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Changes in Distribution, Morphology, and Tumor Necrosis Factor-α Secretion of Alveolar Macrophage Subpopulations During the Development of Bleomycin-induced Pulmonary Fibrosis

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Previous studies indicate that heterogeneous alveolar macrophages (AM) play a pivotal role in events associated with bleomycin-induced pulmonary fibrosis. A critical role has been suggested for tumor necrosis factor-alpha (TNF-α), a product of activated macrophages, in this fibrotic process. The present study examined whether the characteristics and function (TNF-α secretion) of rat AM subpopulations were altered during the development of bleomycin-induced fibrosis. After intratracheal bleomycin treatment, AM were separated into 18 density-defined subpopulations. Bleomycin treatment altered the distribution and morphology of AM subpopulations of densities 1.017 to 1.061 g/ml at all time points studied (4, 14, and 28 days). Subpopulations of densities 1.090 to 1.141 g/ml were affected only at 4 days after bleomycin treatment. Tumor necrosis factor-α secretion increased with time in 14- and 28-day samples of bleomycin-treated rats, particularly in subpopulations of densities 1.075 to 1.097 g/ml. These data indicate that alterations in the distribution, morphology, and function of AM subpopulations accompany the development of bleomycin-induced pulmonary fibrosis. When coupled with previous studies suggesting that TNF-α plays a role in the fibrotic process in this disease model, these data indicate that AM of densities 1.075 to 1.097 g/ml are responsible for the production of TNF-α associated with bleomycin-induced pulmonary fibrosis. (Am J Pathol 1992, 140:503–512)

Fibrotic processes are generally associated with inflammatory responses. Factors that contribute to the control and modulation of pulmonary fibroses are poorly understood, however, and the relationship between inflammatory responses and subsequent fibrogenesis is not clear. Nonetheless recent studies have demonstrated that cells of the inflammatory/immune system are under the regulation of alveolar macrophages (AM).[1–3] Alveolar macrophages are the major mononuclear phagocytes of the lung, the primary defense against airborne particles, and exhibit a variety of functions, including modulation of pulmonary inflammation and fibrosis.[4,5] The number of AM in the lung is increased after exposure to aerosolized particles and in many inflammatory lung disorders.[6–8] Moreover, using bleomycin-induced pulmonary fibrosis[9,10] as a model for human interstitial fibrosis, previous studies have shown alterations in the numbers and functions of AM.[3,11,12]

Recent data indicate that AM represent a heterogeneous population of cells that can be separated into a number of discrete subpopulations that differ biochemically, morphologically, and immunologically.[13–15] The possibility is raised that these characteristics of the AM population (and subpopulations thereof) are altered during lung disease. Several studies suggest that there are changes in the composition of the AM population in lung disease; however, the characterization of specific subpopulations was not determined.[16–19] A recent study has suggested that tumor necrosis factor-alpha (TNF-α) plays a key role in bleomycin-induced pneumopathy and fibrosis; however the cell types responsible for increased lung TNF-α mRNA and for secretion of TNF-α were not determined.[20] When coupled with previous studies, these data suggest an involvement of macrophages and

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their TNF-α products in pulmonary fibrosis; however, the precise role for macrophages in mediating this process has not been critically evaluated. Therefore in efforts to elucidate the role of AM and AM-derived TNF-α in the development of bleomycin-induced fibrosis, the present study was undertaken to determine whether physical and functional characteristics of AM subpopulations were altered during the progression of pulmonary fibrosis. The data demonstrate that the AM subpopulation composition, morphology, and function (TNF-α secretion) are indeed altered during evolution of the fibrotic process.

Materials and Methods

Animals

Male Fisher 344 rats (Charles Rivers, Kingston, NY) weighing 200 to 225 g were used. Animals were derived from a pathogen-free colony transported behind biologic filters and maintained in specific pathogen-free colonies with standard laboratory food and water *ad libitum*.

Cell Preparation

Under pentobarbital anesthesia, animals received intratracheally either 1.5 U bleomycin (Blenoxane; a gift from Bristol-Myers, Syracuse, NY) in 0.5 ml sterile saline or an equal volume of saline lacking drug (controls) by established techniques.[12] Animals were killed 4, 14, and 28 days after bleomycin or saline treatment and AM was collected by bronchoalveolar lavage as previously described.[13–15] Briefly, under pentobarbital anesthesia, animals were killed by exsanguination. The trachea was cannulated with polypropylene tubing and lungs were lavaged *in situ* with saline (0.9% NaCl) accompanied by gentle massage of the thoracic cavity. The lavage fluid then was filtered to remove mucus and was centrifuged. Cell pellets from two to three animals for each control or test group were combined and washed twice with cold Dulbecco's phosphate-buffered saline (PBS) without Ca^{+2} and Mg^{+2}, pH 7.4 (PBS) and resuspended in 5 ml RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell numbers and viability then were quantified by trypan blue exclusion using a hemocytometer.

Generation of Iso-osmotic Continuous Percoll Density Gradients

Continuous iso-osmotic density gradients were generated as previously described.[13–15] Briefly, an iso-osmotic working solution of colloidal silica (Percoll; Pharmacia, Piscataway, NJ) was generated by mixing 45 ml Percoll with 5 ml 10X Media 199. A continuous-density gradient then was generated at room temperature by diluting the iso-osmotic working solution with RPMI 1640 in a two-chamber gradient maker. This linear gradient was formed on ice over a 6-ml Percoll cushion. The density of the gradient varied from 1.010 to 1.141 g/ml as determined by refractive index and density marker beads (Pharmacia).[13]

Fractionation of AM

Previous experiments have demonstrated that AM can be separated into 20 fractions using density gradients.[13–15] The first two fractions contained few cells and most were nonviable. Therefore the experiments presented here were performed using fractions 3 through 20. The densities of the gradients and of each fraction have been shown to be reproducible, and the total recovery of cells placed on each gradient was similar to previous reports.[13–15]

Alveolar macrophages were separated by virtue of their buoyant density as previously described.[13–15] Briefly, bronchoalveolar cells ($\leq 5 \times 10^7$) were layered over continuous-density gradients and centrifuged. Fractions containing density-defined AM (DD-AM) then were collected using a sterile, dense sucrose solution to displace the gradient. Fractionated DD-AM then were washed and quantified for viable cells by trypan blue exclusion. Differential counts of 200 to 300 cells were performed using Wright-Giemsa–stained cytopreps.

