Quantification of Bleomycin Pulmonary Toxicity in Mice by Changes in Lung Hydroxyproline Content and Morphometric Histopathology

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ABSTRACT

Bleomycin treatment produced dose-dependent changes in lung collagen content and in several measurable histopathological parameters. NIH/Swiss mice were treated twice weekly for 6 weeks with bleomycin, 0, 1, 20, or 40 mg/kg s.c. The two highest doses produced mortalities of 35 and 100%, respectively, as well as loss of body weight and increase in lung wet weight. Lung hydroxyproline content, an index of collagen, increased to 40 to 50% above control levels at 6 and 8 weeks after initiation of treatment with bleomycin 20 mg/kg. Morphometric analysis was applied to the following parameters at light microscopy: number of intraalveolar macrophages and leukocytes, total pulmonary cell count, alveolar wall thickness, and percentage of consolidation of lung parenchyma. The two highest doses produced increases in all of these parameters as compared to controls. The most marked changes occurred in the number of intraalveolar cells, which in the group given 20 mg/kg rose to 150, 190, and 210% of controls at 4, 6, and 8 weeks. The lowest dose of bleomycin, 1 mg/kg twice weekly for 6 weeks, evoked no pulmonary or other toxicity by the parameters examined. This model of chronic pulmonary toxicity may be useful in analog development, in testing potential antidotes, and in examining the effects of other factors that might modify the pulmonary toxicity of bleomycin.

INTRODUCTION

BLM, a mixture of antibiotic and antineoplastic glycopeptides, produces only minor hematological and gastrointestinal toxicity but is limited in its clinical usefulness by a dose-related pulmonary toxicity. This drug-induced interstitial pneumonitis and subsequent pulmonary fibrosis have been described extensively in patients and in several animal species, including monkeys, dogs, rats, mice, and pheasants. Previous descriptions of the sequential development of this lesion in mice provided the basis for our studies. Since interstitial and intraalveolar fibrosis is a prominent histological finding after BLM treatment, we estimated total lung collagen by measuring its OH-Pro content. This was correlated in each animal at several time points and doses of BLM with morphometric analysis of the following features with light microscopy: number of intraalveolar macrophages and leukocytes, total pulmonary cell density, mean alveolar wall thickness, and percentage of consolidation of lung parenchyma.

MATERIALS AND METHODS

Animals. Male NIH/Swiss albino mice weighing 20 to 25 g were obtained from the NIH colony and provided with Purina rodent chow and water ad libitum.

Drug Doses and Administration. BLM was obtained from Nippon Kayaku Co., Inc., Lot U11AS, through the courtesy of Dr. John Douros of the Natural Products Section, Developmental Therapeutics Program, National Cancer Institute. This material is known to be a mixture of 13 related glycopeptides elaborated by the actinomycete Streptomyces verticillus. BLM was administered s.c. at doses of 1, 20, and 40 mg/kg twice weekly for up to 6 weeks. Control animals received 0.9% NaCl solution s.c. Six mice/group were sacrificed 2, 4, 6, and 8 weeks after initiation of treatment for OH-Pro assay of the right lung and histopathological studies of the left lung.

OH-Pro Assay. Since lung OH-Pro is almost exclusively derived from collagen, whole lung collagen content was estimated by measuring OH-Pro levels as described by Weibel. OH-Pro was measured by the method of Peck and Daniel.

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Lung Fixation. After the right lung had been removed for OH-Pro assay, the trachea of each mouse was cannulated with a 20-gauge Teflon i.v. catheter (Abbott Laboratories, North Chicago, Ill.) and ligated in situ. The tracheal cannulas were connected via plastic i.v. tubing to a reservoir of fixative at 30 cm of fluid pressure, as recommended by Dungworth et al. (10). A manifold attachment of 3-way stopcocks allowed 7 mouse lungs to be perfused simultaneously with fixative. This procedure resulted in consistent expansion and fixation of the left anterior lobe of each lung for 1 hr at 30 cm of fluid pressure. A modified Karnovsky's fixative, suitable for both light and electron microscopy, was used (10). After immersion in fixative for at least 48 hr, the left anterior lobes were processed, embedded in paraffin, serially sectioned (6 µm) in the anterior-posterior plane, mounted on glass slides, and stained with hematoxylin and eosin, van Gieson's, and Masson's trichrome stains.

