The role of IL-5 in bleomycin-induced pulmonary fibrosis

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Abstract: Eosinophils are known to express cytokines capable of promoting fibrosis. Interleukin-5 (IL-5) is important in regulating eosinophilopoiesis, eosinophil recruitment and activation. Lung IL-5 expression is elevated in pulmonary fibrosis, wherein the eosinophil is a primary source of fibrogenic cytokines. To determine the role of IL-5 in pulmonary fibrosis, the effects of anti-IL-5 antibody were investigated in a model of bleomycin-induced pulmonary fibrosis. Fibrosis was induced in mice by endotracheal bleomycin treatment. Animals were also treated with either anti-IL-5 antibody or control IgG. Lungs were then analyzed for fibrosis, eosinophil influx, chemotactic activity, and cytokine expression. The results show that a primary chemotactic activity at the height of eosinophil recruitment is IL-5. Furthermore, anti-IL-5 antibody caused significant reduction in lung eosinophilia, cytokine expression, and fibrosis. These findings taken together suggest an important role for IL-5 in pulmonary fibrosis via its ability to regulate eosinophilic inflammation, and thus eosinophil-dependent fibrogenic cytokine production. *J. Leukoc. Biol.* 64: 657–666; 1998.

Key Words: *eosinophils · cytokine expression · inflammation*

INTRODUCTION

Pulmonary inflammation, bronchial hyperrreactivity, and eosinophil infiltration into the airway are pathological features of asthma [1–3]. Analysis of lung tissue from asthmatic patients who died demonstrated tissue eosinophilia basement membrane thickening and mucus plugging [4, 5]. There is a suggestion that the airway wall thickening may be a fibrotic response [6]. However, it is unclear whether eosinophils directly play a role in pulmonary fibrosis. There is substantial evidence for the presence of eosinophils in lung fibrotic lesions and their ability to elaborate cytokines [7–16]. More recently, studies of a rodent model of bleomycin-induced pulmonary fibrosis show that infiltration by eosinophils parallels the development of the fibrotic lesion, and that they represent a key source for cytokines such as monocyte chemotactic protein-1 (MCP-1), transforming growth factor β_1 (TGF-β_1), and interleukin-5 (IL-5) [8, 9, 16]. IL-5 is important in eosinophil accumulation in the lung [17]. Based on these previous observations, the importance of eosinophils in pulmonary fibrosis is suggested by two lines of evidence. First, these cells are now known to be important sources of cytokines with known inflammatory and fibrosis-promoting activities [8–12, 16], representing a key cellular source of TGF-β_1 and MCP-1 (the *JE* gene product in mice) as well as a significant source of IL-5 in bleomycin-induced pulmonary fibrosis [8, 9, 16]. Second, the presence of eosinophils in lungs of patients with pulmonary fibrosis correlates with worst prognosis and/or resistance to therapy [18, 19]. Thus there is compelling evidence to suggest that eosinophils may play an important role in pulmonary fibrosis via its ability to elaborate the cytokines capable of driving the fibrogenic response.

The mechanism for recruitment of eosinophils into the lung has been extensively studied primarily in animal models of airway reactivity. These studies have identified the importance of certain cytokines and chemokines in this process. Among the many cytokines, IL-5 is the most closely correlated with eosinophilia, presumably because of its role in eosinophilopoiesis, eosinophil chemoattraction, prolongation of eosinophil survival, and eosinophil activation [20–22]. In addition, administration of antibody to IL-5 inhibits eosinophil recruitment into bronchoalveolar lavage fluid of antigen-challenged previously sensitized animals [23–27]. Furthermore, lung IL-5 expression is elevated at sites of active fibrosis in the bleomycin model, with most of the expression localized to T cells and secondarily to eosinophils, and the kinetics of its up-regulation correlates with that for lung eosinophil infiltration [16, 28]. There are, however, other eosinophil chemoattractants that have been more variably reported to be involved in lung eosinophil recruitment. Among these, IL-4, RANTES, macrophage inflammatory protein 1α (MIP-1α), and most recently, eotaxin have been reported to have chemotactic activity for eosinophils [29–31]. However, the role of these cytokines has not been directly examined *in vivo* in pulmonary fibrosis-associated lung eosinophil infiltration.

This investigation was designed to extend these IL-5 studies to test the hypothesis that IL-5 may drive pulmonary fibrosis by its ability to recruit eosinophils into the lung and up-regulating their expression of cytokines previously demonstrated to be

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Abbreviations: IL-5, interleukin-5; MCP-1, monocyte chemotactic protein 1; TGF-β_1, transforming growth factor β_1; MIP-1α, macrophage inflammatory protein 1α; PBS, phosphate-buffered saline; PMSF, phenymethysulfonyl fluoride.

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important in fibrosis. To test this hypothesis, the effects of anti-IL-5 antibodies were investigated to evaluate their ability to inhibit lung tissue eosinophil chemotactic activity, pulmonary eosinophilia, cytokine expression, and fibrosis in a murine model of bleomycin-induced lung injury.

MATERIALS AND METHODS

Animals and tissue sampling

Specific pathogen-free, female CBA/J mice weighing 20–25 g were obtained from Jackson Labs (Bar Harbor, ME). These animals arrived in filtered cages and were maintained in clean animal quarters separate from other laboratory animals. Mice were randomly assigned into 12 different groups of 15 animals each. Six of these groups were treated on day 0 with 26 μg/mouse of bleomycin (Blenoxane®, Bristol-Myers Co., Evansville, IN) by endotracheal injection, as previously described [8, 9]. The remaining groups, representing non-fibrotic control animals, received sterile saline on day 0. On days 0, 3, and 5, two groups each of the control and bleomycin-treated groups were given intravenous injections of 1 mg/mouse of a monoclonal rat anti-mouse IL-5 antibody (TRFK-5), prepared as previously described [32, 33]. This antibody at this dose has previously been found to be effective in suppressing eosinophilic inflammation in mice [20]. Preliminary studies using 0.1 mg/mouse of TRFK-5 antibodies failed to inhibit lung eosinophilia or fibrosis (data not shown). Two other groups each of the control and bleomycin-treated groups were injected with the same amount of control IgG. The remaining two groups from both control and bleomycin-treated groups received equal volumes of sterile saline intravenously.

