

## Oral N-acetylcysteine reduces bleomycin-induced collagen deposition in the lungs of mice

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*Oral N-acetylcysteine reduces bleomycin-induced collagen deposition in the lungs of mice. S. Shahzeidi, B. Sarnstrand, P.K. Jeffery, R.J. McNulty, G.J. Laurent.*

**ABSTRACT:** N-acetylcysteine (NAC) has been employed in the treatment of acute lung injury but its therapeutic value is as yet unproven. In the present study we examined the effect of both L- and D-forms of NAC as inhibitors of bleomycin-induced fibrosis in mice. We hypothesized that, because of the D-form is not metabolized, it may be more effective than the L-form in ameliorating lung injury and fibrosis.

Both drugs were given daily in the drinking water at a dose of approximately 400 mg·kg<sup>-1</sup> body weight commencing one week prior to a single intratracheal instillation of bleomycin at a dose of 6 mg·kg<sup>-1</sup> body weight. Lung injury was assessed 35 days later by measuring total lung collagen content, lung wet weight and examination of tissues by light and electron microscopy.

Total collagen content and lung wet weight of animals receiving bleomycin together with L-NAC were 2.90±0.03 mg and 0.23±0.01 g, respectively. The values for collagen content, but not wet weight, were significantly less (p<0.05) than those given for bleomycin alone (3.90±0.02 mg and 0.260±0.005 g, respectively), but greater than (p<0.05) controls (2.10±0.01 mg and 0.160±0.002 g, respectively). Values for collagen content and wet weight of animals given bleomycin together with D-NAC (3.10±0.02 mg and 0.21±0.01 mg, respectively) were both significantly greater than values for control animals but lower than animals given bleomycin alone. The histological assessment of drug-treated groups compared with those receiving bleomycin alone did not reveal obvious differences, probably due to the patchy distribution of the lesions.

These results suggest that oral N-acetylcysteine may be useful in the prevention of acute lung injury and fibrosis associated with bleomycin-induced damage of the lung, but that D- and L-forms exert similar effects.

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The term bleomycin refers to a group of related agents derived from *Streptomyces verticillus*, which has been successfully used in treatment of lymphoma, squamous cell carcinoma and testicular tumours [1]. It is not toxic for bone marrow [2] but may cause impaired pulmonary function [3-5] and in those patients with respiratory involvement a significant minority go on to develop acute respiratory distress syndrome [6], the majority of which after survival develop an impaired lung function characteristic of interstitial pulmonary fibrosis [7, 8].

Bleomycin is currently widely used in experimental studies, producing lung injury leading to pulmonary fibrosis, employing many species including dogs [9], baboons [10], rabbits [11], rats [12] and mice [13]. These models have been used by several groups to assess the effects of putative therapeutic agents including β-amino

propionitrile, D-penicillamine and p-aminobenzoic acid [14], desferrioxamine [15, 16] and N-acetylcysteine [17].

The L-form of N-acetylcysteine (L-NAC) is known to have oxygen radical scavenger properties. It is rapidly absorbed from the gut and taken into the lungs via the pulmonary circulation [18]. The therapeutic value of L-NAC has been examined by several investigators in various models of lung injury, but its ability to prevent lung fibrosis remains controversial [17, 19]. Most studies, thus far, have only examined the development of fibrosis histologically. We have found no reports of the effects of L-NAC on collagen accumulation in the lung. It was considered appropriate to study in parallel both the biochemistry and histology in response to bleomycin and the capacity of L-NAC to alter deposition of lung collagen subsequent to bleomycin administration. Since L-NAC is readily metabolized,

particularly in the liver, we also employed D-NAC. D-NAC is also a reducing agent but resistant to metabolic break down processes. We investigated whether D-NAC might be more effective than the L-isomer. The results indicate that both forms of the drug are partially effective in ameliorating collagen deposition in this model of pulmonary fibrosis, but that both drugs have similar effects.

## Methods

### Animals

Experiments were performed on mice (strain, B<sub>6</sub>D<sub>2</sub>F<sub>1</sub>), aged 8–9 weeks and weighing 24–26 g. In the initial study of the dose-response to bleomycin, animals were divided into untreated normal, saline control groups, and four experimental groups receiving 5, 6, 7 and 10 mg·kg<sup>-1</sup> body weight of bleomycin. There were six or seven mice in each group. Animals were anaesthetized by alphaxolon 0.9% w/v and alphadolon 0.3% w/v (Glaxo, UK) intraperitoneally at a dose of 7.5 ml·kg<sup>-1</sup> body weight. Bleomycin (Lundbeck, UK) was administered in 0.05 ml of 0.14 M NaCl. Control groups received 0.05 ml of 0.14 M NaCl alone. The trachea was intubated and the appropriate solution introduced using a Hamilton syringe with a 25 gauge needle. A small volume (0.2 ml) of air was then quickly injected to flush the airways, followed by another 0.05 ml of 0.14 M NaCl and a further 0.2 ml of air.

