No Change in Repair Capacity of Mouse Lung Irradiated Three Months after a Single Dose of Cyclophosphamide

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ABSTRACT

The repair capacity of mouse lung was determined at 3 months after a single i.p. injection of cyclophosphamide (Cy) at a maximally tolerated dose of 275 mg/kg. Mice were irradiated to the whole thorax only with 1, 2, 9, or 15 fractions of X-rays using doses/fraction ranging from 1.2 to 11 Gy. Breathing rate (breaths per minute), histology and pulmonary mortality were used to assess lung damage. Raw breathing rate data were converted to quantal response data by scoring the number of mice in each dose group in each fractionation schedule with a breathing rate 1.3 times the breathing rate of control mice. Dose-response curves of mortality and the converted breathing rate data were constructed at 15 weeks after irradiation (approximately 28 weeks after drug treatment) fitted by logit analysis and 50% effective doses with 95% confidence limits obtained. Values of a/ß were obtained by using the direct analysis method of H. D. Thames et al. (Int. J. Radiat. Biol., 49: 999–1009, 1986).

The a/ß for mice given Cy 3 months before radiation was 3.69 Gy (95% confidence limits, 2.83, 4.69 Gy) and 3.06 Gy (95% confidence limits, 2.31, 3.99 Gy) for the lethality data and breathing rate data, respectively. These a/ß values are in good agreement with the previously published ranges of a/ß of 3 to 4 Gy for mouse lung not given Cy previously. Because the repair capacity of the target cells of a tissue govern the fractionation response and choice of fractionation regimen in clinical radiotherapy, these data indicate that the fractionation regimen used can remain the same as that used in non-drug-treated lungs when the lung is irradiated 3 months after exposure to Cy.

INTRODUCTION

Cyclophosphamide can by itself, induce idiopathic pneumonitis in mice as well as in humans (1–3). When given with irradiation, Cy enhances the radiation dose response of the lung (4), shortens the latent period before radiation pneumonitis is manifested (4, 5), and reduces the isoeffect dose for death (5). Usually, Cy is given within 1 month before or after irradiation. It has been shown that the effects of Cy appear very soon after injection (4 days), but that these effects persist up to death (5). Recently, we reported that the isoeffect dose for radiation pneumonitis in mouse lung, either as a single dose or a fractionated regimen, was significantly smaller when a tolerance dose of Cy was given 1, 3, or 6 months before whole thorax irradiation compared with control (i.e., non-drug-treated) lungs (5). These studies suggest that some of the original drug damage is "remembered" by the lung at long intervals after treatment. Although the mechanisms responsible for this residual drug damage are unknown, one hypothesis is that the initial course of therapy, in this case Cy, caused a permanent depletion of the critical target cells for radiation, which then was manifested as a reduced radiation dose for isoeffect at retreatment. Because conventional radiotherapy is most often given as a multifractionated schedule, allowing for repair of sublethal damage in the target cells, the question arises as to whether the repair capacity of those target cells surviving the first treatment has changed. Although there are reports in the literature on the radiation response of normal tissues previously treated with drugs or radiation, there are no data available on the repair capacity of tissues previously treated with drugs or radiation.

The purpose of the studies here was to determine the radiation repair capacity of mouse lung treated 3 months previously with a single tolerance dose of Cy. This time interval between drug and radiation was chosen based on our previous experiments which showed that the maximum reduction in isoeffect dose for radiation pneumonitis was 3 months after a single maximally tolerated dose of Cy (5).

MATERIALS AND METHODS

Animals. Female C3H/Kam mice, bred and maintained in a specific pathogen-free colony, were used. They were housed 4 to 5 mice/cage and were given sterilized food and sterilized acidified water ad libitum. The mice were 3 months old at the time of irradiation. Because mice treated with Cy are known to develop abnormalities of the incisor teeth (7, 8), the teeth of all mice were checked weekly, and, if abnormalities were found, the mice were given softened food twice a week. Teeth were clipped to normal lengths with dental rongeurs on a weekly basis, if necessary.

Drug. Cy from Mead Johnson Pharmaceuticals was used. A single dose of 275 mg/kg was injected i.p. Previous experiments have shown that this dose is three-fifths of the dose for lethality in 50% of the animals at 30 days for this strain of mice (5).

Irradiation. The irradiation procedure has been described in detail elsewhere (9, 10). Briefly, unanesthetized mice were restrained in special Lucite jigs with supports behind the forelegs. Groups of 4 mice each were irradiated simultaneously to the whole thorax through a 30- x 25-mm portal on the ventral surface of the mouse. The rest of the body was shielded with 3 mm of lead. X-rays were generated by a 250 kVp X-ray machine operating at 250 kV, 15 mA with a total filtration of 0.5 mm Cu and a dose rate of 1.19 Gy/min.

Assays of Lung Damage. Breathing rates and mortality were used to assess lung damage (11). Breathing rates of unanesthetized mouse lung treated with Cy and then at intervals of 2 or 4 weeks, starting 2 weeks after irradiation, throughout the duration of the experiment (11 months after irradiation).

