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Cyclophosphamide-induced pulmonary toxicity

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Cyclophosphamide (Cy) is an important anticancer agent used in the treatment of a variety of human neoplasia (Calabresi & Parks, 1980). While its primary side effect has been marrow suppression, it is now well established that this chemotherapeutic agent also can cause significant pulmonary toxicity in both man and rodents (Calabresi & Parks, 1980; Batist & Andrews, 1981). Like radiation therapy, treatments with chemotherapeutic agents such as Cy or bleomycin may lead to pulmonary pneumonitis and progressive fibrosis. However, unlike radiation, pulmonary toxicity following drug treatments may develop soon after exposure.

The mechanisms underlying lung damage resulting from radio- or chemotherapy are poorly understood. Both epithelial and endothelial cell injury have been implicated (Rubin & Casarett, 1968; Withers *et al.*, 1980). With respect to the latter cell type, increased vascular permeability has been suggested as a possible mechanism of radiation-induced lung damage. In support, it recently has been reported that protein leakage from blood vessels following irradiation of the thorax might be related to both the pneumonitic (Anderson *et al.*, 1985) and fibrotic (Law, 1985) phases of lung injury.

The aim of the present investigations was to assess pulmonary toxicity following Cy exposure and to determine to what extent increased leakage of proteins occurs in the lungs after such a treatment. C3H mice were injected with a range of Cy doses and lung damage was quantified using the functional endpoint of increased breathing frequency as developed by Travis *et al.* (1979). This method of assessing lung injury has provided a reproducible assay for radiation induced injury (Travis *et al.*, 1979, 1983; Siemann *et al.*, 1985) and is fast gaining support as an endpoint in studies of cytotoxic drug induced pulmonary toxicity (Collis *et al.*, 1980). Since impairment of lung function could be the consequence of an increase in protein exudate following Cy administration, protein levels in the alveoli were determined by lung lavage at times when significant changes in breathing frequency had occurred.

Materials and methods

Animals and drug treatments

Eight to fourteen-week-old female C3H/HeJ mice obtained from the Jackson Laboratories (Bar Harbor, ME) were used in all studies. Cy was dissolved in 0.9% NaCl and administered i.p. on the basis of animal body weight.

Breathing frequencies

As a functional endpoint of pulmonary toxicity breathing rates were measured in mice using a plethysmograph as previously described (Travis *et al.*, 1979). Measurements were initiated 4 days post Cy exposure and then at weekly intervals after drug treatment.

Biochemistry

After treatments the animals' thoraxes were exposed and the lungs perfused through the heart with 0.9% NaCl until visibly free of blood. The lungs then were removed *en bloc* and lavaged with a total of 2.1 ml ice cold 0.9% NaCl. Recovered lavage fluid was centrifuged and the supernatant analyzed for total protein content (Lowry *et al.*, 1951).

Morphology

At various times after Cy exposure, mice were killed by cervical dislocation and tissue samples immediately immersed in paraformaldehyde-formaldehyde fixative. One mm^3 blocks were dissected and processed as previously described (Penney *et al.*, 1982). Thick sections (1.0 μm) were stained with methylene blue, azure II and basic fuchsin. Thin sections (40–80 nm) were stained with uranyl acetate and lead citrate, and photographed in a Zeiss 10A transmission electron microscope.

Results

Mice were injected with Cy doses ranging from 100–300 $mg\,kg^{-1}$ and their breathing rates determined at various times after treatment. Figure 1 shows the time course of breathing frequency changes for mice treated with 250 or 275 $mg\,kg^{-1}$ doses of Cy respectively. Breathing frequencies increased significantly within a few days after Cy exposure and peaked between 2 and 7 days. A complete dose response curve for breathing frequencies at various Cy doses was therefore

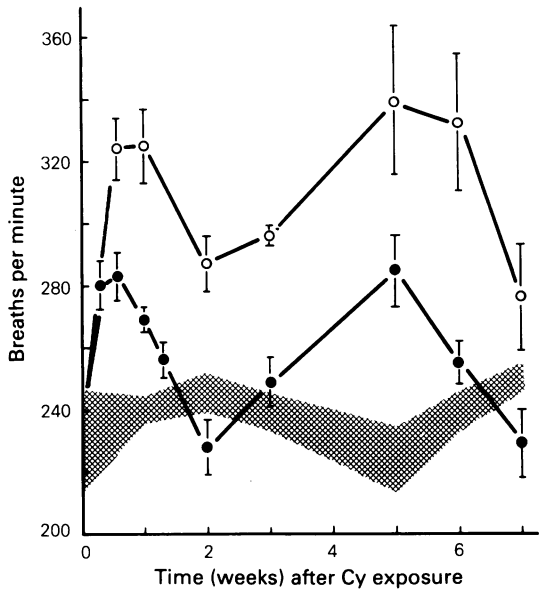

Figure 1 Breathing frequencies as a function of time after treatment with 0 (shaded area), 250 (●) or 275 mg kg^{-1} Cy (○). Data shown are the mean ± s.e. of 8–10 mice.

constructed 4 days after chemotherapeutic agent exposure from data similar to those shown in Figure 1. This dose response curve rises steeply with increasing Cy doses (Figure 2). Light microscope and ultrastructural evaluations performed at this time indicated evidence of pulmonary pneumonitis and the presence of significant levels of proteinaceous material in the alveolar spaces of the lungs of Cy treated mice (Figure 3a).