TNF-α Quantification in AM Supernatant

To generate supernatants for TNF-α quantitation, adherent AM and DD-AM were cultured in serum-free RPMI 1640 at 1×10^6 cells/ml in a 37°C/5% CO_2 incubator as previously described.[15] Consistent with our previous studies, at least 98% of the adherent cells in each fraction were macrophages.[15] After 24 hours of culture, cell-free supernatants were collected, pooled as indicated (ie, fractions 3 through 6, 12 through 14, and 15 through 20), made 5% (vol/vol) with FCS, filter sterilized, and stored at −70°C until assayed. Tumor necrosis factor-α activity in supernatants was quantified in a blinded fashion by comparison with dose–response curves generated using human rTNF-α (2.17×10^7 U/mg; a gift from Cetus Corp., Emeryville, CA) in assays employing the TNF-α–sensitive WEHI 164 clone 13 cell line as previously described.[21] Briefly, 100 μl appropriately diluted AM culture supernatants (routinely a 1:100 dilution was used) or human

rTNF-α was added to 2×10^4 WEHI 164 clone 13 cells in 100 μl medium containing RPMI 1640 supplemented with 5% FCS, 20 μg/ml gentamicin sulfate, and 2 mmol/l (millimolar) L-glutamine in 96-well, flat-bottomed microculture plates and cultured in a 37°C/7% CO_2 incubator. After 20 hours, cultures were pulsed with 10 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) at a concentration of 5 mg/ml in PBS, and incubated for an additional 4 hours at 37°C. Cultures then were developed and plates read as previously described.[22] Percentage of dead cells in replicate assays was determined as previously described[21] using mean ± standard deviation of quadruplicate determinations. Standard deviations were always less than 10% of the mean. Routinely 1.38 ± 0.14 μg/ml human rTNF-α induced 50% maximal TNF-α–induced death of WEHI 164 clone 13 cells. Results are presented in micrograms per milliliter TNF-α activity as obtained by comparisons with dose–response curves. RPMI 1640 lacking AM cells served as a negative control. No irrelevant positive controls were included because a substantial amount (≈100 μg/ml) of TNF-α was produced in particular DD-AM fractions (see Results).

To confirm the identify of MΦ-derived TNF (TNF-α) in DD-AM culture supernatants, DD-AM supernatants and murine rTNF-α (1 mg/ml; provided by Biogen Corp., Cambridge, MA) were assayed for TNF-α activity as described above after a 1-hour preincubation of samples with a 1:400 dilution of heat-inactivated normal rabbit serum or heat-inactivated neutralizing polyclonal rabbit antiserum raised against murine rTNF-α (anti–TNF-α; provided by Dr. Kathleen Sheehan, Washington University School of Medicine, St. Louis, MO). The specificity, neutralization titer, and capacity of this antiserum to neutralize rat TNF have been described previously.[23]

Cellular Volume

Cellular volume determinations were performed on fixed (2.5% glutaraldehyde in PBS) DD-AM fractions collected from control and bleomycin-treated animals. Briefly, cells were placed in a hemocytometer, and the diameter of 100 cells was measured with a calibrated reticle (Zeitz, Wetzlar, Germany) in the eyepiece of a light microscope using a 45× objective.[13] The mean cell volume was calculated by using the formula $V = \pi D^3/6$.[13]

In Vivo *Proliferation of DD-AM*

Density-defined AM proliferation was measured by the incorporation of [^{3}H]TdR (Amersham, Needham Heights, MA) as an indicator of DNA synthesis. Briefly, 48 hours before being killed, control and experimental animals were injected intraperitoneally with 25 μCi [^{3}H]TdR. The animals were anesthetized 48 hours later with pentobarbital (90 mg/kg), blood was collected in a heparinized syringe from the inferior vena cava, and the animals were killed by exsanguination. Alveolar macrophages then were harvested by lavage and fractionated as described above. Mononuclear cells were isolated from heparinized whole blood using Ficoll-Hypaque (Pharmacia) as previously described.[24] Density-defined AM and peripheral blood monocytes then were allowed to adhere to microscintillation vials for 1 hour at 37°C in a humidified, 5% CO_2/95% air incubator. Nonadherent cells then were removed and adherent cells were washed with PBS containing Ca^{+2} and Mg^{+2}. Adherent DD-AM or peripheral blood monocytes then were mixed with 5 ml scintillation fluid, allowed to stand at room temperature for 24 hours, and measured for incorporation of radioactivity by liquid scintillation counting. Control experiments showed that at least 98% of the applied DD-AM and peripheral blood monocytes adhered to the scintillation vials and were identified as macrophages or monocytes by nonspecific esterase staining (data not shown). The results were expressed as counts per minute [^{3}H]TdR incorporation per 10^6 cells.

Statistical Evaluation

All data are expressed as the mean ± standard error of the mean except as noted. Differences between the treatment groups were tested using a one-way analysis of variance (ANOVA). Student's *t*-test was used to determine significant differences between control and bleomycin-treated animals. A value of $P < 0.05$ was considered significant. Duncan's multiple range test was used to assess differences between fractions.