Histological sections were prepared from the lungs of all mice surviving at the end of the study and from any mice killed due to severe growth abnormalities in these experiments.
respiratory distress. Lungs were fixed by intratracheal infusion of 10% neutral buffered formalin and, after fixation, were embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin and examined microscopically.

Experimental Design. Irradiation to the whole thorax was performed 3 months after injection of Cy because our previous studies showed this to be the time when the amount of residual drug damage was maximal (5). At this time, groups of at least 40 mice each were given a range of 1, 2, 9, or 15 equal doses of X-ray; there were at least 5 dose groups in each fractionation arm, and 8 to 10 mice were used in each dose group. Doses per fraction varied from 1.2 to 11 Gy, and thus, total doses ranged from 8 to 30 Gy (Table 1). Six mice received cyclophosphamide and no irradiation to serve as drug-only controls. A group of 10 age-matched mice received no treatment, serving as controls.

All mice in all fractionation schedules were irradiated 3 times a day at 6-h intervals to allow for complete repair of sublethal damage between fractions. The longest overall treatment time was 5 days for the 12-fraction arm to minimize the influence of slow repair in the results (12).

Because of our previous extensive experiments on the repair capacity of mouse lungs after radiation treatment only, the experiments presented here did not include groups of mice given only radiation with no prior Cy treatment. The estimates of the α/β ratio for normal lungs is well established in our laboratory in our mice, as well as in other laboratories. All of these previous studies report α/β ratios between 3 and 4 Gy for radiation pneumonitis (13–17).

Statistical Analysis. Dose-response curves of pulmonary lethality were constructed and fitted by a logit program and LD50 values with 95% confidence limits were determined at 15 weeks after irradiation (105 days). Breathing rate data, as BPM, were plotted as a function of time after irradiation. Dose-response curves for increases in breathing rate can be constructed either from the raw data or by calculating the proportion of mice in each dose group that exceed a specified rate of breathing. We have chosen this latter method to analyze the BPM data as it has the advantage of allowing the resultant curves to be fitted by logit methods, providing error estimates of the isoeffect doses. We have constructed dose-response curves for the proportion of mice exceeding 1.3 times the sham irradiated, Cy-treated controls, although the data also were analyzed at 1.1 and 1.2 time control values. Breathing rate data were analyzed biweekly between 10 and 16 weeks, the time of maximum increases in breathing rates. Any mice that died prior to these assay times but with symptoms of radiation pneumonitis, i.e., increased BPM, which was confirmed histologically, were counted as responders at all subsequent assay times. Dose-response curves were then constructed of these quantal breathing rate data and ED50s were obtained with 95% confidence limits.

The repair capacity of the lung was assessed from both the mortality data and the quantal breathing rate data by calculating the α/β ratio, using the direct analysis method of Thames et al. (18).

### RESULTS

Mortality. Deaths occurred as early as 6 weeks in the mice in this study who were given Cy 3 months prior to lung irradiation. Mortality in this group peaked between 8 and 12 weeks after irradiation and declined slowly thereafter (data not shown). No further deaths occurred at 44 weeks after irradiation, when the experiments were terminated. These data are in agreement with our previously published data for mice given Cy at intervals up to 6 months before irradiation (5). There was no difference in the mortality pattern in any of the fractionation schedules in this study. Dose-response curves of mortality from pneumonitis were constructed at 15 weeks in this study, when the peak mortality had ended. Histological examination of the lungs of all mice killed up to this time exhibited a typical pneumonitis, characterized by cellular infiltrate in the alveoli and septum, septal edema, and widening of the interstitium.

Breathing Rate. Fig. 1 shows breathing rates expressed in BPM, plotted as a function of time for only two of the radiation schedules, 1 fraction (top) and 9 fractions (bottom). Changes in BPM occurred as early as 4 weeks after radiation and peaked between 10 and 14 weeks consistent with the mortality data.

There were no subsequent “waves” of increased BPM, although persistent elevations in breathing rate were observed in the higher dose groups which contained surviving mice. However, none of these mice died. The BPM of the mice given Cy alone was significantly lower than that of the sham-irradiated age-matched controls.

Dose-Response Curves. Dose-response curves for increased breathing rates and mortality from pneumonitis, both at 15 weeks after irradiation, are shown in Fig. 2, top and bottom, respectively. The curves are steep and well defined for both assays. The LD50 and ED50 data for the mice from this study treated with radiation 3 months after Cy administration are given in Table 1. The LD50 and ED50 for pneumonitis is significantly lower when mice are treated with Cy 3 months prior to irradiation than after radiation alone, 9.88 versus 12 to 13 Gy, respectively (5).

