

# MACROPHAGE PRODUCTION OF TRANSFORMING GROWTH FACTOR $\beta$ AND FIBROBLAST COLLAGEN SYNTHESIS IN CHRONIC PULMONARY INFLAMMATION

BY NASREEN KHALIL,\*<sup>†</sup> OLIVER BEREZNY,<sup>†</sup>  
MICHAEL SPORN,<sup>§</sup> AND ARNOLD H. GREENBERG\*

*From the \*Manitoba Institute of Cell Biology, <sup>†</sup>Department of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0V9; and the <sup>§</sup>Laboratory of Chemoprevention, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892*

Transforming growth factor  $\beta$  (TGF- $\beta$ )<sup>1</sup> is a 25-kD homo- and heterodimer that is a multifunctional regulator of cell growth and differentiation (1). It is present in a wide variety of tissues and interacts with specific cell membrane receptors (2) that are found on virtually all cells (3).

A number of functions of TGF- $\beta$  have been described that suggest a role for it in tissue repair. Platelets, which are a major storage site for TGF- $\beta$  (4), release the peptide at sites of injury (5). TGF- $\beta$  is a powerful chemoattractant for monocytes, and stimulates monocytes to increase production of IL-1, which is a potent fibroblast mitogen (6). Resting macrophages maintain high steady-state TGF- $\beta$  mRNA levels and will secrete TGF- $\beta$  upon activation (7). TGF- $\beta$  has also been shown to be a chemoattractant for fibroblasts (8), and stimulates the growth of immature fibroblasts (9, 10), suggesting that at sites of injury the presence of TGF- $\beta$  can recruit fibroblasts from the surrounding tissues and also stimulate their proliferation and differentiation. Furthermore, TGF- $\beta$  has the combined effect of enhancing the synthesis of collagen, fibronectin (10, 11), and proteoglycans (12), while suppressing protease secretion (13, 14). When injected subcutaneously or applied to incisions, TGF- $\beta$  rapidly stimulates granulation tissue and new blood vessel formation (15, 16).

Chronic pulmonary fibrosis represents a group of fibrotic lung diseases that result from a variety of pathological conditions, including chronic infectious and noninfectious inflammation (17). In the following study, we have used a well-established rodent model of chronic pulmonary fibrosis induced by a single intratracheal administration of the antineoplastic antibiotic, bleomycin (18, 19). Bleomycin-induced pulmonary fibrosis resembles the chronic human fibrotic lung disease histologically and physiologically (19). The resultant injury is characterized by necrosis of type I pneumocytes with proliferation of type II pneumocytes (20). As well, there is evidence of interstitial edema, sequential accumulation of inflammatory cells, fibroblasts (21), and finally, collagen and fibronectin synthesis (22-24).

Because of the well-described effects of TGF- $\beta$  on inflammatory cells, fibroblasts,

---

This work was supported by the Medical Research Council of Canada.

<sup>1</sup> *Abbreviations used in this paper:* BPF, bleomycin-induced pulmonary fibrosis; FBP, fetal bovine plasma; TGF- $\beta$ , transforming growth factor  $\beta$ .

and connective tissue, we examined the role of TGF- $\beta$  in the genesis of chronic pulmonary fibrosis induced by bleomycin.

### Materials and Methods

**Animals.** Female Sprague-Dawley rats, which were free of respiratory disease and weighed between 250 and 300 g, were obtained from the University of Manitoba vivarium. In each experiment, all rats were matched for age and weight.

**Reagents.** Rabbit anti-TGF- $\beta$  antibodies LC and CC, used for immunohistochemistry, have been described previously. Both were raised by immunizing with synthetic peptide 1-30 of the TGF- $\beta_1$  (25). LC stains intracellular TGF- $\beta$  and CC stains extracellular TGF- $\beta$  associated with matrix (26). Porcine TGF- $\beta_1$  and neutralizing anti-TGF- $\beta$  antiserum were obtained from R & D Systems (Minneapolis, MN). TGF- $\beta_1$  peptide 1-30 and sepharose-linked TGF- $\beta$  for antiserum neutralization have been described previously (27).

**Bleomycin Instillation.** Rats were anesthetized by an intraperitoneal injection of 40 mg/kg of Nembutal (Abbott Laboratories, Toronto, Ontario). Tracheostomy was performed and sterile normal saline containing 1 U of bleomycin sulfate (Blenoxane; Bristol Laboratories, Syracuse, NY) was instilled into the lungs using a 25-gauge needle inserted between cartilagenous rings of the trachea. The tracheal site of surgery was sutured and the rats were allowed to recover until the time of lung harvesting.

At appropriate time intervals, the rats were killed by cervical dislocation. Lungs to be used for TGF- $\beta$  extraction were removed and flushed through the right ventricle with heparinized (5 U/ml) PBS. The lungs were then frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ .

**Immunohistochemical Staining.** TGF- $\beta$  was localized in sections of lungs of rats treated with intratracheal bleomycin at a number of time intervals. Lungs were exposed through a mid-thoracotomy incision and were filled with 10% neutral buffered formalin injected through the trachea. The trachea was then tied and the lungs were immersed in the buffered formalin for 24 h. The lungs were then transferred to Bouin's solution (0.9% picric acid, 9% (vol/vol) formaldehyde, 5% acetic acid) for 4-6 h at room temperature and then rinsed with 70% ethanol. They were stored in 70% ethanol until ready for embedding in paraffin.

