

Response of Mouse Lung to Carbon Deposition During Injury and Repair

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Increased respiratory disease and daily mortality rates are associated with higher levels of fine particulate air pollutants. We examined the possibility that deposition of even inert particles to previously injured lungs may accentuate pulmonary damage by investigating how the lung handles small carbon particles delivered during acute injury or during fibrotic repair. Mice received 2 mg carbon by intratracheal instillation into lungs already showing acute injury, 3 days after bleomycin (BL), or into lungs with fibrosis, 4 weeks after BL. At 3 days after BL, injury to the type I alveolar epithelium resulted in high protein levels in lavage fluid. Instilling carbon at this time induced a large increase in inflammatory cells, though many particles reached the interstitium, and a high proportion was retained up to 16 weeks later. However, fibrosis in these mice was equal to that found after BL alone. In the mice that received carbon 4 weeks after bleomycin, fibrotic repair had already occurred, and the epithelial surface was restored before particle instillation. After carbon, the subsequent inflammatory reaction cleared most particles, little reached the interstitium, and carbon retained at 16 weeks was not different from that in the carbon-only group. Instilling particles into fibrotic lung did not induce additional fibroblast growth or collagen production. The results indicate that instillation of fine particulates to the alveoli at a time of epithelial injury results in increased translocation to the interstitium. However, deposition of pure carbon into injured lungs does not further stimulate an ongoing fibrotic process, although it alters the patterns of particle deposition and retention in the lung. *Key words:* air pollution, carbon particulates, bleomycin, lung injury, phagocytosis. *Environ Health Perspect* 103:72-76 (1995)

Human exposure to various inhaled particulates may result in lung disease, which has been attributed to the nature and toxicity of specific particle types, for example silica-induced pulmonary fibrosis (1-3). One of the most common inhaled air pollutants is the carbon particle, and deposits are frequently seen upon autopsy of human lungs, particularly lungs of city dwellers (1,4). It is generally assumed that carbon itself is inert, and any associated lung disease arises from the effects of co-inhaled toxic substances, such as mineral dusts, metal ions, or acidic gases. Recent epidemiologic evidence, however, points to the need for more investigation into the health effects of inhaled particulate matter in general. Studies now link exposure to fine par-

ticles with increased mortality, and there is evidence that diminished lung function in persons with lung disease accompanies inhalation of particulate air pollutants (5-8). In a recent study, the daily mortality rate in six U.S. cities was associated with the level of air pollution, after correcting for other factors such as smoking (8). The strongest correlation with mortality was found in levels of fine respirable particles rather than with aerosol acids or gases such as nitrogen dioxide (8).

Many experimental studies have been published to describe the deposition, clearance, and pathologic effects, if any, of various particles introduced into normal lungs (9,10), but not much is known about possible synergistic effects of depositing particles, whether toxic or inert, into abnormal lungs. Fine particulates (< 2.5 μm diameter) such as soot can reach the most distal regions of the lung, and although such particles are usually considered inert, there is little information on their effects on previously injured lungs. If fine particles are deposited into alveoli during an acute injury phase, damage at the air-blood barrier may increase particle translocation to the interstitium (11). On the other hand, it is also possible that the increased number of inflammatory cells recruited to the alveoli could enhance phagocytosis and subsequent clearance. There may also be differences in deposition and clearance if particles are introduced into a fibrotic lung in which function as well as structure is altered (12). In many examples of fibrosis, activation of macrophages is believed to be a key event in cytokine production (13,14). How these activated pulmonary macrophages then respond, even to an inert particle, is not known. During phagocytosis and attempted clearance, there may be synergistic effects within the macrophage to increase cytokine secretion so that phagocytosis of an inert particle may become an extra fibrogenic stimulus.

We studied these questions on how the injured lung responds to deposition of fine particulates using intratracheal (IT) instillation of colloidal carbon as an inert particle. Carbon was given to mice a few days after IT bleomycin, which induces acute injury to the alveolar wall, and to mice 4 weeks after bleomycin when the epithelial surface is restored but fibrosis has developed. Lung responses such as injury, inflammatory cell numbers, particle

retention, and the production of fibrosis were compared over a subsequent 16-week period.