Groups of animals containing one each of the control and bleomycin-treated groups with or without anti-IL-5 antibody injections were killed [8, 9] on days 7 and 14 after treatment. At each time point, the lungs from 10 animals per group were used for extraction of mRNA for Northern hybridization analysis and hydroxyproline assay. For the remaining animals in each group, the lungs were rapidly dissected out and immediately inflated with phosphate-buffered saline (PBS), 10% neutral buffered formalin, pH 7.2, or methacarn (methanol, chloroform, glacial acetic acid 6:3:1 v/v). After overnight fixation, lungs were embedded in paraffin [8, 9]. PBS-inflated lung tissues were used for frozen sections. Three-to four-micrometer serial sections of formalin, methacarn-fixed tissue or frozen specimens (using AO Microtome) were used for routine histology or immunohistochemistry [8, 9].

For studies of eosinophil chemotactic activities, separate groups of animals (five per group) were treated with saline or bleomycin on day 0 and killed at various time points. The lungs after rapid perfusion with PBS were homogenized in PBS supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), and the homogenate centrifuged at 20,000 *g* for 30 min at 4°C. The supernate was collected and stored frozen until assay for eosinophil chemotactic activity.

RNA extraction

At each indicated time point, lungs from control and treated animals were rapidly perfused with sterile PBS via the right ventricle until blanched. The lungs were rapidly dissected out, promptly suspended into guanidine isothiocyanate solution [8], and rapidly homogenized with a polytron (Brinkmann Instruments, Westbury, NY). Total RNA was isolated as previously described and the poly(A)-rich fraction purified by oligo (dT) cellulose chromatography [34–38].

Northern hybridization

To determine and quantify lung IL-5, TGF-β_1, and MCP-1 mRNA expression, Northern hybridization analysis was performed on total RNA isolated from mouse lungs of treated and control groups as previously described [34–38]. Briefly, equal amounts of total lung RNA (20 μg/lane) were electrophoresed on 1.0% agarose gels containing formaldehyde. Uniformity of RNA concentration, and thus equal loading, was confirmed by examination of 18S and 28S rRNA bands on separate ethidium bromide-stained mini gels under ultraviolet light. The separated RNA was then transferred overnight onto nitrocellulose filters, and the blots were confirmed for equal loading by viewing the 28 and 18S rRNA bands after ethidium bromide staining. Blots were then baked, prehybridized, and hybridized with ^{32}P-labeled oligonucleotide antisense probes for mouse IL-5, TGF-β_1, and MCP-1. The sequences for the 30-mer oligonucleotide antisense probes were as follows: mIL-5, 5′-TCCAGGACCTGCCTCGTCCTCC-GTCTCTCC-3′ [16]; TGFβ_1, 5′-GAACTTGGCATGGTAGCCCTTGGGCTC-GTG-3′ [39]; MCP-1, 5′ TGTCTGGACCCATTCCTTCTTGGGGTCAGC-3′ [40]; and GAPDH, 5′-CACCCTGTTGCTGTAGCCGTATTCATTGTC-3′ [41].

These probes were synthesized on an automated DNA synthesizer and purified by high-performance liquid chromatography followed by sodium acetate and ethanol precipitation. After overnight hybridization at 56°C, blots were washed [8, 9, 16, 34–38], and autoradiograms developed from these blots were quantitated using an Ambis Optical Imaging System (Ambis, San Diego, CA) [32–38]. The blots were reprobed with the GAPDH probe to confirm equivalent RNA loading.

Lung hydroxyproline assay

The total lung hydroxyproline concentration was determined as previously described [42].

Antibodies and immunohistochemistry

Rat monoclonal anti-mouse IL-5 was prepared as previously described [25, 27, 32, 33] or purchased commercially from Genzyme (Cambridge, MA). The commercial antibody was used only for the *in vitro* chemotactic assays. Rat and rabbit affinity-purified non-immune IgG, chromotrope 2R, and toluidine blue were purchased from Sigma Chemical Co. (St. Louis, MO). Polyclonal rabbit anti-TGF-β antibody (AB-100-NA) was from R & D Systems (Minneapolis, MN), and is a pan-specific antibody that recognized all the major TGF-β isoforms. Hamster monoclonal anti-mouse MCP-1 antibody was from Pharmingen (San Diego, CA). Antibodies to MIP-1α and RANTES were prepared as previously described [43]. Anti-human eosinophil major basic protein antibody (BMK-13, IgG1) was from Monosan (Uden, The Netherlands). Anti-murine eotaxin antibodies were purchased from PeproTech (Rocky Hill, NJ). Secondary antibodies and related reagents were purchased from Vector Laboratories (Burlingame, CA). Mouse myeloma IgG1 was from Zymed (South San Francisco, CA).

Immunohistochemistry for analysis of IL-5, TGF-β, and MCP-1 protein expression and cellular localization was performed using two different methods, alkaline phosphatase and immunoperoxidase. The alkaline phosphatase method was undertaken as previously described [44] with some modification. Briefly, sections were deparaffinized, rehydrated, and digested with proteinase K, followed by blocking with normal rabbit or goat serum to reduce nonspecific binding of the secondary antibody. The primary antibodies were added at the following concentrations: anti-IL-5 at 1:5 to 1:10 dilution, anti-TGF-β 10 μg/mL, anti-MCP-1 5 μg/mL, and BMK-13 at 1:10 dilution. They were then allowed to incubate at 4°C overnight. The appropriate biotinylated and affinity-purified secondary antibodies were then added and allowed to incubate for 1 h at room temperature, followed by addition of a red substrate (Vector Labs, Burlingame, CA) plus levamisole solution to inhibit endogenous alkaline phosphatase.

Immunoperoxidase staining was done as previously described [44–46] with some modification, using microwave procedures. Briefly sections were deparaffinized, rehydrated, washed in PBS, and endogenous peroxidase activity in section was inactivated with 3% H_2O_2. After washing with deionized water, slides were microwaved in deionized water for 2 min and 30 s, and washed for 1 min with 70–80°C deionized water. After repeating this twice, the slides were washed with deionized water and PBS, followed by incubation with normal rabbit or goat serum to reduce nonspecific binding of secondary antibody. The primary and secondary antibodies were added in the same manner as the alkaline phosphatase method, followed by avidin-biotin amplification (ABC Elite) for 30 min. After incubation with the DAB substrate, the reaction product was enhanced by exposure to DAB enhancing solution, followed by counterstaining with hematoxylin, and sequential dehydration. Mouse spleen cells stimulated with Con A [47] were used as positive controls for the anti-IL-5 antibody. Negative controls included (1) substitution of primary antibody for corresponding nonimmune IgG isotype, (2) omission of primary antibodies, and (3) omission of respective secondary antibodies.

