Bleomycin-induced Pulmonary Fibrosis Is Attenuated in γ-Glutamyl Transpeptidase–Deficient Mice

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To investigate repair mechanisms in bleomycin-induced pulmonary fibrosis, we used mice deficient in γ-glutamyl transpeptidase ($GGT^{-/-}$), a key enzyme in glutathione (GSH) and cysteine metabolism. Seventy-two hours after bleomycin (0.03 U/g), $GGT^{-/-}$ mice displayed a different inflammatory response to wild-type mice as judged by a near absence of neutrophils in lung tissue and bronchoalveolar lavage and a less pronounced rise in matrix metalloproteinase-9. Inflammation in $GGT^{-/-}$ mice consisted mainly of lymphocytes and macrophages. At 1 month, lungs from bleomycin-treated $GGT^{-/-}$ mice exhibited minimal areas of fibrosis compared with wild-type mice(light microscopy fibrosis index: 510 ± 756 versus 1975 ± 817, $p < 0.01$). Lung collagen content revealed a significant increase in bleomycin-treated wild-type (15.1 ± 3.8 versus 8.5 ± 0.7 μg hydroxy(OH)-proline/mg dry weight, $p < 0.01$) but not in $GGT^{-/-}$ (10.4 ± 1.7 versus 8.8 ± 0.8). Control lungs from $GGT^{-/-}$ showed a significant reduction of cysteine (0.03 ± 0.005 versus 0.055 ± 0.001, $p < 0.02$) and GSH levels (1.24 ± 0.055 versus 1.79 ± 0.065, $p < 0.002$). These values decreased after 72 hours of bleomycin in both $GGT^{-/-}$ and wild-type but reached their respective control values after 1 month. Supplementation with N-acetyl cysteine partially ameliorated the effects of GGT deficiency. These findings suggest that increased neutrophils and matrix metalloproteinase-9 during the early inflammatory response and adequate thiol reserves are key elements in the fibrotic response after bleomycin-induced pulmonary injury.

Keywords: glutathione; matrix metalloproteinase-9; neutrophils; cysteine

Pulmonary fibrosis, a common final pathway of numerous acute and chronic lung injuries, is characterized by fibroblast proliferation, extracellular matrix accumulation, and distortion of the parenchymal architecture (1). The pathogenic mechanisms underlying the fibrotic response have not been elucidated, but a number of studies have implicated an imbalance between reactive oxygen species and available antioxidant defenses, principally glutathione (GSH) (2–4).

γ-Glutamyl transpeptidase (GGT) is a membrane-bound enzyme that plays an essential role in the metabolism of GSH. Being involved in cellular processes dependent on the oxidation/reduction of GSH, GGT is markedly upregulated in response to a variety of lung oxidant injuries, including nitrogen dioxide, hyperoxia, ozone, and quinones (5–8). Likewise, it has been shown that in type 2 epithelial cells, GGT activity is also increased during the inflammatory phase of the bleomycin-induced lung damage in rats (9). On the other hand, although not all of the functions of the γ glutamyl cycle are known, it has been suggested that one of the major functions of GGT is the amino acid supply through glutathione metabolism, especially cysteine (10).

Recently, a GGT-deficient mouse ($GGT^{-/-}$) was generated (11); these animals appear normal at birth but grow and mature slowly and undergo premature death. The $GGT^{-/-}$ mouse phenotype is characterized by glutathionuria and as a result became cysteine deficient (11, 12). Feeding N-acetylcysteine (NAC) to replace lost GSH normalized growth and reversed most of the pathologic effects observed in $GGT^{-/-}$ mice.

Amino acid availability regulates collagen expression, and it has been recently demonstrated that human lung fibroblasts exposed to amino acid deprivation reduces type I collagen mRNA expression by decreasing both transcription rate and transcript stability (13). Moreover, in the absence or with low concentrations of cysteine, fibroblasts produce undetectable levels of α1(I) procollagen protein (14). With these ideas in mind, we designed a study to examine the pulmonary response to a fibrogenic insult in GGT-deficient mice with the idea of evaluating the roles of decreased levels of lung GSH and low levels of cysteine in extracellular matrix turnover. We hypothesized that GGT null mice would develop less fibrosis than the wild-type ($GGT^{+/+}$) mice because cysteine deficiency would impair extracellular matrix synthesis. Some of the results of this study have been previously reported in the form of an abstract (15).

METHODS

Animals

The study was approved by the Ethical Committee at the National Institute of Respiratory Diseases. C57BL/6/129SvEv-GGT–deficient (−/−) mice and wild-type (+/+) littermates 7 to 8 weeks old were used. GGT-deficient mice were generated as previously described (11). Additional detail is provided in the online supplement.

Bleomycin Treatment

Bleomycin (Bristol Laboratories, Syracuse, NY) was administered intratracheally (0.3 U/10 g). Control animals were instilled with saline solution. Mice were killed at 72 hours and 1 month after bleomycin or saline instillation. In parallel experiments, cysteine intake was supplemented with NAC. $GGT^{-/-}$ and $GGT^{+/+}$ mice were instilled with bleomycin, and 2 days after, they were fed with NAC by dissolving it (10 mg/ml) in the drinking water (11). Animals were killed 1 month after bleomycin exposure.

Unless otherwise specified, for every variable analyzed, six to eight control and experimental mice were used.