Materials and Methods

Swiss mice (25-g males) were divided into 5 groups of 40 which received intratracheal injections while under mild nembuthal anesthesia. Group 1 received 2 mg of a carbon suspension (carbon black in hydrolyzed gelatin, 40-nm diameter; Faber-Castell Corp., Newark, New Jersey) in 0.1 ml water; group 2 received 0.15 units of bleomycin (Bristol-Myers Squibb Co., Evansville, Indiana) suspended in 0.1 ml water; group 3 received the same dose of bleomycin, then 3 days later received 2 mg carbon; group 4 was instilled with bleomycin, then the same dose of carbon 4 weeks later; and group 5 received 0.1 ml distilled water only. Animals were killed in groups of four by barbiturate overdose at the following times after bleomycin or water (groups 2 and 5) or after carbon (groups 1, 3, 4): days 0, 1 and 3, weeks 1, 2, 4, 8, 12, and 16; mice received 2 $\mu\text{Ci/g}$ tritiated thymidine 1 hr before death.

A tracheotomy was performed on each animal, and the lungs were washed four times with 1 ml saline. We pooled the bronchoalveolar lavage (BAL) fluid and determined the total cell count by hemocytometer. The cell suspension was centrifuged; a cytospin preparation was made and stained by the Giemsa method; differential counts of polymorphonuclear leukocytes (PMN) and alveolar macrophages (AM) were made on 500 cells per slide. We determined the total number of cells of each type for each time studied, and the mean \pm SE for four mice per group was calculated. We used the supernatant of the lavage fluid from each mouse to determine total protein content as an index of lung injury (15,16).

After lavage, the bronchus leading to the right lung was clamped; this lung was removed, weighed, and frozen for biochemical analysis. The left lung was inflated with 0.5 ml of 2% buffered glutaraldehyde and removed; most of the tissue was processed for embedding in glycol methacrylate. Sections (0.75 μm thick) from three random blocks per animal were prepared for histology and for autoradiography with the use of Kodak NTB2 emulsion. We determined the percentages of ^3H -thymidine-labeled cells at each time by

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counting 3000 alveolar lung cells per animal. The means and SEs were calculated for each group and plotted as time after carbon instillation. Significant differences were determined using ANOVA or Student's *t*-test as appropriate.

The right lung of each mouse was homogenized in water, and biochemical assays were performed on duplicate samples. We determined total protein, and as an index of collagen content, we determined hydroxyproline (HYP) levels after hydrolysis with hydrochloric acid (16,17). In addition, six extra mice per group were killed at the end of the experiment (16 weeks) to assess the amount of carbon retained in the whole lung. The lungs were removed, immediately chopped, and incubated in 40% potassium hydroxide overnight in a 80°C waterbath. When the tissue was completely digested, the solution was centrifuged at 1500 rpm for 15 min, and a residue was obtained. This was washed twice in distilled water, resuspended in water, and transferred to a weighed tube. The residue was dried to constant weight. Means \pm SEs were calculated for each group of six, and the significance of any difference from control and from carbon only groups was determined.

Results

Lung Morphology

Mice that received only water showed normal lungs by light microscopy. The lungs of animals instilled with only carbon had increased cellularity, particularly in the first 2 weeks when most carbon was phagocytized in alveoli by AM and PMN, as previously described (18,19). A few large AM with carbon persisted for several weeks, and some carbon was seen in interstitial macrophages, particularly in peribronchiolar locations.

Lungs examined 3 days after bleomycin showed diffuse injury to the alveolar wall and an inflammatory response of AM and PMN (Fig. 1A). After these mice received carbon, additional inflammatory cells were seen in the alveoli and, as the experiment progressed, it appeared that more carbon was present in the pulmonary interstitium and remained there as fibrosis developed (Fig. 1B). Lungs examined 4 weeks after bleomycin only showed areas of extensive fibrosis (Fig. 2A). When these animals received carbon, an inflammatory response with increased AM and PMN in the alveoli was seen a few days later, particularly in less fibrotic areas of lung. Most of the carbon appeared to be cleared by 4–8 weeks when the inflammatory response was reduced, and little carbon was found in fibrotic areas of interstitium (Fig. 2B). There were no apparent differences in the

severity of fibrosis seen morphologically in any of the bleomycin groups at the end of the experimental period.