The immunohistochemical staining procedure has been described previously (28). Briefly, the sections were incubated overnight at  $4^{\circ}\text{C}$  with 15-20  $\mu\text{g/ml}$  of pretitrated anti-TGF- $\beta$  IgG. After extensive washing, the sections were blocked with normal goat serum, then incubated with affinity-purified goat anti-rabbit IgG and avidin-enzyme-complex (Vector Laboratories, Inc., Burlingame, CA). Staining was visualized by incubating with alkaline phosphatase red (Vector Laboratories, Inc.), as per manufacturer's instructions, and counterstained with 1% methyl green for 1 min. Controls consisted of replacing anti-TGF- $\beta$  by normal rabbit IgG and using antisera depleted by incubation with TGF- $\beta$  sepharose resin (27). In blocking experiments, TGF- $\beta$  peptide 1-30 was added to sections before TGF- $\beta$  antibody, as described previously (26).

**TGF- $\beta$  Extraction Procedure.** To previously frozen lungs, 4 ml of an acid-ethanol solution (95% ethanol, 0.2 M HCl) containing 1  $\mu\text{g/ml}$  each of PMSF (Sigma Chemical Co., St. Louis, MO) and pepstatin (United States Biochemical Corp., Cleveland, OH) was added as described (27). The lungs were then homogenized using a Polytron tissue homogenizer (Brinkmann Instruments Co., Westbury, NY). The homogenate was stirred overnight at  $4^{\circ}\text{C}$  and centrifuged at 10,000  $g$  for 10 min. The supernatant was diluted with two parts distilled water and lyophilized. It was then resuspended in 2 ml of 4 mM HCl with 0.1% BSA per lung, centrifuged to remove insoluble particles, then stored at  $-80^{\circ}\text{C}$  until assayed. Samples were neutralized with 2-3  $\mu\text{l}$  of 5 N NaOH and 1 M Hepes before use, and any precipitate was removed by a 5-min centrifugation at 10,000 rpm on a microfuge (Beckman Instruments, Inc., Palo Alto, CA).

**Lung Fibroblast Isolation.** Bleomycin-treated rats were killed at different time intervals by cervical dislocation and the lungs with the heart were removed under sterile conditions and placed in cold PBS and heparin (5 U/ml). After flushing lungs through the right ventricle with heparinized PBS, the heart and major bronchi were removed. The lungs were manually minced with fine scissors and digested in 0.04% trypsin EDTA (Gibco Laboratories, Grand

Island, NY) and 0.1% collagenase I (Sigma Chemical Co.) for 20 min at 37°C in 5% CO<sub>2</sub>. After rigorous vortexing, the trypsin was inactivated by addition of F12 medium (Gibco Laboratories) with 20% FCS (Gibco Laboratories). The digest was passed through sterile nylon mesh, centrifuged, and the cell pellet was retained. RBC in the pellet were removed by hypotonic lysis and the remaining cells were resuspended as 10<sup>5</sup> cells/ml in  $\alpha$ -MEM with 5% fetal bovine plasma (FBP) with gentamycin (40  $\mu$ g/ml) (Schering, Pointe Claire, Quebec) and Fungizone (0.25  $\mu$ g/ml) (Gibco Laboratories). FBP was used because it contained very low levels of TGF- $\beta$  compared with serum and did not interfere with measurements of collagen synthesis. It also enhanced fibroblast adhesion to the microwells. The cells were seeded as 10<sup>5</sup> cells/well in triplicate in a 24-well tissue culture plate and incubated for 16–20 h at 37°C in 5% CO<sub>2</sub>.

*Assay for Collagen Synthesis In Vitro.* After 24 h in culture, lung fibroblasts were assayed for collagen synthesis as described by Roberts et al. (15). Briefly, cells were washed with  $\alpha$ -MEM to remove nonadherent cells. To each well, 300  $\mu$ l of 10% (FCS) MEM with ascorbate (0.25 mM), Hepes (20 mM), and NaHCO<sub>3</sub> (350 mg/liter) was added. Then, 25  $\mu$ l of L-[2,3-<sup>3</sup>H] proline (29-1 A/mmol; 1 Ci, 37 GBq) (Amersham International, Amersham, UK) was added and the cells were incubated for 3 h at 37°C. Media were pooled from the triplicates and the unincorporated radioactive amino acids were separated from the proteins by 5% TCA (Fisher Scientific Co., Pittsburg, PA) precipitation. The TCA-precipitated protein was redissolved in 0.2 M NaOH. Equal portions (150  $\mu$ l) were neutralized with 0.7 N HCl and 1 M Hepes and incubated with or without collagenase III (Advanced Biofactures, Lynbrook, NY) at 37°C for 90 min. The proteins were reprecipitated with 40% TCA and 1% tannic acid (Sigma Chemical Co.) After centrifugation the supernatant containing peptides derived from collagen was removed into a counting vial. The pellet containing the noncollagen protein was dissolved in 200  $\mu$ l of 80% formic acid and placed in the protein fraction vial. Then, 10 ml of Scintiverse II (Fisher Scientific Co.) was added and all vials were counted in a  $\beta$  Scintillation Counter (Beckman Instruments, Inc.). The remaining cells were used to calculate micrograms of DNA by the method of Burton. The data were calculated as described by Roberts et al. (15) and presented as dpm/min/ $\mu$ g DNA.