General histological appearance of lung tissue was assessed after routine hematoxylin and eosin (H & E) staining. Eosinophil was identified by staining with carbol chromotrophe 2R as previously described [8], and by their

polymorphonuclear morphology. IL-5-, TGF-β-, and MCP-1-positive cells were identified by immunostaining with their respective specific antibodies [8, 9, 16].

Morphometric analysis

The number of lung eosinophils were counted in tissue sections stained with either chromotrope 2R or BMK-13 antibody as previously described [8, 9]. IL-5- or TGF-β-positive cells were similarly counted by light microscopy using the ×40 objective and ocular grid. At least 30 randomly chosen noncontiguous and non-overlapping high power fields were counted in each lung segment. Five segments from each mouse lung and a total of five mice per group were analyzed. Cell counts were expressed as the number of cells per high power field.

Eosinophil chemotactic assay

Lung extracts from control and bleomycin-treated animals were prepared as described above for assay and identification of eosinophil chemotactic activity. Mouse peritoneal eosinophils were isolated from mice previously sensitized to, and challenged with schistosome egg antigen as previously described [47–49]. The final preparation was at least 80% eosinophils, with the remainder comprised of neutrophils and mononuclear cells. Chemotactic assay was performed in microwell modified Boyden chambers using 5-μm pore size polycarbonate filters as previously described [31, 50]. Various dilutions of the lung extracts were analyzed for eosinophil chemotactic activity. Migrated cells in the bottom surface of the filter were counted after identification as eosinophils with the use of chromotrope 2R staining, and expressed as cells migrated per high power field. A minimum of 100 cells were counted in randomly selected, non-overlapping high power fields. After subtracting the negative control value (migration to media alone), the activity was expressed as a percentage of the activity of the positive control (50 ng/mL MIP-1α). A unit of activity was defined as 1% of the activity found in the positive control sample. Checkerboard analysis was used to determine chemotactic versus chemokinetic activities.

To identify the nature of any detected chemotactic activity, selected lung extract samples with high activity were preincubated for 1 h with specific antibodies (100 μg/mL) to IL-4, IL-5, RANTES, eotaxin, MIP-1α, or a combination of antibodies. These pretreated samples were then used in the chemotactic assay to see if any of these antibodies or combination of antibodies were able to inhibit the eosinophil chemotactic activity. To confirm specificity of the inhibition, negative controls using non-immune isotype-matched IgG or sera were included.

Statistical analysis

All data were expressed as means ± SE, with *N* being the number of animals for each group. Differences between mean values from the various treatment and control groups were assessed for statistical significance by analysis of variance followed by *post hoc* analysis using Scheffé's test for comparison between any two groups [8, 9]. A P value < 0.05 was considered to indicate statistical significance.

RESULTS

Lung eosinophil chemotactic activity

There is substantial evidence supporting a role for IL-5 in lung eosinophil recruitment in models of hyperreactive airway disease [16, 24, 47]. However, the role of other cytokines and other non-protein chemoattractants is not as clear or consistently documented. Evidence for any of these factors in eosinophil recruitment in pulmonary fibrosis is lacking. To identify the nature of any eosinophil chemotactic activity, lung tissue extracts from bleomycin-injured and control lungs were first examined for the presence of such activity. The results of eosinophil chemotactic assays of these lung extracts show detectable activity in samples from both control and bleomycin-treated mice at all time points examined (**Fig. 1**). The relatively low levels of activity on day 1 after bleomycin injury became significantly elevated on days 2, 4, and 8 in the bleomycin-treated animals, and only on days 2 and 4 for the saline-treated control animals. However, the activities at all time points were greater in the bleomycin-treated lung samples relative to those from the saline controls, with the difference being statistically significant for days 2, 4, and 8. The activity remained high on day 8 for the bleomycin-treated lung extracts, but declined to baseline levels for the saline controls. Checkerboard analysis of lung extracts from day 4 bleomycin-treated animals revealed the activity to be chemotactic with only minimal chemokinetic activity (**Table 1**).

Based on these results, lung extract samples from the day 4 time point were chosen for further study in order to identify the source of this activity. Both control and bleomycin-treated samples were preincubated with the indicated cytokine antibodies or their respective control immunoglobulins, and then assayed for eosinophil chemotactic activity. Based on previous studies of their chemotactic activity and the availability of antibodies directed against these proteins, antibodies against MIP-1α, eotaxin, RANTES, IL-4, and IL-5 were used for this experiment. The results indicated that all five antibodies had some specific inhibitory activity against the chemotactic activity in both control and bleomycin-treated samples (**Fig. 2**). Against the activity in control samples, these antibodies were able to inhibit from <40%, for anti-RANTES antibody, up to as high as 52% for the anti-IL-5 antibody. The inhibition by these antibodies was greater against the bleomycin-treated samples,

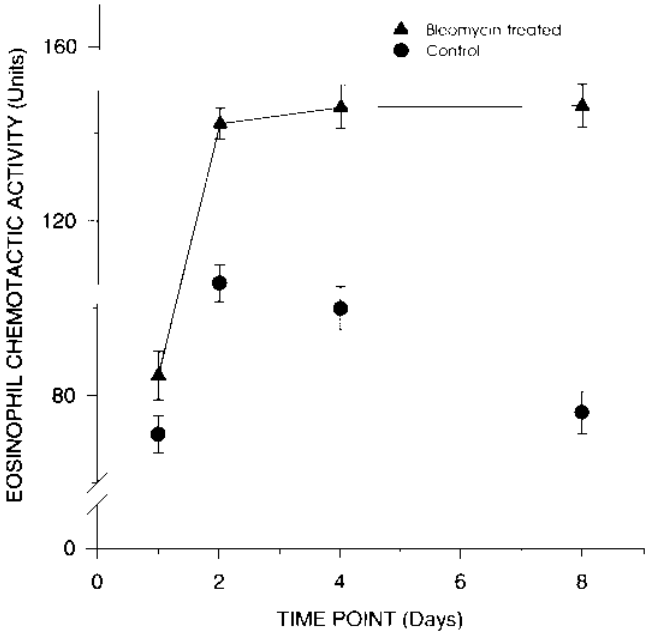

Fig. 1. Kinetics of lung eosinophil chemotactic activity. Lung extracts from control and bleomycin-treated mice were prepared at the indicated time points and then assayed for eosinophil chemotactic activity as described in Materials and Methods. Each data point represents the mean of activity obtained from five different mice per group, and standard error (SE) bars are shown. The activities in samples from bleomycin-treated animals were significantly ($P < 0.05$) higher than those from control mice at all time points examined after day 1.