Bronchoalveolar Lavage

Values obtained for the numbers of AM and PMN and for the protein level in water-injected controls were not different from noninjected (time 0) mice and so are not shown at each time to simplify the figures. The number of AM recovered after bleomycin alone increased fourfold in the first week, and, though it fell somewhat, it was still greater than baseline over the 16-week period (Fig. 3). After carbon only, AM numbers peaked at 8–10 times normal, then fell slowly. A similar response in AM numbers was seen in groups that received bleomycin 3 days or 4 weeks before

carbon instillation (Fig. 3). A similar pattern was seen when the PMN response was quantitated. A small increase in lavaged cells was found soon after bleomycin alone, whereas the increase was much greater after carbon injection (Fig. 4). A comparable PMN response in intensity and duration was found after instilling carbon 3 days after bleomycin or 4 weeks later in fibrotic lungs.

To quantitate lung injury, particularly any permeability change at the air–blood barrier, protein levels were measured in BAL fluid. Normal values were low, around 90 μ g/ml, and carbon instillation caused a sharp but transient increase (Fig. 5). A similar pattern was seen in mice with fibrotic lungs due to bleomycin administration 4 weeks before receiving carbon.

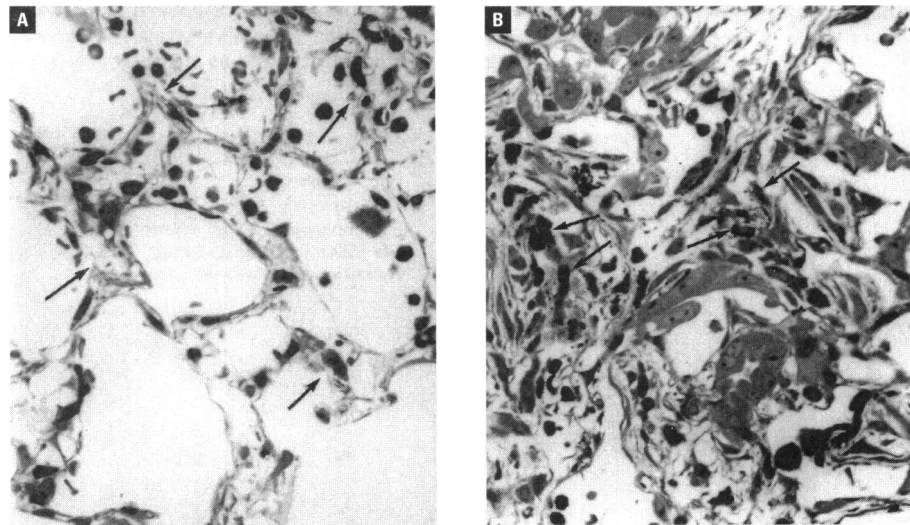


Figure 1. (A) Mouse lung, 3 days after bleomycin, shows diffuse alveolar injury (arrows) with cell debris, red blood cells, and inflammatory cells in alveoli. (B) Lung section from mouse to which carbon was instilled 3 days after bleomycin, killed 8 weeks later. Many carbon-laden macrophages (arrows) are seen in fibrous interstitium. $\times 655$.

