

Bleomycin-induced pulmonary fibrosis is independent of eosinophils

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Abstract: Eosinophils have been shown to increase in tissues during many fibrotic conditions and consequently have been suggested to contribute to the development of fibrosis. This study tested the hypothesis that eosinophils are essential in the development of lung fibrosis in mice in response to bleomycin (BLM). Anti-IL-5 antibody was administered intraperitoneally into mice 2 h prior to endotracheal BLM inoculation and thereafter, every other day. Lung eosinophilia was evaluated by measurement of eosinophil peroxidase activity and confirmed by eosinophil counts in histologic sections. Lung fibrosis was evaluated by hydroxyproline content and confirmed by collagen staining in histological sections. Results demonstrated that BLM induced pronounced lung eosinophilia, which was maximal 7 days after BLM treatment and remained elevated through day 14, in C57Bl/6 SCID mice and CBA/J mice. In contrast, eosinophilia was a minor component in the lungs of wildtype C57Bl/6 mice after BLM treatment, although lung fibrosis developed similarly in all three strains of mice. Treatment with anti-IL-5 completely abrogated eosinophilia but failed to block pulmonary fibrosis induced by BLM in all mouse strains, including C57Bl/6 SCID, wildtype C57Bl/6 mice, and CBA/J mice. Analysis of cytokine mRNA by RNase-protection assay in C57Bl/6 SCID mice indicated that BLM treatment caused enhanced expression of the cytokines, TNF- α , and IL-6 at days 3, 7, and 14 post-BLM inoculation, regardless of whether eosinophils were depleted by anti-IL-5. Finally, the importance of eosinophils in lung fibrosis was examined in IL-5 gene knockout mice (IL-5^{tm1Kopf}). BLM treatment induced significant lung fibrosis in IL-5 knockout mice in the absence of eosinophilia. These findings indicate that eosinophils are not an absolute requirement for BLM-induced pulmonary fibrosis in the mouse. *J. Leukoc. Biol.* 68: 515–521; 2000.

Key Words: lung eosinophilia · cytokines · knockout mice

INTRODUCTION

Bleomycin (BLM) consists of a group of glycopeptides isolated from a strain of *Streptomyces verticillus* and is a therapeutically

important drug for treatment of a variety of tumors [1]. One of the major adverse and sometimes lethal effects associated with treatment with BLM is pulmonary fibrosis [2]. Pathogenesis of this disease has been studied extensively in mouse models [3], however the precise mechanisms involved in the induction of this disease remain incompletely understood.

A series of recent studies have associated eosinophilia with pulmonary fibrosis. Accumulation of eosinophils in alveolar space and parenchyma has been observed in fibrogenic lesions, such as idiopathic pulmonary fibrosis in humans [4] and BLM-induced lung fibrosis in rats [5] and mice [6]. Moreover, we have recently shown that BLM induces T cell-independent lung fibrosis, which is accompanied by prominent lung eosinophilia [7], in C57Bl/6 severe combined immunodeficiency (SCID) mice. The observed absence of a critical role for T and B lymphocytes and the presence of a prominent eosinophilia in these studies suggested a potentially important role for eosinophils in this murine disease model. Studies by several labs [8–11] demonstrated that treatment of mice with anti-tumor necrosis factor (TNF)- α resulted in a significant reduction in lung fibrosis as measured by hydroxyproline content. In addition, treatment with anti-TNF- α has been shown to block lung eosinophilia in BLM-treated mice as determined by histology [11]. Using *in situ* and northern hybridization, and immunohistochemical and histochemical techniques, monocyte chemoattractant factor-1 (MCP-1), a potent chemoattractant for monocytes, and interleukin (IL)-5, a major cytokine involved in development of eosinophilia [12], were shown to be upregulated in eosinophils of BLM-induced fibrotic mouse lungs [6, 13]. These observations suggested that eosinophils might exert fibrogenic effects via recruitment of monocytes and additional eosinophils. Finally, eosinophils could potentially serve as an important cellular source of fibrogenic cytokines, such as transforming growth factor (TGF)- β 1 and TGF- α , and have been shown to produce TGF- β 1 in BLM-induced fibrotic lungs in rats [14]. Furthermore, the majority of the eosinophils present in healing wounds have been shown to express TGF- α mRNA and protein [15]. Thus, there is substantial evidence in rodent models to suggest that eosinophils may contribute to lung fibrosis. In spite of the fact that many studies have shown a correlation of eosinophilia with fibrosis, direct evidence is needed before

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eosinophils can be documented as an essential component in the development of fibrosis.