Semiquantitative Evaluation of Lung Lesions

Lung sections were stained with hematoxylin and eosin and Masson trichrome and were coded and scored blindly for severity and extent

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of the lesions and percentage of fibrosis as previously described (16). Additional details are provided in the online data supplement. A fibrosis score was determined by multiplying extent of the lesion × percentage of fibrosis.

Immunohistochemistry

Sections were incubated with antimouse anti–matrix metalloproteinase (MMP)-9 (Chemicon, Temecula, CA) at 4°C overnight. A secondary biotinylated anti-immunoglobulin followed by horseradish peroxidase-conjugated streptavidin (BioGenex, San Ramon, CA) was used according to manufacturer. 3-Amino-9-ethyl-carbazole (BioGenex) was used as substrate (16). The primary antibody was replaced by nonimmune serum for negative control slides. Additional detail is provided in online supplement.

Bronchoalveolar Lavage

In a parallel experiment, bronchoalveolar lavage (BAL) was performed in control wild-type and $GGT^{-/-}$ mice and after 3 days after 1 month of bleomycin instillation. Lungs were lavaged by flushing with two aliquots of 250 μL of saline solution. Additional detail is provided in the online supplement. Hematoxylin and eosin stained slides were used for differential cell counting.

Measurement of Hydroxyproline

Lungs were analyzed for collagen content, as described by Woessner (17). Data are expressed as micrograms of hydroxyproline per milligram dry weight. Additional detail is provided in the online supplement.

Lung Tissue and BAL Gelatin Zymography

Lung samples from $GGT^{+/+}$ and $GGT^{-/-}$, both control subjects, and after 72 hours of bleomycin instillation were homogenized and supernatants containing 5 μg of protein were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (8.5%) containing gelatin (1 mg/ml) and a final concentration of 0.3 mg/ml heparin (18). For BAL zymograms, fluid samples containing 5 μg of protein also from control subjects and 72 hours of bleomycin were used. Additional detail is provided in the online supplement.

GSH and Cysteine Measurements

GSH and cysteine levels of lungs from bleomycin-treated and control mice were determined as described by Kleinman and Richie (19) using HPLC equipped with an electrochemical detector (ESA, Boston, MA). Additional detail is provided in the online supplement.

Statistical Analysis

Values presented here are mean ± SD. Differences between genetically altered mice and littermates were analyzed by using the Tukey's test. A p value of less than 0.05 was considered statistically significant.

RESULTS

As previously described (11), GGT-deficient mice grew and gained weight slowly and by 6–8 weeks weighed significantly less than wild-type littermates (13.7 ± 3.7 versus 20.9 ± 2.9 g, respectively).

GSH and Cysteine Determinations

Both cysteine and GSH levels were significantly decreased in the lungs from control saline-instilled GGT-deficient mice in comparison to the control wild-type mice (Table 1). After 72 hours of bleomycin instillation, GSH and cysteine levels were significantly decreased in both wild-type and $GGT^{-/-}$ mice, but levels reached their respective control values at 1 month after bleomycin treatment (Table 1). With the exception of the GSH levels at 72 hours after bleomycin, mutant mice always had values that were significantly lower than their littermates.

Lung Histology and BAL

Normal nontreated lungs from GGT null mice were smaller but did not display any differences compared with normal lungs of wild-type animals (Figures 1A and 1B). At 3 days after bleomycin instillation, GGT-deficient mice and wild-type mice developed patchy areas of inflammation throughout the lung parenchyma. Figures 1C and 1D illustrate $GGT^{-/-}$ and wild-type mice early response to bleomycin. As shown in Table 2, the severity and extent of the inflammatory response to bleomycin showed no significant differences among $GGT^{-/-}$ and wild-type at 72 hours after instillation. However, the type of inflammatory cell involved was different. At 3 days after instillation, the inflammatory process in the wild-type mice was primarily characterized by accumulation of neutrophils and lymphocytes (Figure 1E). In contrast, inflammation in $GGT^{-/-}$ showed a predominance of lymphocytes and macrophages with few polymorphonuclear cells (Figures 1F and 1G). Similar results were revealed by the cell profile analysis from BAL fluid (Table 3). GGT-deficient mice displayed approximately half of the total cells recovered in BAL compared with the wild type, but in both, a similar percentage of increase was observed after 72 hours and 1 month after bleomycin instillation. The difference in cellularity probably correlates with the GGT-null mouse phenotype, as it also has been reported that they present a 50% reduction in thymus and spleen cellularity (20). However, although an important increase of lymphocytes and neutrophils was observed in the wild-type after 72 hours of bleomycin instillation, $GGT^{-/-}$ exhibited a noteworthy increase of lymphocytes (approximately 40% of the total cells recovered) with a mild increase of neutrophils (Table 3). This divergence cannot be attributed to a difference in circulating leukocytes as similar numbers are found in $GGT^{+/+}$ and $GGT^{-/-}$ per mm^3 (5,498 ± 1,056 versus 4,750 ± 688, n = 5).