### L- and D-isomers of NAC

Both L- and D-forms of NAC were obtained from AB Draco, Lund, Sweden. Animals were divided into five control and three experimental groups. One control group received bleomycin alone. The remaining four groups received D-NAC, L-NAC, vehicle alone (0.9% NaCl, 0.06% Na<sub>2</sub> edetic acid (EDTA) pH7.0) and plain drinking water, respectively. Experimental groups included those animals receiving bleomycin and L-NAC, bleomycin and D-NAC, bleomycin and vehicle. Drug administration in the drinking water commenced one week prior to bleomycin instillation, both L- and D-NAC were administered as a solution containing 2 mg·ml<sup>-1</sup> of these agents. Based on the volume estimates, performed each day, the maximal water consumption was about 200 ml·kg<sup>-1</sup> body weight for each animal, suggesting a 24 h dose of NAC at approximately 400 mg·kg<sup>-1</sup> body weight per day. Drinking water was changed each day during the 35 days of the experiment. There were eight animals in each group, at least two of which were examined histologically.

### Tissue and blood analysis

Animals were killed by inhalation of CO<sub>2</sub>. In the initial dose-response study, this occurred 21 days after bleomycin administration. In the time course study,

animals were killed at 3, 10, 21 and 35 days, and 2 and 7 months after bleomycin. Blood was collected from the abdominal aorta and levels of NAC determined in serum using techniques described previously [20]. Residual blood was flushed from the lung by severing the abdominal aorta and perfusing lungs *via* the right side of the heart with two ml of phosphate buffered 0.9% NaCl at 4°C. The trachea and larynx were cut and the thoracic contents removed en bloc; lungs were dissected free of major airways and vessel and blotted dry before weighing. Total lung collagen content was assessed as described previously, by measurement of hydroxyproline, determined spectrophotometrically after oxidation with chloramine-T and extraction of the toluene miscible product [21].

### Histological examination

The lungs of randomly selected bleomycin-treated animals were assessed by both light and electron microscopy. For the former, animals were killed at the same times as mentioned above, by overdose with sodium pentobarbitone and the lungs fixed by intratracheal inflation with 10% neutral-buffered formalin at a pressure of 25 cmH<sub>2</sub>O. The trachea was ligated just caudal to the larynx and the thoracic content removed en bloc. The lungs were further fixed by immersion in formalin, prior to routine dehydration and paraffin embedding. Three to 5 µm thick sections were cut from both right and left lungs and step sections at several levels of the lung were stained either by haematoxylin and eosin (H&E), Verhoeff's elastic van Gieson (EVG), Gordon & Sweet's stain for reticulin, martius-scarlet-blue (MSB) for connective tissue and fibrin or picro sirius red in an attempt to increase collagen birefringence and distinguish young from mature collagen [22]. For electron microscopy, animals were killed as above, and the cardiac lobe of the right lung of each animal fixed by intrabronchial instillation of glutaraldehyde (2% in 0.1 M sodium cacodylate buffer at pH 7.2) until pleural margins were sharp. The lobar bronchus was ligated to maintain inflation and the cardiac lobe removed. If nodular or whitened areas about the hilum or periphery of the lung were present they were dissected and the equivalent areas removed from control animals for comparison. After 2 h fixation 1 mm<sup>3</sup> diced tissue was post-fixed in 1% osmium tetroxide (in 0.1 M cacodylate buffer) and embedded in epoxy-resin (Araldite). One µm survey sections were stained with toluidine blue and ultra-thin sections prepared for examination by electron microscopy.

### Statistical evaluation

For biochemical measurements median values were compared using the Mann-Whitney U test and probability (p) values of less than 0.05 were taken as statistically significant. Values are presented as means±standard errors of the mean (SEM).