*CCL-64 Mink Lung Growth Inhibition Assay for TGF- $\beta$ .* CCL-64 mink lung epithelial cells were maintained in DME (Gibco Laboratories) with 10% FBP. Subconfluent cells were used in the TGF- $\beta$  growth inhibition assay as described by Danielpour et al. (27). Cells were trypsinized and washed with  $\alpha$ -MEM in 0.2% FBP, and resuspended in DME, 0.2% FBP, 10 mM Hepes at pH 7.4, penicillin (25 U/ml), and streptomycin (25  $\mu$ g/ml), and seeded as 5  $\times$  10<sup>5</sup> cells per 0.5 ml in 24-well Costar dishes (Flow Laboratories, Inc., Mississauga, Ontario). Aliquots of neutralized acid ethanol-extracted lung preparations with and without TGF- $\beta$  antibody (R & D Systems) were added 3 h later. Antibody was always placed in the wells before lung samples. After 22 h, the cells were pulsed with 0.25  $\mu$ Ci (5 Ci/mg) of 5'-[<sup>125</sup>I]iodo 2'-deoxyuridine for 2–3 h at 37°C. Cells were then fixed with 1 ml of methanol-acetic acid (3:1) (vol/vol). After 1 h at room temperature the wells were washed twice with 2 ml of 80% methanol. The cells were lysed with 1 ml of 1 N NaOH for 30 min at room temperature and the <sup>125</sup>I-UdR was counted in a gamma counter (LKB Instruments, Inc., Gaithersburg, MD). A standard curve of porcine TGF- $\beta$  was included in each assay and data were expressed as micrograms of TGF- $\beta$ , as described (27).

## Results

To determine whether TGF- $\beta$  might be involved in the genesis of pulmonary fibrosis after bleomycin injury, we initially examined the total TGF- $\beta$  content of rat lungs at increasing time intervals after instillation of bleomycin into the trachea of Sprague-Dawley rats. After acid-ethanol extraction of lungs, lyophilization, and reconstitution, as described in Materials and Methods, we measured TGF- $\beta$  levels using the CCL-64 mink epithelial lung cell inhibition assay (27). By assaying inhibition in the presence or absence of polyclonal rabbit anti-TGF- $\beta$  antiserum, which blocks both TGF- $\beta$ <sub>1</sub> and - $\beta$ <sub>2</sub>, we were able to estimate the total TGF- $\beta$  content of the lung.

Fig. 1 presents the TGF- $\beta$  levels per lung at different time points after bleomycin administration. Detectable levels were found within 2 h, with a progressive but slow increase to about day 4. A 30-fold increase was seen at day 7, which was the peak response, followed by a gradual decrease over the following weeks. TGF- $\beta$  levels remained significantly elevated above control levels for the full 28 d of the experiment.

If TGF- $\beta$  was a major contributor to the induction of pulmonary fibrosis, then one would expect it to either precede or be coincidental with the synthesis of collagen by pulmonary fibroblasts. We have assayed collagen synthesis by measuring collagenase-sensitive [ $^3$ H]proline incorporation in fibroblasts isolated from bleomycin-treated lungs at the same time intervals at which we assayed TGF- $\beta$  levels. By evaluating isolated lung fibroblasts within 24 h of recovery from the lung, we were able to identify enhanced collagen synthesis in the bleomycin-treated rat lungs. The methodology also allowed us to compare the relative synthesis of collagen and noncollagen protein per microgram DNA, which is presented in Fig. 2. Collagen synthesis increased gradually and was minimally but detectably above untreated controls 7 d after bleomycin administration. It reached a peak of synthesis by 14 d, which was past the point at which TGF- $\beta$  content of lungs was at its maximum. Collagen synthesis decreased but remained significantly above untreated controls for the full 28 d of the assay, paralleling the TGF- $\beta$  content of the lungs. Changes in the level of [ $^3$ H]proline incorporation into noncollagenous protein followed the identical pattern with that of collagen.

To determine the source of TGF- $\beta$  production during the course of the bleomycin-induced lung injury, we examined lungs at all time points by immunohistochemical staining. The two antibodies used for this procedure have been described (26, 28) and are able to distinguish between intracellular TGF- $\beta$  (LC), and extracellular TGF- $\beta$  (CC) that is associated with stromal tissue or matrix. To confirm that all staining was specific, each experiment and each time point was controlled using the IgG fraction from normal rabbit serum. Furthermore, examples of positively stained tissue were re-examined with anti-TGF- $\beta$  antibodies after preabsorption with insolubilized TGF- $\beta$ , or blocked with specific peptides corresponding to the NH<sub>2</sub>-terminal amino acids 1-30 of TGF- $\beta$ , which were used as antigens for the generation of these polyclonal antibodies (27). The fixation procedure described in the methodology, using