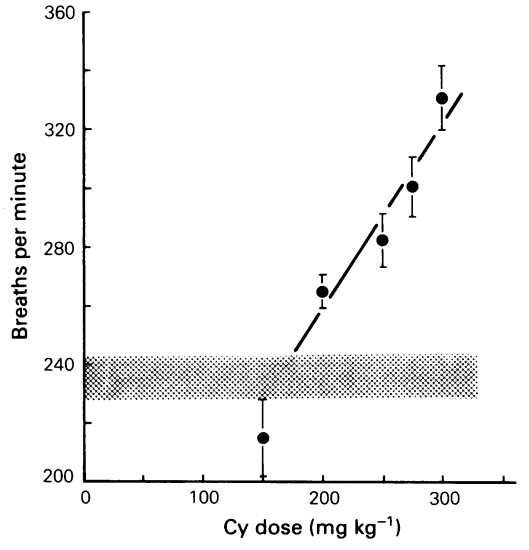

Figure 2 Breathing frequencies as a function of Cy dose measured 4 days after drug treatment. Data shown are the mean (± 1 s.e.) of 8–15 mice.

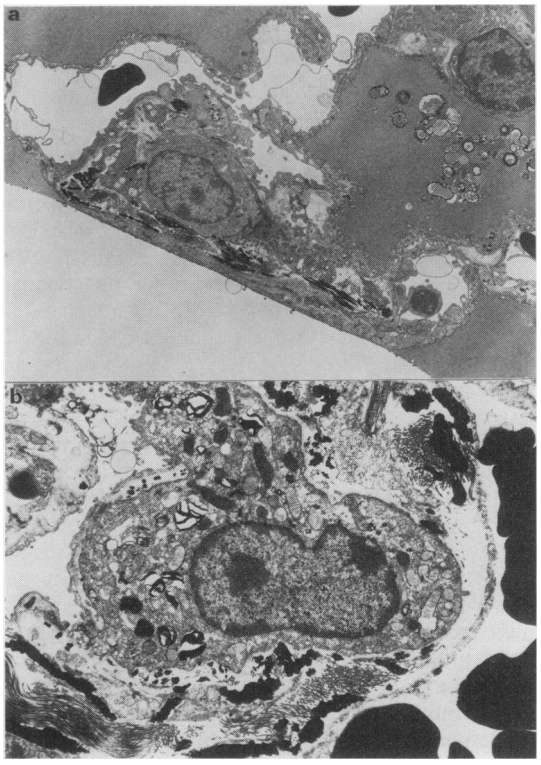

Figure 3 Electron micrographs of portions of the distal lungs from animals given 300 mg kg^{-1} Cy and recovered 4 days (a) or 50 days (b) later. Note the alveolar flooding with proteinaceous material and endothelial blebbing by 4 days, and the increase in septal collagen and fibrin by 50 days. Magnifications: (a) = × 4800; (b) = × 11,200.

Because of these functional and histological observations, it was of interest to quantify the levels of alveolar protein in the lungs of the animals 4 days after Cy exposure. This was done by performing bronchoalveolar lavages. Figure 4 shows changes in the level of alveolar protein that follow treatment with a range of Cy doses. The data represent pooled results of several experiments. There is a clear relationship with increasing Cy doses corresponding to increased levels of alveolar protein. Recovery of soluble protein from lung lavages on day 4 increased with Cy dose in a manner similar to that seen with breathing rates (Figure 2) although elevations in recovered alveolar protein could be observed at drug doses yielding no change in breathing frequency.

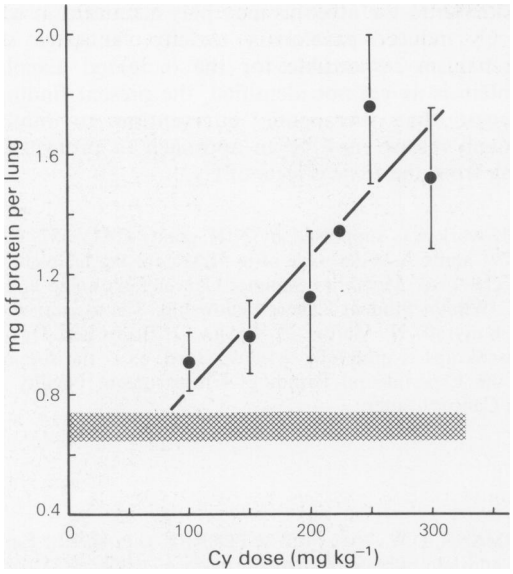

Figure 4 Alveolar protein recovered from lung lavages 4 days after treating mice with a range of Cy doses. Results are the mean (±1 s.e.) of 9–12 animals.