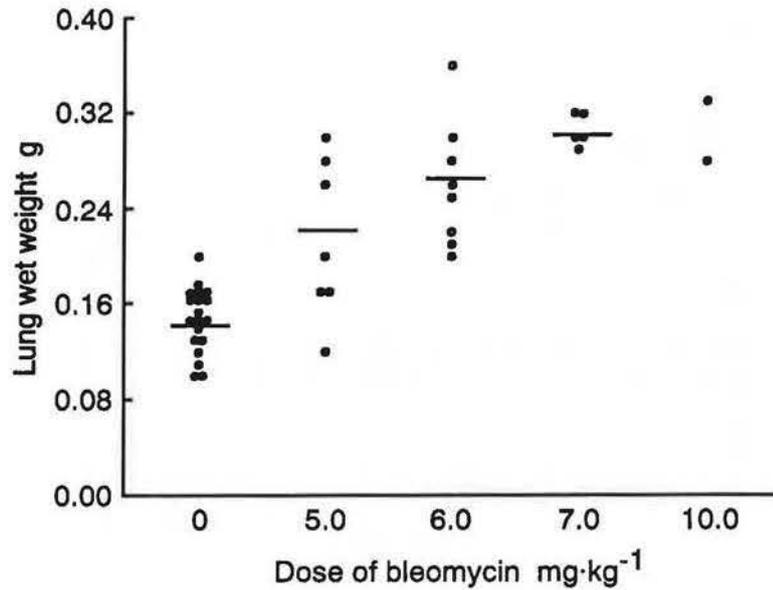


Fig. 1. – Dose-response curve, showing changes in lung wet weight with increased doses of bleomycin, 21 days after intratracheal instillation.

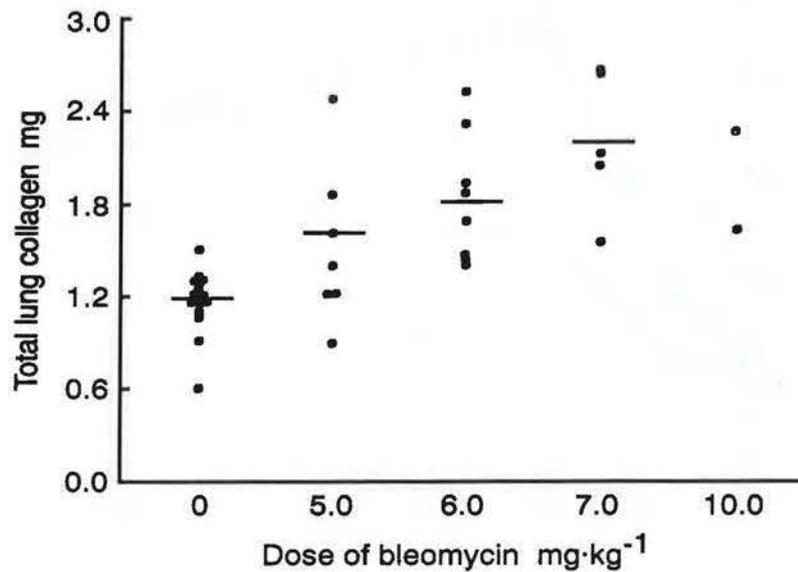


Fig. 2. – Relationship between lung collagen content and dose of bleomycin, 21 days after a single dose.

## Results

### Dose response

Figures 1 and 2, show changes in lung wet weight and collagen content for mice given four doses of bleomycin ranging from 5–10 mg·kg<sup>-1</sup> body weight. When compared with controls, the mean lung weights of bleomycin treated animals as well as their collagen content increased significantly at doses of 6 and 7 mg·kg<sup>-1</sup> body weight ( $p < 0.01$  and  $p < 0.001$ , respectively).

At 10 mg·kg<sup>-1</sup> body weight only two animals survived.

Figure 3 shows the lung wet weight at 3, 10, 21, 35, 60 and 210 days after bleomycin (6 mg·kg<sup>-1</sup> body weight) compared with controls. Increases were significant at all times except at 3 days ( $p < 0.001$ ,  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.05$ ,  $p < 0.05$ , respectively, for subsequent times). Within 10 days there was almost a doubling in wet weight. After the acute phase, the lung weight appeared to return towards control values, although after 210 days the weight was still about 30% above control.