Results

Effects of Bleomycin Treatment of AM Cell Number

Intratracheal administration of 1.5 U bleomycin resulted in significant increases in numbers of rat heterologous (unfractionated) AM at all time points examined (Table 1). The number of DD-AM in most density gradient fractions was significantly varied relative to control values (Figure 1). No significant differences were found between the AM cell numbers in each fraction for the control (saline-treated) groups (4-, 14-, and 28-day samples); therefore, the control fraction data (fractions 3 through 20) were pooled. At 4 days after bleomycin treatment (Figure 1A),

Table 1. *Relative Numbers of AM After Bleomycin Treatment*

	Time after bleomycin treatment		
Control	4 Days	14 Days	28 Days
100.0 ± 5.6*	145.2 ± 6.7†‡	139.2 ± 3.3†‡	265.1 ± 13.2†‡

* Mean ± SEM of numbers of AM derived from 11 control experiments (three animals per datum point) arbitrarily assigned a value of 100% for comparisons. The absolute mean cell count in this control group was 11.1 ± 0.6 × 10^6.
† Mean ± SEM of numbers of AM from three experiments (three animals per datum point) expressed as percent of control.
‡ $P < 0.005$ as compared with control.

there was a significant increase in the number of AM in fraction 3 through 8 and fractions 12 through 20, and a significant decrease in the number of AM in fraction 10. The number of AM in fractions 3 through 6 and fraction 12 was found to remain significantly elevated at 14 days after bleomycin treatment. The number of AM in fraction 9 and 10 was significantly decreased to below control values. In contrast, at 28 days after bleomycin treatment, there were significant elevations in AM numbers in fractions 3 through 9, 13, and 19, and no fraction was significantly decreased relative to control values. These data indicate that AM distribution, ie, the composition of DD-AM subpopulations, undergoes dynamic alterations during the development of bleomycin-induced fibrosis.

Effects of Bleomycin Treatment on AM Cellular Volume

We examined the effects of bleomycin treatment on the cellular volume of DD-AM (Figure 2). Bleomycin treatment significantly increased the cellular volume of DD-AM in all fractions except fraction 17 at 4 days (Figure 2A) as compared with the cellular volume of pooled control DD-AM (Figure 2D); these control fraction data were pooled (ANOVA, $P > 0.05$) in a fashion analogous to that reported above for cell number determinations. The cellular volume of DD-AM was significantly elevated at 14 days after bleomycin treatment only in fractions 3 through 10 and 19 (Figure 2B), and in fractions 3 through 7, 9, and 10 at 28 days after bleomycin treatment (Figure 2C). With regard to cell growth, uptake of [^{3}H]TdR (≥2 × background counts per minute) was present only in fractions 9 through 12 at 4 days after bleomycin treatment and in fractions 9 and 10 at 14 days (data not shown). No substantial uptake of [^{3}H]TdR (<2 × background counts per minute) was observed in blood monocytes, controls, or any fractions at 28 days after bleomycin treatment. Taken together, these data suggest that DD-AM subpopulations have differential divisional properties that contribute to the change in AM population composition. Moreover changes in cell volume in the absence of cell growth may indicate changes in activation state or function of a particular DD-AM subpopulation or subpopulations.

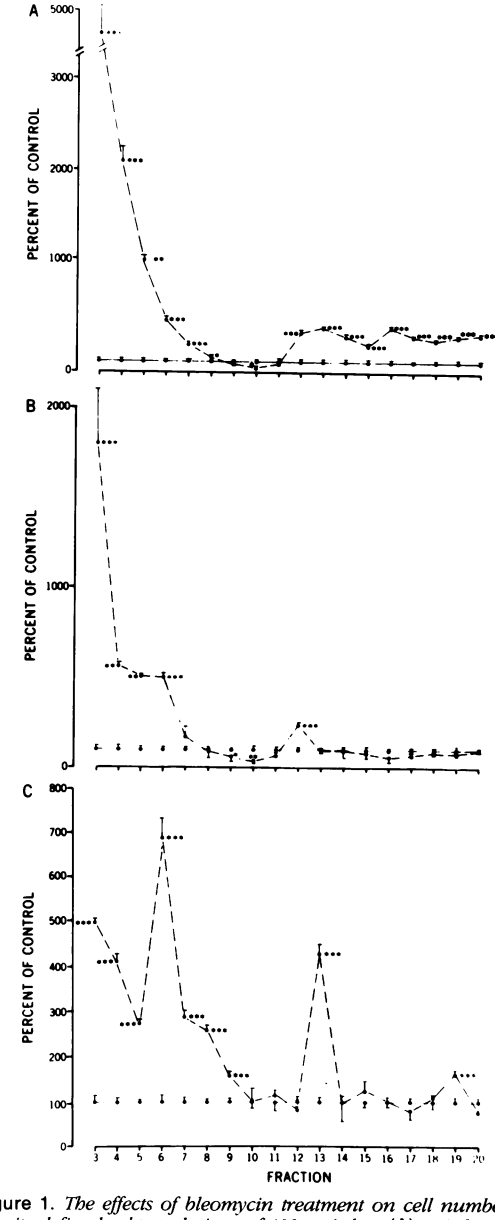

Figure 1. *The effects of bleomycin treatment on cell number in density-defined subpopulations of AM at 4 days* **(A)**, *14 days* **(B)**, *and 28 days* **(C)** *depicted as percent of control. Each datum point represents the mean ± SEM for three experiments and is depicted as a normalized percent of total cells applied to Percoll for fractionation. Control values (n = 11 experiments; normalized to 100% of control) are represented by the solid line in all panels. ANOVA for all time points* $P < 0.01$. *Student's t test values for comparisons of test and control fraction data are represented as follows:* *$P < 0.05$; **$P < 0.02$; *and* ***$P < 0.01$.

