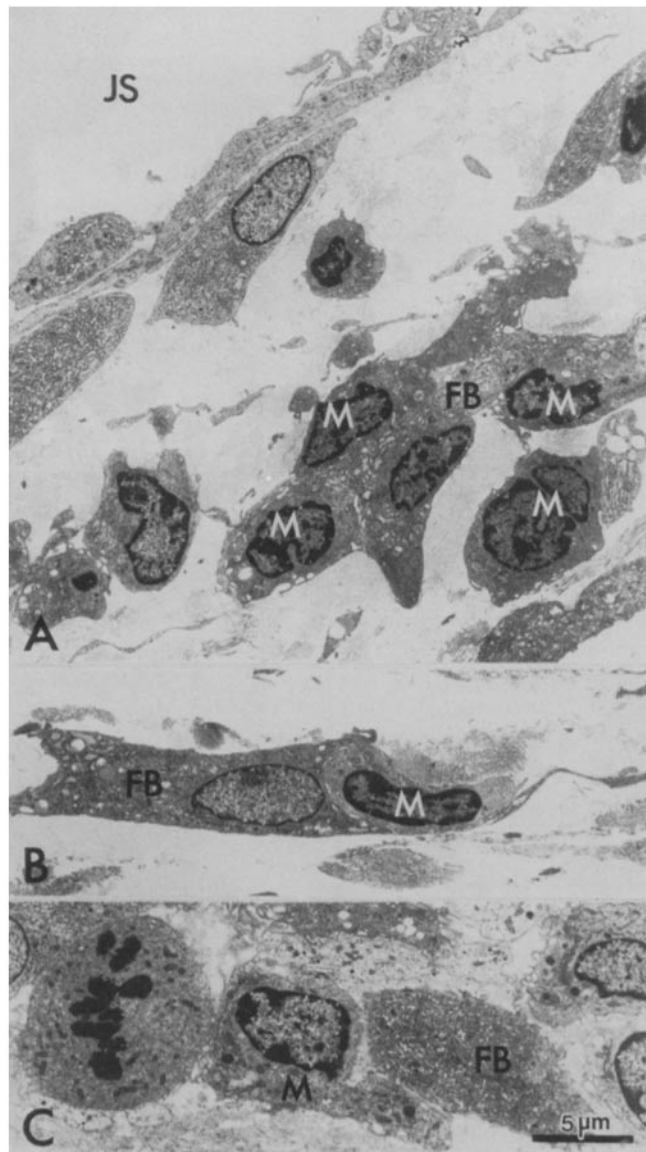


FIGURE 6. Mononuclear cells in subsynovial connective tissue. (*A* and *B*) 3 d after TGF- $\beta$ 1. Numerous mononuclear cells are seen in close association with fibroblasts (*FB*). Disruption of the subsynovial collagenous tissue is evident. Joint space (*JS*). (*C*) 2 d after TGF- $\beta$ 2. A mononuclear cell (*M*) lies adjacent to a fibroblast (*FB*) and a mitotic cell (*left*).



