Early response to bleomycin is characterized by different cytokine and cytokine receptor profiles in lungs

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Cavarra, Eleonora, Fabio Carraro, Silvia Fineschi, Antonella Naldini, Barbara Bartalesi, Annalisa Pucci, and Giuseppe Lungarella. Early response to bleomycin is characterized by different cytokine and cytokine receptor profiles in lungs. Am J Physiol Lung Cell Mol Physiol 287: L1186–L1192, 2004. First published August 20, 2004; doi:10.1152/ajplung.00170.2004.—The sensitivity to the fibrosis-inducing effect of bleomycin varies considerably from species to species, the reasons for which are unknown. The variability of the response in different strains of mice is well documented. Recent evidence indicates that the upregulated expression of cytokines and cytokine receptors may be involved. We evaluated the expression pattern of some cytokines and their receptors in C57Bl/6J bleomycin-sensitive and Balb/C bleomycin-resistant mice. Animals from both strains received, under ether anesthesia, either saline (50 μl) or bleomycin (0.1 U/50 μl) intratracheally. At various times after the treatment, the lungs were analyzed for cytokines and cytokine receptors by histochemistry and their mRNA by RNase protection assay. A significantly increased expression of TNF-α and IL-1β was observed in both strains. However, an upregulated lung expression for TNF-α and IL-1 receptors was observed in C57Bl/6J-sensitive animals only. This profile is evident from 63 h onward. In addition to TNF-α, bleomycin administration also resulted in the upregulated expression of TGF-β in the lungs of both strains at 8 h and in an enhanced expression of TGF-β receptors I and II in C57Bl/6J mice only. The upregulation of TGF-β receptor expression was preceded in this strain by an increased expression of IL-4, IL-13, and IL-13 receptor-α (at 8 h after bleomycin) and followed by an upregulation of gp130 and IL-6. The difference we observed in the cytokine receptor profile may offer an additional explanation for the different fibrogenic response of the two mouse strains to bleomycin.

Previous studies have demonstrated a genetic susceptibility to bleomycin-induced pulmonary toxicity based on the close association between mouse strain and the fibrotic outcome. C57Bl/6J and C3H/HeN mice are considered to be fibrosis prone, and Balb/c and C3H/f mice are relatively fibrosis resistant (5, 42).

The mechanisms behind this genetic susceptibility are not completely understood but may include differences in bleomycin pharmacokinetics, susceptibility to oxidative stress, ability to repair DNA damage, immune system responses to lung injury, or the pattern of cytokines produced during the inflammatory and/or repair process (14–15, 42).

Recent evidence supports the hypothesis that changes in the expression of cytokines and cytokine receptors may play a role in the development of bleomycin-induced pulmonary toxicity in the rodent model. The cytokine network may influence the fibrotic response through fibroblast activation and proliferation and collagen deposition (6).

In the present study, we evaluated the expression pattern of some important cytokines and of their receptors in the early phase of the bleomycin response in C57Bl/6J-sensitive and Balb/c-resistant mice. The results obtained demonstrate that a different pattern of cytokines and their receptors can characterize bleomycin-prone and -resistant mice as early as a few hours after the fibrogenic stimulus.

MATERIALS AND METHODS

Bleomycin treatment. Male C57Bl/6J and Balb/C mice (supplied by Charles River, Calco, Italy) of 3–4 mo of age were used in this study. All animal experiments were conducted in conformance with the “Guiding Principles for Research Involving Animals and Human Beings” and was approved by the Local Ethics Committee of the University of Siena. For each strain of mice, different groups of animals were used. A group received a single intratracheal instillation of 0.1 μg bleomycin (Rhone-Poulenc Rorer, Milan, Italy) in saline solution (50 μl). Another group was instilled intratracheally with the same amount of saline. All intratracheal instillations were performed under ether anesthesia. At various times after the treatment, the animals from all groups were injected with an overdose of pentobarbital sodium and exsanguinated by cutting the abdominal aorta. The lungs were then excised and processed for conventional light microscopy and immunohistochemistry or RNase protection assay (RPA).