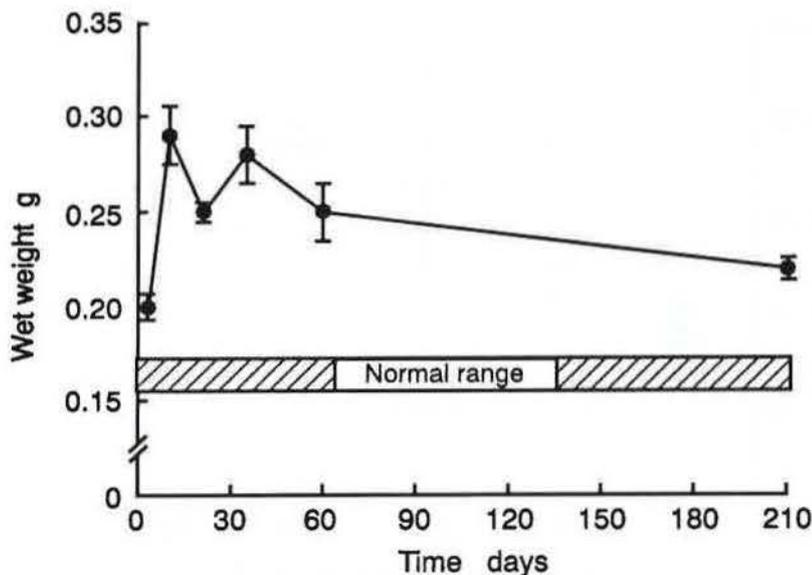


Fig. 3. - Lung wet weight after  $6 \text{ mg}\cdot\text{kg}^{-1}$  body weight of bleomycin given as a single intratracheal dose.

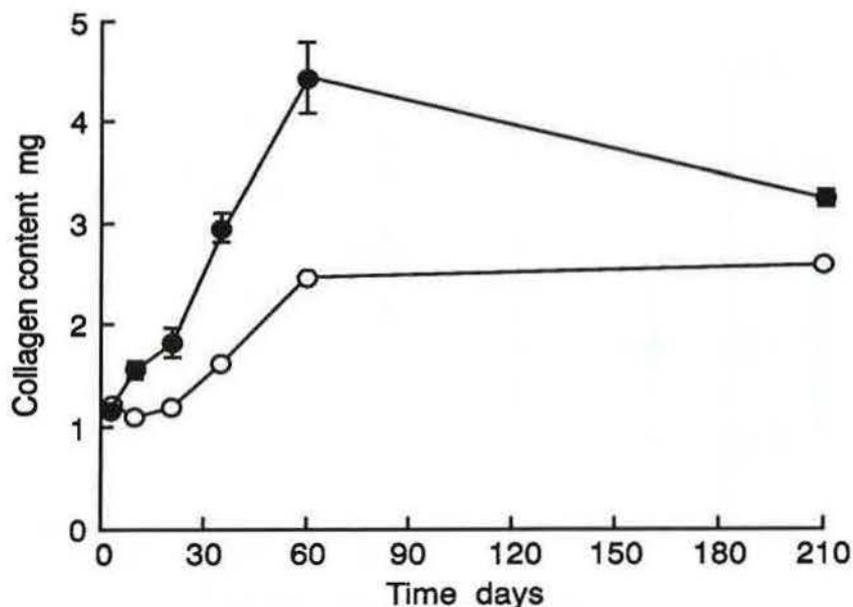


Fig. 4. - Alteration of collagen content at six time points after giving  $6 \text{ mg}\cdot\text{kg}^{-1}$  body weight of bleomycin. Open and closed circles are representative of controls and bleomycin treated animals, respectively.

Figure 4 shows the total lung collagen content at six times following administration of bleomycin. There was a relatively rapid increase in collagen for controls and bleomycin-treated mice over the first two months, although at all times, except at 3 days, the increase was greater in bleomycin-treated animals compared with controls ( $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.01$ ,  $p < 0.001$ ,  $p < 0.01$  at 10, 21, 35, 60, and 210 days respectively). For bleomycin-treated animals, there was a fall in collagen content between 60 and 210 days, although the difference between these last times was not statistically significant.

Untreated and saline-treated controls showed mild congestion of pulmonary capillaries but were otherwise normal with little evidence of inflammatory cell infiltrate. The connective tissue stains revealed interstitial collagen centred around major airways and pulmonary vessels and to a lesser extent present in the pleura and thin alveolar septa.