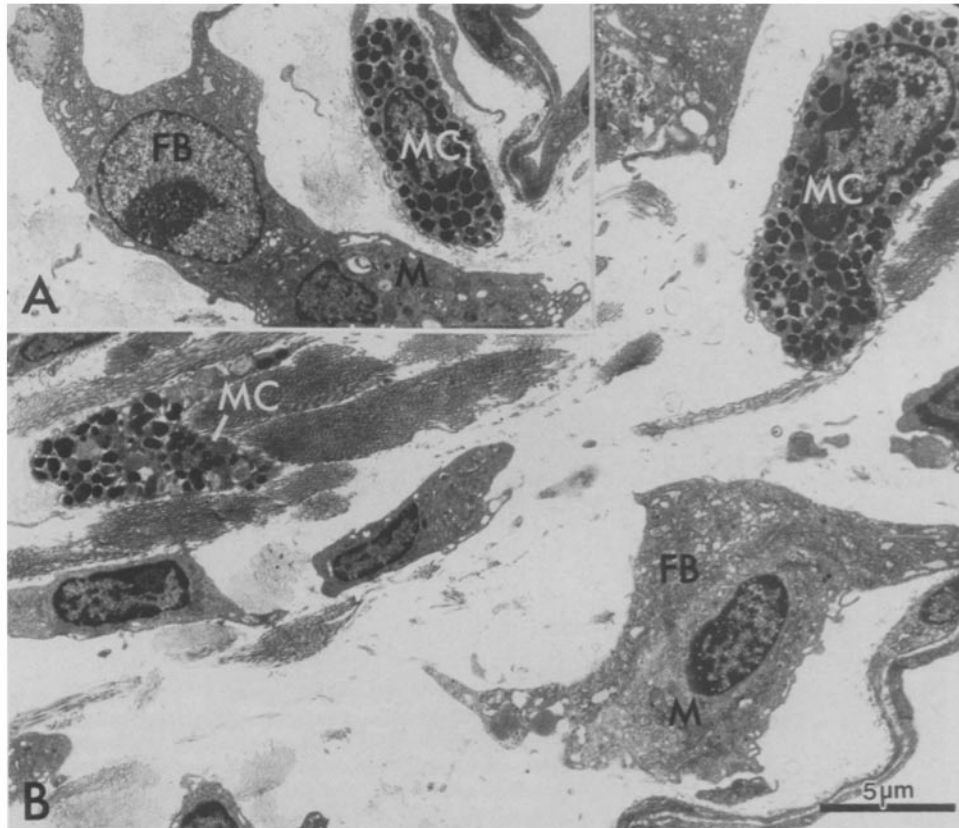


FIGURE 7. Synovium 3 d after TGF- $\beta$ 1. Mast cells (MC) are frequently observed in the sub-synovial connective tissue. (A and B) Mononuclear cells (M) are closely associated with fibroblasts (FB). The collagenous tissue is disrupted.

*Monocyte and Fibroblast Chemotaxis to TGF- $\beta$ .* The predominant effects of local administration of TGF- $\beta$  to the synovium appeared to be monocyte recruitment and activation, fibroblast recruitment and proliferation, and the deposition of extracellular matrix. Our next series of experiments, therefore, focused on delineating in vitro the mechanisms whereby TGF- $\beta$  might be mediating these tissue responses. Natural TGF- $\beta$ 1, recently shown to be a potent chemoattractant for human peripheral blood monocytes (35, 36) was assayed for its ability to induce rat monocyte-macrophage chemotaxis in an in vitro assay in parallel with TGF- $\beta$ 2 and the recombinant form of TGF- $\beta$ 1. TGF- $\beta$ 1, whether natural or recombinant, induced peripheral blood monocyte (Fig. 8) and peritoneal macrophage (data not shown) chemotaxis at 0.1 pg/ml. Furthermore, TGF- $\beta$ 2, not previously tested for chemotactic activity, induced a parallel response in the in vitro assay, consistent with its in vivo ability to initiate monocyte recruitment into the synovium. As previously shown for human fibroblasts (37), TGF- $\beta$ -stimulated rodent fibroblast chemotaxis at femtomolar concentrations in an in vitro assay (Fig. 9).

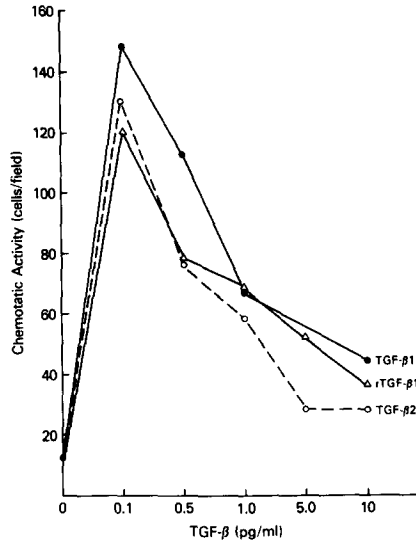


FIGURE 8. Monocyte chemotactic activity of TGF- $\beta$ . Rat peripheral blood monocytes ( $1.1 \times 10^6$ /ml) were assayed for their ability to respond to TGF- $\beta$ 1, TGF- $\beta$ 2, and rTGF- $\beta$ 1 at the indicated concentrations. The data represent the mean number of monocytes that migrated in three fields for triplicate filters (nine determinations/point). Standard errors were routinely <10% of the mean and data are representative of four experiments.