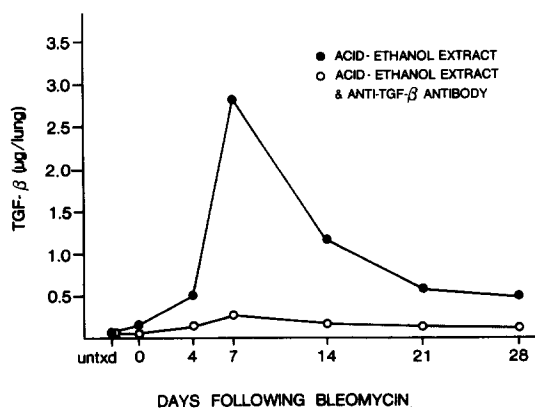


FIGURE 1. Total extractable TGF- $\beta$  per rat lung at increasing time intervals after intratracheal administration of bleomycin. Lungs were acid-ethanol extracted, lyophilized, then resuspended and assayed in the CCL-64 lung epithelial cell growth inhibition assay (27). Each point (●) represents two to three lungs. The ED<sub>50</sub> growth inhibition was converted to TGF- $\beta$  levels on the basis of a porcine TGF- $\beta$ <sub>1</sub> standard curve. Each extract was also treated with anti-TGF- $\beta$  antiserum (○) to confirm the growth inhibition was due to TGF- $\beta$ .

first neutral formalin, the Bouin's solution, was critical for the detection of the TGF- $\beta$  staining outlined below (28).

Within 2 h of bleomycin instillation in the trachea, intracellular staining with LC antibody was detected in bronchiolar epithelium (Fig. 3 A). Staining in the subepithelial matrix was seen at the same time and up to 4 d later with the CC TGF- $\beta$  antibody (Fig. 3 B). Although some staining with these antibodies was seen in normal animals, the extent and intensity were much increased in the bleomycin-treated group. Rare macrophages stained with the LC antibody were also detected in normal animals. By 4 d after bleomycin installation, increasing numbers of macrophages that stained intensely with the LC TGF- $\beta$  antibody were seen associated with areas of increased cellularity in the alveolar walls. The distribution was still patchy and little macrophage organization was observed until 7 d. At this time point, there was widespread evidence of collagen staining with Masson's trichrome (data not shown) and widespread intense staining of macrophages (Fig. 3 C), which was the most pronounced throughout the entire time course, and coincided with the peak levels of TGF- $\beta$  in the lung (Fig. 1). Besides observing TGF- $\beta$  containing macrophages as isolated cells, areas of highly organized but less intensely stained macrophages were now evident (Fig. 3 D). By 14 d the isolated intensely staining macrophages had disappeared, and only the organized macrophages remained. At this time, TGF- $\beta$  levels in the lung were decreasing. As early as 7 d, and clearly by 14 d, a second pattern of staining

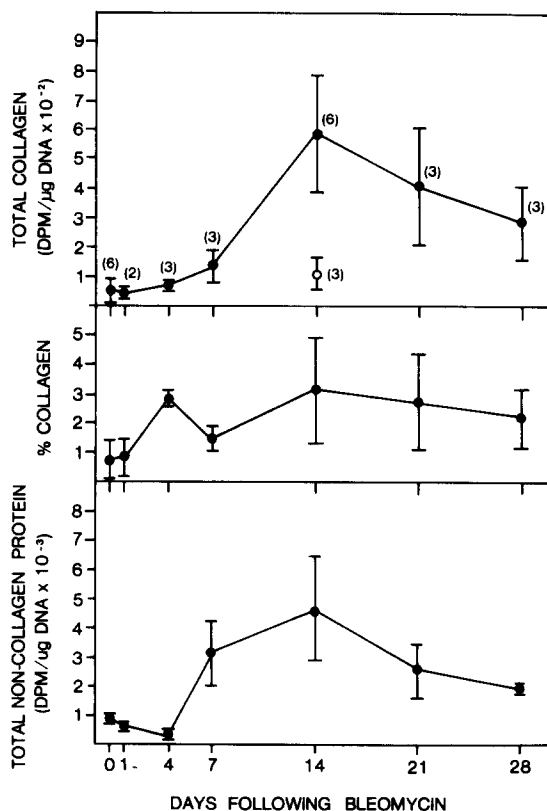


FIGURE 2. [ $^3\text{H}$ ]Proline incorporation into collagen and noncollagen protein of lung fibroblasts recovered from bleomycin-treated animals. At sequential time points ( $\bullet$ ) after intratracheal bleomycin instillation, progressively increasing [ $^3\text{H}$ ]proline incorporation into both collagen (*upper panel*) and noncollagen protein (*lower panel*) was observed. Peak [ $^3\text{H}$ ]proline incorporation occurred at 14 d after bleomycin. Collagen synthesis decreased after 14 d but remained above baseline to 28 d. The proportion of [ $^3\text{H}$ ]proline incorporated into collagen remained two- to threefold higher than background (*middle panel*). Total collagen synthesis in rats 14 d after intratracheal injection with saline ( $\circ$ ) instead of bleomycin was not different from untreated controls (*upper panel*). The number of animals in each group is indicated in brackets in the upper panel.