Following bleomycin alone, inflammatory changes were seen at both low and high doses, *i.e.* around central airways, spreading peripherally and present as subpleural foci consisting of predominantly mononuclear cells with fewer numbers of neutrophils and

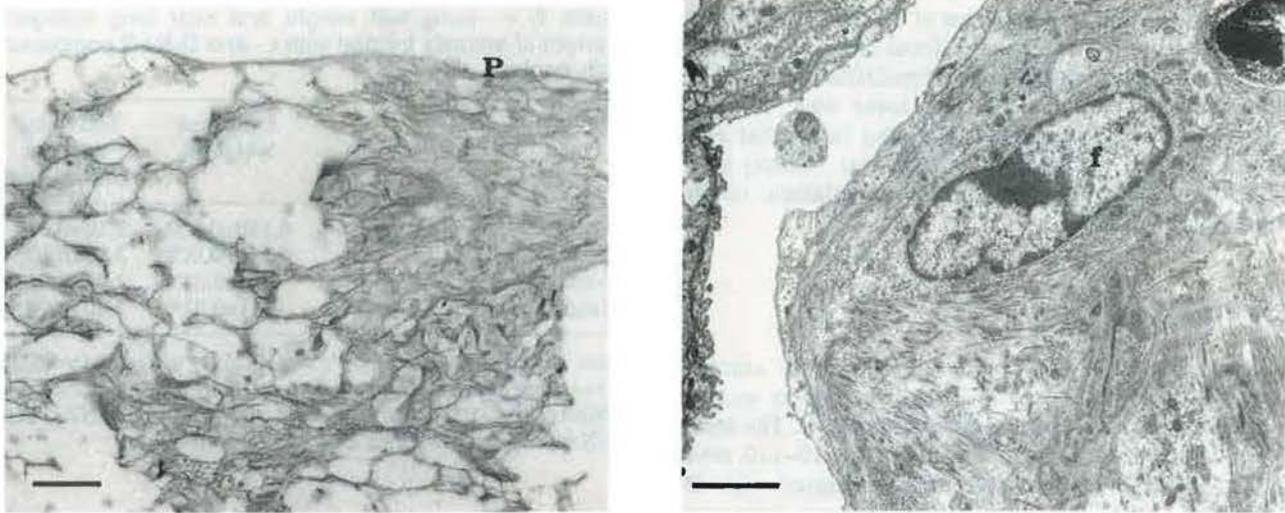


Fig. 5. - a) Gordon & Sweet's method showed an increase in the intensity and amount of reticulin staining and encroachment of alveolar lumina by connective tissues. (X100; bar scale= 100  $\mu$ m). b) Electron micrograph showing interstitial deposition of collagen (X5,600; bar scale=2  $\mu$ m).

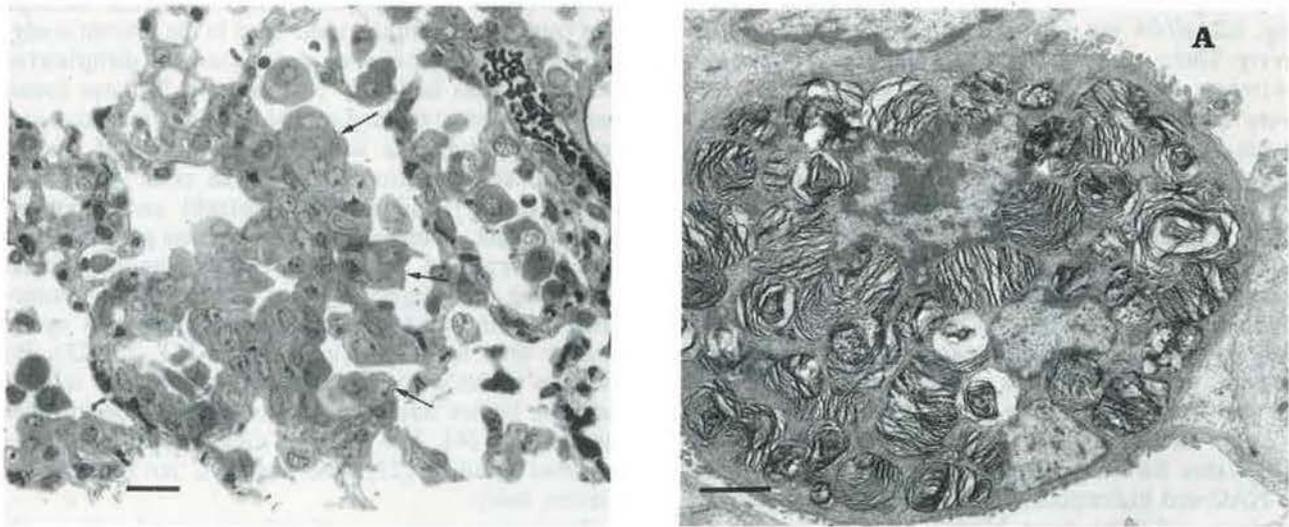


Fig. 6. - a) Plastic-embedded lung viewed by light microscopy to demonstrate focal cuboidal metaplasia of alveolar lining cells (arrows). (X350; bar scale=20  $\mu$ m). b) Electron micrograph appearance of enlarged type II cells with abundant cytoplasm and numerous lamellar bodies. (X4500; bar scale=2  $\mu$ m).