At 1 month after bleomycin exposure, the lungs of wild genotype mice showed mostly a mononuclear inflammation with large areas of fibrosis characterized by young connective tissue rich in fibroblasts together with more dense bands of collagen replacing the lung parenchyma, as observed by Masson trichrome staining (Figures 2A and 2C). In contrast, bleomycin-injured GGT-deficient mice showed usually minimal areas of lesion and scarce collagen accumulation (Figures 2B and 2D). The semiquantitative analysis of the histologic changes revealed a significant decrease in the extent of the lesions in $GGT^{-/-}$ mice compared with wild-type littermate (Table 2). Likewise, the fibrosis score determined multiplying the extent of the lesion by the percent of fibrosis was also significantly reduced in the transgenic mice (510 ± 756 versus 1,975 ± 817 from wild-type mice, p <

TABLE 1. THIOL LEVELS IN BLEOMYCIN-TREATED AND CONTROL MICE

Treatment	Cysteine	GSH
Wild type	0.055 ± 0.001*†	1.79 ± 0.065¶**
$GGT^{-/-}$	0.030 ± 0.005‡	1.24 ± 0.055††
Wild-type +Bleo 72 h	0.036 ± 0.001§	0.74 ± 0.034
$GGT^{-/-}$ + Bleo 72 h	0.017 ± 0.002	0.616 ± 0.030
Wild-type +Bleo 1 mo	0.064 ± 0.008‖	1.77 ± 0.039‖
$GGT^{-/-}$ + Bleo 1 mo	0.030 ± 0.002	1.38 ± 0.057

Definition of abbreviations: GGT = γ-glutamyl transpeptidase; GSH = glutathione.

Values presented here are mean ± SD.

* $p < 0.02$ compared with $GGT^{-/-}$.

† $p < 0.0003$ compared with wild-type plus bleomycin 72 hours.

‡ $p < 0.05$ compared with $GGT^{-/-}$ plus bleomycin 72 hours.

§ $p < 0.01$ compared with $GGT^{-/-}$ plus bleomycin 72 hours.

‖ $p < 0.01$ compared with $GGT^{-/-}$ plus bleomycin 1 month.

¶ $p < 0.002$ compared with $GGT^{-/-}$.

** $p < 0.0004$ compared with wild-type plus bleomycin 72 hours.

†† $p < 0.001$ compared with wild-type plus bleomycin 72 hours.

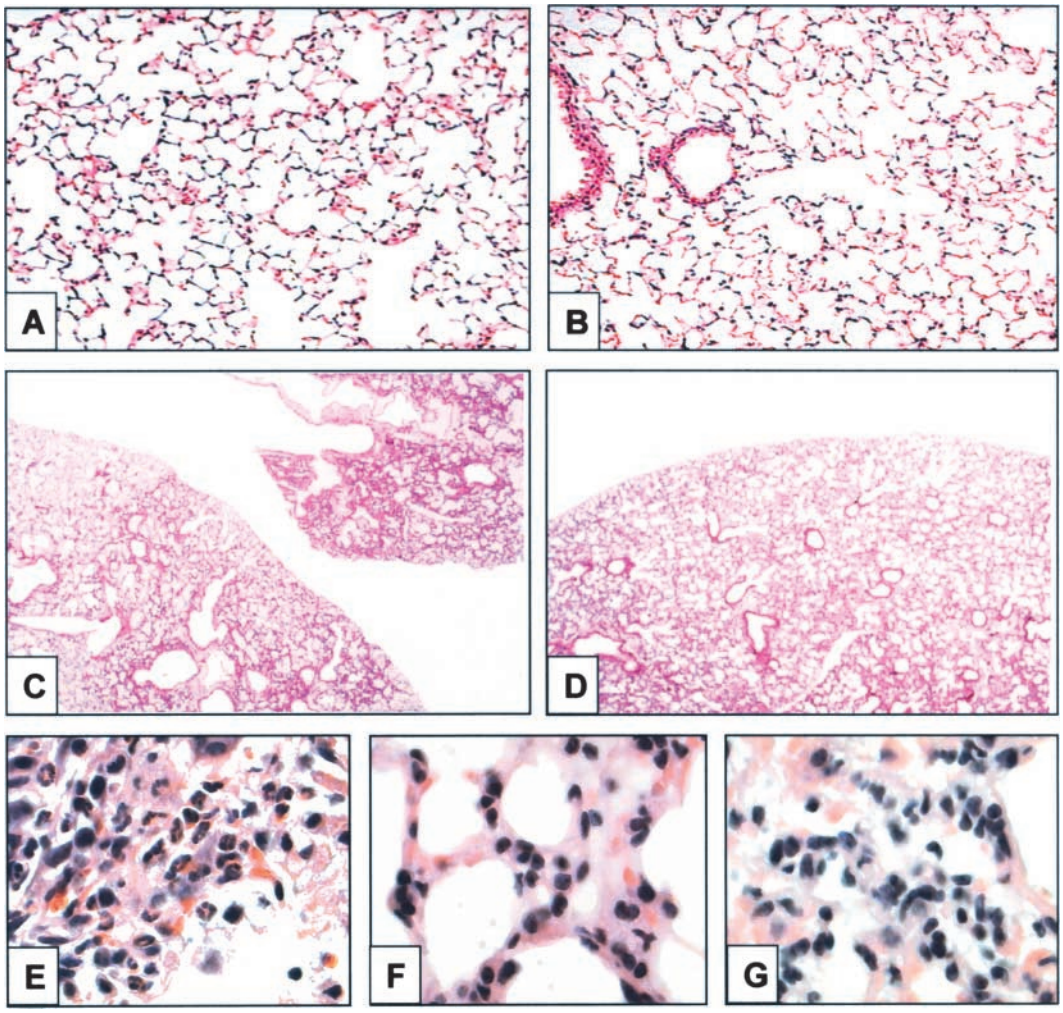

Figure 1. Photomicrographs of tissue sections from normal lungs and 72 hours after bleomycin instillation. (*A* and *B*) Wild genotype and GGT-null lungs at baseline (hematoxylin and eosin, original magnification ×10). (*C* and *E*) Wild-type mice 72 hours after bleomycin (hematoxylin and eosin, ×2.5 and ×40). (*D, F,* and *G*) GGT-deficient mice 72 hours after bleomycin (hematoxylin and eosin, ×2.5 and ×40).