The studies presented in this paper were designed to examine the hypothesis that eosinophils are an essential component for BLM-induced lung fibrosis. Anti-IL-5 monoclonal antibody (TRFK-5) or IL-5 gene knockout mice were used to prevent eosinophilia in mice injected intratracheally with BLM. Lung fibrosis and eosinophilia were evaluated by hydroxyproline content and eosinophil peroxidase activity, respectively. The results indicated that pulmonary fibrosis and pronounced eosinophilia developed in BLM-treated C57Bl/6 SCID mice and CBA/J mice. In contrast, minimal eosinophilia was detected by eosinophil peroxidase activity in BLM-treated wildtype C57Bl/6 mice; however, pulmonary fibrosis was equivalent to that observed in C57Bl/6 SCID and CBA/J strains of mice. Treatment of C57Bl/6 SCID and CBA/J mice with anti-IL-5 antibody blocked completely pulmonary eosinophilia but failed to block pulmonary fibrosis. IL-5 gene knockout C57Bl/6 IL-5^{tm1Kopf} mice failed to develop eosinophilia but still developed profound lung fibrosis following BLM treatment. These data demonstrated that eosinophils are not essential for the development of lung fibrosis in BLM-treated mice and that their presence in fibrotic lesions may indicate a role in post-induction events in lung fibrosis.

MATERIALS AND METHODS

Animals

Pathogen-free mice at 6–7 weeks of age were used in all studies. C57Bl/6 and C57Bl/6 SCID mice were purchased from National Cancer Institute (Harlan-Sprague Dawley, Frederick, MD). CBA/J and IL-5 gene knockout (C57Bl/6 IL-5^{tm1Kopf}) mice were obtained from Jackson Laboratory (Bar Harbor, ME). After arrival, mice were allowed to acclimate for 1–2 weeks in microisolator cages provided with sterile acidified water and sterile food ad libitum.

Intratracheal inoculation of BLM and treatment with anti-IL-5 antibody

Each mouse was anesthetized with pentobarbital sodium (26.5 mg/Kg of body weight, Butler Company, Columbus, OH). The trachea was exposed by a 1.5 cm incision in the neck and injected with 0.075 units (U; C57Bl/6 and C57Bl/6 SCID mice) or 0.025 U (CBA/J mice) BLM (Blenoxane, BLM sulfate, Bristol Myers Pharmaceuticals, Evansville, IN) dissolved in 20 μ l phosphate-buffered saline (PBS). The incision was closed with a 9 mm Mikron Autoclip (Becton Dickinson, Sparks, MD). Postsurgically, the mice were warmed under a table lamp until recovered from anesthesia. Mortality as a result of toxic effects of BLM was <15% in all strains of mice used in these studies. All procedures were conducted in a sterile environment and approved by the Institutional Animal Care and Use Committee at the University of Kentucky (Lexington).

TRFK-5 (rat anti-mouse IL-5) ascites was prepared in pristane-primed Balb/C mice with the TRFK-5 hybridoma, kindly provided by Dr. Robert Coffman (DNAX, Palo Alto, CA). Mice were injected intraperitoneally with TRFK-5 ascites (1 mg protein/mouse in 200 μ l) 2 h before BLM treatment and thereafter, every other day. Normal rat immunoglobulin G (IgG) was used as a control for the anti-IL-5 antibody.

Eosinophil peroxidase (EPO) activity measurement

A biochemical method described by Schneider and Issekutz [16] was modified to quantify lung EPO activity. Briefly, right, upper-lung lobes (wet weight, about 40 mg) were collected and stored at -70°C until processing. Lung tissue was homogenized with a motor-driven, 1 ml glass homogenizer in 1 ml of pH

8.0 HEPES buffer followed by centrifugation at 10,000 g for 30 min at 4°C . The pellet was rehomogenized in 350 μ l 0.5% CTAC (cetyltrimethylammonium chloride) and centrifuged as above. The supernatant (tissue extract) was diluted 1:1 with pH 8.0 HEPES buffer, and 75 μ l of the diluted sample was pipette into a 96-well plate in duplicate. HEPES buffer served as a negative control. The substrate solution, which consisted of 50 mM HEPES, pH 8.0, 6 mM KBr, 3mM OPD (O-phenylenediamine), and 8.8 mM H_2O_2 , was freshly prepared, and 75 μ l was added to each well. The reaction was allowed to occur at room temperature and was stopped after 1.5 min with 75 μ l 4 N sulfuric acid containing 2 mM resorcinol. OD_{490} was determined for EPO activity.

Hydroxyproline content determination

A modified method from Witschi *et al.* [17] was used to determine hydroxyproline content. At day 14 after BLM administration, mice were killed, and lungs were perfused with PBS via the right ventricle. The left lung lobes were dissected out and hydrolyzed with 6 N HCl at 110°C for 72 h. The hydroxyproline content was determined by oxidizing the samples with 0.2 M chloramine-T extracting with toluene and reacting with Ehrlich's reagent. The colored product was measured at 560 nm and compared to a standard curve.