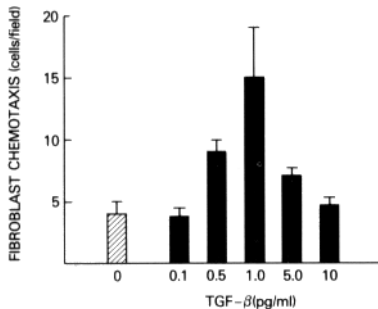


FIGURE 9. Fibroblast chemotaxis to TGF- $\beta$ . Dermal fibroblasts ( $2.5 \times 10^5$ /ml) were assayed in vitro for chemotactic responsiveness to TGF- $\beta$ 1 at the indicated concentrations. Data represent the mean  $\pm$  SEM fibroblasts per field for triplicate filters.

*Direct and Indirect Effects of TGF- $\beta$  on Fibroblast Proliferation.* In addition to the recruitment of fibroblasts into the synovium, the increased fibroblast population was also attributed to proliferation as evidenced by the increased frequency of mitotic figures (Figs. 3 E and 6 C). To determine whether TGF- $\beta$  was directly mitogenic for synovial fibroblasts, fibroblasts were cultured in the presence or absence of TGF- $\beta$  and proliferation monitored by incorporation of [ $^3$ H]TdR into DNA (Table I). The addition of TGF- $\beta$  to serum-free cultures of fibroblasts did not induce fibroblast proliferation and furthermore, the addition of TGF- $\beta$  to proliferating fibroblasts (NRS-induced) often suppressed the proliferative response. These data suggested that the TGF- $\beta$ -mediated fibroblast proliferative response in vivo might be mediated indirectly, possibly through induction of monocyte growth factor production. In fact, TGF- $\beta$  added to monocyte cultures stimulated these cells to release products capable of stimulating fibroblast proliferation (Table I).

*Induction of IL-1 Production by TGF- $\beta$  In Vitro and In Vivo.* One monocyte product that can influence fibroblast growth is IL-1, and therefore we evaluated the effects of TGF- $\beta$  on the induction of IL-1. Messenger RNA was obtained from PEC ex-

TABLE I  
*Direct and Indirect Effects of TGF- $\beta$  on Rat Synovial  
 Fibroblast Proliferation In Vitro*

Addition to fibroblast culture	Amount	Fibroblast proliferation
		( $^3\text{H}$ ]TdR incorporation)
	<i>ng/ml</i>	<i>cpm</i>
DME + TGF- $\beta$ 1*	0	697 <sup>§</sup>
	10	681
	20	363
NRS + TGF- $\beta$ 1*	0	38,409
	0.1	30,381
	1	25,396
	10	19,759
	20	19,412
Monos <sup>†</sup>	0	3,500
Monos + TGF- $\beta$ 1 <sup>‡</sup>	1	6,825
	5	20,430
	10	16,930
	20	14,380
Monos + TGF- $\beta$ 2 <sup>‡</sup>	1	20,395
	5	13,506
	10	5,735
	20	5,456

\* Synovial fibroblasts ( $5 \times 10^4/\text{ml}$ ) were cultured without (DME) or with 5% NRS to which was added 0–20 ng/ml TGF- $\beta$ 1.

<sup>†</sup> Fibroblasts were cultured in the presence of cell-free supernatants obtained from rat monocytes that had been stimulated for 36 h with 0–20 ng/ml TGF- $\beta$ 1 or TGF- $\beta$ 2.

<sup>§</sup> After 48 h, the fibroblast cultures were pulsed 4 h with [ $^3\text{H}$ ]TdR and evaluated for incorporation of [ $^3\text{H}$ ]TdR into DNA. Data are the mean of triplicate cultures from a representative experiment ( $n = 4$ ).

posed to TGF- $\beta$ 1 or  $\beta$ 2 in vitro and probed with a cDNA probe for IL-1 $\beta$  (Fig. 10 A). After 4 h, IL-1 $\beta$  steady-state mRNA levels were elevated in TGF- $\beta$ 1- or TGF- $\beta$ 2-treated cell cultures. The induction of IL-1 $\beta$  mRNA was concentration dependent with augmentation at 1 ng/ml and increasing mRNA levels at higher concentrations. Constitutively expressed GAPDH levels were not altered by TGF- $\beta$ .