spindle-shaped cells (*i.e.* fibroblasts) and accumulations of "foamy" macrophages. Neutrophils were seen particularly around airways and blood vessels. In the alveolar region there was mild reactive cuboidal cell hyperplasia up to 21 days. By H&E staining there were atypical epithelial-like cells each with a large vesicular nucleus and an abundance of eosinophilic cytoplasm. The MSB stain clearly demonstrated the increase in deposition of connective tissue and occasionally showed evidence of subpleural fibrinoid alveolar thickening. The

increase of interstitial collagen content detected histologically by the trichrome stain was accompanied by increases of elastin and reticulin (fig. 5a) and intra-alveolar buds of immature (reticular) collagen. Electron microscopy confirmed many of the above findings and demonstrated foci of collagen deposition (fig. 5b) and mononuclear cellular infiltrate of the interstitium; there was cuboidal cell (fig. 6a) hyperplasia and an increase in the number of type II cell lamellar bodies (fig. 6b). The first definitive evidence of interstitial fibrosis seen

histologically, became apparent at 35 days after a dose of 6 mg·kg<sup>-1</sup> body weight. The focal changes remained at 210 days following bleomycin instillation. Occasional lining cells were binucleate and some were enlarged with "mucoid-like" granules. Dividing interstitial cells were seen and nonciliated bronchiolar (Clara) cells showed a proliferation of smooth endoplasmic reticulum at their apices.

#### Effect of NAC

Serum levels of NAC were measured in animals treated with both L- and D-forms of drug as well as untreated animals and those given bleomycin. The mean serum concentration was 50 mM (range 10–110 mM) and 7.5 mM (0–10 mM) for animals treated with D-NAC and L-NAC, respectively. Bleomycin treatment did not significantly affect serum levels of NAC.

Table 1 shows the lung wet weight and total lung collagen content of animals treated with L- and D-NAC compared with controls. The mean values ( $\pm$ SEM) from the four control groups not given bleomycin (*i.e.* saline, vehicle, L-NAC or D-NAC) were 0.170 $\pm$ 0.002 g, 0.160 $\pm$ 0.002 g, 0.160 $\pm$ 0.002 g, 0.160 $\pm$ 0.002 g for lung weight and 2.03 $\pm$ 0.03 mg, 2.13 $\pm$ 0.06 mg, 2.13 $\pm$ 0.05 mg, 2.24 $\pm$ 0.04 mg for total collagen content, respectively. There were no statistically significant differences between these groups and for this reason they were combined for comparison with treated animals (table 1).

Bleomycin caused a significant increase in both wet weight and total lung collagen content of about 50 and 75%, respectively, measured 35 days after bleomycin instillation. Animals given L-NAC or D-NAC with bleomycin also had increased lung wet weight and collagen content, although the increase was less marked than in animals given bleomycin alone. For collagen content, this was reflected in the significant difference between the values seen in L- and D-NAC treated animals compared with those given bleomycin alone. The value for lung weight in animals given L- and D-NAC and bleomycin was also intermediate between controls and those given bleomycin alone but the difference was statistically significant only for animals given D-NAC.

The lungs from animals given saline, L-NAC or D-NAC alone were all similar histologically to the untreated control lungs reported above for dose-response experiments.

In mice given L-NAC, the lungs of one of three animals showed similar bleomycin-induced changes and indications of alveolar bronchiolization: foci of affected areas were still present in the remaining two animals. Following the addition of the D-form, two of three lungs remained affected, depending on the particular lobe there was extensive cellular thickening of alveolar septa and focal obliteration of alveoli with subpleural deposition of fibrin and thickening of reticular fibres. There were no obvious differences between the NAC-treated and untreated groups.