Histology examination

Hematoxylin-eosin staining and Masson's Trichrome staining for collagen were performed by the University of Kentucky Histology Service in the Department of Pathology.

Measurement of lung tissue cytokine mRNA levels

Lower lobes of right lungs were collected and immediately frozen in liquid nitrogen until extraction of RNA. Total RNA was isolated by TRIzol, according to the methods described by the manufacturer (Life Technologies, Grand Island, NY). The purified RNA was used for analysis of mRNA expression using the Riboquant Multi-Probe RNase Protection Assay System (PharMingen, San Diego, CA), according to manufacturer's instructions. Briefly, ^{32}P -labeled antisense RNA probes were synthesized with the mouse cytokine/chemokine multiprobe template set 3b by polymerase T7RNA. The labeled probe (3×10^5 cpm/ μ l) was hybridized in solution overnight in excess of the target RNA (10 μ g total RNA/treatment) in a total reaction volume of 8 μ l. The free probe and other single-strand RNA were digested with RNases A and T1, per instructions provided by the manufacturer. The remaining RNase-protected probe was precipitated and dissolved in loading buffer and denatured by heating. After resolution of protected probes in 5% acrylamide gel at 45 watts for 2 h, the gel was dried in a slab gel dryer (Savant Instruments, Farmingdale, NY). After autoradiography by phosphorimage screen exposure for 36 h, cytokine mRNA was quantified by densitometry and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Data expressions and statistics analysis

All data in this paper were presented as mean \pm SEM. The difference between two groups was tested with Student's *t*-test for unpaired data. The difference among multiple groups was tested with one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be significant.

RESULTS

BLM induces lung fibrosis in C57Bl/6 SCID and C57Bl/6 wildtype mice

Lung fibrosis was assessed by quantifying the hydroxyproline content of the entire left lung lobe of individual mice 14 days after BLM treatment. BLM induced a significant increase in hydroxyproline content in C57Bl/6 SCID and wildtype C57Bl/6 mice (**Fig. 1**). The BLM-induced increases in hydroxyproline content were approximately the same extent in both strains of mice, 41% increase in C57Bl/6 wildtype ($P < 0.005$), and 43% increase in C57Bl/6 SCID ($P < 0.005$). The observed increase

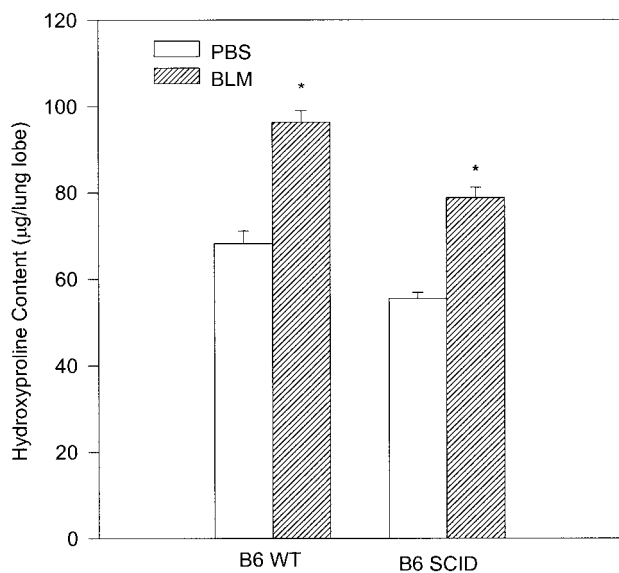


Fig. 1. BLM-induced lung fibrosis in C57Bl/6 SCID and wildtype C57Bl/6 mice. C57Bl/6 SCID and wildtype mice were injected i.t. with 0.075 U BLM and were killed 14 days later. Hydroxyproline content of their left lung lobes was determined as described in Materials and Methods. Data are representative of three similar experiments, each containing 8–12 animals per group. * $P < 0.005$ indicates a significant difference from the PBS-treated control group.

in hydroxyproline was similar to that observed by other investigators in the murine BLM model 2 weeks after BLM treatment [8]. C57Bl/6 SCID and C57Bl/6 wildtype mice developed a similar degree of alveolar-wall consolidation and thickening, which stained positively for collagen by Masson's Trichrome staining, whereas the lungs of PBS-treated mice lung did not show an increase in collagen staining compared with untreated mice (unpublished results).