Morphology and immunohistochemistry. The lungs from the different groups of mice were fixed intratracheally with buffered formalin (5%) at a constant pressure of 20 cmH2O for 4 h and then immersed in the same fixative for an additional hour. The samples...
were then dehydrated, cleared in toluene, and embedded in paraffin. For the morphological studies, transversal sections (7 μm) of each pair of lungs were cut and stained with hematoxylin and eosin. For the immunohistochemical studies, tissue sections (8 μm) were stained for TNF-α, IL-1β, TGF-β1, IL-4, IL-13, TGF-β RI, and TGF-β RII by an immunoperoxidase method. The sections were pretreated with 3% hydrogen peroxide to inhibit the activity of the endogenous peroxidase. For antigen retrieval, the sections were heated in a microwave for 20 min in 0.01 M citrate buffer, pH 6.0, and allowed to cool slowly to room temperature.

All sections were incubated with 3% BSA for 30 min at room temperature to block nonspecific antibody binding. They were then incubated overnight at 4°C with the primary antibodies (Ab). The primary polyclonal Ab used were rabbit Ab to mouse TNF-α diluted 1:20 (Immunogenetics, Ghent, Belgium), rabbit Ab to mouse IL-1β diluted 1:100 (Antigenix America, New York, NY), rabbit Ab to mouse TGF-β1 diluted 1:20 (Insight Biotechnology, Wembley, UK), goat Ab to mouse IL-4 diluted 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA), goat Ab to mouse IL-13 diluted 1:400 (R&D Systems, Minneapolis, MN), goat Ab to mouse TGF-β RI diluted 1:100 (R&D Systems), and goat Ab to mouse TGF-β RII diluted 1:100 (R&D Systems). All sections were rinsed and incubated with sheep anti-rabbit IgG or rabbit anti-goat IgG Ab for 30 min at room temperature. The staining was revealed by adding peroxidase-antiperoxidase complex prepared from rabbit or goat serum. Detection was accomplished by incubation in diaminobenzidine freshly dissolved in 0.03% H2O2 in 50 mM Tris-HCl, pH 7.6. As negative controls for the immunostaining, the primary Ab were replaced by nonimmunized rabbit or goat serum. The sections were counterstained with hematoxylin.

RPA. Cytokine and cytokine receptor mRNAs were evaluated by RPA, as previously described (29), using the murine ribosomal protein L32 as housekeeping gene. Total RNA was isolated from lung homogenates by RNA Wizard, according to the suggested procedures (AMBION, Austin, TX). The RNA probes were synthesized using T7 phage polymerase (MAXiScript; AMBION) and a biotin RNA labeling mixture (Boehringer, Mannheim, Germany). DNA templates were purchased from Pharmingen (San Diego, CA) as a multiprobe template set (Riboquant RPA system). Total RNA and appropriate RNA probes were hybridized, and the products of the RPA were separated for analysis on a denaturing polyacrylamide gel (5% acrylamide-8 M urea) by running the gel at 200 volts for 1 h. The nucleic acids were transferred from the gel to a positively charged nylon membrane, using a semidy transfer unit (Hoefer Pharmacia Biotech, San Francisco, CA). The nucleic acids were then immobilized by ultraviolet cross-linking. Nonisotopic detection was performed with the BrightStar BioDetect kit (AMBION), following the manufacturer’s instructions. The identity and quantity of each mRNA species in the original sample were then determined from the signal intensities given by the appropriately sized protected probe fragment bands. Antiensese L32 (housekeeping gene) probe transcripts were synthesized and used in a series of parallel reactions to normalize the amounts of RNA present in the samples. Gel electrophoretic autoradiographs were then quantitated by Sigma Gel analysis software (Jandel Scientific, Chicago, IL).

Statistical Analysis. For each parameter, either measured or calculated, the values for individual animals were averaged, and the SD was calculated. The significance of the differences between groups was calculated using one-way ANOVA (F-test). A P value <0.05 was considered significant.

RESULTS

RPA. Cytokine and cytokine receptor mRNA levels were assessed in lung tissue from both strains of mice at different times after bleomycin administration. The various time points at which the most significant changes were observed are reported in Figs. 1 and 2.