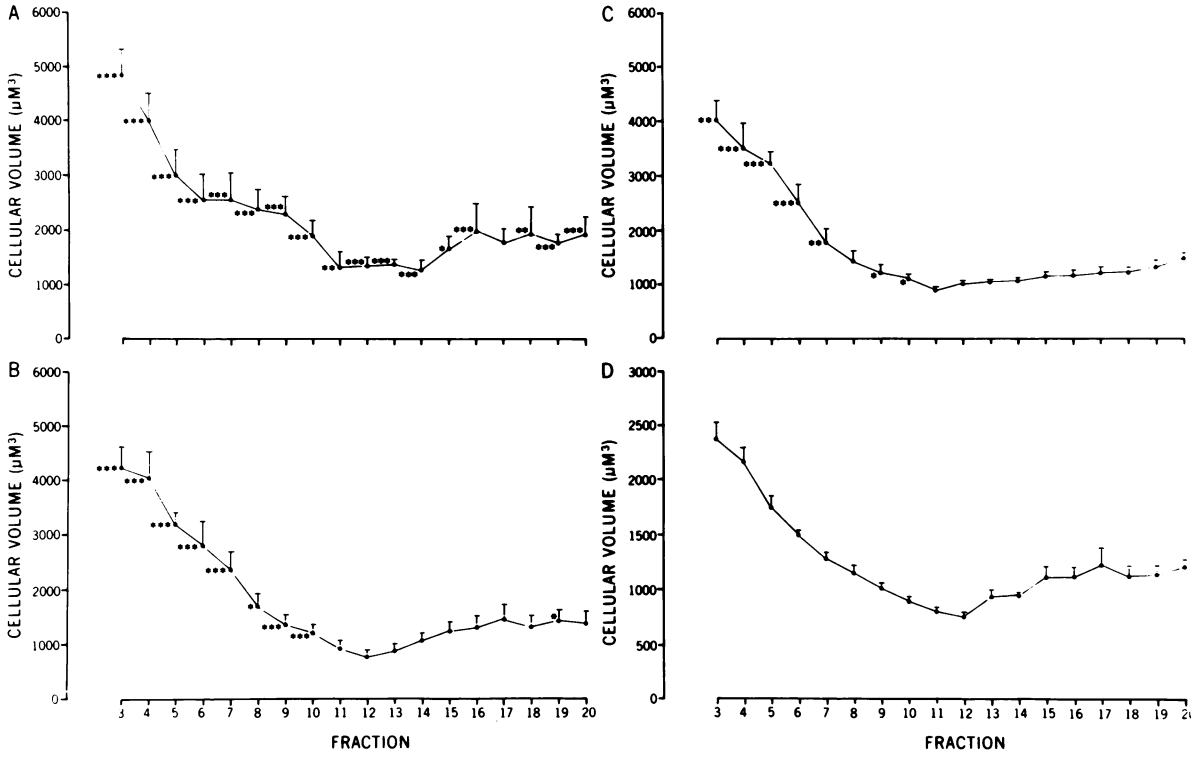

Figure 2. *The effects of bleomycin treatment on AM subpopulation cellular volume. Cellular volume was determined as described in Materials and Methods. Each point represents the mean ± SEM for three experiments with control values (n = 11) presented in* (D). **A**: *Cellular volume for AM subpopulations at 4 days, ANOVA,* P < *0.01. Duncan's multiple range test* (P < *0.05): fractions 3 or 4 versus fractions 5–20; fraction 5 versus fractions 10–20; fraction 6, 7, or 8 versus fractions 11–14; and fraction 9 versus fraction 14. The* P *values as compared with control values are depicted as:* *P < *0.05;* **P < *0.02; and* ***P < *0.01.* **B**: *Cellular volume for AM subpopulations at 14 days, ANOVA,* P < *0.01. Duncan's multiple range test* (P < *0.05): fraction 3 or 4 versus fractions 5–20, fraction 5 versus fractions 7–20, fraction 6 or 7 versus fraction 8–20, and fraction 8 versus fraction 12. The p values as compared with control values are depicted as:* *P < *0.05 and* ***P < *0.01.* **C**: *Cellular volume for AM subpopulations at 28 days, ANOVA,* P < *0.01. Duncan's multiple range test* (P < *0.05): fraction 3 versus fractions 5–20, fraction 4 or 5 versus fractions 6–20, fraction 6 versus fractions 7–20, and fraction 7 versus fractions 11–14. The* P *values as compared with control values are depicted as:* *P < *0.05;* **P < *0.02; and* ***P < *0.01.* **D**: *Cellular volume for control AM subpopulations, ANOVA,* P < *0.01. Duncan's multiple range test* (P < *0.05): fraction 3 or 4 versus fractions 5–20; fraction 5 versus fraction 7–20; fraction 6 versus fractions 8–20; fraction 7 versus fractions 10–14; fraction 17 versus fractions 10–12; fraction 8, 15, 16, 18, 19, or 20 versus fractions 11–12.*

Effects of Bleomycin Treatment on TNF-α Secretion by AM

The possibility was examined that bleomycin treatment of rats induced the production and secretion of TNF-α by AM. To this end, 24-hour supernatants collected from cultures of heterologous (unfractionated) populations of AM derived from saline-treated (control) or bleomycin-treated animals were tested for the presence of TNF-α (Figure 3). Although TNF-α levels in control supernatants remained relatively low, TNF-α levels in AM supernatants collected from 4-, 14-, and 28-day bleomycin-treated rats trended upward, albeit not significantly ($P > 0.05$), as a function of time after bleomycin treatment. Although these data are suggestive of enhanced TNF-α secretion by MΦ subsequent to bleomycin treatment of the animals from which they were derived, they do not differentiate whether this function is particular to a given subpopulation of AM. Therefore in efforts to differentiate whether this enhanced TNF-α secretion was a generalized phenomenon of AM or due to a specific subpopulation of AM, DD-AM supernatants were tested for TNF-α secretion. Similar to results obtained using heterologous AM, the control values for each DD-AM fraction did not differ as a function of time of death (ANOVA, $P > 0.05$; Figure 4A). Striking differences in TNF-α secretion profiles were observed, however, in DD-AM supernatants derived from animals treated with bleomycin for 4, 14, and 28 days (Figure 4B–D). Tumor necrosis factor-α secretion remained relatively low and unaffected at 4 days after bleomycin treatment (Figure 4B), but increased substantially and as a function of time at 14 (Figure 4C) and 28 (Figure 4D) days after bleomycin treatment, particularly in fractions 11 and 12 through 14. Although the trend toward increased TNF-α secretion began at 14 days after bleomycin treatment, a statistical significance of $P < 0.005$ was achieved only at 28 days after bleomycin treatment in fraction 11.