Repair Capacity. Reciprocals of the LD50s and ED50s from the mortality and breathing rate data, respectively, from the current study are plotted as a function of dose per fraction in Fig. 3. This plot compares the reciprocal total dose for isoeffect versus the dose per fraction for the same isoeffect (e.g., LD50), and allows an estimation of the repair capacity, or α/β ratio, as the ratio of y intercept to slope of the regression line (19). Also shown in Fig. 3 is the reciprocal dose plot (“Fe” plot) from previously published fractionation data from this laboratory in these mice (13). It is clear from these plots that there is no difference between the values of α/β for the lungs of mice irradiated 3 months after a tolerance dose of Cy as compared with those treated only with radiation. The values of α/β also were calculated from the mortality and breathing rate data by the direct analysis method of Thames et al. (18), giving values of 3.69 Gy (95% confidence limit, 2.83, 4.69 Gy) and 3.06 Gy (95% confidence limit, 2.31, 3.99 Gy), respectively (Table 1), in good agreement with the values obtained from the Fe plots. The α/β ratio for mouse lungs not previously treated with Cy from this laboratory in these same mice is 3.7 Gy (95% confidence limit, 3.2, 4.3 Gy) (13).

### DISCUSSION

Clinically, it is sometimes necessary to retreat recurrent tumors that have already received a full course of radiation...
therapy or chemotherapy, which means further exposure of critical normal tissues. More data now are available on changes in radiation dose for isoeffect after previous irradiation of acutely responding normal tissues, i.e., skin (20-22), and late-responding normal tissues, e.g., lung (23), kidney (24-26), spinal cord (27, 28), and bladder (29, 30). However, knowledge of changes in radiation dose for a given effect at re-treatment, although critical, is not sufficient for the radiotherapist since few treatments use large single fractions of radiation. A second critical factor in avoiding complications in normal tissues treated previously with drugs or radiation is the repair capacity of these tissues at re-treatment. To our knowledge, such data are not available for any normal tissue.

The $\alpha/\beta$ ratio for mouse lung treated 3 months previously with Cy in our studies are in remarkably good agreement with the $\alpha/\beta$ range of published values for “normal” mouse lung. The $\alpha/\beta$ ratio for radiation pneumonitis in mouse lung has been well defined by ourselves as well as many other investigators and is always in the range of 3 to 4 Gy, regardless of mouse strain. For this reason we chose not to repeat these experiments but rather to compare our data after Cy with existing published data. In fact the $\alpha/\beta$ ratio was virtually identical for the lungs of mice given the Cy in the current study compared to published values of $\alpha/\beta$ for the lungs of normal mice. This was true for both assays of lung damage-mortality from pneumonitis and breathing rate, $\alpha/\beta$ of 3.7 and 3.1 Gy, respectively. The breathing rate data analyzed here used a 30% increase above Cy-treated controls as the cut-off for responders versus non-responders; analysis at other levels of damage did not change the conclusions. In addition, analysis at 1.15 times the BPM of the non-drug-treated control mice, gave similar values of $\alpha/\beta$.

The $\alpha/\beta$ ratio for mice given Cy was calculated at 15 weeks because none of the drug-treated mice given radiation died after this time, in agreement with published data. Histological examination of the lungs of those mice treated with both agents showed a pneumonitis similar to that seen after irradiation.
can remain the same. Such data are relevant clinically to patients with advanced lymphomas who fail their initial chemotherapy, necessitating treatment with radiation portals involving the lung, or in patients in whom radiation is part of their consolidation therapy following a full course of chemotherapy.

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REFERENCES


Fig. 3. Reciprocal of total dose as a fraction of dose/fraction for 50% incidence of deaths (¶) or 50% incidence of BPM ≥ 30% above controls (•) from the present studies compared with historical controls using radiation only. Bar on each point are the reciprocals of the 95% confidence limits. The lines are linear regression fits to the data. The bar on the abscissa labeled 95% c.l. are the 95% confidence limits on the alpha/beta value after lung irradiation only (13, 18).

The interval between drug and radiation used in these studies was chosen, based on our previous data which indicated that the biggest reduction in the single radiation dose for isoeffect was 3 months after the maximally tolerated single dose of Cy (5). When Cy was given in a more clinically relevant regimen in these earlier studies, i.e., as 5 daily doses, the radiation dose for pneumonitis also was reduced at all times tested up to 6 months after Cy, but this did not depend on the time interval between the two treatments. Based on these data, we would predict that the repair capacity of the lung will remain the same at other times after Cy treatment, at least up to 6 months after drug.

The amount of damage remaining in normal tissues at long times after initial treatment with drugs and the influence of this residual damage on retreatment with another modality is being investigated by many laboratories. It is now clear that the amount of damage remembered by a tissue and the influence of this on subsequent retreatment depends on the initial treatment (i.e., radiation or drug), the specific tissue, the size of initial dose, and the interval between these treatments (20-30). However, there are no data published on the repair capacity of normal tissues previously exposed to either drugs or radiation.

We have conducted studies on the repair capacity of mouse lung previously irradiated at intervals up to 1 year with a sublethal dose of radiation. These studies show that the repair capacity of the lung decreased, i.e., the alpha/beta ratio increased (31).

This, although it has been suggested that these two cytotoxic agents target the same population of cells in the lung, the differences in the repair capacity indicate that these cells respond differently to the two types of damage. Alternatively, the data suggest that the target cells for radiation damage in the lung are not the same as those for Cy.

Since the repair capacity of the target cells of a tissue govern the fractionation response and choice of fractionation regimens in clinical radiotherapy, these data show that although the total dose of radiation given must be lowered to avoid complications in lungs previously treated with Cy, the fractionation schedule...
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