In parallel cultures, 36-h supernatants were assayed for IL-1 bioactivity (Fig. 10 B). At 1 ng/ml TGF- $\beta$ 1 or TGF- $\beta$ 2, IL-1 could be detected in the monocyte supernatants with increased levels at 10–20 ng/ml TGF- $\beta$ . Aliquots of these culture supernatants were also assayed for TNF, another monocyte product with pleiotropic effects in inflammation and repair. A similar concentration-dependent induction of TNF was observed in response to either TGF- $\beta$ 1 or TGF- $\beta$ 2 (data not shown), indicating that TGF- $\beta$  is an effective inducer of IL-1, TNF, and likely other monocyte-derived growth factors (6).

Because of the ability of TGF- $\beta$  to stimulate monocytes to produce growth factors such as IL-1 in vitro, we processed synovial tissue obtained after TGF- $\beta$  administration to determine whether TGF- $\beta$  was similarly active in vivo. As demonstrated in Fig. 11, TGF- $\beta$  not only was an effective inducer of IL-1 $\beta$  message in vitro, but also

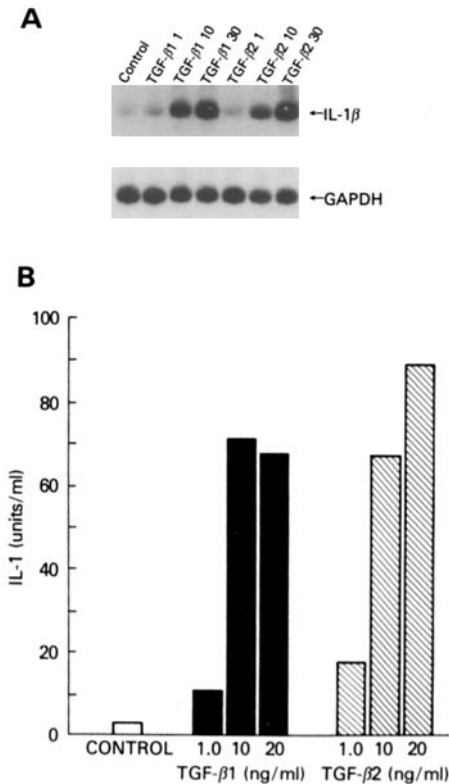


FIGURE 10. Induction of IL-1 by TGF- $\beta$ . (A) RNA was isolated from peritoneal macrophages after 4 h incubation with the indicated concentrations of TGF- $\beta$ 1 or TGF- $\beta$ 2. After extraction, the mRNA was analyzed by RNA hybridization with  $^{32}$ P-labeled IL-1 $\beta$  and GAPDH probes. (B) Peritoneal macrophages were cultured at  $10^6$ /ml in the presence or absence of 1–20 ng/ml TGF- $\beta$ 1 or TGF- $\beta$ 2. Cell-free supernatants were harvested at 36 h and assayed for IL-1 levels in the thymocyte costimulator assay. Units were determined by comparison with a standard curve with 100 U/ml IL-1.

in vivo. Synovial tissue obtained 24 h after a single injection of TGF- $\beta$ , which is characterized by an infiltrate of activated (Ia $^+$ ) monocytic cells, expressed significantly elevated steady-state levels of IL-1 $\beta$ . Furthermore, mRNA levels of TGF- $\beta$ 1 were also elevated in these tissues indicating autoinduction of TGF- $\beta$ . In addition to the 2.4-kb mRNA of TGF- $\beta$ , a recently identified 1.9-kb transcript (38) was also apparent in the TGF- $\beta$ -injected synovial tissues, but not in the control synovium.