Confirmatory experiments indicated that the observed

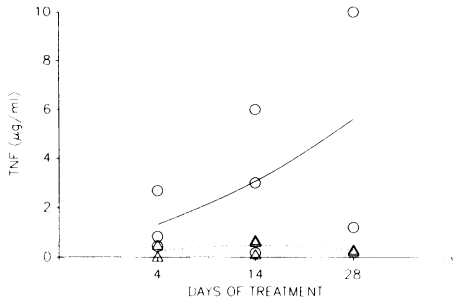

Figure 3. *The effects of bleomycin treatment on TNF-α secretion by heterologous AM. TNF concentrations were quantitated as described in* Materials and Methods *in 24-hour supernatants derived from heterologous (unfractionated) AM samples from animals treated with saline (△) or bleomycin (○) for the indicated durations. Data points are expressed as the mean of quadruplicate TNF determinations for each of two to three different experimental samples per time point. Each experimental sample was derived from AM pooled from two to three animals. Data means for animals treated with saline or bleomycin are depicted by dashed and solid curves, respectively.*

TNF activity was indeed mediated by MΦ-derived TNF-α. Specifically, the inclusion of polyclonal anti-murine TNF-α antiserum neutralized the activity of representative supernatants containing substantial levels of TNF activity (derived from bleomycin-treated animals) and murine rTNF-α in TNF assays (Table 2). Similar neutralizations were observed in selected control supernatants (derived from saline-treated control animals, which contained trivial, but detectable, levels of TNF), in multiple samples of those depicted in Table 2, and in representative supernatants derived from heterologous (unfractionated) AM cultures (data not shown). In conjunction with the evidence above, these data indicate that bleomycin treatment of rats induces specific DD-AM subpopulations to secrete high levels of TNF-α and that these levels increase as a function of time after bleomycin treatment.

Discussion

Recent studies have focused on the capacity of AM to act as modulators of events associated with pulmonary fibrosis.[1,11,25–27] Several studies have shown that AM produce factors that suppress fibroblast growth and collagen production by proliferating lung fibroblasts.[25,27] In more recent studies, AM have been demonstrated to synthesize factors that stimulate collagen production by lung fibroblasts.[11,26] In another study, it was found that after bleomycin treatment AM synthesized and released a growth factor for fibroblasts that was modulated by the lipoxygenase inhibitor nordihydroguiaretic acid.[28] These diverse capacities of AM suggest that they are composed of discrete subpopulations of cells having different functions. Recent findings by our laboratories and others have shown that the AM population is composed of several subpopulations that differ biochemically, morphologically, and immunologically.[13–15] The results of the present study indicate that there are dramatic shifts in the composition of AM subpopulations and in their morphologic and functional characteristics subsequent to bleomycin treatment. In this regard, general differences were observed between test (bleomycin-treated animals) and control (saline-treated animals) data using heterologous (unfractionated) AM. Several possible mechanisms present themselves for alveolar macrophage heterogeneity and alterations in alveolar macrophage population composition. Alveolar macrophages could reflect maturational states in a continuum of cellular functions. Research into colony-forming ability of alveolar macrophages could arise from multiple committed progenitor cells. Our data do not allow us to determine which mechanisms are generating these subpopulations or shifts in subpopulations. Specific differences, however, were made evident by analyzing DD-AM separated using Percoll density gradients.

Previous studies have shown that the composition of unfractionated AM changes during inflammatory or disease states. Alveolar macrophages harvested by bronchoalveolar lavage from patients with acute and chronic lung disease exhibit differences in size, soluble mediator synthesis, and antigenic determinants.[1,2,16–19] Acute lung injury was characterized by the predominance of small and medium-sized AM. In chronic lung disease, however, the cellular size of the AM population was increased.[17] In patients with idiopathic pulmonary fibrosis, the number of AM was increased, and a neutrophil chemotactic factor and a growth factor for lung fibroblasts was evident.[1,2] In sarcoidosis, AM expressed higher amounts of the human leukocyte antigen-DR (Ia antigen) membrane marker and expressed monocyte surface antigens, suggesting changes in the composition of the AM population.[16] Similarly changes have been noted in the bleomycin model for pulmonary fibrosis in which AM have been characterized as immature in appearance and increased in number; however, examination of possible shifts in AM subpopulation was not reported.[12,29]

Changes in the AM subpopulation composition and function could have dramatic effects on the inflammatory and fibrotic responses of the lung. In previous studies, we have shown that AM subpopulations have differential abilities to synthesize and release cyclo-oxygenase products such as prostaglandin (PG) E, PGI_2, and thromboxane A_2.[15] This is interesting in view of earlier findings indicating that circulating levels of PGE, PGI_2, and thromboxane A_2 were altered during the development of bleomycin-induced pulmonary fibrosis.[30] Furthermore PGE has been shown to be inhibitory to the synthesis of col-