TABLE 1. Checkerboard Analysis of Eosinophil Chemotactic Activity

Top (%) → / Bottom (%) ↓	0	20	40	60	80
0	1.94 ± 0.12	2.78 ± 0.19	1.82 ± 0.29	1.88 ± 0.19	1.72 ± 0.14
20	15.1 ± 0.40	5.14 ± 0.31	3.62 ± 0.26	2.84 ± 0.3	2.42 ± 0.28
40	26.04 ± 0.72	17.24 ± 0.30	5.66 ± 0.31	3.8 ± 0.42	3.28 ± 0.10
60	18.90 ± 0.53	21.26 ± 0.78	17.74 ± 0.54	5.42 ± 0.36	4.82 ± 0.48
80	18.34 ± 0.7	17.24 ± 0.33	20.7 ± 0.48	16.66 ± 0.48	5.92 ± 0.30

Lung extracts from day 4 bleomycin-treated animals were placed in the upper (Top) and lower (Bottom) compartments of the modified blind-well Boyden chambers at the indicated concentrations. Eosinophils were placed in the upper compartment. The results were expressed in units of activity and shown as means ± SE with $N = 5$.

ranging from 50% for anti-RANTES antibody to >80% for anti-IL-5 antibody. A combination of anti-eotaxin and anti-IL-5 antibodies did not significantly increase the inhibition by anti-IL-5 alone. Similarly, a combination of all antibodies failed to significantly increase the inhibition of activity by anti-IL-5 or anti-eotaxin alone (data not shown). The respective IgG-negative controls did not have a significant effect on the observed chemotactic activities of both control and bleomycin-treated lung samples (data not shown). Thus the chemotactic activity in the bleomycin-treated lungs was both qualitatively and quantitatively different from that in the control lung samples. The greatest suppression in the bleomycin lung samples by the anti-IL-5 and anti-eotaxin antibodies suggested that these two cytokines may be the key eosinophil chemoattractants (or as co-chemotactins) in bleomycin-injured lungs, although the contribution of MIP-1α and IL-4 were not insignificant.

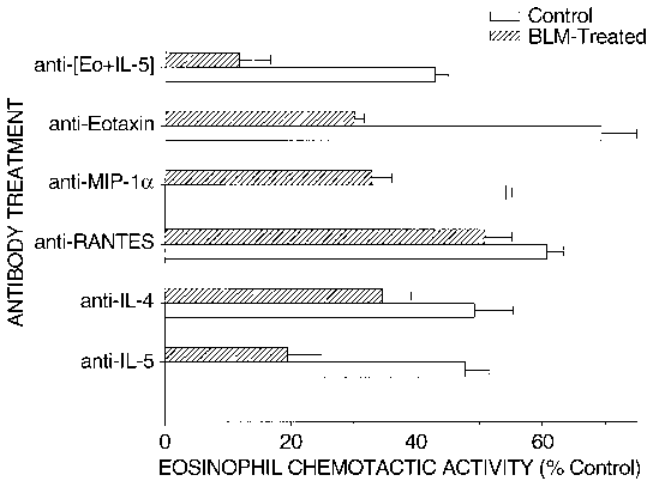

Fig. 2. Effects of various anti-chemokine antibodies on lung eosinophil chemotactic activity. Lung extract samples from day 4 control and bleomycin-treated mice were pretreated with the indicated antibody or combination of antibodies, and then assayed for eosinophil chemotactic activity. The results were expressed as a percentage of the respective control mean value using the corresponding immunoglobulin control for each antibody. The various control IgGs had no significant effects on the chemotactic activity (data not shown). The means ± SE of triplicate assays are shown.

Effect of anti-IL-5 antibodies on lung eosinophils, histopathology, and fibrosis

Examination of fixed lung tissue from control mice treated with control IgG or anti-IL-5 antibodies at various time points did not show any sign of inflammation or other morphological changes (data not shown). In contrast, lungs from bleomycin treated animals injected with control IgG showed severe distortion of lung parenchyma, which became more extensive on day 14. This was characterized by loss of normal alveolar architecture by prominent disorganized thickening of the alveolar septae, infiltration of the alveolar space by organizing inflammatory infiltrate and fibroblasts. Peribronchiolar, perivascular, and submesothelial inflammatory and fibrotic lesions became prominent with dramatic increases in lung cellularity. In accord with previous studies [8, 9, 16, 25], immunostaining of frozen lung sections with BMK-13 antibody (data not shown) or histochemical staining with chromotrope 2R of formalin or methacarn fixed lung tissue, showed significant numbers of eosinophils at days 7 (data not shown) and 14 in bleomycin-treated mice (**Fig. 3**, **A** and **C**). The eosinophils were clustered in cellular areas where early fibrosis was evident (Fig. 3A). In view of the results from the chemotactic studies, the effects of anti-IL-5 antibodies on eosinophil recruitment *in vivo* and fibrosis were examined. Lungs from bleomycin-treated animals, which were also treated with anti-IL-5 antibodies, showed markedly reduced inflammation and fibrosis on day 14 (Fig. 3B). Although the antibodies did not completely suppress inflammation at this time point, it was much less than that seen in lungs from bleomycin-treated animals receiving control IgG (Fig. 3A). Examination of chromotrope-2R-stained sections revealed that lungs from bleomycin-treated animals that also received anti-IL-5 antibody contained noticeably fewer eosinophils distributed in cellular areas, which were much smaller than those treated with control IgG, and with diminished evidence of early fibrosis (Fig. 3, B and D). Morphometric analysis of the lung tissue sections confirm the reduction in eosinophil recruitment by anti-IL-5 treatment at both the day 7 and day 14 time points (**Fig. 4**). Thus neutralization of IL-5 activity was effective in inhibiting eosinophil recruitment to the lung, which correlated with suppression of pulmonary inflammation and fibrosis. Control IgG had no detectable effects on the

