Residual Damage in Mouse Lungs at Long Intervals after Cyclophosphamide Treatment

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ABSTRACT

The purpose of these studies was to quantify the effects of radiation given to mouse lungs at intervals up to 6 months after injection of the maximally tolerated dose of cyclophosphamide. In one set of experiments a single i.p. injection of 300 mg/kg of cyclophosphamide was followed at either 1, 3, or 6 months by a range of single doses of γ-rays delivered to the whole thorax only. In a second set of experiments mice were given five daily i.p. injections of cyclophosphamide, 100 mg/kg, followed at 1, 3, and 6 months by a range of fractionated doses of X-rays. Breathing rate, histology, and mortality were used to assess lung damage. These data were compared with age-matched animals given either the drug alone or single doses of radiation alone. Dose-response curves of lethality were constructed and fitted by a logit program, and 50% lethal doses with 95% confidence limits were determined at monthly intervals after irradiation. Dose enhancement factors were then calculated at this isoeffect for the mice given the drug and radiation. Deaths from radiation pneumonitis occurred as early as 6 weeks in mice given cyclophosphamide before irradiation; few deaths occurred after 26 weeks. However, in the mice given radiation alone, deaths from pneumonitis did not occur before 12 weeks. Cyclophosphamide given as either single doses or fractionated doses at all three times before irradiation enhanced radiation pneumonitis in mouse lungs. Dose enhancement factors of 1.2, 1.4, and 1.3 were obtained when single doses of radiation followed single doses of cyclophosphamide at 1, 3, and 6 months, respectively. The dose enhancement factor for radiation pneumonitis after the fractionated exposures was less, 1.1, and was independent of time between the two treatments. An enhancement factor of 1.2 was observed for the later wave of lung damage in those few studies available for analysis at this time. These data clearly show that prior treatment of the animal with cyclophosphamide significantly reduces the radiation dose that can be given to the lung for as long as 6 months after drug treatment. In addition, lung damage occurred sooner when the drug was given prior to irradiation. These data indicate that the lung will be sensitive to retreatment with radiation when a full tolerance dose of cyclophosphamide precedes radiation.

INTRODUCTION

The primary dose-limiting factor in clinical radiotherapy is normal tissue toxicity. For tissues that respond long after treatment is completed, e.g., lung and kidney, few methods are available to spare these tissues. In addition, many patients referred for radiotherapy have been treated previously with chemotherapy, sometimes months or years before referral. The long-term effects of chemotherapeutic drugs on normal tissues are not well known. Even less is known about the effects of chemotherapy and subsequent radiotherapy when time intervals greater than 4 weeks separate the two treatments.

The purpose of this study was to quantify the effects of radiation given to mouse lung at intervals up to 6 months after maximally tolerated doses of cyclophosphamide. These studies also allowed the time course of tissue effects at long times after cyclophosphamide treatment to be quantified. Cyclophosphamide was chosen because of its widespread clinical use and documented toxicity to lung tissue both in experimental animals and humans (1–8).

In one set of experiments, a single MTD3 of drug was followed by a range of single doses of X-rays. To make our study more relevant to clinical situations, another set of experiments used a fractionated MTD of drug followed by a range of fractionated doses of X-rays.

MATERIALS AND METHODS

Animals. Female C3H/Kam mice, bred and maintained in a specific-pathogen-free colony, were used. The mice were 8 to 9 weeks old at the time of initial treatment (either with the drug or with saline), except for one experiment in which the mice were 13 weeks old. Mice were housed four or five per cage and given sterilized food and sterilized, acidified water ad libitum. Some mice treated with cyclophosphamide developed loss of or growth derangement of incisor teeth. Because these mice could not masticate hard food pellets, food was soaked in sterile water until softened. Softened food was administered at least twice a week and helped prevent loss of mice from starvation. Teeth were clipped to normal lengths with dental rongeurs on a weekly basis, if necessary.

Drug. Cyclophosphamide from Mead Pharmaceuticals was used for all but one experiment, in which Neosar from Adria Labs was used. The single and fractionated MTDs for cyclophosphamide in our mice were determined from preliminary LD50 values after other toxicological insults. The data obtained from the functional assay have been shown to agree well with histological and radiobiological assays. Breathing rate, histology, and mortality were used to assess lung damage. Breathing rates of unanesthetized mice at 4- to 6-week intervals throughout the experiment were used to assess lung damage. Breathing rates of unanesthetized mice at rest were measured at 4- to 6-week intervals throughout the experiment starting from Week 2 after drug administration and Week 2 after irradiation. The breathing rate assay used most extensively in radiation studies was used (10–14). Briefly, each mouse was placed in a sealed plethysmograph chamber with a capacity of about 200 ml, and the mouse's rate of breathing (BPM) was determined using a capacitance manometer microphone, as described previously (15). This noninvasive assay provides a quantitative assessment of lung damage following radiation as well as after other toxicological insults. The data obtained from the functional assay have been shown to agree well with histological and radiobiological assessments of damage (10). Breathing rates were calculated as BPM per minute; LD50, 50% lethal dose; Cy, cyclophosphamide.