A significantly increased expression (over saline-treated animals) of TNF-α and IL-1β mRNA was observed in both strains at 8 h (Fig. 1). However, bleomycin caused upregulation of TNF-α and IL-1 receptor expression in the lungs of C57Bl/6J-sensitive animals only. The expression of these receptors progressively increased from 8 h onward, reaching a significant difference in respect to controls only at 63 h after bleomycin challenge (Fig. 2). In addition to TNF-α, bleomycin also caused upregulation of TGF-β expression in the lungs of both strains at 8 h (Fig. 1), and at a later time (6 days) an enhanced expression of TGF-β RI and RII in C57Bl/6J mice only (Fig. 2). In particular, TGF-β RII expression can be found significantly higher as early as the 15th h after the treatment. The upregulation of the TGF-β receptor expression was preceded in this strain by an increased expression of IL-4, IL-13, and IL-13 receptor-α1 at 8 h after bleomycin administration (Figs. 1 and 2). Additionally, an increased expression of IL-6 (Fig. 1) and the gp130 receptor for IL-6 family cytokines was observed in C57Bl/6J-sensitive mice only, from 8 and 63 h onward, respectively. IL-6 and its receptor (Fig. 2) appeared significantly elevated up to 6 days after bleomycin challenge.

Immunohistochemistry. A faint reaction for TNF-α was observed on alveolar cells with morphological features of macrophages and type II pneumocytes in control mice of both strains. At 15 h, 21 h, and 3 days after bleomycin challenge, a clear reaction was seen in both strains on the above-mentioned alveolar cells, and on parenchymal areas showing an inflammatory reaction. At 6 days, the staining was also distributed on extracellular matrix components associated with an inflammatory reaction (data not shown).

With regard to IL-1β, a faint and diffuse staining of all alveolar structures was seen in control mice of both strains. At 15 h after bleomycin administration, a marked positive staining on alveolar cells was detected in the subpleural regions of both mouse strains. Subpleural regions with an inflammatory reaction and extracellular matrix components also stained positively at 21 h and 3 days. A weaker staining was seen on the above-mentioned structures at 6 days (data not shown).

No reaction for TGF-β could be appreciated in lung slices from control animals of both strains. After bleomycin admin-
istration (15 h), a first positive staining for TGF-β was seen throughout the parenchyma of both strains. A more intensive staining of alveolar wall cells was evident at 6 days (data not shown).

According to RPA data, immunostaining for IL-1β RI in C57Bl/6J mice treated with bleomycin showed a positive reaction on various components of lung parenchyma, in particular in subpleural regions. This reaction became more pronounced 6 days after the treatment (Fig. 3A). No reaction for IL-1β RI was seen in Balb/C mice treated with bleomycin (Fig. 3B) and in control animals from both strains.

In control animals from both strains, there was a mild reaction for TGF-β RI on interstitial cells and on bronchial epithelial cells. After bleomycin administration (6 days), a positive staining for TGF-β RI was seen on epithelial cells and on parenchymal areas showing an inflammatory reaction (Fig. 4A) and alveolar macrophages (Fig. 4B) in C57Bl/6J mice. No reaction for TGF-β RI was seen in Balb/C mice treated with bleomycin (Fig. 4, C and D).

Immunostaining for TGF-β RII revealed a faint reaction on all pulmonary parenchyma in control animals. After bleomycin treatment (6 days), an intensive staining was shown on subpleural regions and in peripheral areas of C57Bl/6J mice (Fig. 5A). This reaction was most evident on parenchymal areas showing an inflammatory reaction (Fig. 5C). As shown for TGF-β RI, no reaction for TGF-β RII was seen in Balb/C mice treated with bleomycin (Fig. 5B).

A positive staining for IL-13 was found in both Balb/C and C57Bl/6J mice from 15 h after bleomycin treatment onward. IL-13 was detected on inflammatory infiltrate and in peribronchiolar areas (data not shown).

Immunostaining for IL-4 showed an intensive reaction on inflammatory cells infiltrating lung parenchyma 15 h after bleomycin treatment in C57Bl/6J mice (Fig. 6A). On the contrary, no reaction for IL-4 was shown in Balb/c mice at the same time (Fig. 6B).

**DISCUSSION**

The etiology and pathogenesis of IPF are still unknown. All available evidence is consistent with the concept that the disease results from an uncontrolled, chronic inflammatory process, but it is unclear what initiates and maintains the alveolitis. The most plausible hypothesis suggests that the inflammation caused by a variety of insults to the alveoli results in pulmonary fibrosis only in susceptible individuals (13). Studies in humans and in animal models indicate that a persistent interstitial inflammation leading to, and modulating development of, fibrosis has therefore developed. In favor of this hypothesis, there are many studies carried out in experimental models of fibrosis that highlight some cytokines involved in acute and chronic inflammation [i.e., TNF-α, IL-1β, platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), and TGF-β] that are of critical importance in determining the outcome of pathogenic events (26). These mediators were found to be upregulated during the late fibrotic phase in bleomycin-prone (or silica-prone) animals. Unfortunately, few studies have been carried out to characterize the cytokine profile soon after bleomycin challenge (within the first 7 days).