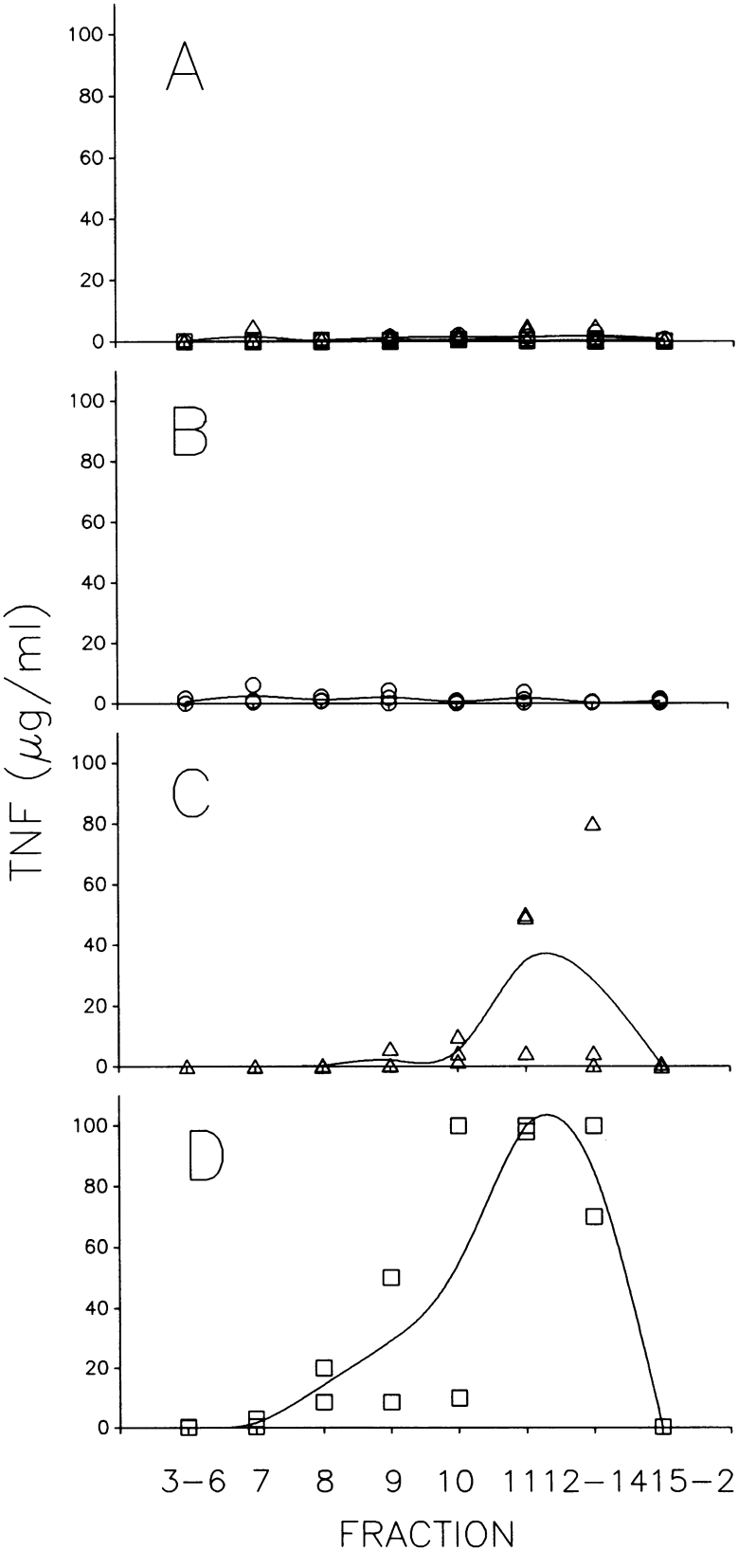

Figure 4. *The effects of bleomycin treatment on TNF-α secretion by AM subpopulations. TNF concentrations were quantitated as described in Materials and Methods in 24-hour supernatants derived from DD-AM samples (fractions 3–20) from saline-treated animals* (**A**, ○, *4 days;* △, *14 days; and* □, *28 days) or bleomycin-treated animals* (**B**, *4 days;* **C**, *14 days; and* **D**, *28 days). Data points are expressed as described in Figure 3. Data means are depicted by solid curves.*

Table 2. *Neutralization of TNF-α Activity by Anti-TNF-α Antiserum*

Sample	Concentration	Percent TNF-α-mediated cell death of WEHI 164 clone 13 cells (mean ± SD)		
		Additions to culture		
		Media	NRS*	Anti-TNF-α
Medium	—	0 ± 0	0 ± 0	0 ± 0
Murine rTNF-α	5 ng/ml	62.8 ± 2.0	60.4 ± 1.0†	0 ± 0‡
Culture supernatant from bleomycin-treated animals‖ (day 14 sample)	1:100 dilution	35.1 ± 1.4	33.5 ± 1.6†	0 ± 0‡
Culture supernatant from bleomycin-treated animals‖ (day 28 sample)	1:100 dilution	40.8 ± 1.6	41.6 ± 2.5†	0.8 ± 0‡

* Normal rabbit serum.
† ≤18% nonspecific effect by 1:400 dilution of heat-inactivated NRS by comparison to dose-response curve.
‡ >99% neutralization by 1:400 dilution of heat-inactivated polyclonal rabbit anti-TNF-α antiserum by comparison to dose-response curve.
‖ Supernatants were generated from pooled cells derived from two to three animals as described in *Materials and Methods*. Representative samples containing substantial levels of TNF activity were tested for TNF-α specificity by neutralization with anti-TNF-α antiserum as described in *Materials and Methods*.