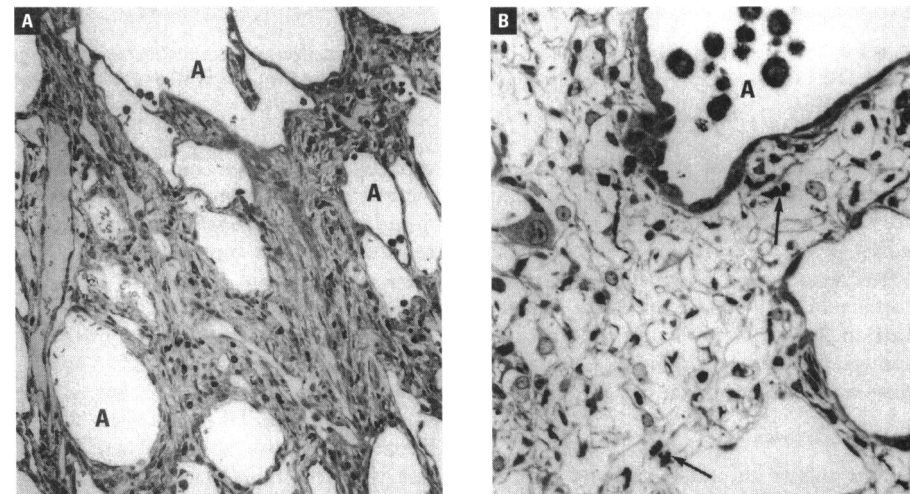


Figure 2. (A) Mouse lung, 4 weeks after bleomycin only, shows extensive interstitial fibrosis while alveoli (A) are mostly clear of cell debris and inflammatory cells. (B) Lung section from mouse to which carbon was instilled 4 weeks after bleomycin and killed 8 weeks later. Little carbon (arrows) is seen in the fibrotic interstitium, while a few carbon-laden alveolar macrophages are seen in the airspace (A). $\times 655$.

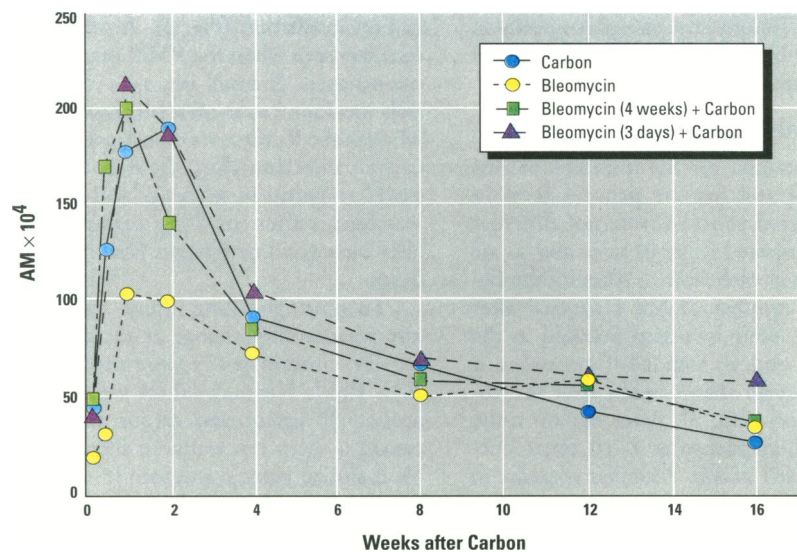


Figure 3. The number of alveolar macrophages (AM) recovered by bronchoalveolar lavage (BAL) from the four treatment groups up to 16 weeks after receiving carbon. There were no statistical differences among the three groups that received carbon.

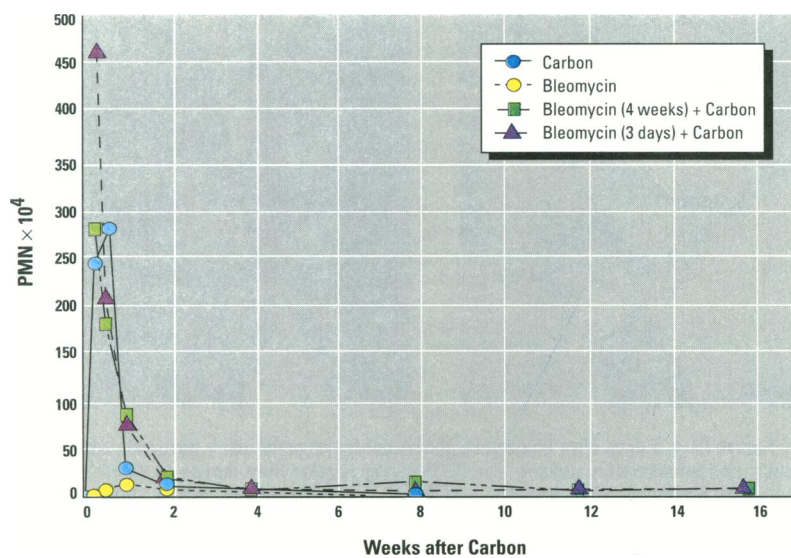


Figure 4. The number of polymorphonuclear leukocytes (PMN) recovered by bronchoalveolar lavage (BAL). The 3-day bleomycin-plus-carbon value was significantly higher than that in other groups at 1 day only ($p < 0.01$).