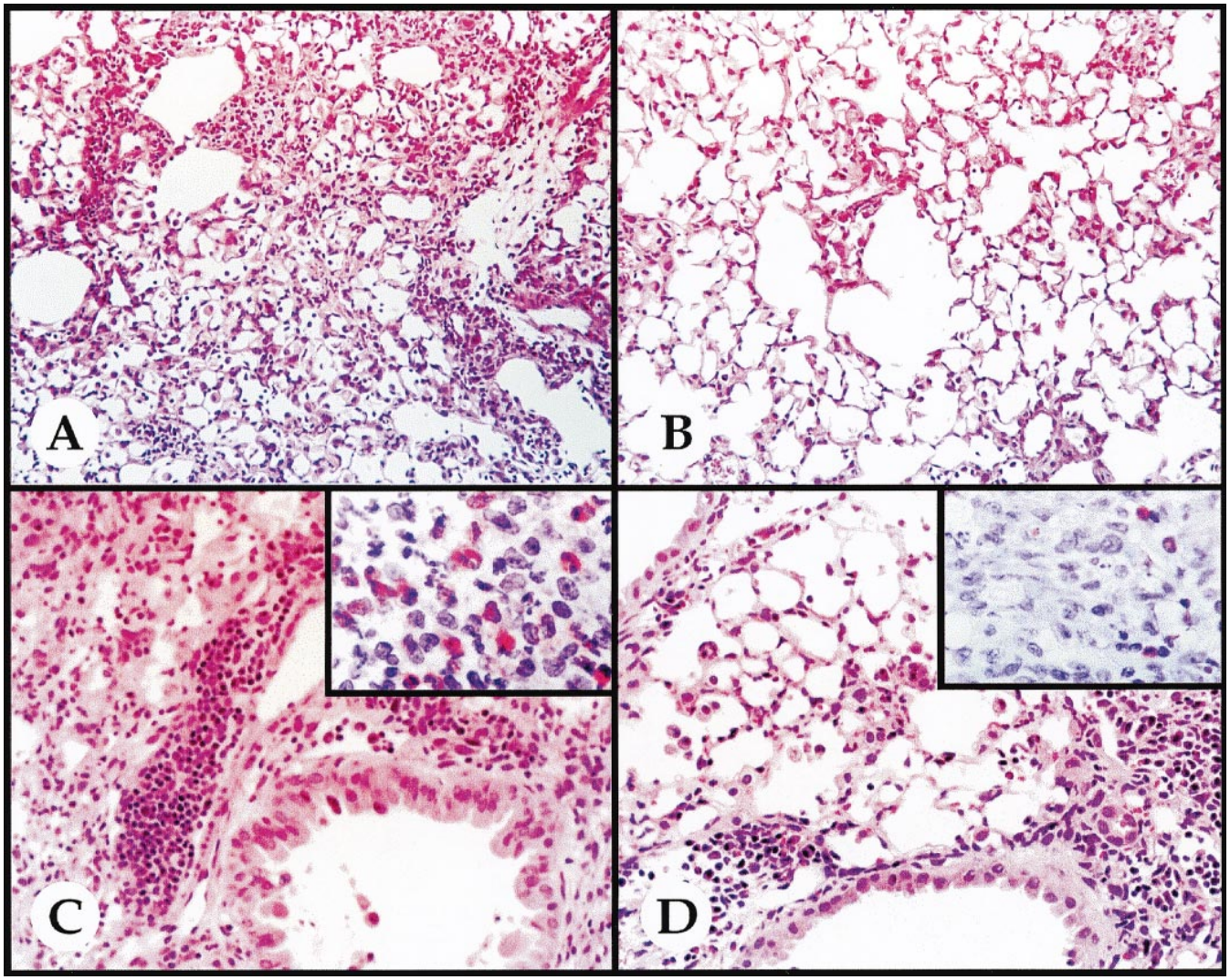

Fig. 3. Effects of anti-IL-5 antibody treatment on lung histopathology. Lung sections from day 14 control and treated animals were stained with chromotrope 2R to identify eosinophils and counterstained with hematoxylin as described in Materials and Methods. Representative lung sections from bleomycin-treated animals and injected with either control IgG (A, C) or anti IL-5 antibody (B, D), are shown. Low-power views (original magnification ×200) show inflammatory infiltrate and early fibrosis in control lungs (A), which were substantially diminished in anti-IL-5 antibody-treated mice (B). A higher-magnification (original magnification ×400) view of the cellular areas show dense clusters of polymorphonuclear cells, which stained red with the chromotrope 2R (inset in C), which became markedly reduced by the anti-IL-5 antibody treatment (D and inset).

morphology of saline or bleomycin-treated lungs (data not shown).

To confirm the morphological evidence of reduced pulmonary fibrosis, separate lung tissue samples were subjected to hydroxyproline assay. The results showed that the lung hydroxyproline content of control mice treated with control IgG were not significantly different from those treated with anti-IL-5 antibodies (**Fig. 5**). In contrast, lungs from bleomycin-treated animals injected with control IgG showed significant increases in lung hydroxyproline content (Fig. 5) consistent with the histopathological findings (Fig. 3). Treatment with anti-IL-5 antibodies significantly suppressed the bleomycin-induced increase in lung hydroxyproline content (Fig. 5). Control IgG had no significant effect on hydroxyproline content of control or bleomycin-treated lungs (data not shown).

Effects on lung cytokine expression

Previous studies have shown that eosinophils represent a key source of several fibrogenic cytokines, such as TGF-β_1 and MCP-1 [1–3, 5, 6, 8, 9, 15, 16]. Because anti-IL-5 can effectively suppress eosinophil recruitment, the effects of this antibody on lung cytokine expression after bleomycin-induced injury were examined to assess the contribution of eosinophils to overall lung cytokine expression. Lung mRNA content for two cytokines, namely TGF-β_1 and MCP-1, were quantitated by Northern blot analysis. Consistent with previous studies [8, 9], the results show significant increases in expression of both cytokines as a result of bleomycin-induced lung injury in animals treated with control IgG (**Fig. 6**). These increases were inhibited by treatment with anti-IL-5 antibodies (Fig. 6).