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3 The abbreviations used are: MTD, maximally tolerated dose; BPM, breaths per minute; LD50, 50% lethal dose; Cy, cyclophosphamide.
and plotted as a function of time for each dose group.

Mice judged as terminally sick were killed. These deaths were recorded with other lethality as they occurred. Any mice that died due to tooth growth abnormalities were excluded from mortality analyses.

Histological sections of mouse lungs were prepared from all mice surviving at the end of each experiment and from mice killed due to severe respiratory distress or recent death from other causes. Lungs were fixed by intratracheal infusion of 10% neutral buffered formalin. Fixed lung sections were embedded in paraffin, and 5-μm sections were stained with hematoxylin/eosin.

Hematological Assays. Blood was collected from mice by a tail vein nick; the fluid was immediately collected in a heparinized capillary tube. Twenty μl of blood (measured by glass micropets) were transferred to 19,980 ml Isoton II blood diluent. Erythrocytes were lysed by a commercial blood-lysing liquid (Zapoglobin). A Coulter ZBI counter was used for leukocyte counts. Hemoglobin was measured by a Coulter hemoglobinometer. Commercial hematology controls were included in each run. In most cases, the first dilution (before lysing) was further diluted for erythrocyte count and hematocrit determination. Because erythrocyte counts and hematocrits mirrored the hemoglobin levels, only hemoglobin levels are reported.

Experimental Design. Single drug doses were always followed by single doses of radiation (Experiments S-1, S-3, and S-6), and fractionated drug doses were always followed by fractionated radiation (Experiments F-1, F-3, and F-6). Two groups of 210 mice each received either a single i.p. dose or five daily doses of cyclophosphamide as described above. At 1, 3, and 6 months following cyclophosphamide treatment, groups of 70 mice each were given either single doses of radiation ranging from 8.5 to 12.75 Gy (Table 1) or 10 equal fractions ranging from 1.8 to 3.5 Gy/fraction (Table 2) of γ-rays to the whole thorax. Each dose group contained at least 8 mice. The choice of radiation doses in both the single and fractionated regimens was designed to detect cyclophosphamide enhancement factors for lung damage ranging from 1.0 (no effect) to 1.7 (up to a 70% reduction in radiation dose following cyclophosphamide treatment). In the fractionated experiments, the interval between fractions was 12 h so as to allow "complete" repair of sublethal radiation damage and the overall treatment time was 5 days to minimize the influence of slow repair on the results (11, 16). At the 1- and 6-month irradiation time, two groups, each with 60 age-matched mice, received either a range of single doses of γ-rays (10.5 to 13.5 Gy) or 10 equal fractions ranging from 2.0 to 3.8 Gy/fraction of γ-rays to the whole thorax and served as age-matched radiation-only controls. In addition, two groups of eight mice each were given a single dose or five daily doses of cyclophosphamide, serving as drug controls. One group of eight mice was given an i.p. injection of saline and sham-irradiated, thus serving as age-matched controls.

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 monthly intervals after irradiation. Dose enhancement factors [the ratio of isoeffect dose (i.e., LD₅₀) for radiation alone to that for drug and radiation combined] with 95% confidence limits were calculated using the method of Pike and Alper (17) for the mice given before the drug and radiation.

RESULTS

Mortality

Fig. 1 shows a histogram of numbers of mice dead as a function of weeks after single dose (left) or fractionated dose (right) irradiation. Top row, pattern of death as a function of time after radiation alone; bottom three rows, pattern of death when cyclophosphamide precedes radiation by 1, 3, or 6 months (S-1, S-3, and S-6 or F-1, F-3, and F-6, respectively).

A similar pattern was observed after fractionated doses of cyclophosphamide and radiation; i.e., deaths occurred sooner than after radiation alone, particularly when the two treatments were separated by 1 month (Fig. 1). This trend was less pronounced when 3 or 6 months elapsed between Cy and radiation. Neither the single nor the fractionated doses of drug on their own killed any mice from lung damage.