Fig. 2. Cytokine receptor mRNAs evaluated on bleomycin-treated lungs by RPA. Data represent mean values ± SD from 3 different experiments carried out on different pools of 5 lungs each, from C57Bl/6J (gray bars) or Balb/C (black bars) mice. Time points represent those at which the maximal change in cytokine receptor expression was observed. Results are expressed as %values from control mice. *P < 0.05 vs. respective controls.

Fig. 3. Immunostaining for IL-1β receptor (R) I. A: C57Bl/6J 6 days after bleomycin treatment. B: Balb/C 6 days after bleomycin treatment (original magnification ×250).
The results presented here indicate that a different pattern of expression of cytokines and their receptors can characterize bleomycin-prone and -resistant mice as early as a few hours after bleomycin treatment.

In particular, an upregulation of IL-1β, TNF-α, TGF-β, IL-4, IL-13, and IL-13R has been found soon after bleomycin administration. At later times, an enhanced expression of TNF-R75 and IL-1 R1 (63 h) and TGF-β RI and II (6 days) was demonstrated in fibrosis-prone mice. On the contrary, these receptors were downregulated in bleomycin-resistant mice. Additionally, an enhanced expression of IL-6 and gp130 accompanied and followed the upregulated expression of TGF-β in bleomycin-sensitive mice.

A number of human and animal studies have revealed the presence of IL-1β in chronic inflamed tissues and in tissues undergoing fibrogenesis, with accumulation of myofibroblasts and matrix deposition (8, 21, 35). Although the direct effect of IL-1 on fibroblasts still has to be determined, it is known that IL-1 is a potent inducer of TGF-β, and this suggests that at least a part of its profibrotic effect is mediated through this growth factor (24). A recent study shows that, in transgenic mice, a transient overexpression of IL-1β accompanied by an increase of TNF-α, PDGF, and TGF-β was associated with severe progressive tissue fibrosis in the lung (24). Moreover, administration of the IL-1 receptor antagonist markedly diminishes the extent of fibrosis observed in both bleomycin and silica mouse models, suggesting a causative link between cytokines involved in the acute phase of inflammation, such as IL-1, and the conversion to chronic inflammation and fibrosis (37). It has been shown that IL-1 mediates the production of matrix metalloproteinase (MMP)-1 and -3. These enzymes, degrading the basal membrane, may facilitate fibroblast recruitment during the inflammatory process in the presence of appropriate chemotactic stimuli (22). The damage to the basal membrane induced by IL-1β could be an early key factor in the development of the fibrotic response. Although higher levels of IL-1β mRNA were found in both C57Bl/6J and Balb/c mice with respect to their own controls, an increased expression of IL-1β receptor was observed after bleomycin only in C57Bl/6J-sensitive mice. Thus, in our opinion, the IL-1β receptor signal may contribute to the susceptibility of this strain to fibrosis.

TNF-α has been shown to play an important role in the pathogenesis of bleomycin-induced lung fibrosis (36). Neutralization of TNF-α with anti-TNF-α Ab (28) or the administration of soluble TNF-α receptors (p75 and p55; see Ref. 38) can prevent or diminish the development of bleomycin-induced lung fibrosis in mice. The importance of the TNF-α receptors in the pathogenesis of bleomycin-induced lung fibrosis is demonstrated by the fact that mice in which both of these receptors have been deleted are protected from the inflammatory and fibrotic effects of bleomycin (31–32). TNF-α has been shown to induce collagen mRNA expression in cultured human fibroblast (10, 47). However, the effects of TNF-α on collagen transcription may be mediated indirectly by upregulating the expression of cytokines such as PDGF, which promotes migration and proliferation of fibroblasts, and TGF-β (44), which in turn can influence collagen metabolism via CTGF expression (25). Furthermore, TNF-α activity promotes induction of matrix-degrading metalloproteinases that can enhance basal membrane disruption and can facilitate fibroblast migration (33, 43).

We found a significant increase in expression of TNF-α mRNA in both fibrosis-prone and fibrosis-resistant mice soon after bleomycin treatment. However, bleomycin treatment resulted in upregulated expression of TNF-R75 in the lung, only in C57Bl/6J-sensitive animals. Lack of TNF-R75 overexpression after bleomycin treatment may preserve Balb/c mice from developing pulmonary fibrosis. It has recently been demon-
strated that the signaling by TNF-α via its p75 receptor contributes to the regulation of TGF-β1 expression (32).