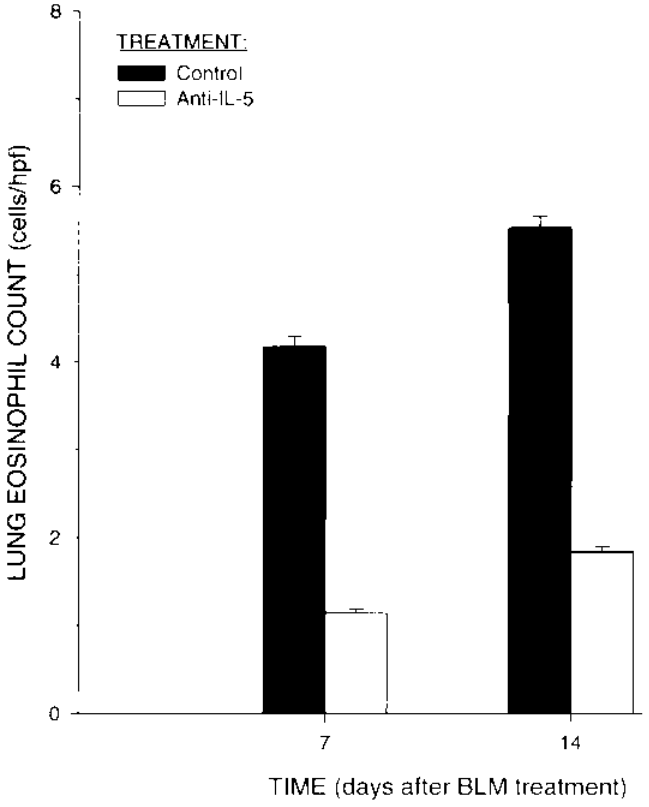

Fig. 4. Effects of anti-IL-5 antibody on lung eosinophil recruitment. Eosinophils in lung sections from bleomycin-treated mice with or without anti-IL-5 antibody treatment were identified by chromotrope 2R staining and polymorphonuclear morphology, and counted as described in Materials and Methods. Results were expressed as the number of cells per high power field. Values represent means ± SEM ($N = 5$ per group). Antibody treatment significantly reduced ($P < 0.01$) the number of lung eosinophils at both time points examined.

To explore the effects of the anti-IL-5 antibody on the cellular distribution of bleomycin-induced lung TGF-β_1 and IL-5 expression, immunohistochemical evaluation of cytokine expression was performed. Immunostaining with anti-IL-5 antibody of lung sections from bleomycin-treated mice showed clusters of IL-5-expressing cells in fibrotic areas. The predominant IL-5-expressing cells were mononuclear cells, consistent with the previous observation that T lymphocytes are the primary source of IL-5 in bleomycin-injured murine lungs, whereas the eosinophil is a secondary source [16, 51–57]. When lung sections from bleomycin- and anti-IL-5-treated animals were examined, there was a substantial decrease in the number of IL-5-expressing cells, which was confirmed by morphometric counting (**Fig. 7A**). Control lungs from mice receiving control IgG or anti-IL-5 antibody did not show any detectable IL-5 expression.

Immunostaining with anti-TGF-β and anti-MCP-1 antibodies revealed similar results. As previously reported [8, 9, 16], there was a substantial increase in the number of cells expressing these cytokines in bleomycin-injured lungs, which was also inhibited by treatment with anti-IL-5 antibody as confirmed by morphometric counting of cytokine-positive cells (Fig. 7, B and C, respectively).

DISCUSSION

A previous study has demonstrated that induction of lung IL-5 mRNA and protein expression in bleomycin-injured lungs is primarily localized to T lymphocytes and secondarily to eosinophils at sites of active fibrosis [16]. Furthermore, at early time points during the period of active fibrosis, expression of TGF-β_1 and MCP-1 is seen primarily in eosinophils [8, 9]. Considering the importance of these cytokines to pulmonary inflammation and fibrosis, plus the importance of IL-5 in eosinophil recruitment [31, 55–59], this study was undertaken to see whether neutralization of endogenous IL-5 activity could down-regulate lung eosinophilia associated with bleomycin-lung injury and the consequent fibrotic response. Initial studies confirmed the importance of IL-5 as an important eosinophil chemotactic factor or cofactor in bleomycin-injured lung tissue because anti-IL-5 antibody inhibited most of the activity found in extracts of injured lungs. Significant inhibition was also evident with antibodies to eotaxin, MIP-1α, and IL-4, but when these were added to the anti-IL-5 antibody, no significant further inhibition from that obtained by the anti-IL-5 antibody alone was observed. This seems to favor the role of IL-5 as a cofactor in the chemotactic activity present in the lung extracts. Using TRFK-5, a neutralizing monoclonal antibody to murine IL-5 [20, 32, 33, 60] in a murine model of bleomycin-induced lung injury and fibrosis [8, 9], the results show significant reduction in severity of lung inflammation, tissue damage, and fibrosis by

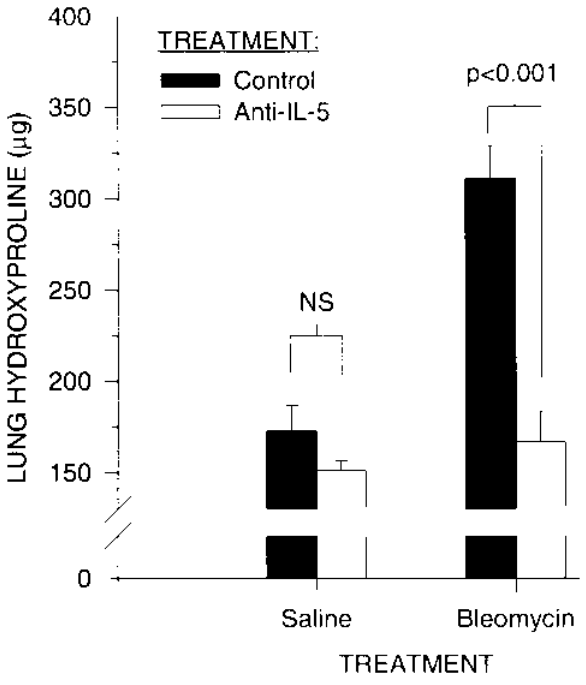

Fig. 5. Effects of anti-IL-5 antibody on lung hydroxyproline content. Pulmonary fibrosis was biochemically assessed by measurement of lung hydroxyproline content on the indicated days post-bleomycin treatment. Results were expressed as total lung hydroxyproline content. Means ± SE ($N = 5$) for each experimental group are shown. "Control" treatment group consisted of mice treated with control IgG. Bleomycin-induced pulmonary fibrosis was significantly inhibited by treatment with anti-IL-5 antibody. The hydroxyproline contents of control untreated and bleomycin-treated (without control IgG or anti-IL-5) lungs were 154 ± 9 and 302 ± 17 μg, respectively. These were not significantly affected by treatment with control IgG.