### Discussion

The potential of TGF- $\beta$  to initiate synovial inflammation was examined by direct injection of recombinant or natural TGF- $\beta$ 1 or natural TGF- $\beta$ 2 into the synovial space of rat hind ankle joints. Each form of TGF- $\beta$  rapidly induced a profound synovitis. Administration of as little as 20 ng rTGF- $\beta$ 1 initiated an inflammatory response in the synovium. Early events included edema and leukocyte infiltration. Within 24 h after the first injection, mononuclear cells were the dominant infiltrating inflammatory cell and immunohistochemical analysis indicated that they were primarily Ia $^+$  macrophages. Lesser numbers of T lymphocytes (OX19 $^+$ ) were detected. Increased cellularity of the synovial lining layer and fibroblast proliferation in the synovial connective tissue were also apparent, and expansion of the fibroblast population and deposition of collagen continued over the entire period of peptide administration. At the apex (days 3–4) of the response, the formation of new capillaries was

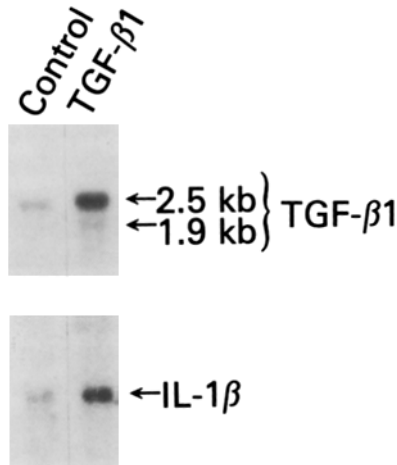


FIGURE 11. Induction of IL-1 $\beta$  and TGF- $\beta$ 1 gene expression in synovium by TGF- $\beta$ . RNA was isolated from synovial tissue 24 h after a single i.a. injection of TGF- $\beta$  or vehicle. After extraction, the mRNA was analyzed by RNA hybridization with  $^{32}$ P-labeled IL-1 $\beta$  and TGF- $\beta$ 1.

discernable. Both the mononuclear cell infiltration and the associated fibrosis were reversible when i.a. injection of TGF- $\beta$  was discontinued.

The complement of events induced by TGF- $\beta$  was remarkable in its complexity and diversity. Some, but not all, of the events can be ascribed to the direct activities of TGF- $\beta$ . For example, TGF- $\beta$  has been shown to be approximately three orders of magnitude more potent than C5a as a chemoattractant for human monocytes (35, 36), and as shown in the present study, TGF- $\beta$ 1, TGF- $\beta$ 2, and rTGF- $\beta$ 1 are also chemotactic for rat monocytes. Thus, it appears likely that the mononuclear cell infiltration seen in the synovium after i.a. injection of TGF- $\beta$  largely reflects the direct chemoattraction of monocytes and/or tissue macrophages by these peptides. However, the possibility that TGF- $\beta$  might also induce additional chemotactic signals in vivo cannot be excluded. Although TGF- $\beta$  has not been shown to be chemotactic for T lymphocytes, the appearance of T cells after TGF- $\beta$  administration may indicate the induction of lymphocyte chemoattractants such as IL-8 (39). Furthermore, TGF- $\beta$  is directly chemotactic for fibroblasts (37) (Fig. 9) that increase in number soon after TGF- $\beta$  administration.

Other sequelae of i.a. TGF- $\beta$  cannot as readily be attributed to direct activity of this cytokine. For example, fibroblast and endothelial cell proliferation is inhibited, not promoted, by TGF- $\beta$  in culture (reviewed in reference 1). It seems probable, therefore, that other signals, induced in situ by TGF- $\beta$ , may also play important roles in the development of TGF- $\beta$ -induced synovial pathology. In this regard, it has been recently reported that TGF- $\beta$ 1 and - $\beta$ 2, at picomolar concentrations, induce human monocytes to express several cytokines including IL-1 $\beta$ , TNF- $\alpha$ , platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) (6). In the present study, we report that whereas TGF- $\beta$ 1 and TGF- $\beta$ 2 were not directly mitogenic for synovial fibroblasts, supernatants from TGF- $\beta$ 1- and TGF- $\beta$ 2-stimulated rat monocyte/macrophages did stimulate synovial fibroblast growth. These supernatants contained significant levels of IL-1 $\beta$  and TNF- $\alpha$ , and Northern blot analysis demonstrated induction of the specific mRNA for IL-1 $\beta$ . Consistent with this in vitro

observation was the elevation of IL-1 $\beta$  mRNA in the synovial tissue within 24 h after TGF- $\beta$  administration. Furthermore, these synovial tissues expressed increased message levels for TGF- $\beta$ 1, implicating an autoinduction of this peptide that may further promote mononuclear cell recruitment and activation. Of interest is the identification of the recently described 1.9-kb species of the TGF- $\beta$ 1 mRNA, the function of which is unknown, but which has been linked to conditions of tissue injury and inflammation (20, 38).