BLM caused pronounced pulmonary eosinophilia in C57Bl/6 SCID mice

Our previous studies in C57Bl/6 SCID mice showed BLM treatment caused lung fibrosis and prominent eosinophilia, as determined by histological staining. In this study, EPO was used as a more reliable quantitative method to quantify eosinophils in lung tissue. Lung EPO activity was measured in C57Bl/6 SCID and C57Bl/6 wildtype mice at days 3, 7, and 14 after BLM intratracheal inoculation (0.075 U). In C57Bl/6 SCID mice, EPO activity was not elevated at day 3 but was significantly elevated (3.2-fold of control, $P < 0.005$) at day 7 and remained elevated at day 14 (2.2-fold of control, $P < 0.05$). In contrast, C57Bl/6 wildtype mice showed only a minor increase in EPO activity on days 7 and 14 after BLM treatment, which was not significantly increased compared with PBS-treated control mice (Fig. 2). That the measurement of EPO activity was specific for eosinophils was shown in preliminary studies in which the EPO assay failed to detect the influx of neutrophils in the lungs 24 h after intratracheal administration of lipopolysaccharide. In contrast, neutrophils were detected when a myeloperoxidase (MPO) enzyme assay was used (unpublished results).

Eosinophils are not necessary for development of BLM-induced lung fibrosis

To test the hypothesis that eosinophils are involved in pulmonary fibrosis induced by BLM, TRFK-5, a neutralizing antibody specific for IL-5, was used to block the development of eosinophilia. Mice were injected intraperitoneally with TRFK-5 ascites (1 mg protein/mouse) 2 h before BLM inoculation and thereafter, every other day; normal rat IgG served as an antibody control. Only a few diffusely distributed eosinophils were visible among the inflammatory cells in the lungs of BLM-treated C57Bl/6 wildtype injected with control antibody (Fig. 3A). Treatment with anti-IL-5 prevented the appearance of eosinophils and led to an increase in the number of neutrophils present in the lungs (Fig. 3B). The increase in neutrophils in lung tissue was confirmed by measurement of MPO activity (unpublished results). In contrast, distinct pulmonary eosinophilia was observed in the lungs of BLM-treated C57Bl/6 SCID mice, which were treated with the control antibody (Fig. 3C). However, eosinophils were not present in lungs of BLM-treated C57Bl/6 SCID mice treated with anti-IL-5 (Fig. 3D). Lung inflammation was still present in anti-IL-5-treated SCID mice, but it was predominantly mononuclear. Lung EPO activity was measured in BLM-treated mice injected with anti-IL-5 or control antibody also. EPO activity was increased at 7 days after inoculation of 0.075 U BLM into C57Bl/6 SCID mice and 14 days after 0.025 U BLM in CBA/J mice (Fig. 4). Treatment with anti-IL-5, but not control antibody, completely blocked the elevation of EPO activity induced by BLM in C57Bl/6 SCID and CBA/J mice (Fig. 4). Similar trends were observed in anti-IL-5-treated C57Bl/6 wildtype mice (Fig. 4); however, statistical significance was not achieved because of the minor eosinophilia that resulted in this mouse strain in response to BLM.

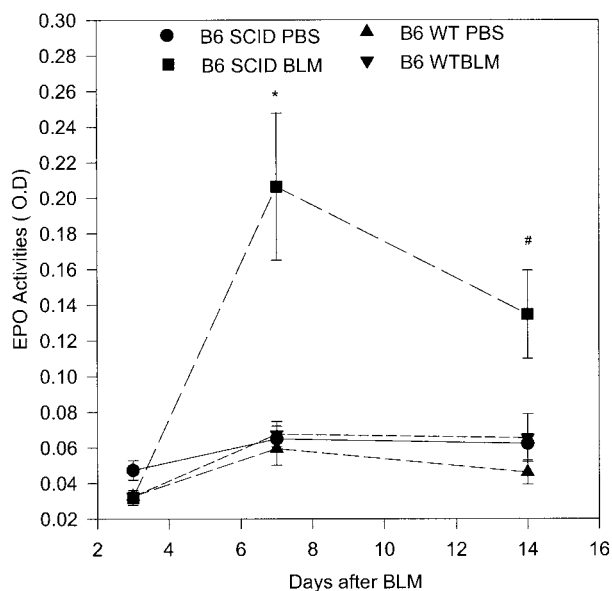


Fig. 2. Lung eosinophilia in C57Bl/6 SCID and C57Bl/6 wildtype mice after BLM treatment. Mouse lung EPO activity was measured at 3, 7, and 14 days after i.t. BLM (0.075 U) inoculation. Data represent three similar experiments with 4–10 mice per group in each experiment. * $P < 0.005$ and # $P < 0.05$ indicate significant differences from the PBS-treated control group.