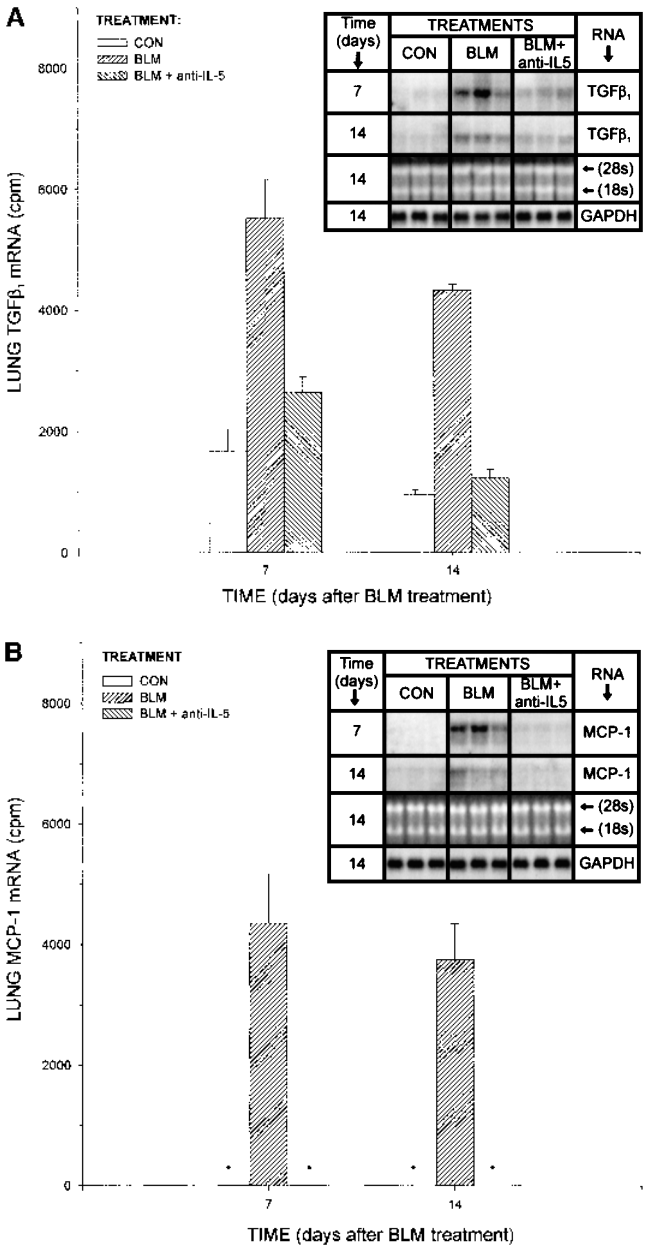

Fig. 6. Lung cytokine mRNA expression by Northern blotting analysis. Total lung RNA from IgG or anti-IL-5 antibody-treated control and bleomycin-treated animals at the indicated time points were subjected to Northern blot analysis for determination of TGF-β_1 (A) and MCP-1 (B) mRNA as described in Materials and Methods. The autoradiograph for each cytokine mRNA is shown in the inset in the upper right portion of each panel. The corresponding 28S and 18S ribosomal RNA bands, and the GAPDH mRNA results are shown for the day 14 specimens to document uniform RNA loading. Similar results were obtained with the day 7 samples (data not shown). The results of direct quantitation of cytokine mRNA by a radio-imaging system (Ambis) are shown in the graph. Each lane represented an RNA sample from a single animal. The time points refer to the number of days after bleomycin or saline treatment on day 0. The plotted data represent means ± SE of results from three animals at each time point. The increases (relative to respective controls) in bleomycin-treated animals were statistically significant ($P < 0.05$) for days 7 and 14. The bleomycin-induced increase in mRNA of both cytokines were significantly inhibited (bleomycin plus control IgG versus bleomycin plus anti-IL-5 treated groups) by treatment with anti-IL-5 antibodies at both time points studied.