Two to three weeks after Cy treatment, breathing rates decreased (Figure 1) in concert with a clearing of protein from the lungs (data not shown), before rising once again. Ultrastructural studies performed at times greater than 5 weeks post-drug treatment demonstrated a thickening of the septal walls as well as collagen and fibrin deposition (Figure 3b). Such findings are typically associated with pulmonary fibrosis. To further quantitate the extent of lung damage at this later time, measurements of areas available for gaseous exchange were made on tissue sections obtained from mice treated with different Cy doses. This technique has previously been used to assess the impairment of function in the lungs of irradiated mice (Penney *et al.*, 1982). Evaluations of tissue sections obtained in the present experiments indicate a progressive decrease in available air space with increasing Cy dose (Penney *et al.*, in preparation).

Discussion

The present study reports on Cy induced lung damage in mice. Histological evaluation demonstrated that pulmonary pneumonitis can be detected within the week after exposure to this alkylating agent while animals which survive 5 to 7 weeks showed clear evidence of lung fibrosis. These histological observations were associated with impaired lung function as measured by increased breathing rates and reduction in the lung area available for gaseous exchange. These histological and functional changes confirm the effects of Cy on lung tissue previously reported by Collis *et al.* (1980).

Mechanisms of pulmonary injury following insult with radiation or chemotherapy are currently under debate. Both parenchymal and vascular damage have been implicated. A prominent feature of the initial response of the lung to therapy is the exudation of plasma proteins associated with interstitial pneumonitis. Two recent studies have focused on this aspect of lung injury following localized lung irradiation (Law, 1985; Anderson *et al.*, 1985). The aim of the present investigation was to evaluate the role of alveolar protein in Cy induced pulmonary toxicity. The data show a dose dependent increase in alveolar protein levels 4 days after Cy exposure (Figure 4); i.e. at a time when pulmonary pneumonitis can be demonstrated morphologically. In conjunction with the elevated alveolar protein levels, a dose response relationship between Cy dose and breathing frequency also was observed (Figure 2). These data suggest that elevated alveolar protein levels may lead to impaired lung function. The present findings are similar to the results recently reported by Anderson *et al.* (1985), who observed a dose dependent increase in alveolar proteins 16 weeks after lung irradiation. Although breathing frequencies were not measured in that investigation, dose dependent changes in breathing rates are known to occur at this time of radiation induced pulmonary pneumonitis (Travis *et al.*, 1983; Siemann *et al.*, 1985).

Attempts also were made in the present experiments to relate the functional, biochemical and ultrastructural changes observed following Cy exposure to animal lethality as has been done effectively with radiation induced lung injury (Travis *et al.*, 1983; Siemann *et al.*, 1982, 1985). However, due to the systemic nature of the drug's action, such evaluation is difficult. At the highest doses administered a few mice died within 30 days after treatment; the time period usually associated with marrow toxicity. At later times (7 to 13 weeks), mice died in a dose dependent fashion (Figure 5) but many of these animals exhibited extensive damage to their teeth as has been previously observed (Travis *et al.* 1981). Such deaths could be reduced or avoided by maintaining the animals on a semi-solid diet. Progressive lung injury nevertheless occurred in these animals as indicated by the ultrastructural evidence for pulmonary fibrosis (Figure 3b) and loss of area available for gaseous exchange (Penney *et al.*, in preparation).

In summary, these data indicate that release of

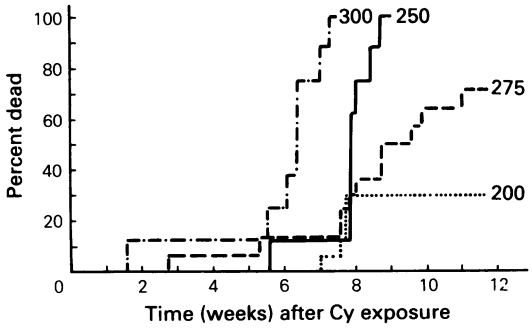

Figure 5 Percent of animals dead as a function of time after treatment with Cy. Numbers indicated are Cy doses administered in $mg\,kg^{-1}$.

protein into the alveolus may play a significant role in Cy induced pulmonary toxicity. Although the mechanism responsible for the increased alveolar protein is as yet not identified, the present findings suggest that therapeutic intervention to inhibit protein release may be an approach to protect the lung from the toxic effects of Cy.

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