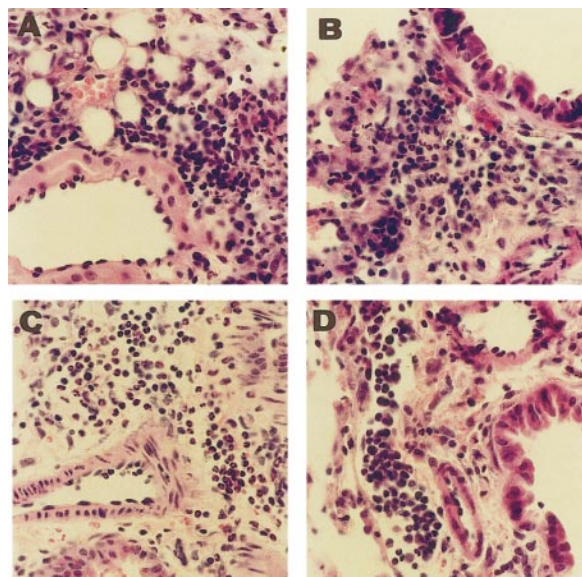


Fig. 3. Hematoxylin- and eosin-stained histological sections of lungs from BLM-treated mice. (A) C57Bl/6 wildtype, treated with BLM and normal rat IgG; (B) C57Bl/6 wildtype, treated with BLM and anti-IL-5; (C) C57Bl/6 SCID, treated with BLM and normal rat IgG; and (D) C57Bl/6 SCID treated with BLM and anti-IL-5.

In contrast to the effect of anti-IL-5 on EPO activity, lung fibrosis, as measured by hydroxyproline content 14 days after BLM treatment, was not altered by the treatment with TRFK-5 compared with BLM treatment alone in any of the three mouse strains tested (**Fig. 5**). Histological staining with Masson's Trichrome for collagen in BLM-treated

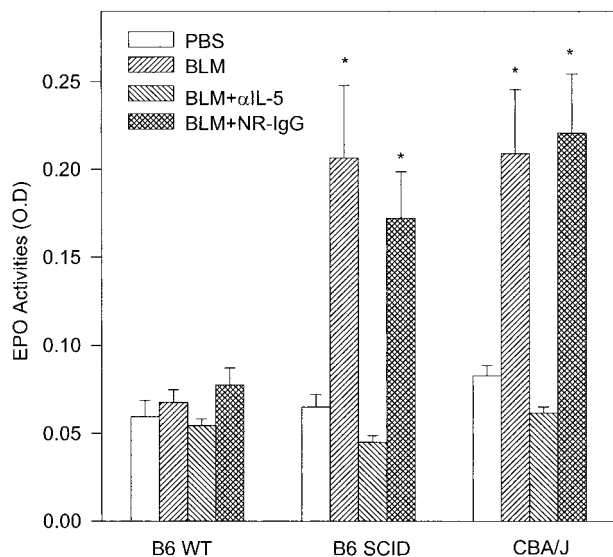


Fig. 4. Anti-IL-5 antibody completely blocks lung eosinophilia induced by BLM. C57Bl/6 wildtype, C57Bl/6 SCID, and CBA/J mice were injected i.t. with BLM (0.075 U for C57Bl/6 and 0.0275 U for CBA/J) and i.p. with TRFK-5, anti-IL-5 antibody, or normal rat IgG 2 h before BLM and thereafter, every other day. Lung EPO activity was measured in C57Bl/6 SCID and C57Bl/6 wildtype mice 7 days, and in CBA/J mice, 14 days, post-BLM inoculation. Data are representative of three similar experiments with 4–10 mice per group in each experiment. * $P < 0.05$ indicates a significant difference from the related PBS control group.

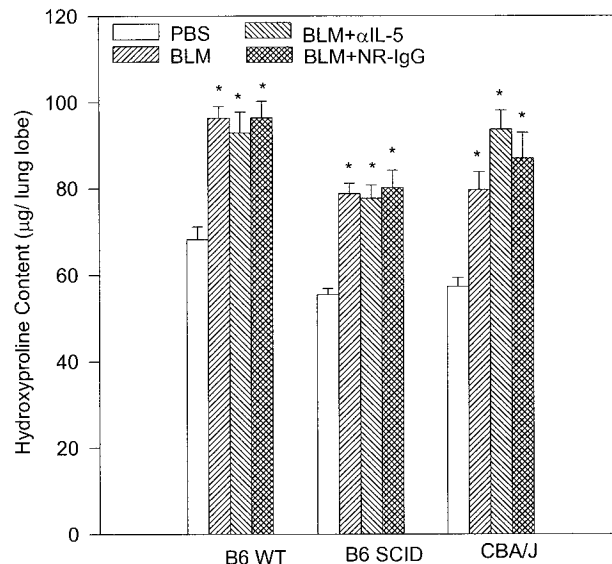


Fig. 5. Anti-IL-5 antibody failed to reduce BLM-induced lung fibrosis in C57Bl/6 SCID, C57Bl/6 wildtype mice, or CBA/J mice. Mice were treated with BLM and anti-IL-5 as described in Figure 4. Hydroxyproline content was measured 14 days post-i.t. BLM inoculation. No significant difference was observed among different treatment groups of BLM-injected mice in all three strains of mice tested. These data are representative of three similar experiments with 4–10 per group in each experiment. * $P < 0.005$ indicates a significant difference from the related PBS-treated group.