Several studies have documented that TGF- $\beta$  promotes connective tissue matrix synthesis by fibroblasts *in vitro* and *in vivo*. However, induction of monokine synthesis by TGF- $\beta$  may also contribute to regulation of fibroblast matrix component synthesis (reviewed in reference 1). In this regard, analysis of the mRNA from TGF- $\beta$ -injected synovium revealed a marked increase in type I procollagen mRNA (Manthey, C., unpublished observation) consistent with the morphological identification of matrix deposition in these tissues. TGF- $\beta$  also suppresses the expression of proteases while promoting the synthesis of protease inhibitors (40) contributing to the profibrotic influence of this peptide.

It is noteworthy that in contrast to the pannus invasion and destruction of cartilage and bone that is characteristic of streptococcal cell wall (SCW)-induced arthritis in this rat model, the synovitis induced by TGF- $\beta$  eventually resolves with no evidence of permanent pathologic alterations of the joint. However, the transient nature of the TGF- $\beta$  stimulus administered in these studies may not be reflective of the chronic lesions in antigen-induced arthritis and the continued presence of TGF- $\beta$  and other cytokines that promote pannus formation and tissue erosion. Thus, although TGF- $\beta$  may be a key mediator of the events associated with SCW-induced arthritis, we cannot determine in these studies whether it is a causative factor in joint destruction. Intriguing indirect support for a protective role for TGF- $\beta$  in SCW-induced arthritis is the suggestion that retinoids, which are effective in suppressing SCW-induced joint destruction (41; Allen, J. B., H. L. Wong, G. L. Costa, and S. M. Wahl, manuscript in preparation), may exert some of their biological actions through the induction of TGF- $\beta$  (1). TGF- $\beta$  has been identified not only in the tissues, but in the synovial effusions of patients with rheumatoid arthritis (18, 22, 42). Thus, the contribution of TGF- $\beta$  to the pathogenesis and/or regulation of this disease is as yet speculative, since the potent immunosuppressive actions of TGF- $\beta$  (7), coupled with its ability to promote tissue healing (43), suggest that it may be beneficial, whereas the ability of TGF- $\beta$  to promote inflammation through its effects on mononuclear phagocytes (7) implicates this peptide as a pathogenic factor.

### Summary

After intraarticular injection of TGF- $\beta$ 1 or TGF- $\beta$ 2, marked swelling and erythema of the injected joints were apparent within 12–24 h. On a scale of 0 to 4, by day 3, the TGF- $\beta$ -treated joints had articular indices (AI) of  $3.6 \pm 0.5$  to  $4.0 \pm 0.0$  compared with no response for the vehicle-injected contralateral joints. Histopathologic evaluation revealed a predominantly mononuclear phagocyte infiltrate with some neutrophils and T lymphocytes, consistent with active inflammation. The monocytic pattern of leukocyte infiltration at 2–3 d was comparable to that seen in animals with antigen-induced arthritis after 2–3 wk. Extensive synovial fibroblast hyperplasia

became apparent within 48 h, likely as a result of TGF- $\beta$  induction of growth factor synthesis by the accumulating monocytes. TGF- $\beta$ 2, a homologue of TGF- $\beta$ 1, was found to induce a similar level of synovitis and synovial hyperplasia consistent with its parallel monocyte and fibroblast chemotactic properties and ability to induce transcription and translation of monocyte/macrophage-derived growth factors. These data suggest that TGF- $\beta$ , released by platelets and activated inflammatory cells, may play a direct role in leukocyte recruitment and activation in arthritic and other chronic inflammatory lesions.

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