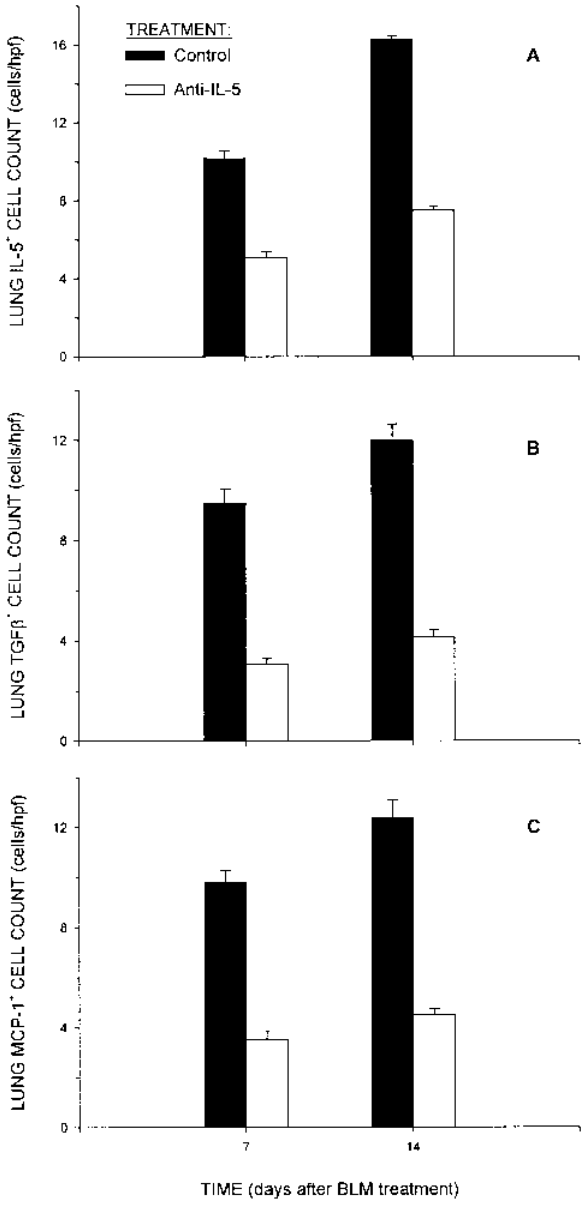

Fig. 7. Effects on lung cellular cytokine antigen expression. Lung sections from day 7 bleomycin-treated animals treated with either control IgG or anti-IL-5 antibodies, were immunostained with anti-IL-5 (A), anti-TGF-β (B), or anti-MCP-1 (C) antibodies to quantitate the number of cytokine-expressing cells as described in Materials and Methods. Cytokine expression was significantly elevated in bleomycin-treated lungs treated with control IgG. This bleomycin-induced increase in cytokine-expressing cells was significantly ($P < 0.01$) reduced in animals treated with anti-IL-5 antibodies for both time points studied. Results are shown as means ± SE with $N = 5$ animals per group. Control untreated lungs did not show detectable staining for any of these cytokines, except for TGF-β staining localized primarily to epithelial cells. Control IgG did not significantly alter lung cellular expression of cytokines in bleomycin-treated animals (data not shown).

this antibody. This effect was accompanied by inhibition of bleomycin-induced lung eosinophil influx, cytokine expression, and elevation in lung hydroxyproline content. The antibody effect was specific because control IgG did not have such an inhibitory effect. Taken together these findings provide some support for the conclusion that IL-5 is involved in the lung eosinophilia seen in mice undergoing bleomycin-induced pul-

monary fibrosis, and that this eosinophilia is important to the overall fibrotic response.

The ability to suppress eosinophilia with anti-IL-5 antibodies is not unexpected because previous reports in mice have shown that blood eosinophilia induced by a parasite infection or systemic administration of IL-2 can be abolished by the administration of similar anti-IL-5 monoclonal antibodies [33, 60]. Previous studies have also reported on the efficacy of similar anti-IL-5 antibodies in suppressing the lung airway eosinophilia associated with antigen-challenged lungs in previously sensitized animals [33], but the results described here represent the first report of such suppression of eosinophilia in the lung parenchyma associated with lung injury and interstitial as well as alveolar fibrosis. The eosinophil chemotactic activity of IL-5 is well documented [31, 56, 57], hence the ability to suppress eosinophilia with this antibody may be mediated via this mechanism. However, the data presented cannot distinguish between its role *in vivo* as a cofactor (i.e., in conjunction with other chemotactic factors, such as RANTES, MIP-1α, eotaxin, etc.) or as a primary chemoattractant in its own right. In addition, IL-5 is known to prolong and/or promote eosinophil survival [20, 61, 62], thus providing another mechanism by which neutralization of endogenous IL-5 activity could diminish lung eosinophil accumulation. With respect to the fibrotic process itself, IL-5 is a well-known stimulator of eosinophils with direct implications on their expression of such fibrogenic cytokines as TGF-β and MCP-1 [8, 9, 63].

There is mounting evidence that the eosinophil is a key source of many cytokines, including those with direct relevance to the inflammatory and fibrotic responses. First, with regard to the cellular source of IL-5 itself, the evidence indicates that it is primarily restricted to the T lymphocyte and secondarily to the eosinophil and mast cells [52, 53]. Consistent with this is the demonstration that both T cells and mast cells are involved in eosinophil accumulation in the lungs of allergic mice have been documented [52, 53]. These results implicate a role for T cells and IL-5 in the eosinophilic inflammatory response in the lung. The findings in this study are consistent with these and other recent studies [52, 54], which demonstrated that IL-4 and IL-5 mRNA were expressed predominantly by T cells, and suggested that T_H2 cells were activated. A role for T cells in bleomycin-induced and other models of pulmonary fibrosis has been previously suggested [16, 54], which may be mediated by T cell-derived IL-5 in those lesions characterized by eosinophil infiltration. Indeed this may be a mechanism by which neutralization of TNF-α activity results in abrogation of pulmonary fibrosis [63]. However, there is some controversy as to the exact role of T cells in this model of fibrosis [64]. A more direct role of eosinophils *vis-á-vis* fibrosis is their importance as sources of TGF-β, TGF-α, and other cytokines [9–11, 65, 66]. There is evidence that TGF-β is highly expressed in bleomycin-induced pulmonary fibrosis, and at the peak of active fibrosis, a key source of TGF-β_1 is the eosinophil in this model [9]. Hence prevention or diminution of eosinophilia by anti-IL-5 antibody treatment effectively suppresses expression of these cytokines in the lung, which are critical for fibrosis.

In addition to IL-5, IL-3 and granulocyte-macrophage colony-stimulating factor are also capable of regulating several functions of eosinophils, such as proliferation, adhesion, chemotaxis, and activation [12, 67, 68]. However, given the effectiveness of IL-5 neutralization alone in suppressing fibrosis, these other cytokines may play secondary roles. Alternatively, because the eosinophil is a key source of both TGF-β_1 and MCP-1 in this model, suppression of eosinophilia by the anti-IL-5 antibody may be sufficient to suppress both inflammation and fibrosis, by effectively inhibiting expression of the cytokines essential for both processes [60]. Although IL-5 has some effects on B cell function in the mouse, there is no evidence of an important role for B cells in this model of lung injury and fibrosis, despite some evidence for T cells. This plus the relatively specific effects on eosinophil-function and the eosinophil's ability to express TGF-β_1 and MCP-1 in this model [8, 9] argues for the conclusion that IL-5 is important for pulmonary fibrosis via its ability to promote eosinophilia, which in turn is responsible for the full expression of an array of cytokines critical for promoting the inflammation and fibrosis that ensues.

On the basis of these observations in animals, it is tempting to speculate about the potential utility of anti-IL-5 monoclonal antibodies or other modes of suppressing eosinophil recruitment/activation in treatment of patients with comparable forms of lung injury, inflammation, and fibrosis, especially those with an eosinophil component. Previous reports have suggested that the presence of eosinophils may be quite widespread even in those lung diseases without a demonstrable allergic component [13, 15, 68]. The possibility of using the TRFK-5 antibody in human immunotherapy studies may become more feasible with the production of humanized forms of the antibody [60].

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