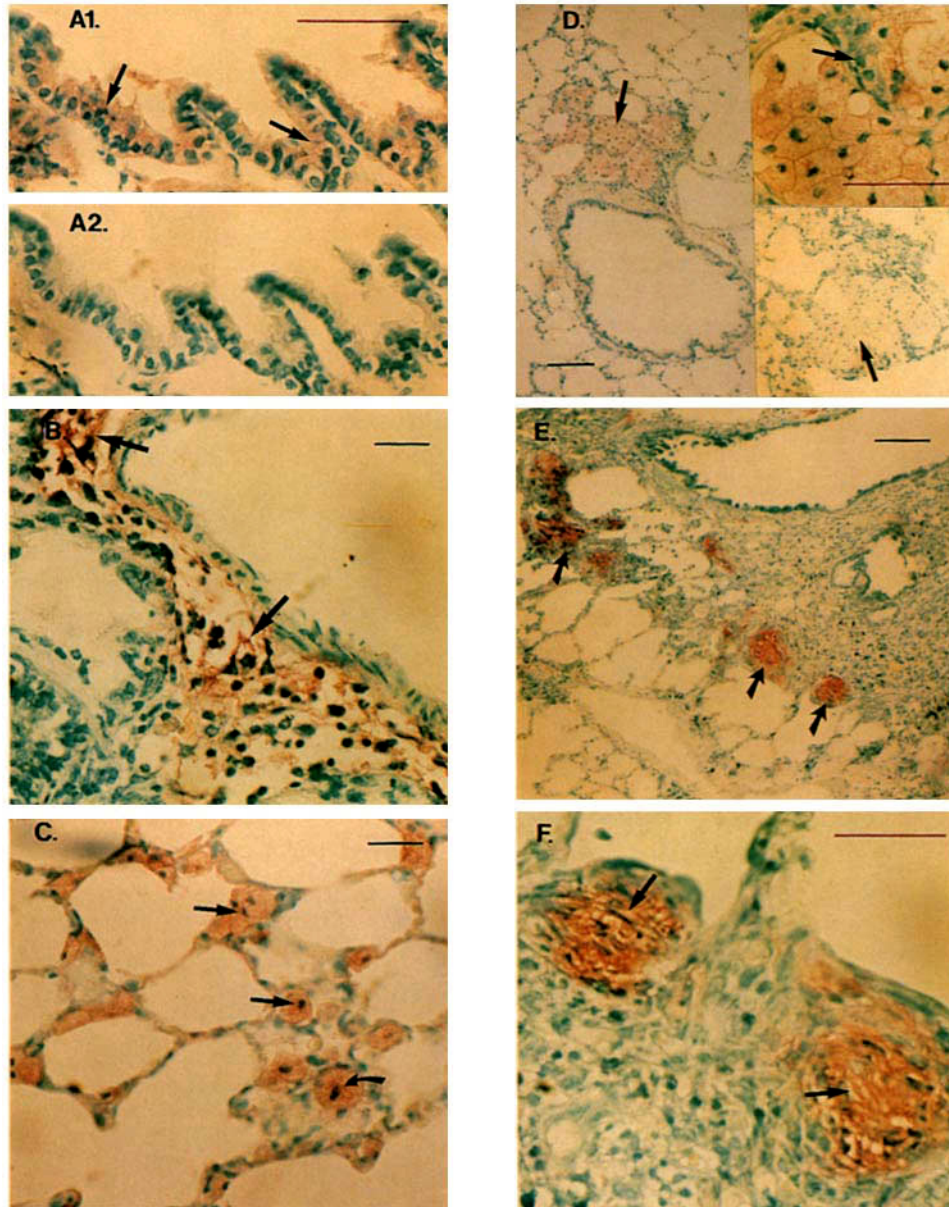


FIGURE 3. Immunolocalization of TGF- $\beta$  in rat lung after intratracheal bleomycin induction of chronic pulmonary inflammation. (A.1) Antibody to TGF- $\beta_1$  NH<sub>2</sub>-terminal synthetic peptide (anti-LC-[1-30]) localizes in the cytoplasm of bronchiolar epithelium (arrows). Avidin-biotin-alkaline phosphatase (red) detection system was used with methyl green counter stain (bar, 50  $\mu$ M). (A.2) TGF- $\beta$  staining was blocked by preabsorption with sepharose on which TGF- $\beta$  had been coupled. (B) Anti-CC (1-30) antibody to TGF- $\beta_1$  localizes to subendothelium primarily staining connective tissue 1 d after bleomycin administration (arrows) (bar, 50  $\mu$ M). (C) Anti-LC (1-30) localizes to macrophages 7 d after bleomycin. The cytoplasm of the macrophages is intensely positive (arrows). Staining was blocked or greatly diminished by preincubation with 20-fold molar excess of peptide LC (1-30) (not shown) (bar, 50  $\mu$ M). (D) Anti-LC (1-30) antibody localizes to macrophages organized in clusters between 7 and 14 d after bleomycin (arrow) (bar,

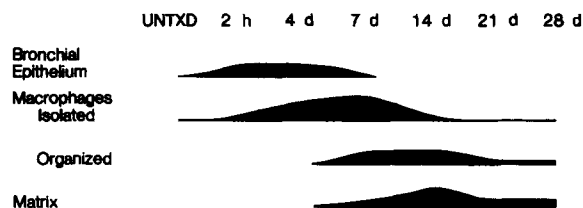


FIGURE 4. Summary of the distribution and intensity of TGF- $\beta$  immunohistochemical staining in lungs after bleomycin administration.

was evident with the CC antibodies. This antibody detected intense TGF- $\beta$  staining associated with areas of repair with hypercellularity and collagen deposition (Fig. 3, *E* and *F*). The staining was associated with matrix and occurred at the edges of these intensely hypercellular areas. This pattern of staining, a combination of weakly staining organized macrophages, and areas of matrix-associated TGF- $\beta$  staining in focal regions of hypercellularity, persisted through to day 28. Throughout this period, TGF- $\beta$  levels in the lung were above background, and significant collagen synthesis was detected in isolated lung fibroblasts. The pattern of staining in all lung tissues over the 28 d of the experiment is summarized in Fig. 4.