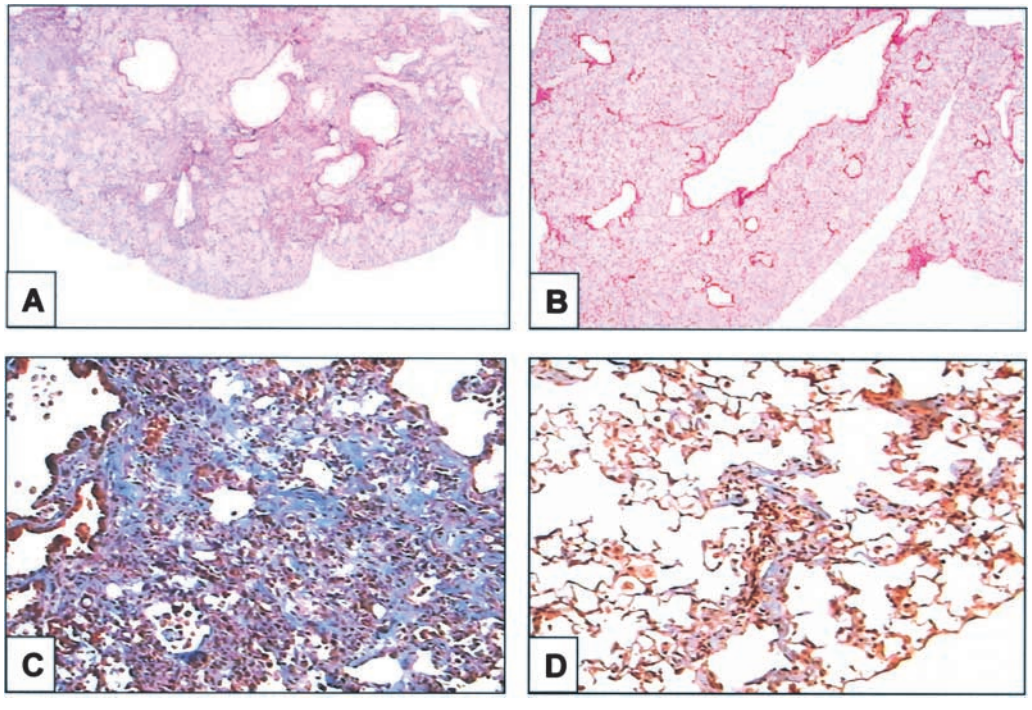

Figure 2. Light micrographs of lung sections from bleomycin-treated mice killed at 1 month. (*A* and *C*) Wild-type mice. (*B* and *D*) GGT-deficient mice. (*A* and *B*) hematoxylin and eosin staining, original magnification ×2.5. (*C* and *D*) Masson trichrome staining ×10. The photomicrographs are representative of six to eight animals from each experimental group and were selected to illustrate the extent and pattern of lung fibrosis.

TABLE 2. SEMIQUANTITATIVE ANALYSIS OF THE MORPHOLOGIC CHANGES

	Extent	Severity	Percentage of Fibrosis	Fibrosis Score
Wild-type 72 h	35.7 ± 9.8	1.2 ± 0.4	< 5	
GGT$^{-/-}$ 72 h	35.0 ± 10.5	1.6 ± 0.5	< 5	
Wild-type 1 mo	43.8 ± 10.6*	2.5 ± 0.5	44.4 ± 11.2†	1,975 ± 817*
GGT$^{-/-}$ 1 mo	13.3 ± 13.7	1.9 ± 0.7	26.7 ± 18.5	510 ± 756

Definition of abbreviation: GGT = γ-glutamyl transpeptidase.
Values presented here are mean ± SD.
* $p < 0.01$ compared with GGT$^{-/-}$ 1 month.
† $p < 0.05$ compared with GGT$^{-/-}$ 1 month.

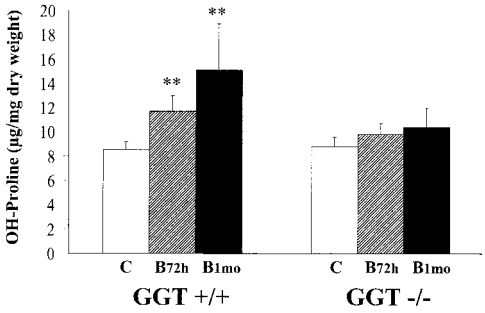

Figure 3. Effect of bleomycin instillation on lung hydroxyproline in wild-type (GGT$^{+/+}$) and GGT-deficient (GGT$^{-/-}$) mice at 72 hours and 1 month. **$p < 0.01$.

0.01). BAL cell profile showed no difference, with macrophages being most of the recovered inflammatory cells (Table 3).

Collagen Content of Normal and Bleomycin-treated Lungs

Lung collagen accumulation was quantified by measuring hydroxyproline content (Figure 3). Lung hydroxyproline in noninjured wild-type and GGT-deficient mice did not show significant differences (8.5 ± 0.7 and 8.8 ± 0.8 μg OH-proline/mg dry weight). At 72 hours after bleomycin instillation, a moderate but significant increase in lung hydroxyproline was noticed in wild-type mice when compared with nonexposed littermates (11.7 ± 1.3 μg OH-proline/mg dry weight, $p < 0.01$). In contrast, no changes were observed in GGT-null mice.