C57Bl/6 SCID mouse lungs confirmed that anti-IL-5 had no effect on the development of pulmonary fibrosis because collagen fiber staining was equivalent in lungs of mice treated with anti-IL-5 or rat IgG (**Fig. 6**). Similar results were observed in C57Bl/6 wildtype and CBA/J mice (unpublished results).

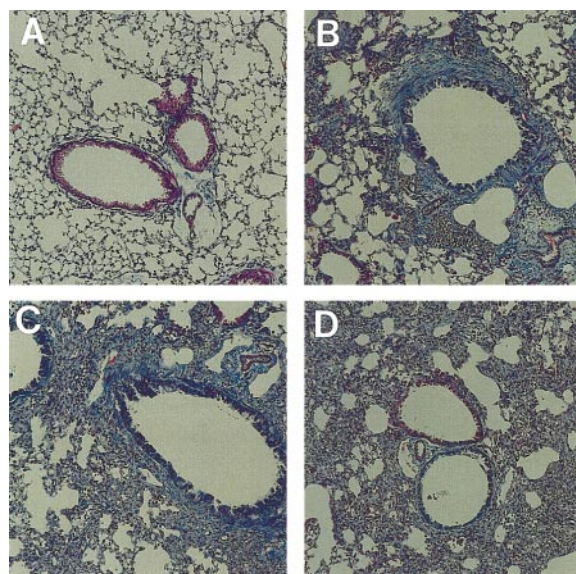


Fig. 6. Masson's Trichrome-stained histological sections of lungs from C57Bl/6 SCID mice 14 days after BLM treatment. (A) Control, PBS-treated; (B) BLM-treated; (C) BLM- and anti-IL-5-treated; (D) BLM- and normal rat IgG-treated.

TABLE 1. IL-5 Knockout (KO) Mice Developed Lung Fibrosis in Response to BLM

Mouse	Treatment	n	EPO activities (O.D.)	Hyp content (mg/lung lobe)
IL-5 KO	PBS	3	0.0217 ± 0.0032	84.67 ± 2.71
IL-5 KO	BLM	4	0.0260 ± 0.0010	123.65 ± 5.36 ^a
WT	PBS	3	0.065 ± 0.0091	74.85 ± 5.31
WT	BLM	3	0.073 ± 0.0110	113.39 ± 7.62 ^b

IL-5 knockout C57Bl/6 mice and wildtype C57Bl/6 mice were injected i.t. with BLM (0.075 U). Lung EPO activity and hydroxyproline content were measured at day 10 post-BLM treatment. ^a*P* < 0.005, and ^b*P* < 0.02 indicate significant changes from the related PBS-treated group.

BLM induces significant pulmonary fibrosis in IL-5 knockout mice

To confirm that eosinophils are not essential for BLM-induced lung fibrosis, IL-5 gene knockout mice (C57Bl/6 IL-5^{tm1Kopf}) were treated with BLM (0.075 U) and evaluated for development of fibrosis and eosinophilia. Compared with C57Bl/6 wildtype mice, the basal level of EPO was reduced 67% in IL-5 KO mice and was not elevated by BLM treatment (Table 1). However, hydroxyproline content was increased 46% compared with PBS-treated controls 10 days post-BLM inoculation in IL-5 KO mice. As a control, C57Bl/6 wildtype mice were shown to develop a similar degree of lung fibrosis in response to BLM treatment (Table 1).

Cytokine mRNA levels in BLM-treated C57Bl/6 SCID mice

Given the lack of lymphocytes and eosinophils in SCID mice treated with anti-IL-5 antibody, it was of interest to determine if similar changes in proinflammatory cytokine mRNA expression were observed in C57Bl/6 SCID mice and anti-IL-5-treated SCID mice after BLM treatment. mRNA levels of TNF- α and IL-6 (cytokines known to be elevated in response to BLM) were examined by RNase protection assay and normalized to GAPDH mRNA at days 3, 7, and 14 after BLM treatment. The results in Figure 7 demonstrate that TNF- α and IL-6 mRNA levels were increased significantly on days 3 and 7 after BLM treatment. Moreover, both cytokines were increased to a similar extent after BLM treatment in C57Bl/6 SCID mice regardless of whether IL-5 was neutralized to prevent eosinophilia, indicating that BLM-induced changes in TNF- α and IL-6 expression were independent of the presence of eosinophils. By day 14, the levels of TNF- α and IL-6 mRNA had returned normal and were not significantly different from PBS-treated control mice.