### Discussion

The histological and physiological changes associated with bleomycin-induced pulmonary fibrosis (BPF) have been extensively studied and reported (18–24). Although many lymphokines have been implicated in the inflammatory and fibroblast proliferative response consequent to bleomycin-induced pulmonary injury, the regulation of fibrosis and repair remains unresolved. Recently, Hoyt and Lazo (24) found that early after bleomycin infusion they detected an elevation of TGF- $\beta$  mRNA in lungs of bleomycin-susceptible but not bleomycin-resistant mice. These changes preceded increases in pulmonary fibronectin, procollagen  $\alpha_2$ (I) and  $\alpha_1$ (III) mRNA content and suggested that TGF- $\beta$  may participate in the repair response. The TGF- $\beta$  mRNA increase could have resulted from either altered synthesis/degradation, or simply from changes in pulmonary cell content. For example, TGF- $\beta$  steady-state mRNA levels are high in normal macrophages, and translation does not occur until the cells are activated (7), consequently, entry of non-TGF- $\beta$ -secreting macrophages into the lung would alter mRNA content. It was, therefore, unclear from this study whether TGF- $\beta$  was being actively produced. It was the purpose of our study to examine the production of TGF- $\beta$ , identify its source, and correlate it with lung collagen synthesis. We have observed a rapid and substantial increase in TGF- $\beta$  production that was temporally related to enhanced synthesis of collagen and noncollagen protein in pulmonary fibroblasts. Furthermore, TGF- $\beta$  was identified in three tissue sites: (a) macrophages, whether isolated or organized in clusters; (b) bronchiolar epithel-

100  $\mu$ M). Inset (*top*) shows a higher power magnification of organized stained macrophages adjacent to areas of hypercellularity (*arrow*) (bar, 50  $\mu$ M). Inset (*bottom*) shows absence of macrophage staining (*arrow*) using normal rabbit IgG in place of LC antibody. (*E*) Anti-CC (1–30) antibody intensely staining localized areas at the edge of regions of hypercellularity 14 d after bleomycin (*arrows*) (bar, 100  $\mu$ m). (*F*) High power of *E* illustrating staining primarily in interstitial connective tissue in whorl patterns (*arrows*). Staining was blocked or greatly reduced by preabsorption of antiserum on sepharose-linked TGF- $\beta$  (not shown) (bar, 50  $\mu$ M).

lium and subepithelial matrix; and (c) extracellular matrix associated with areas of repair and collagen synthesis.

The initial TGF- $\beta$  response to bleomycin is localized to the bronchiolar epithelial cells and subepithelial matrix. Since little TGF- $\beta$  was seen in the bronchiolar epithelium of untreated animals, and minimal amounts were extracted from untreated animals, it suggests that this is a response to the bleomycin-induced injury. The source of TGF- $\beta$  in subepithelial matrix is likely from the bronchiolar epithelial cells themselves, or possibly from platelets that have degranulated in response to bleomycin vascular injury. TGF- $\beta$  is a chemoattractant to monocytes and macrophages (6), and the early TGF- $\beta$  production by bronchiolar epithelial cells may represent a mechanism for recruiting macrophages into the damaged area.

Once in damaged tissue, and activated, macrophages have been shown to secrete TGF- $\beta$  (7). This is the first report that macrophages recruited to an area of lung injury are producers of TGF- $\beta$ . Since the intense intracellular TGF- $\beta$  staining in alveolar macrophages after bleomycin coincides with the peak extractable total lung TGF- $\beta$  activity (Fig. 1) and total TGF- $\beta$  mRNA (24), this suggests that the main source of TGF- $\beta$  in bleomycin-damaged lungs is from pulmonary macrophages.

By 14 d after bleomycin instillation, [ $^3\text{H}$ ]proline incorporation into collagenous protein was at its peak. This point of maximal collagen production in our study corresponded to the rise in mRNA of procollagen  $\alpha_2(\text{I})$  and  $\alpha_1(\text{III})$  reported by others (22, 23). The biochemical and immunohistochemical evidence of TGF- $\beta$  production preceding collagen synthesis and deposition suggests that TGF- $\beta$  may be regulating collagen production in BPF. Although we have shown elevation of extractable lung TGF- $\beta$ , and described its presence in macrophages, we have provided no direct evidence of its secretion. However, the changing pattern of TGF- $\beta$  distribution from initial intracellular staining in macrophages to association with extracellular matrix (Fig. 4) suggests it is being actively secreted and deposited on matrix. Furthermore, the most intense matrix staining occurs at the peak of collagen synthesis. Therefore, it is possible that once TGF- $\beta$  has become associated with extracellular matrix, it remains localized, recruiting fibroblasts to the site and subsequently inducing them to produce collagen. It is of interest that histological studies of lung sections show that when collagen is detectable by Massons Trichrome stain it is often in a localized whorl-like or nodular pattern. This suggests that collagen is laid down in a similar pattern of distribution to TGF- $\beta$  (see Fig. 3, *E* and *F*). This speculation is supported by the observation that granulation formation and increased collagen synthesis occur at sites of TGF- $\beta$  instillation into subcutaneous tissue (15) or when applied to wounds (16).