lagen,[25,27] whereas PGI_2 was correlated with collagen accumulation in bleomycin-induced pulmonary fibrosis.[30] Therefore it is possible that alterations in AM subpopulations in the early stages of the development of bleomycin-induced fibrosis could contribute to changes in levels of cyclo-oxygenase products and consequent changes in collagen deposition. With regard to other mediators that can affect fibroblast growth and function, AM have been found also to produce compounds that stimulate fibroblast replication and collagen synthesis.[1,11] In a study by Clark and Greenberg,[26] it was shown that AM produced a factor or factors that increased collagen production by fibroblasts, but only after treatment of fibroblast cultures with indomethacin.[26] Another factor that could modulate fibroblast activity is TNF. Alveolar macrophages have been identified as a primary source of TNF.[31] The exact role that alterations in TNF play in the genesis of bleomycin-induced fibrosis is not well understood. Nonetheless TNF could be an important factor in modulating prostaglandin synthesis or fibroblast proliferation, because TNF has been demonstrated to stimulate the synthesis and release of prostaglandins.[32–35] The relatively low levels of TNF in the early phases of bleomycin-induced fibrosis could allow for increased collagen synthesis by lung fibroblasts. This hypothesis is indirectly supported by the data of Clark and Greenberg mentioned above.[26] It is thus possible that *in vivo* TNF levels modulate the synthesis of prostaglandins and thus collagen synthesis by lung fibroblasts. Alternatively TNF could directly affect fibroblast function and collagen synthesis. These findings are controversial, however, because both inhibition and stimulation of fibroblast proliferation and collagen synthesis have been reported.[36–39]

The data in the present study support the ability of TNF to stimulate fibroblast proliferation and collagen synthesis. Previous work by this laboratory has shown that the number of fibroblasts increases dramatically during the period of 4 to 21 days after bleomycin treatment,[40] the same period characterized by increased TNF-α activity in supernatants derived from *in vitro*–cultured AM. Although these data indicate that AM derived from bleomycin-treated animals secrete high levels of TNF, they do not exclude the possibility that fibrosis is mediated by production of a TNF-induced secondary factor or factors. Nonetheless these data parallel *in vivo* increases in fibroblast numbers. Moreover the data presented here corroborate the work of Piguet and colleagues,[20] which indicated that lung TNF mRNA was increased in bleomycin-induced fibrosis, and injection of anti-TNF antibodies markedly prevented development of pulmonary fibrosis. These workers further suggested that perhaps alveolar and interstitial macrophages were the source of TNF; however, the cellular source of TNF was undetermined. Therefore our data extend their findings to indicate that, at least in part, it is indeed the AM that secrete high levels of TNF in bleomycin-induced fibrosis and suggest that the ability of AM to modulate the fibrotic response is at least partially a result of this enhanced TNF-α secretion.

The present study demonstrates alterations in AM subpopulation composition after bleomycin treatment. Although the source of these alterations is unknown, several possibilities exist. The changes in the AM subpopulation composition could result from the maturation of specific subpopulations resulting in numeric increases in a more mature subpopulation or subpopulations. Alternatively the increase in a specific subpopulation could be due to the selective recruitment of interstitial macrophages or blood monocytes that rapidly mature into AM once they have entered the alveolus. This hypothesis is supported by the findings of Hance and co-workers[16] indicating that the AM population in sarcoid patients exhibited higher expression levels of monocyte membrane

markers. Lastly the changes in AM subpopulation composition could be derived from growth within the alveolar compartment itself. Indeed division was observed in fractions 9 through 12 (densities 1.061 to 1.083 g/ml) and 9 through 10 (densities 1.061 to 1.068 g/ml) at 4 and 14 days, respectively, after bleomycin treatment. These changes could constitute a decrease in the cell number of AM subpopulations in fractions 9 and 10 at these same time points with the progeny cells contributing to one of the other fraction subpopulations.

In summary, the AM subpopulation composition and function (TNF-α secretion) are altered during the development of bleomycin-induced fibrosis. Therefore these data are consistent with a key role for the function of specific subpopulations of AM in mediating bleomycin-induced pulmonary fibrosis.

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References

1. Bitterman PB, Adelberg S, Crystal RG: Mechanisms of pulmonary fibrosis. Spontaneous release of the alveolar macrophage-derived growth factor in the interstitial lung disorders. J Clin Invest 1983, 72:1801–1813
2. Hunninghake GW, Gadek JE, Fales HM, Crystal RG: Human alveolar macrophage-derived chemotactic factor for neutrophils. J Clin Invest 1980, 66:473–483
3. Kaelin RM, Center DM, Bernardo J, Grant M, Snider GL: The role of macrophage-derived chemoattractant activities in the early inflammatory events of bleomycin-induced pulmonary injury. Am Rev Respir Dis 1983, 128:132–137
4. Hocking WG, Golde DW: The pulmonary alveolar macrophage. Part 1. N Engl J Med 1979, 310:580–587
5. Hocking WG, Golde DW: The pulmonary alveolar macrophage. Part 2. N Engl J Med 1979, 310:639–645
6. Daniele RP, Dauber JH, Rossman MD: Immunological abnormalities in sarcoidosis. Ann Intern Med 1980, 92:406–416
7. Hunninghake GW, Kawanami O, Ferrans VJ, Young RC, Roberts WC, Crystal RG: Characterization of the inflammatory and immune effector cells in the lung parenchyma of patients with interstitial lung disease. Am Rev Respir Dis 1981, 123:407–412
8. Weinberger SE, Kelman JA, Elson NA, Young RC Jr, Reynolds HY, Fulmer JD, Crystal RG: Bronchoalveolar lavage in interstitial lung disease. Ann Intern Med 1978, 89:459–466
9. Snider GL, Hayes JA, Kortly AL: Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin: Pathology and sterology. Am Rev Respir Dis 1978, 117:1099–1108
10. Snider GL, Celli BR, Goldstein RH, O'Brien JJ, Lucey EC: Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal injection of bleomycin. Lung volumes, volume-pressure relations, carbon monoxide uptake, and arterial blood gas studied. Am Rev Respir Dis 1978, 117:289–297
11. Kovacs EL, Kelley J: Secretion of macrophage-derived growth factor during acute injury induced by bleomycin. J Leukoc Biol 1985, 37:1–14
12. Thrall RS, Barton RW, D'Amato DD, Sulavik SB: Differential cellular analysis of bronchoalveolar lavage fluid obtained at various stages during the development of bleomycin-induced pulmonary fibrosis in the rat. Am Rev Respir Dis 1982, 126:488–492
13. Chandler DB, Fuller WC, Jackson RM, Fulmer JD: Fractionation of rat alveolar macrophages by isopycnic centrifugation: Morphological, cytochemical, biochemical and functional properties. J Leukoc Biol 1986, 9:371–383
14. Chandler DB, Fuller WC, Jackson RM, Fulmer JD: Studies of membrane receptors and phagocytosis in subpopulations of rat alveolar macrophages. Am Rev Respir Dis 1986, 133:461–467
15. Chandler DB, Fulmer JD: Prostaglandin synthesis and release by subpopulations of rat alveolar macrophages. J Immunol 1987, 139:893–898
16. Hance AJ, Douches S, Winchester RJ, Ferrans VJ, Crystal RG: Characterization of mononuclear phagocyte subpopulations in the human lung by using mononuclear antibodies: Changes in alveolar macrophage phenotype associated with pulmonary sarcoidosis. J Immunol 1985, 134:284–292
17. Lenzini L, Heather DJ, Rottoli L, Rottoli P: Studies on bronchoalveolar cells in humans: I. Preliminary morphological studies in various respiratory diseases. Respiration 1978, 36:145–152
18. Razma AG, Lynch JP III, Wilson BS, Ward PA, Kunkel SL: Expression of Ia-like (DR) antigen on human alveolar macrophages isolated by bronchoalveolar lavage. Am Rev Respir Dis 1984, 129:419–424
19. Rennard SI, Hunninghake GW, Bitterman PB, Crystal RG: Production of fibronectin by the human alveolar macrophage: Mechanism for the recruitment of fibroblasts to sites of tissue injury in interstitial lung diseases. Proc Natl Acad Sci U S A 1981, 78:7147–7151
20. Piguet PF, Collart MA, Grau GE, Kapanci Y, Vassalli P: Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. J Exp Med 1989, 170:655–663
21. Espevik T, Nissen-Meyer J: A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. J Immunol Methods 1986, 95:99–105
22. Mosmann TR, Fong TAT: Specific assays for cytokine production by T cells. J Immunol Methods 1989, 116:151–158
23. Sheehan KCF, Ruddle NH, Schreiber RD: Generation and characterization of hamster monoclonal antibodies that neu-