Table 1. — Lung wet weight and total lung collagen content of animals treated with L- and D-NAC compared with controls

Groups (n)	Lung wet weight g	Total lung collagen content mg
Combined controls (28)	0.160 $\pm$ 0.002	2.10 $\pm$ 0.01
Bleomycin (6)	0.260 $\pm$ 0.005*	3.90 $\pm$ 0.02*
Bleomycin with L-NAC (6)	0.23 $\pm$ 0.01*	2.90 $\pm$ 0.03**
Bleomycin with D-NAC (6)	0.21 $\pm$ 0.01**	3.10 $\pm$ 0.02**

Data are given as mean $\pm$ sd. \*: significantly different ( $p < 0.05$ ) when compared with bleomycin treated alone; \*\*: significantly different ( $p < 0.05$ ) when compared with controls; L-NAC and D-NAC: L- and D-forms of N-acetylcysteine.

#### Discussion

The fibrosis seen after intratracheal instillation of bleomycin, into the B<sub>6</sub>D<sub>2</sub>F<sub>1</sub> strain of mice, was patchy and tended to be bronchocentric. Large areas of pulmonary parenchyma were of normal appearance, consistent with reports in other strains [21, 23]. The predominance of epithelial over endothelial damage was compatible with the route used in the present study. Fibrinous exudate was restricted to subpleural interstitial sites in a very few animals. We also found indications of alveolar bronchiolization, cuboidal and squamoid metaplasia with the appearance of greatly enlarged eosinophilic epithelial-like cells, previously reported [24] and of both normal and "foamy" macrophages. A mural pattern of fibrosis predominated and, as reported for other species, the interstitial thickening was highly cellular, predominantly mononuclear with patchy obliterative patterns and cystic change seen in a very few animals [21, 23, 25]. By electron microscopy the increase of alveolar type II lamellar bodies has been previously reported in other strains [24, 26]. The alveolar ciliated cell metaplasia reported by others [27] was, however, not found in the present study.

Biochemical estimates of collagen were based on the total amount of hydroxyproline in lungs of the mice. We believe total collagen levels are most appropriate in this type of study because they reflect the net result of the balance between synthetic and degradative changes. An alternative way of expressing collagen is as a concentration, with respect to wet weight, dry weight or some other constituent, such as deoxyribonucleic acid (DNA). However, when this is done the denominator introduces problems in interpretation of the data, particularly in the early stages when there is a net influx of cells and fluids from the circulation [28]. Nevertheless, in the current study when collagen was expressed per unit wet weight, it revealed an increase from 0.70 $\pm$ 0.03 mg·g<sup>-1</sup> in controls at the beginning of the study to 1.47 $\pm$ 0.60 mg·g<sup>-1</sup> at the end. Such age related changes have been observed previously in rats, along with changes in the relative amount of the collagen types

also seen [29]. In the current study, mice given bleomycin showed no change in collagen concentration, as was seen previously in rabbits [21].

Both L- and D-isomers of NAC partially blocked the enhanced collagen deposition seen in bleomycin treated animals. If, in the current study, collagen was expressed per unit wet weight, there was no significant change, comparing bleomycin treated animals (with or without NAC) with controls. This might suggest that the drugs are not selectively inhibiting collagen deposition, rather that they are influencing events more upstream in the damage process, and preventing subsequent damage which is manifested by the deposition of components of fibrous tissue.

The effects of drugs on the increased lung collagen deposition which follows bleomycin have previously been examined for various agents [14-16, 30], but we are unaware of any study examining the effects of NAC on collagen content in this experimental model. Both L- and D-isomers of NAC demonstrated a capacity to partially inhibit lung collagen deposition. The potential of L-NAC to prevent lung damage and subsequent fibrosis is controversial. Studies by BEREND [19] and BEREND *et al.* [31] in rats showed NAC given by intratracheal instillation, abolished the acute pulmonary damage assessed by changes in lung wet weight, but when NAC was given as a bolus dose or by continuous intraperitoneal infusion, it was shown to be ineffective. However, studies by JAMIESON *et al.* [17] suggest NAC, when given intraperitoneally, prevented the increase in lung wet weight one week following bleomycin. There are likely to be several possible reasons for these differing results. The effects of bleomycin will depend on the form in which the drug is administered, as well as its mode of administration [32] and the timing between giving the bleomycin and the NAC. Furthermore, where the criteria for detection of lung injury differs this may explain apparent discrepancy. One simple explanation for differing results obtained from various laboratories may relate to the final concentration of drug attained in lung cells. Another determinant may be the strain of the animal. For example, B<sub>6</sub>CF<sub>1</sub> (the parent strain of the group employed here) develop fibrosis, assessed histologically and biochemically whereas another mouse strain (BALBc) was unresponsive to bleomycin [24].