At 1 month after bleomycin exposure, lung hydroxyproline in wild-type mice was further increased as compared with 72-hour or saline-exposed animals (15.1 ± 3.8 μg of OH-proline/mg dry weight; $p < 0.05$ versus 72 hours, and $p < 0.01$ versus saline, respectively). In contrast, GGT-deficient mice showed a marginal but not significant increase of hydroxyproline content when compared with 72-hour or saline-exposed animals (Figure 3).

Zymography of Lung Tissue Extracts and BAL

To analyze gelatinases behavior, lung tissue obtained from wild-type and GGT$^{-/-}$ mice in both control animals and 72-hour bleomycin-treated animals was examined. A lung tissue zymogram showing two representative samples per group is illustrated in Figure 4A. ProMMP-9 band was significantly increased in lung samples from bleomycin-injured wild-type as compared with bleomycin-injured GGT null mice. A twofold increase was revealed by densitometric quantification of the surface and intensity of lysis bands of zymograms derived from five different animals in each group. Likewise, a twofold increase of MMP-9 active form was also observed in wild-type bleomycin-treated mice as compared with GGT-null mice. When samples were treated with aminophenylmercuric-acetate, which promotes processing of proMMPs to lower molecular weight active forms, it became evident that total MMP-9 was higher in bleomycin wild-type compared with GGT$^{-/-}$ mice (data not shown). ProMMP-2 and MMP-2 seemed to be increased in some bleomycin-treated mice mainly in wild-type animals, but no statistical significant differences were found. Lower molecular weight activity bands that could represent MMP-13 because they run at the same level of MMP-13 standard (data not shown) were also increased in bleomycin treated wild-type animals as compared with both control and bleomycin-treated GGT-null mice (Figure 4A). All bands were inhibited when the gels were incubated in the presence of 20 mM of ethylenediaminetetraacetic acid (data not shown).

BAL fluid samples were also examined by gelatin zymography (Figure 4B). ProMMP-9 was not revealed in BAL from control GGT$^{+/+}$ and GGT$^{-/-}$. After 72 hours of bleomycin injury, wild-type exhibited an important increase of proMMP-9 and its lower molecular weight active form. In contrast, bleomycin-treated GGT$^{-/-}$ mice displayed a small increase of proMMP-9. By densitometric analysis, an approximately ninefold difference was quantified ($p < 0.01$, $n = 4$; Figure 4B). MMP-9 active form was increased in bleomycin-injured animals both wild-type and GGT$^{-/-}$ mice as compared with respective control subjects. ProMMP-2 activity was increased in both wild-type and GGT$^{-/-}$ as compared with control BAL.

Localization of MMP-9 Immunoreactive Protein in Lung Tissue

The cellular source of MMP-9 was examined by immunohistochemistry on tissue sections from wild-type and GGT$^{-/-}$ control subjects and after 72 hours of bleomycin injury. Positive staining was always more prominent in wild-type bleomycin-injured mice, and a strong signal was observed in numerous macrophages as well as neutrophils and bronchiolar epithelial cells (Figure 5A). In GGT-null bleomycin-treated lungs, MMP-9 was found pri-

TABLE 3. BRONCHOALVEOLAR LAVAGE CELL PROFILE

Experimental Groups	Total Cells ($\times 10^3$)	Macrophages	Lymphocytes	Neutrophils	Eosinophils
Wild-type saline	10.4 ± 1.4	9.7 ± 0.56	0.7 + 0.56	0	0
GGT$^{-/-}$ saline	5.7 ± 0.9	5.36 ± 0.1	0.3 ± 0.14	0	0.04 ± 0.05
Wild-type bleomycin 72 h	19.7 ± 6.5	10.2 ± 2.3	4.1 ± 3.9	5.3 ± 2.5	0
GGT$^{-/-}$ bleomycin 72 h	10.1 ± 1.4	5.18 ± 3.5	4.17 ± 3.1	0.67 ± 0.8	0.07 ± 0.1
Wild-type bleomycin 1 mo	38.6 ± 8.3	35.0 ± 3.2	3.3 ± 2.0	0.19 ± 0.3	0.07 ± 0.15
GGT$^{-/-}$ bleomycin 1 mo	23.5 ± 3.9	20.9 ± 1.6	2.51 ± 1.41	0	0.07 ± 0.14

Definition of abbreviation: GGT = γ-glutamyl transpeptidase.
Values presented here are mean ± SD.