The highest levels of alveolar protein were found in mice in the acute injury phase up to 1 week after bleomycin instillation, when levels were 16–20 times normal. These values were obtained in BAL after bleomycin only and in mice that received carbon 3 days after bleomycin. In these groups, protein levels did not return to normal for several weeks (Fig. 5).

Lung Fibrosis

We examined autoradiographs of lung to determine if there was evidence of extended cell proliferation, particularly in the fibroblast population. In the first week after injecting carbon, the percentage of thymidine-labeled cells increased in all

experimental groups (Fig. 6). This early increase appeared mainly due to interstitial monocyte/macrophage labeling, though mice that received bleomycin also showed some epithelial and endothelial cell labeling in the first 2 weeks. Labeling in the carbon-only group dropped sharply to control values by 2 weeks, while DNA synthesis was higher in all bleomycin groups (Fig. 6). After 1 week, labeling in the various bleomycin groups was similar, and in each case, the predominant labeled cell type was the fibroblast.

The similar fibrotic response in the three bleomycin groups was confirmed by the HYP measurements. Mice that received carbon only showed a small

increase in HYP, not different from water controls, and consistent with aging (Fig. 7). Animals in groups that received bleomycin, with or without carbon at different stages of the post-bleomycin response, showed a rapid rise in tissue HYP beginning at 2 weeks. Thereafter, all bleomycin groups were equivalent, and HYP levels were all significantly greater than the control or carbon-only groups (Fig. 7).

Lung Residue

An amorphous, nonparticulate residue weighing $0.2 + 0.05$ mg was obtained from control lungs, and a similar weight of residue was obtained from fibrotic lungs after bleomycin alone (Fig. 8). Particle retention in mouse lung 16 weeks after instilling the large dose of carbon could be quantitated; the residue was black and weighed significantly more than that from controls. A similar weight of residue was measured from lungs that received carbon only and carbon 4 weeks after bleomycin. The highest weight of residue was recovered from lungs that received carbon during the acute injury phase, 3 days after bleomycin. The residue from this group weighed significantly more than that obtained from all other groups (Fig. 8).

Discussion

Baseline studies on deposition, clearance, and pathologic effects that determine whether a particle is “toxic” or “inert,” are usually carried out on normal animal lungs after inhalation or intratracheal instillation. However, in human terms, exposure to particulate air pollutants often occurs when the lung is compromised. It is possible that exposure to a particle such as carbon, which causes no effects in normal lung, may induce a different reaction when introduced to lungs at different stages of an injury-repair cycle. In general, particles deposited in the lung are mostly cleared by the mucociliary system, which can eliminate free particles as well as those engulfed by AM. As the dose increases, an adaptive increase in AM occurs, and at higher levels PMN also migrate to the alveoli to augment phagocytosis and clearance (18,19). These initial responses occur whether the particle is considered inert or toxic (3) and were seen here in all carbon-injected groups. There were differences, however, in the subsequent fate of particles, which can be related to the integrity of the alveolar epithelium.

Little is known about particle handling by the lung when there is alveolar epithelial cell injury. The normal epithelium is a barrier to particle translocation, and it is only crossed in overload situations when particles are transported across type I cells

to reach the interstitium, where they are phagocytized by interstitial macrophages (18–20). Particles may remain in this location for long periods, and in the case of carbon, no fibrotic reaction ensues. In the present study we created a diffuse alveolar injury by instilling bleomycin 3 days before giving a large dose of carbon. Because transepithelial passage increases with particle number (18,20), we used a level near the maximum deliverable in a single dose to a mouse to help detect any possible changes in the fibrotic response due to particle exposure of injured lung. Intratracheal bleomycin induces type 1 epithelial necrosis (21) and results in a high level of leaked protein that is measured in BAL fluid. Although carbon does not increase this level of damage, the

epithelial injury present for about 2 weeks allows many free particles to reach the interstitium for phagocytosis by interstitial macrophages. This was seen morphologically, and later the increased translocation and retention of carbon was confirmed by the increased weight of a black lung residue recovered at 16 weeks. The increase in particle translocation to the interstitium occurred despite a brisk inflammatory cell response to carbon that increased the number of AM and PMN in the alveoli. Most of these cells would have recently arrived in the alveoli and so were likely immature and poor phagocytes. The observation that acute inflammatory lung injury retards particle clearance has also been reported in calves with viral pneumonitis, in which clearance of cobalt oxide

was reduced and particles were sequestered in the interstitium (9).