The concept that attainment of high intracellular concentrations of L-NAC (or active metabolites) might be critical to its efficacy, lead us to seek related molecules with greater activity. The D-isomer of NAC is not metabolized and attains blood levels at least 10 times greater than the L-form [20] and the data obtained here for plasma levels of D-NAC compared with the L- form confirmed this. For this reason, we hypothesized that the D-form would be more effective than the L-form in preventing collagen deposition and fibrosis. The biochemical and histological results of the present short-term study do not, however, support this hypothesis. Both forms were similarly effective, and there was no apparent difference in the efficacy of the two drugs. Furthermore, qualitative histological

examination of randomly selected animals did not distinguish between the animals treated with either drug; indeed, differences between bleomycin alone and those given bleomycin with either form of NAC were difficult to detect histologically due to the patchy distribution of the lesion and the variation between lobes and animals.

The mechanism by which NAC limits fibrosis is uncertain, but is likely to be *via* its ability to limit damage to lung structures in the acute stage of disease. NAC is a cell permeable sulphhydryl compound [33], and despite the conformational differences between L- and D-forms of NAC, both isomers can readily enter cells [34, 35]. Once in cells, L- and D-NAC may act directly as oxygen radical scavengers. Furthermore, D-NAC could stimulate acetylation of the L-cysteine, promoting its entry into cells in the gut or other tissues including the lung. It would then act as a reducing agent and would be expected to protect against damage by bleomycin which, after forming activated complex with iron and oxygen, is capable of generating oxidants. These products can cause cleavage of DNA and other macromolecules and can break down membrane structures by inducing lipid peroxidation (see [32] for review).

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- La N-acétylcystéine par voie orale réduit les dépôts de collagène induits par la bleomycine dans les poumons de souris.* S. Shahzeidi, B. Sarnstrand, P.K. Jeffery, R.J. McAnulty, G.J. Laurent.
- RÉSUMÉ: La N-acétylcystéine (NAC) a été utilisée pour le traitement des atteintes pulmonaires aiguës, mais sa valeur thérapeutique est encore non démontrée. Dans l'étude actuelle, nous avons examiné les effets des formes L et D de NAC, comme inhibiteurs de la fibrose induite par la bleomycine chez la souris. Nous avons émis l'hypothèse que, puisque la forme D n'est pas métabolisée, elle pourrait être plus efficace que la forme L pour améliorer les accidents pulmonaires et la fibrose.
- Les deux médicaments ont été administrés quotidiennement dans l'eau de boisson à une dose approximative de 400 mg·kg<sup>-1</sup> de poids corporel, en commençant une semaine avant une administration intra-trachéale unique de bleomycine à la dose de 6 mg·kg<sup>-1</sup> de poids corporel. Le lésion pulmonaire a été appréciée 35 jours plus tard en mesurant le contenu pulmonaire total en collagène, le poids pulmonaire humide, et en examinant les tissus à la microscopie optique et électronique.
- Le contenu total en collagène et le poids humide du poumon des animaux recevant la bleomycine en même temps que L-NAC, ont été respectivement de 2.90±0.03 mg et de 0.23±0.01 g. La valeur du contenu en collagène, mais non celle du poids humide, était significativement plus faible (p<0.05) que celle obtenue après bleomycine seule (3.90±0.02 mg et 0.26±0.005 g respectivement), mais plus élevée que celle des contrôles (p<0.05) (2.10±0.01 mg et 0.16±0.002 g respectivement). Les valeurs pour le contenu en collagène et pour le poids humide chez les animaux chez lesquels la bleomycine a été administrée en même temps que la D-NAC (3.10±0.02 mg et 0.21±0.01 g respectivement) furent significativement plus élevées que les valeurs chez les animaux contrôle, mais plus faibles que celles des animaux auxquels la bleomycine avait été administrée isolément. L'étude histologique des groupes traités par les médicaments, en comparaison avec ceux recevant la bleomycine seule, n'a pas montré de différence évidente, sans doute à cause de la distribution irrégulière des lésions.
- Ces résultats suggèrent que la N-acétylcystéine orale peut être utile pour la prévention des atteintes pulmonaires aiguës et de la fibrose associée à des lésions pulmonaires induites par la bleomycine, mais que les formes D et L ont des effets similaires.
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