Although diminished in amount, extractable TGF- $\beta$  levels and collagen synthesis persist chronically up to 28 d after bleomycin instillation. Immunohistochemical staining suggests that much of the TGF- $\beta$  in the chronic phase of injury is extracellular in distribution, although areas of organized weakly stained macrophages do remain. The persistently elevated amounts of extractable TGF- $\beta$  in the later phase may be derived both from previously synthesized, secreted, and extracellular matrix-associated TGF- $\beta$ , as well as from macrophages. This continued association of TGF- $\beta$  production and collagen synthesis supports the hypothesis that chronic fibrosis is mediated by TGF- $\beta$ . Furthermore, this would suggest that in diseases characterized by chronic pulmonary fibrosis, such as active idiopathic pulmonary fibrosis, sar-



coidosis, or Hamman-Rich syndrome, in which a continuous inflammatory response is evident, TGF- $\beta$  production may be etiologic.

In conclusion, we have presented evidence that TGF- $\beta$ , a peptide well recognized for its effects on inflammatory cells, fibroblasts, and collagen production, is produced in 30-fold elevated levels during the course of BPF and is found primarily in pulmonary alveolar macrophages. The increase in macrophage TGF- $\beta$  production precedes collagen synthesis while the peak of collagen production coincides with the presence of extracellular TGF- $\beta$ . This temporal relationship and histological distribution suggest a role for the aberrant production of TGF- $\beta$  in the pathogenesis of pulmonary fibrosis.

### Summary

A rat model of bleomycin-induced pulmonary inflammation and fibrosis was used to examine the relationship between collagen synthesis and transforming growth factor  $\beta$  (TGF- $\beta$ ) production, and cellular distribution. Total lung TGF- $\beta$  was elevated within 2 h of intratracheal bleomycin administration and peaked 7 d later at levels 30-fold higher than controls. This was followed by a gradual decline with lower but persistent levels of production in the late phase of the response between 21 and 28 d later. The peak TGF- $\beta$  levels preceded the maximum collagen and noncollagen protein synthesis measured by [ $^3$ H]proline incorporation into lung fibroblast explants of bleomycin-treated rats. The pattern of immunohistochemical staining localized TGF- $\beta$  initially in the cytoplasm of bronchiolar epithelium cells and subepithelial extracellular matrix. The peak of lung TGF- $\beta$  levels at 7 d coincided with intense TGF- $\beta$  staining of macrophages dispersed in the alveolar interstitium and in organized clusters. Later in the course of the response, TGF- $\beta$  was primarily associated with extracellular matrix in regions of increased cellularity and tissue repair, and coincided with the maximum fibroblast collagen synthesis. This temporal and spatial relationship between collagen production and TGF- $\beta$  production by macrophages suggests an important if not primary role for TGF- $\beta$  in the pathogenesis of the pulmonary fibrosis.

We thank Anita Roberts for many helpful discussions and Nancy Thompson, David Danielpour, and Nan Roche for their advice.

*Received for publication 6 April 1989 and in revised form 18 May 1989.*

### References

1. Roberts, A. B., and M. B. Sporn. 1989. The transforming growth factor betas. *In* Peptide Growth Factors and their Receptors. Handbook of Experimental Pharmacology, Vol. 95. Springer-Verlag, Heidelberg. In press.
2. Massagué, J., and B. Like. 1985. Cellular receptors for type- $\beta$  transforming growth factor. *J. Biol. Chem.* 260:2636.
3. Wakefield, L. M., D. M. Smith, T. Masui, C. C. Harris, and M. B. Sporn. 1987. Distribution and modulation of the cellular receptor for transforming growth factor-beta. *J. Cell Biol.* 105:965.
4. Assoian, R. K., A. Komoriya, C. A. Meyers, D. M. Miller, and M. B. Sporn. 1983. Transforming growth factor-beta in human platelets. *J. Biol. Chem.* 258:7155.