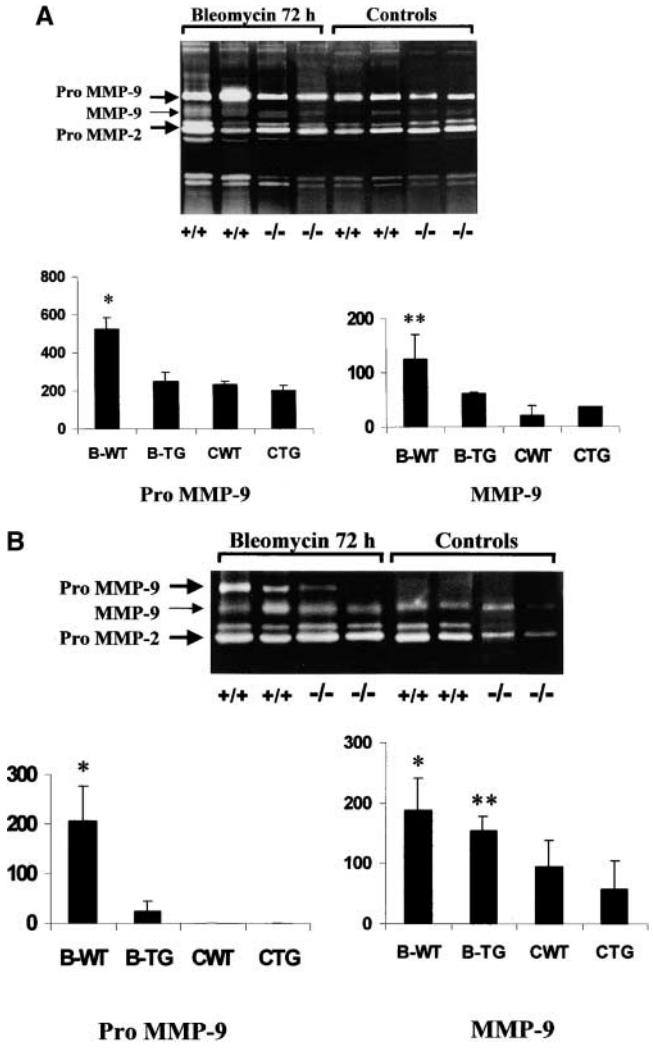

Figure 4. Identification of gelatinolytic activities in lung tissues (*A*) and BAL fluid (*B*). Supernatants from lung tissue extracts and BAL fluid derived from bleomycin-instilled wild-type (+/+), GGT-deficient mice (−/−), and control animals were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (8.5%) containing gelatin (1 mg/ml) and a final concentration of 0.3 mg/ml heparin. Zones of enzymatic activity appear as clear bands over a dark background. Densitometric analysis of four to five animals in each group is shown in the graphics. Results are expressed as activity in arbitrary units. (*A*) *$p < 0.01$; **$p < 0.05$. (*B*) ^$p < 0.001$; *$p < 0.05$ compared with the control wild type. **$p < 0.05$ compared with the control GGT-deficient mice. *B-TG* = Bleomycin, GGT-deficient mice; *B-WT* = Bleomycin, wild-type mice; *CTG* = control, GGT-deficient mice; *CWT* = control, wild-type mice.

marily in scattered alveolar macrophages (Figure 5B). Immunohistochemical staining for MMP-9 was negative in normal lungs of both wild-type and GGT-null mice (Figure 5C). Lung tissue samples incubated with nonimmune sera were also negative (Figure 5D).

NAC Supplementation and Response to Bleomycin

Supplementation of cysteine intake with NAC normalizes the growth curve and partially reverses the phenotype of GGT$^{-/-}$ mice (11). Likewise, NAC supplementation of GGT-deficient mice reverses low GSH levels (21). On the other hand, because it has been claimed that NAC may decrease the lung fibrotic response in normal rodents (22), we fed wild-type and GGT-null mice with NAC starting 2 days after bleomycin instillation. Thus, GGT$^{-/-}$ mice received bleomycin with their GGT-null phenotype, and after the initial lung damage, they received NAC. Results of this experiment are shown in Table 4. NAC supplementation increased the extent of the lesion in the GGT$^{-/-}$ mice, but the fibrotic index was still significantly lower 1 month after bleomycin than the wild-type plus NAC.

Regarding collagen content, bleomycin injury in NAC-treated GGT$^{-/-}$ mice provoked a significant increase of lung hydroxyproline as compared with control saline-treated mice (11.8 ± 1.4 versus 8.8 ± 0.8, $p < 0.01$). However, lung hydroxyproline was still significantly lower in NAC-treated GGT$^{-/-}$ mice compared with NAC-treated wild type (Table 4).

DISCUSSION

In this study, we compared the initial inflammatory and late fibrotic responses to bleomycin in GGT-deficient mice and wild-type littermates. Bleomycin administration to wild-type mice caused an initial pneumonitis that evolved to fibrosis. In contrast, GGT$^{-/-}$ developed a similar early inflammatory response as measured by severity and extent but appeared to be largely protected from the late fibrotic effects of bleomycin.

Interestingly, the type of inflammatory cells observed at 72 hours after bleomycin instillation exhibited important differences. Thus, although in the wild-type mice there was a neutrophilic/lymphocytic inflammatory response, GGT-deficient mice displayed a predominantly mononuclear cell reaction with few polymorphonuclear cells. The early alveolitis noticed in wild-type mice is similar to that reported in this and other species where a remarkable infiltration of neutrophils characterizes the initial inflammatory response to bleomycin (23–25).

A prominent finding of this work was the demonstration that GGT-deficient mice develop a markedly lesser fibrotic response than the wild-type mice after 1 month bleomycin-induced lung injury. Thus, lung morphology in GGT$^{-/-}$ mice showed only few small lesions with reduced collagen staining, and lung hydroxyproline content was similar to GGT-deficient nontreated mice.