Morphometric Analysis. Based on previous descriptions of BLM toxicity in mice (1, 2) and on our own studies, the following parameters were chosen for morphometry at the light microscopic level: number of intraalveolar macrophages and leukocytes, total pulmonary cell density, mean alveolar wall thickness, and percentage of consolidation of lung parenchyma. Each slide was projected onto a rectangular grid that represented 0.033 sq mm of actual lung area with a Leitz-Wetzlar projection microscope, Model 396, that produced ×1100 image magnification. Three representative areas in each lung were selected by the following criteria: only lung parenchyma with alveoli and smaller alveolar ducts was included within the counting grid; larger blood vessels, bronchioles and bronchi, and areas of dense consolidation were carefully excluded; and 1 grid was examined in each of the anterior, middle, and posterior thirds of the left anterior lobe. Only this lobe of the left lung was measured to further standardize the measurement procedure. The width of 10 alveolar walls in each of the 3 projected grids was measured so that the mean alveolar wall thickness represents the average of 30 such measurements in each lobe.

All intraalveolar macrophages and leukocytes within each grid and all cell nuclei in the same area, as an estimate of total parenchymal cell density, were counted. Because BLM does not produce a uniformly distributed lesion throughout the lung, counting 3 separate sites in each lobe resulted in less sampling error. Percentage of consolidation of each left anterior lobe was estimated by projecting a low-power ×70 image of each slide onto a point-counting grid system to determine the total area of each specimen and the area of consolidation (23). Consolidation was defined as the area of lung parenchyma in which alveolar air space was replaced by cellular infiltrate, fluid exudate, and/or connective tissue.

All slides were coded, randomized, and read by 2 observers without knowledge of type or duration of treatment. The statistical significance of the results was calculated by Student's t test.

RESULTS

Overall Toxicity. The highest BLM dose, 40 mg/kg, produced 100% mortality before the end of the 6-week treatment period (Chart 1). The intermediate dose, 20 mg/kg, produced 35% mortality during the 8-week course of the experiment, while 1 mg/kg resulted in no deaths. Body weights show a similar pattern of no evident toxicity in the low-dose group and progressive losses of body weight in the 2 higher-dose groups (Chart 2).

Mice in the 2 highest-dose groups exhibited typical der-
matological toxicity, with sclerodermatous changes, patchy alopecia, and brittle nails leading to onychoptosis. Gross evaluation of tissues at necropsy revealed normal-appearing livers, kidneys, and intestinal tract in the 2 higher-dose groups. Histological examination after 3 weeks of high-dose treatment revealed normal liver and kidneys, some flattening of small intestinal epithelium, and relatively normal bone marrow and spleen. As expected, the lungs were the site of major pathological changes.

**Lung Collagen.** Wet weight of the lungs increased at 4 weeks in the groups given 20 and 40 mg/kg and rose to almost twice the control levels in surviving animals at 6 and 8 weeks (Chart 3). Whole lung collagen, as measured by OH-Pro content, increased above controls at 6 and 8 weeks in the group given 20 mg/kg (Chart 4). Histological examination of lungs with van Gieson’s and Masson’s stains in animals with increased lung OH-Pro content clearly revealed increased numbers of collagen bundles, most prominent in areas of consolidation. In animals with increased lung wet weights prior to Week 6, consolidated areas consisted mainly of interstitial and intraalveolar edema and a cellular exudate with macrophages and leukocytes.

**Morphometric Analysis.** Areas of consolidation were found in the 20 and 40 mg/kg groups beginning at 6 and at 4 weeks, respectively (Table 1). These areas ranged from focal lesions, frequently subpleural, to almost complete loss of recognizable alveolar architecture in moribund animals (Fig. 1). The transition from consolidated areas to areas of normal-appearing lung was characteristically abrupt, as illustrated in Fig. 3. Consolidation in the 40 mg/kg group at 4 weeks exhibited the features of an acute inflammatory response, with large amounts of intraalveolar fluid exudate and leukocytes, including neutrophils, lymphocytes, and plasma cells, as well as foamy macrophages (Fig. 5). In the intermediate-dose group, 20 mg/kg, consolidation did not appear until the sixth week, with fewer neutrophils, the appearance of fibroblasts, and evidence of scattered collagen bundles on Masson’s and van Gieson’s stains.