5. Assoian, R. K., and M. B. Sporn. 1986. Type  $\beta$  transforming growth factor in human platelets: release during platelet degranulation and action on vascular smooth muscle walls. *J. Cell Biol.* 102:1217.
6. Wahl, S. M., D. A. Hunt, L. M. Wakefield, N. McCartney-Francis, L. M. Wahl, A. B. Roberts, and M. B. Sporn. 1987. Transforming growth factor type  $\beta$  induces monocyte chemotaxis and growth factor production. *Proc. Natl. Acad. Sci. USA.* 84:5788.
7. Assoian, R. K., B. E. Frolik, H. C. Stevenson, D. K. Miller, E. W. Medtes, R. R. Raines, and M. B. Sporn. 1987. Expression and secretion of type beta transforming growth factor by activated human macrophages. *Proc. Natl. Acad. Sci. USA.* 84:6020.
8. Postlethwaite, A. E., J. Keski-Oja, H. L. Moses, and A. H. Kang. 1987. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor  $\beta$ . *J. Exp. Med.* 165:251.
9. Hill, D. J., A. J. Strain, S. F. Elstow, I. Svenne, and R. D. G. Milner. 1986. Bi-functional action of transforming growth factor- $\beta$  on DNA synthesis in early passage human fetal fibroblasts. *J. Cell. Physiol.* 128:322.
10. Fine, A., and R. H. Goldstein. 1987. The effect of transforming growth factor- $\beta$  on cell proliferation and collagen formation by lung fibroblasts. *J. Biol. Chem.* 262:3897.
11. Ignatz, R., and J. Massagué. 1986. Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J. Biol. Chem.* 261:4337.
12. Chen, J. K., H. Hoshi, and W. L. McKeehan. 1987. Transforming growth factor type  $\beta$  specifically stimulates synthesis of proteoglycan in human adult arterial smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* 84:5287.
13. Laiho, M., O. Sakesela, and J. Keski-Oja. 1987. Transforming growth factor- $\beta$  induction of type-1 plasminogen activator inhibitor. *J. Cell Biol.* 262:17467.
14. Edwards, D. R., G. Murphy, J. J. Reynolds, S. E. Whitham, A. J. P. Docherty, P. Angel, and J. K. Heath. 1987. Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1899.
15. Roberts, A. B., M. B. Sporn, R. K. Assoian, J. M. Smith, N. S. Roche, L. M. Wakefield, U. L. Heine, L. A. Liotta, V. Falanga, J. H. Kehrl, and A. S. Fauci. 1986. Transforming growth factor type  $\beta$ : rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc. Natl. Acad. Sci. USA.* 83:4167.
16. Mustoe, T. A., G. F. Pierce, A. Thomason, P. Gramates, M. B. Sporn, and T. F. Duel. 1987. Accelerated healing of incisional wound in rats induced by transforming growth factor- $\beta$ . *Science (Wash. DC).* 237:1333.
17. Crystal, R. G., J. E. Grilic, V. J. Ferrans, J. D. Fulmer, B. R. Line, and G. W. Hunninghake. 1981. Interstitial lung disease: current concepts of pathogenesis, staging and therapy. *Am. J. Med.* 70:542.
18. Thrall, R. S., J. R. McCormick, R. M. Jack, R. A. McReynolds, and P. A. Ward. 1979. Bleomycin induced pulmonary fibrosis in the rat. *Am. J. Pathol.* 95:117.
19. Snider, G. L., R. C. Bartolosne, H. G. Ronald, J. J. O'Brien, and E. C. Lucey. 1978. Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin. *Am. Rev. Resp. Dis.* 117:289.
20. Adamson, I. R., and D. H. Bowden. 1979. Bleomycin-induced injury and metaplasia of alveolar type 2 cells. *Am. J. Pathol.* 96:531.
21. Chandler, D. B., D. M. Hyde, and S. N. Giri. 1983. Morphometric estimates of infiltrative cellular changes during the development of bleomycin-induced pulmonary fibrosis in hamsters. *Am. J. Pathol.* 112:170.
22. Raghov, R., S. Lurie, J. M. Seyer, and A. H. Kang. 1985. Profiles of steady state levels of messenger RNAs coding for type 1 procollagen, elastin, and fibronectin in hamster lungs undergoing bleomycin-induced interstitial pulmonary fibrosis. *J. Clin. Invest.* 76:1733.

23. Kelley, J., L. Chrin, S. Shull, D. W. Rowe, and F. R. Cutroneo. 1985. Bleomycin selectively elevates mRNA levels for procollagen and fibronectin following acute lung injury. *Biochem. Biophys. Res. Commun.* 131:836.
24. Hoyt, D. G., and J. S. Lazo. 1988. Alterations in pulmonary mRNA encoding procollagens, fibronectin and transforming growth factor- $\beta$  precede bleomycin-induced pulmonary fibrosis in mice. *J. Pharmacol. Exp. Ther.* 246:765.
25. Flanders, K. C., N. L. Thompson, D. S. Cissel, E. Obberghen-Schilling, C. C. Baker, M. E. Koss, L. R. Ellingsworth, A. B. Roberts, and M. B. Sporn. 1989. Transforming growth factor  $\beta_1$ : histochemical localization with antibodies to different epitopes. *J. Cell Biol.* 108:653.
26. Thompson, N. L., K. C. Flanders, J. M. Smith, L. R. Ellingsworth, A. B. Roberts, and M. B. Sporn. 1989. Expression of transforming growth factor-beta<sub>1</sub> in specific cells and tissues of adult and neonatal mice. *J. Cell. Biol.* 108:661.
27. Danielpour, D., L. L. Dart, K. C. Flanders, A. B. Roberts, and M. B. Sporn. 1989. Immunodetection and quantitation of the two forms of transforming growth factor-beta (TGF- $\beta_1$  and TGF- $\beta_2$ ) secreted by cells in culture. *J. Cell. Physiol.* 138:79.
28. Heine, U. I., E. F. Munoz, K. C. Flanders, L. R. Ellingsworth, P. H. Lam, N. L. Thompson, A. B. Roberts, and M. B. Sporn. 1987. Role of transforming growth factor  $\beta$  in the development of the mouse embryo. *J. Cell. Biol.* 105:2861.