An oxidant/antioxidant imbalance has been proposed as a pathogenic mechanism contributing to lung damage and inflammation in patients with pulmonary fibrosis. In this context and at least theoretically, GSH deficiency might enhance bleomycin-induced fibrosis, as this molecule is the most important extracellular antioxidant in the lung. In fact, there is evidence suggesting that an increased oxidant burden and a deficiency of GSH may play a role in the pathogenesis of fibrosing alveolitis (2–4). Moreover, there are several findings supporting that GSH can directly affect inflammation by inhibiting the production or function of several inflammatory cytokines and chemokines, such as tumor necrosis factor-α, monocyte chemotactic protein-1, and some chemokine receptors (26–28). In addition, the lack of GGT impairs the mouse immune system affecting the immune responsiveness, suggesting an important immunoregulatory role for GSH (20). Likewise, the use of exogenous antioxidants attenuates the severity of the fibrosis and suppressed the increases in lymphocyte and neutrophil counts, in bleomycin-induced lung injury (29). Thus, according to these studies, it would be expected that GGT-deficient mice, in opposite to our findings, would develop a more aggressive fibrosis.

Three different mechanisms may at least partially explain this paradox. In part, the reduced fibrotic response of the GGT$^{-/-}$ mice might be related to the differences noticed in the early inflammatory response. As mentioned, wild-type mice displayed a neutrophilic reaction and showed more fibrosis. It has been suggested that persistence of neutrophils may enhance lung fi-

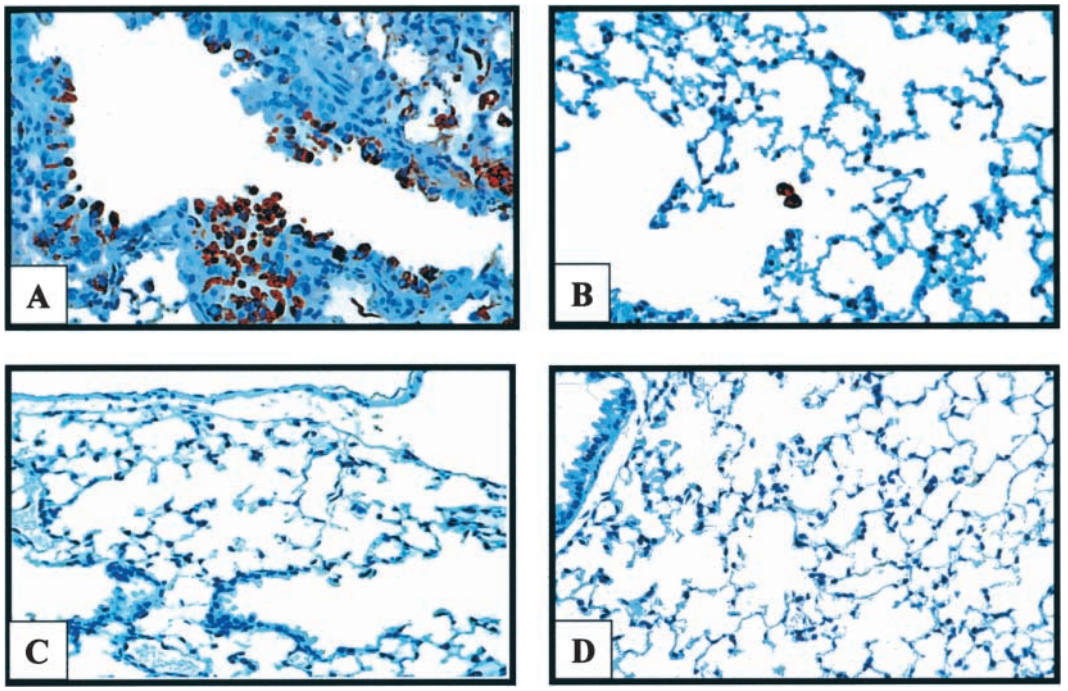

Figure 5. Localization of MMP-9 in wild-type and GGT$^{-/-}$ lung samples. Immunoreactive MMP-9 was revealed by 3,3′-diaminobenzidine. (*A*) Numerous MMP-9 staining cells are noticed in wild-type lung (40×). (*B*) An area of positive stained alveolar macrophages in GGT$^{-/-}$ lung (40×). (*C*) Wild-type control lung. No reactivity was observed in normal lungs of both wild-type and GGT$^{-/-}$. (*D*) Negative control section from a GGT$^{-/-}$ in which the primary antibody was omitted. Samples were counterstained with hematoxylin.

brosis to a number of injuries. Thus, in experimental lung fibrosis induced by silica or bleomycin, the increase of neutrophils and duration of tissue neutrophil activation has been correlated with chronic alveolitis progressing to fibrosis (30).

Likewise, an increase of BAL neutrophils has been found in a number of interstitial lung diseases that result in fibrosis (31, 32). Furthermore, neutrophil migration and activation are usually higher in patients with more aggressive and worse prognosis fibrotic lung disorder (33), and increased tissue neutrophils loaded with MMP-9 and collagenase-2 are implicated in the fibrotic reaction of patients with chronic hypersensitivity pneumonitis (15).

Another potential fibrogenic mechanism that may also contribute to the different fibrotic response found in this study is related with the participation of MMPs. Particularly, an increased lung expression of MMP-9 has been demonstrated in experimental and human pulmonary fibrosis (15, 17, 34, 35). Excessive MMP-9 activity may contribute to the loss of integrity of the basement membranes and subsequently plays a fibrogenic role. Thus, in bleomycin-induced lung fibrosis, a coincidental increase of MMP-9 activity and disruption of the alveolar epithelial basement membrane has been documented (36). On the other hand, cell surface–localized MMP-9 proteolytically cleaves latent transforming growth factor-β, a prototype of profibrotic growth factor, providing a potentially important mechanism for transforming growth factor-β activation and tissue remodeling (37).