The number of intraalveolar macrophages and leukocytes per sq mm of lung area was elevated significantly to more than 3 times control values at 4 weeks in the high-dose group (Chart 5). Increases in intraalveolar cells also were present in the 20 mg/kg group at this time and progressed at 6 weeks. Data for 2 animals in the high-dose group that survived until Week 5 are included in Charts 5 to 7. Alveolar macrophages in toxic animals, which had abundant cytoplasm and the granular, foamy appearance of actively
phagocytosing cells (Fig. 4), were qualitatively as well as quantitatively different from macrophages of controls (Fig. 2).

The total pulmonary cell count was increased in the 2 higher-dose groups, although this was significant at only 2 time points because of greater variability in this parameter.

![Chart 5](image1)

**Chart 5.** Number of intraalveolar cells per sq mm of lung tissue. Three nonconsolidated areas totaling 0.1 sq mm were counted in each left anterior lobe. ∆, control; O, 1 mg/kg; ○, 20 mg/kg; □, 40 mg/kg. * significantly different from control (p < 0.05). Mean ± S.E., n = 3 to 6.

![Chart 6](image2)

**Chart 6.** Total pulmonary cell count during BLM treatment. ∆, control; O, 1 mg/kg; ○, 20 mg/kg; □, 40 mg/kg. * significantly different from control (p < 0.05). Mean ± S.E., n = 3 to 6.

![Chart 7](image3)

**Chart 7.** Alveolar wall thickness during BLM treatment. Thirty alveolar walls were counted in each left anterior lobe. ∆, control; O, 1 mg/kg; ○, 20 mg/kg; □, 40 mg/kg. * significantly different from control (p < 0.05). Mean ± S.E., n = 3 to 6.

This and the other morphometric parameters examined at the high-power ×1100 magnification were measured in nonconsolidated areas of lung, since consolidation was considered as a separate index of toxicity (Table 1). This selection of nonconsolidated areas is a measure of pulmonary toxicity in areas where gross toxicity was not evident at low power and thus is a conservative estimate of total pulmonary and intraalveolar cells in animals with consolidation.

Finally, alveolar wall thickness was significantly increased only at the most distant time point, 8 weeks, in animals receiving BLM, 20 mg/kg. The low-dose group, which received BLM, 1 mg/kg s.c., twice weekly for 6 weeks, did not differ from control animals in any of the parameters studied.

**DISCUSSION**

Although BLM pulmonary toxicity has been extensively reported and described (1–5, 8, 9, 12, 13, 17, 19–22), it has been difficult to quantify in both patients and animal models. In this report, we have derived quantifiable indices for both biochemical and histopathological parameters in an experimental model of BLM lung toxicity. Animals receiving the lowest BLM dose, 1 mg/kg, demonstrated no abnormalities by toxicological, biochemical, and histopathological criteria. An intermediate dose, 20 mg/kg, resulted in the morphological appearance of chronic pulmonary toxicity, with significant increases in lung collagen content by 6 and 8 weeks. The high-dose group exhibited a more acute toxicity, with changes in morphometry, but 100% lethality occurred before significant increases in lung collagen were detectable.

For reproducible development of chronic BLM lung toxicity in mice, our data suggest an optimal dose of 10 to 20
mg/kg s.c. twice weekly for 4 to 6 weeks. The s.c. route is preferred because there is no appreciable local toxicity and because intratracheal injection of BLM may occur in 10 to 20% of i.p. injections of BLM in mice, with subsequent digestion of the glycopeptide (unpublished data).

Several other biochemical approaches to evaluation of pneumotoxicity have been attempted with pulmonary toxins other than BLM, including studies of nucleic acid, protein, and phospholipid synthesis; mixed-function oxidation; intermediary metabolism; and lipid peroxidation (24). Elevation of lung collagen content has been described previously with silica administration (14), hyperbaric oxygen (6), radiation (18), and paraquat (15) but not with BLM. BLM has been reported to produce increases in total phospholipids and disaturated lecithin in the alveolar washings of mice after 1 and 2 weeks of daily administration, but a dose-response effect and a relationship to possible chronic toxicity were not studied (2).

Morphometric studies of lung have been reported with oxygen toxicity and experimental pulmonary calcinosis (11, 16). Unless techniques of automated image analysis become more available on a routine basis, quantitative evaluations will remain rather painstakingly slow and tedious. Maintenance of stringently controlled conditions in preparation of tissues is also essential, since the type of fixative, the degree of lung expansion, and variation in thickness of tissue sections may drastically alter measurements of numbers of cells and anatomical architecture.

Studies are currently underway in our laboratory on the effects of schedule of administration of BLM, as well as on potential antidotes and modifiers, with the parameters presented in this paper as indices of lung toxicity.

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