It is well known that TGF-β is the most potent stimulator of fibroblast collagen production yet described. This enhanced collagen deposition is mediated by increased mRNA transcription and stability, through decreased degradation of procollagen via inhibition of collagenase production, and through increased production of MMP inhibitors (26). Recent studies demonstrate that TGF-β can stimulate fibroblast proliferation and collagen synthesis and deposition via CTGF expression (25). The importance of CTGF in the fibrotic response is demonstrated by a recent study showing that overexpression of TGF-β in Smad3 knockout mice does not induce lung fibrosis resulting from a failure to activate CTGF gene expression (11). Furthermore, Simvastatin, a compound with described antifibrotic properties, also inhibits CTGF expression in isolated IPF patient-derived lung fibroblasts (2).

Indeed, we were not able to detect differences in the expression of TGF-β between the two strains of mice in our experimental conditions. Thus the higher sensitivity to bleomycin of C57Bl/6J in respect to Balb/C mice may be related to the different strain levels of TGF-β receptors after bleomycin treatment.

Recent studies suggest a role in pulmonary fibrosis for the Th2 cytokines IL-4 and IL-13, which can be expressed in the lung by a variety of inflammatory or resident cells (22, 40). The biological effects of these cytokines are mediated via the IL-13 receptor.

In our model, we found a marked increase in production of the IL-4/IL-13 receptor only in C57Bl/6J mice treated with bleomycin.

It is well recognized that IL-4 is an important mediator of fibroblast activation. Recent studies have demonstrated that IL-4 promotes “in vitro” the production of fibroblast-derived extracellular matrix proteins, including type I and III procollagens and fibronectin, to a magnitude similar to that induced...
by an equal amount of TGF-β (39). Moreover, IL-4 has been identified as a chemotactic factor for fibroblasts and can induce fibroblast proliferation and cytokine production. Indeed, the role of IL-4 in the development of lung fibrosis is as yet controversial (20). Recent studies carried out on IL-4-overexpressing and knockout mice (4, 12, 18, 19, 28) suggest that IL-4 does not play a critical profibrotic role after bleomycin challenge. However, studies from other colleagues (16, 17) indicate that IL-4 may play a selective anti-inflammatory role during initial lung injury stages by suppressing T cell infiltration, and a profibrotic role at later stages by modulating fibroblast activation and myofibroblast differentiation. In our opinion, further work on the pleiotropic effects of IL-4 on both immune cells and fibroblasts, and on its different role at the different stages of the disease process, will clarify the overall contribution of this mediator to the pathogenesis of bleomycin-induced lung fibrosis in mice.

IL-13, which shares with IL-4 similar biological properties, has been implicated in the pathogenesis of fibroproliferative disorders (30). In particular, IL-13 induces in vitro the expression of fibroblast-derived type I and III procollagens (to a similar degree as IL-4 and TGF-β), inhibits IL-1-induced MMP-1 and MMP-3 production, and enhances tissue inhibitor of metalloproteinase-1 generation in fibroblast cultures (30). Moreover, IL-13 has been shown to stimulate production and activate in vivo TGF-β (27).

The different responses in terms of the level of expression of the IL-4/IL-13 receptor in the early phase of fibrogenesis may provide a further explanation for the different fibrogenic responses of the two mouse strains to bleomycin.

It is well known that IL-6 is released after stimulation by fibrogenic growth factors such as TGF-β (9). Recent data stress the potential importance of gp130 signaling in mediating cellular effects (cell cycle arrest-apoptosis; proliferation-antiaapoptosis) that may play a pivotal role in fibrogenesis (23). The differences observed in the fibrogenic response between bleomycin C57Bl/6J-sensitive and Balb/c-insensitive mice could also be related in part to the different levels of IL-6/gp130 expression that we observed in these mice. However, further studies are necessary to define the role of the gp130/IL-6 family of cytokines in the late stage of fibrogenesis.

In conclusion, the difference observed in the profile of cytokine receptors may be important in dictating the fibrogenic response to bleomycin in the different mouse strains. This modulation of the cytokine response, not only at the level of cytokines but through regulation of the expression of cytokine receptors, may play a role in fibrogenesis. Many of these receptors are downregulated in fibrosis-resistant mice.

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