tralize murine tumor necrosis factors. J Immunol 1989, 142:3884–3893
24. Boyum A: Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 1968, 21(suppl 97):77–89
25. Clark JG, Kostel KM, Marino BA: Bleomycin-induced pulmonary fibrosis in hamsters: An alveolar macrophage product that increases fibroblast prostaglandin E2 and cyclic adenosine monophosphate and suppresses fibroblast proliferation and collagen production. J Clin Invest 1983, 72:2082–2091
26. Clark JG, Greenberg J: Modulation of the effects of alveolar macrophages on lung fibroblast collagen production rate. Am Rev Respir Dis 1987, 135:52–56
27. Elias JA, Rossman MD, Zurier RB, Daniele RP: Human alveolar macrophage inhibition of lung fibroblast growth. A prostaglandin-dependent process. Am Rev Respir Dis 1985, 131:94–99
28. Phan SH, Kunkel SL: Inhibition of bleomycin-induced pulmonary fibrosis by nordihydroguiaretic acid. The role of alveolar macrophage activation and mediator production. Am J Pathol 1986, 124:343–352
29. Tryka AF, Godleski JJ, Brain JD: Alterations in alveolar macrophages in hamsters developing pulmonary fibrosis. Exp Lung Res 1984, 7:41–52
30. Chandler DB, Giri SN: Changes in plasma concentrations of prostaglandins and plasma angiotensin-converting enzyme during the development of bleomycin-induced pulmonary fibrosis. Am Rev Respir Dis 1983, 128:71–76
31. Decker T, Lohmann-Matthes M-L, Gifford GE: Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages. J Immunol 1987, 138:957–962
32. Elias JA: Tumor necrosis factor interacts with interleukin-1 and interferons to inhibit fibroblast proliferation via fibroblast prostaglandin-dependent and -independent mechanisms. Am Rev Respir Dis 1988, 138:652–658
33. Elias JA, Gustilo K, Freundlich B: Human alveolar macrophage and blood monocyte inhibition of fibroblast proliferation. Evidence for synergy between interleukin-1 and tumor necrosis factor. Am Rev Respir Dis 1988, 138:1595–1603
34. Dayer J-M, Beutler B, Cerami A: Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblasts. J Exp Med 1985, 162:2163–2168
35. Bachwich PR, Chensue SW, Larrick JW, Kunkel SL: Tumor necrosis factor stimulates interleukin-1 and prostaglandin E_2 production by resting macrophages. Biochem Biophys Res Commun 1986, 136:94–101
36. Postlethwaite AE, Smith GN, Mainnardi CL, Seyer JM, Kang AH: Lymphocyte modulation of fibroblast function in vitro: Stimulation and inhibition of collagen production by different effector molecules. J Immunol 1984, 132:2470–2477
37. Kahaleh MB, DeLustro F, Bock W, LeRoy EC: Human monocyte modulation of endothelial cells and fibroblast growth: Possible mechanism for fibrosis. Clin Immunol Immunopathol 1986, 39:242–255
38. Korn JH, Halushka PV, LeRoy EC: Mononuclear cell modulation of connective tissue function: Suppression of fibroblast growth by stimulation of endogenous prostaglandin production. J Clin Invest 1980, 65:543–554
39. Jalkanen M, Penttinen R: Enhanced fibroblast collagen production by a macrophage-derived factor (CEMF). Biochem Biophys Res Commun 1982, 108:447–453
40. Chandler DB, Hyde DM, Giri SN: Morphometric estimates of infiltrative cellular changes during the development of bleomycin-induced pulmonary fibrosis in hamsters. Am J Pathol 1983, 112:170–177