DISCUSSION

The mechanism by which BLM causes pulmonary fibrosis is not fully understood. Recent studies have speculated that eosinophils may serve an important role given the fact that eosinophilia has been associated with fibrosis in many disease conditions. Co-localization of fibrosis and eosinophilia was found in rodent lungs in response to BLM [5, 7] and in human

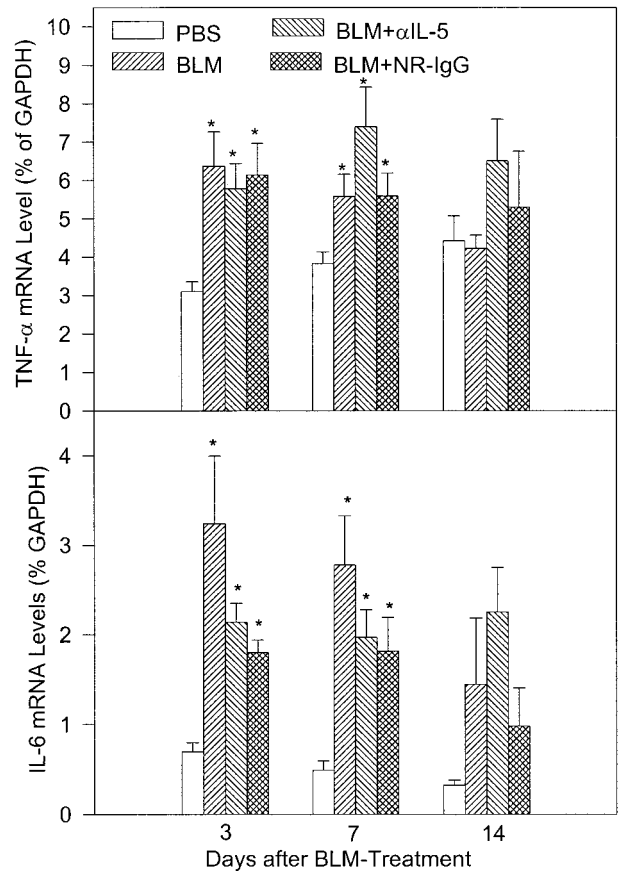


Fig. 7. BLM elevated lung TNF- α and IL-6 mRNA levels in C57Bl/6 SCID mice. C57Bl/6 SCID mice were treated with BLM and anti-IL-5 as described in Figure 3. Lung TNF- α and IL-6 mRNA levels were assayed by RPA and normalized to GAPDH. Data represent three similar experiments with 4–6 mice per group in each experiment. **P* < 0.05 indicates a significant difference from the related PBS-treated group. There is no significant difference among groups treated with BLM.

idiopathic pulmonary fibrosis [18, 19]. Lung fibrosis and eosinophilia induced by BLM were blocked by anti-TNF- α treatment [11], suggesting these two events may be linked functionally. Moreover, eosinophils at the fibrotic site have been shown to express fibrogenic cytokines TGF- α and TGF- β [14, 15].

In agreement with previous studies, our current investigations demonstrate that lung eosinophilia developed in CBA/J mice after BLM treatment. In addition, after BLM instillation, pronounced lung eosinophilia was present in C57Bl/6 SCID mouse lungs but was only minimally present in wildtype C57Bl/6 mice. The fact that all three strains developed equivalent levels of lung fibrosis but displayed discrepant levels of eosinophilia suggested that eosinophils may not be a critical cell type for the development of lung fibrosis.

To evaluate the role of eosinophils in fibrosis and to prevent eosinophilia after BLM treatment, mice were administered anti-IL-5 2 h before BLM inoculation and every other day thereafter. Treatment with anti-IL-5 prevented completely BLM-induced lung eosinophilia in C57Bl/6 SCID mice and in CBA/J mice, as evidenced by reduced EPO activity; however, anti-IL-5 failed to block BLM-induced lung fibrosis (Figs. 4

and 5). Treatment with anti-IL-5 failed also to prevent BLM-induced lung fibrosis in C57Bl/6 wildtype mice, which displayed only minor pulmonary eosinophilia after BLM (unpublished results). These data strongly suggested that eosinophils are not essential for lung fibrosis induced by BLM in any of the mouse strains tested.

In contrast to our finding, Gharaee-Kermani *et al.* [20] demonstrated that anti-IL-5 treatment blocked pulmonary fibrosis induced by BLM in CBA/J mice. The current studies used the same mouse strain (CBA/J), the same route of administration for BLM (intratracheal), the same dose of BLM (0.025 U), and the same anti-IL-5 antibody (TRFK-5). The only difference in the present study was that mice were injected with anti-IL-5 antibody more frequently (every other day) and with a larger dose (1 mg protein). The reason for the discrepancy in outcome between the present study and that of Gharaee-Kermani *et al.* [20] is currently unknown.