The possible role of MMP-9 in lung fibrogenesis is further supported by experiments performed in MMP-9–deficient mice that develop lesser fibrotic lesions compared with MMP-9$^{+/+}$ littermates when exposed to bleomycin (38). Also, transgenic mice overexpressing interleukin-13 develop pulmonary fibrosis associated with MMP-9 upregulation and transforming growth factor-β activation (39). Furthermore, in BAL fluid samples from rapidly progressive idiopathic pulmonary fibrosis, a noteworthy increase of neutrophil-derived MMP-9 activity has been characteristically detected (40).

In this context, our findings of a different MMP-9 expression in both BAL and lung tissues of wild-type compared with GGT$^{-/-}$ mice support a possible role for this enzyme. GGT-deficient mice displayed a lower MMP-9 expression after injury and subsequently a slighter fibrosis than wild type.

Additionally, reduced lung MMP-9 activity in GGT$^{-/-}$ mice can be related to the lesser neutrophilic response, as these cells are a major source of this enzyme. In this context, it has been shown that inhibition of LPS-induced airway neutrophilic infil-

TABLE 4. LUNG RESPONSE TO N-ACETYLCYSTEINE ADMINISTRATION

Bleomycin 1 Month	Extent of the Lesion	Percentage of Fibrosis	Fibrosis Score	Lung Hydroxyproline
Wild-type	43.8 ± 10.6	44.4 ± 11.2	1975 ± 817	15.1 ± 3.8
GGT$^{-/-}$	13.3 ± 10.6	26.7 ± 18.5	510 ± 756	10.4 ± 1.6
Wild-type plus NAC	40.0 ± 21.6	45.0 ± 12.9	2,000 ± 1,397†	13.7 ± 0.8‡
GGT$^{-/-}$ plus NAC	25.0 ± 10.0*	22 0.0 ± 11	600 ± 561	11.8 ± 1.4§

Definition of abbreviations: GGT = γ-glutamyl transpeptidase; NAC = N-acetylcysteine.
Values presented here are mean ± SD.
* $p < 0.05$ compared with GGT$^{-/-}$.
† $p < 0.05$ compared with GGT$^{-/-}$ 1 month plus NAC.
‡ $p < 0.05$ compared with GGT$^{-/-}$ 1 month plus NAC.
§ $p < 0.01$ compared with levels obtained in saline-treated GGT$^{-/-}$ (8.8 ± 0.8, μg OH-proline/mg dry weight; *see* Figure 3).

tration in the guinea pig reduced MMP-9 activity recovered from inflamed guinea pig airways (41).

A third mechanism to consider for explaining the lower lung fibrotic response in GGT$^{-/-}$ mice is related to the role of this enzyme in cysteine homeostasis. GGT catalyzes the first step in the extracellular hydrolysis of GSH and plays a critical role in GSH recycling. GSH is synthesized *de novo* from an intracellular pool of glutamine, glycine, and cysteine and is degraded extracellularly in a two step process catalyzed by GGT and a dipeptidase. As a result of this γ-glutamyl cycle, GSH can be degraded and its constituent amino acids reused. In this context, in normal conditions the extracellular space contain an available pool of cysteine that is the delimiting substrate for GSH biosynthesis.

Actually, results obtained in GGT-null mice support the notion that the cleavage of GSH is a major source of circulating cysteine (11). Without this source of reutilization of cysteine, normal dietary intake is insufficient to compensate its loss as GSH. Supporting this point of view, we found markedly decreased levels of lung cysteine in GGT$^{-/-}$ mice either with saline instillation (control subjects) or after bleomycin exposure. In addition, the increase of the fibrotic lung response after NAC supplementation in GGT-deficient mice supports this notion. GGT-null mice receiving NAC augmented hydroxyproline content after bleomycin injury compared with their nontreated littermates, although continued being lower than the wild-type mice as the fibrotic index did.

A lack of cysteine may have a profound effect on extracellular matrix turnover. There is evidence demonstrating that amino acid deprivation markedly decreases newly synthesized type I collagen by human lung fibroblasts, probably by intracellular degradation of improperly folded molecules (14). Moreover, this effect was shown to be specific for collagen as fibronectin levels, and total protein levels were not affected. In addition, it has been shown that depletion of amino acids also decreases α1(I) collagen mRNA levels and repletion of amino acids induces a rapid re-expression of α1(I) mRNA. More importantly, this effect seems to be critically dependent on cysteine but not on other amino acids (42).

Cysteine deficiency may also affect cysteine-rich proteins implicated in connective tissue remodeling. For example, it has been shown that SPARC (secreted protein acidic and rich in cysteine)-null mice exhibited attenuated collagen accumulation after bleomycin injury (43). SPARC is a unique matricellular gycoprotein that has been implicated as playing an important role in tissue repair (44). SPARC is coexpressed with type I collagen, and it has been associated with tissues undergoing extracellular matrix remodeling and fibrosis (45, 46). Likewise, decreased cysteine may limit synthesis of cysteinyl leukotrienes, which may also contribute to the protection from lung fibrosis, as it has been recently demonstrated in the leukotriene-deficient mice (47).

In conclusion, our results demonstrated that GGT$^{-/-}$ mice develop a markedly decreased fibrotic response after bleomycin injury. This finding was related to a lower neutrophilic alveolitis and MMP-9 expression, and a deficiency of lung cysteine, suggesting potential mechanisms for lung structural remodeling after a fibrogenic insult.

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