The fact that particles deposited in the alveoli during epithelial injury subsequently results in an increased interstitial burden constitutes a potential hazard. In several studies, a clear link has been established between interstitial macrophage activation by particles such as silica or asbestos and the subsequent development of pulmonary fibrosis (22,23). This also occurs in other examples of fibrosis, such as the pulmonary response to bleomycin alone, in which activated macrophages, particularly those in the interstitium, have been identified as the prime source of cytokines that stimulate fibroblast proliferation and collagen deposition (24). It was thought possible that an extra burden on these macrophages, even by an inert particle, might further stimulate an activated cell in a synergistic reaction. However that did not happen in these experiments using pure carbon; despite the extra particulate load retained up to 16 weeks, no additional fibrotic response was seen or measured biochemically. Although we used particle instillation, our results are in agreement with an earlier publication in which adding titanium dioxide as an “inert” particle to inhaled asbestos did not increase fibrosis over that induced by asbestos alone (25).

A different pulmonary response was seen when carbon was instilled to the lung 4 weeks after bleomycin. At this time, epithelial repair was complete, and alveolar protein levels had returned to near normal, even though interstitial fibrosis had been produced. Instilling carbon induces the usual inflammatory response, though particles were mostly deposited in the less fibrotic areas of lung. This may be the result of relative variations in ventilation due to the fibrotic process. In one previous study of inhaled particle retention in fibrotic lungs of hamsters, it was noted that diseased areas of lung received fewer particles (12). Greater deposition of carbon in the more functionally normal regions of lung, together with the restoration of the epithelial barrier before at the time of instillation, allows a more regular pattern of phagocytosis and clearance, which would explain why the retained carbon at 16 weeks was equal to that of the carbon only group (Fig. 8). Despite the likely presence of activated macrophages in these bleomycin-injected lungs (24), few instilled particles reached the interstitium, and there was no additional fibroblast stimulation as judged by the equal levels of HYP after bleomycin, with or without carbon.

The results demonstrate that deposition of a large number of inert particles in a single dose to the lung during a phase of

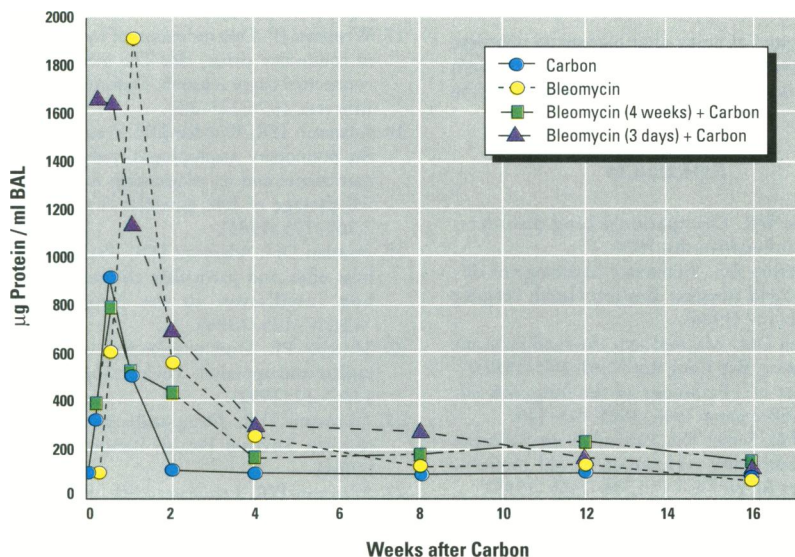


Figure 5. Protein content of bronchoalveolar lavage (BAL) fluid. Values in the first week after bleomycin alone or bleomycin 3 days plus carbon were significantly higher than the other groups ($p < 0.01$).