IL-5 is a well-recognized eosinophilopoietic growth factor capable of stimulating eosinophil differentiation and maturation *in vitro* and *in vivo* [21–23]. TRFK-5, mouse IL-5-specific neutralizing antibody has been shown to prevent effectively or resolve existing tissue eosinophilia in many diseases in which eosinophilia is involved [24, 25]. The present study showed that treatment with TRFK-5 caused EPO activity to fall slightly below basal level at days 7 and 14 after BLM treatment, suggesting suppression of eosinophil differentiation in bone marrow by TRFK-5. In addition to affecting eosinophil differentiation, IL-5 has been shown to cooperate with other chemoattractants, such as eotaxin and RANTES in mediating eosinophil migration to the lung in response to allergen challenge [26–29]. Mice treated with TRFK-5 (0.06 mg/kg 2 h before antigen challenge) resulted in 50% inhibition of bronchoalveolar pulmonary (BALF) eosinophils at 24 h after airway antigen challenge in a lung inflammatory model [25]. Intravenous Sephadex particle injection into guinea pigs mimics intravenous injection of parasite larvae, inducing a pulmonary eosinophilia by 24 h after injection. Treatment with TRFK-5 15 min before the injection of Sephadex completely suppressed eosinophilia in BALF and significantly reduced lung tissue EPO activity [24]. A single dose of TRFK-5 (1 mg/kg, intraperitoneally) reversibly inhibited antigen-dependent lung eosinophilia for at least 12 weeks [25].

Pulmonary eosinophilia, including that induced by BLM, has been suggested to be T cell-dependent [20, 30]. Th2 cells likely play a prominent role in eosinophilia via secretion of IL-5 and IL-4. However, the results of the present study showed that induction of eosinophilia can occur in the absence of T cells, because C57Bl/6 SCID mice developed a prominent eosinophilia after BLM treatment. Moreover, lung eosinophilia in C57Bl/6 SCID mice was still shown to be IL-5-dependent, suggesting that another source of IL-5 was induced by BLM in the absence of T cells, possibly mast cells and/or natural killer (NK) cells [23].

TNF- α has been considered a key factor in the development of lung fibrosis in response to BLM [8–10, 31]. The current study revealed that TNF- α mRNA levels were significantly elevated at days 3 and 7 and declined by day 14 in all BLM-treated groups regardless of whether lung eosinophilia was present. No significant difference in TNF- α mRNA levels

was observed between different treatment groups of BLM-injected mice at any time point examined. The observed changes in TNF- α mRNA levels were consistent with previous findings by Piguet *et al.* [8], where it was shown that a single dose of BLM caused lung TNF- α mRNA to increase between days 5 and 15. The current study further demonstrated that BLM-induced changes in TNF- α could occur also in the absence of T cells and under conditions where eosinophilia failed to occur. It is interesting to note that neutralization of TNF- α was shown by Zhang *et al.* [11] to prevent eosinophilia after BLM treatment; however, our studies have shown that prevention of eosinophilia with anti-IL-5 failed to block TNF- α expression by BLM. Together, these findings suggest that TNF- α may contribute to the development of eosinophilia after BLM but that eosinophils are not required for the expression of TNF- α after BLM treatment.

To confirm our findings that eosinophils were not necessary for the development of pulmonary fibrosis, IL-5 gene knockout mice were evaluated for their response to BLM treatment. Significant lung fibrosis was detected 14 days post-BLM in these eosinophil-deficient mice, again indicating that pulmonary fibrosis can occur in the absence of eosinophils. Thus, in three different mouse strains and via two methods to prevent eosinophilia, we have not been able to demonstrate a critical role for eosinophils in the development of BLM-induced pulmonary fibrosis. Whether eosinophils are critical for other fibrotic diseases in humans or in animal models is not clear from these studies. Certainly, the biology of eosinophils suggests that they could participate in the fibrotic process. Eosinophils have been shown to transcribe and/or translate a wide range of cytokines including IL-1 α , TGF- α , TGF- β , granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF- α , MCP-1, IL-3, IL-5, IL-6, and IL-8. Several of these cytokines have been shown in animal models to participate in the development of fibrosis. However, eosinophils have been shown to synthesize collagenases specific for types I, II, and III collagen also but neither elastase nor nonspecific neutral protease [32–34]. Thus, it is possible that eosinophils may not be involved in initiation of the fibrotic process but rather may serve a role in the remodeling of the fibrotic lesion. Further studies to address whether eosinophils serve this accessory role in remodeling fibrotic lesions in the murine model of fibrosis need to be performed.

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