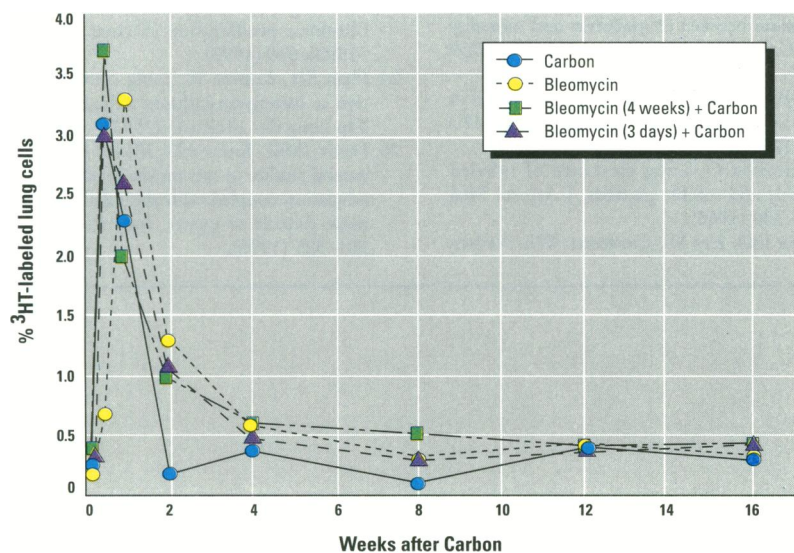


Figure 6. Percentages of all lung cells that incorporated tritiated thymidine (³HT) at various times. No significant differences in labeling pattern after 1 week were seen in the three bleomycin-injected groups.

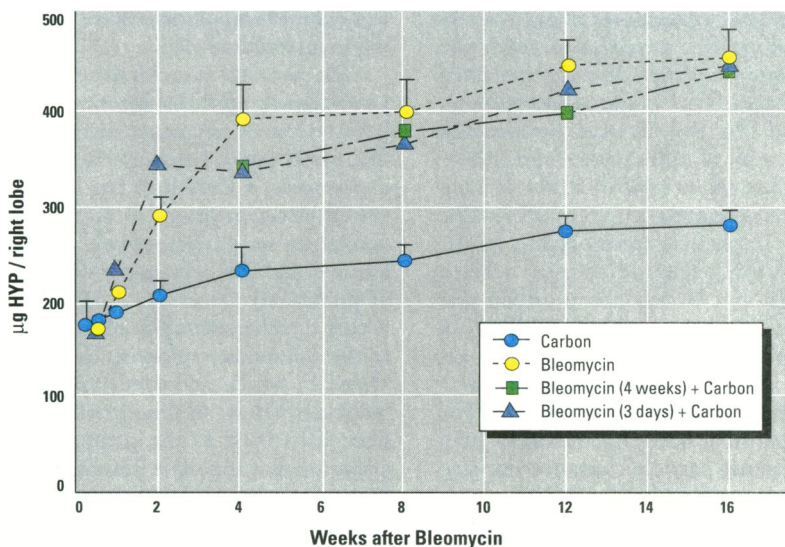


Figure 7. Total hydroxyproline (HYP) content of the right lung plotted at times after bleomycin injection. Mean + SE shown for carbon only and bleomycin groups. From 2 weeks onward, all bleomycin groups are statistically higher than control or carbon-only groups ($p < 0.01$), but no statistical differences among the three bleomycin groups were found.

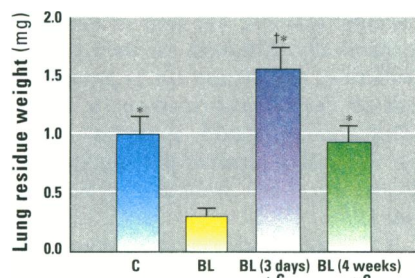


Figure 8. Weight of lung residues (mean + SE) 16 weeks after carbon (C) instillation. $^{\dagger}p < 0.01$ compared to all other groups; $*p < 0.01$ compared to bleomycin (BL) alone or water control.

epithelial injury or of fibrotic repair does not exacerbate the fibrotic response. However, it is possible that repeated exposure of injured lung to inert or to toxic particulates found in urban environments may produce a different outcome. In particular, exposure of the lung at a time of epithelial injury is potentially more serious due to the likelihood of increased particle translocation and retention in the pulmonary interstitium.

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