Breathing Rate

Single Doses. Fig. 2 shows BPM as a function of time for selected dose groups from mice given either single doses of radiation alone (Fig. 2, top) or 3 months (S-3) or 6 months (S-6) after a single dose of 300 mg/kg of cyclophosphamide. The changes in BPM after radiation alone are in agreement with previously published data (10–14, 19, 20). Briefly, both the time of onset and peak response were dose dependent, occurring sooner after the highest doses. No changes in BPM occurred before 10 weeks even after doses that were lethal to 100% of the mice, e.g., 12.75 and 13.5 Gy. After these doses BPM progressively increased after 10 weeks until all mice succumbed from severe respiratory distress. After moderate doses, e.g., 12 Gy, BPM decreased from severe respiratory distress. After moderate doses, e.g., 12 Gy, BPM decreased in those mice surviving the initial peak response which occurred between 20 and 30 weeks.

The breathing rate of mice given cyclophosphamide at any
of the three times before irradiation was increased compared to the BPM of mice given similar or lower doses of radiation alone (see Fig. 2 comparing 11.25 Gy (Fig. 2, top) with 8.5 and 9.5 Gy at 3 months after Cy (S-3) or 11 Gy given 6 months after Cy (S-6)). Although this dose of Cy on its own caused a significant increase in BPM above that of control mice (average BPM of 290 and 250, respectively) (see Fig. 2), the BPM increases in mice given both agents were significantly above those caused by this dose of drug alone and were dependent on radiation dose. Cyclophosphamide also shortened the time of onset and peak response time when given at all three times before irradiation in mice given cyclophosphamide 6 months before irradiation.

The lungs of mice given single or fractionated doses of cyclophosphamide alone exhibited similar changes, except at earlier times. A mild pneumonitis was observed as soon as 1 month after both drug dosing regimens. By 6 months there was diffuse thickening of the interstitium with focal collections of “foamy” cells in the air spaces. Subjectively these changes appeared more severe at all times after single doses than after fractionated doses of cyclophosphamide.

Cyclophosphamide and Radiation. The lungs of mice given both Cy and radiation exhibited pneumonitis up to 4 months after irradiation. All surviving mice from S-3, S-6, and F-6 were sacrificed between 7 and 8 months after irradiation. The lungs of these mice showed a late-resolving pneumonitis with foci of foamy cells. In a few sections, lesions similar to those found 1 year after radiation alone were found. By 1 year after cyclophosphamide and radiation, when all surviving mice from S-1, F-1, and F-3 were sacrificed, the lungs exhibited only these later kinds of lesions, which looked exactly like those found in the lungs of mice sacrificed 1 year after radiation alone. Thus, the peak of deaths between 8 and 12 weeks in mice given both drug and radiation was due to pneumonitis, whereas after radiation alone this same lesion appeared between 12 and 28 weeks. Because few mice given both drug and radiation died after 12 weeks (see Fig. 1), the time taken for construction of all dose-response curves for lethality from pneumonitis was 26 weeks, the standard assay time for pneumonitis after radiation alone.

Dose-Response Relationships

Dose-response curves were constructed at two times, at 26 weeks for deaths from pneumonitis and, where possible, at 48 weeks for deaths from the later phase of lung damage. Fig. 3 (top) shows dose-response curves for death at 26 weeks after single doses of radiation alone or at 1, 3, or 6 months after cyclophosphamide. The curves are logit fits to the data and 95% confidence limits are shown. No age dependency for mortality was found after radiation alone, the LD₅₀ values were virtually the same (see Table 1). Thus, the two age-matched control groups were combined for analysis and are presented as one dose response curve. Smooth, well-defined monotonic dose-response relationships were produced for mice given radiation alone, as well as for mice given cyclophosphamide prior to radiation. There was a clear displacement of the dose-response curves to lower total doses when cyclophosphamide was given at all times before irradiation. This displacement was dependent on the time between the two treatments, with the 3-month curve...
LATE RESIDUAL LUNG DAMAGE AFTER CYCLOPHOSPHAMIDE

Fig. 3. Dose-response curves for pulmonary lethality at 26 weeks from pneumonitis or 48 weeks from the later phase of damage after drug and single doses of radiation (Δ). S-1, S-3, and S-6, months between drug and radiation.

being displaced to lower doses than those curves at 1 or 6 months.

Dose-response curves for mortality after fractionated doses of drug and radiation are shown in Fig. 4 (top). The data are plotted as a function of total dose. The radiation-alone curve consists of two experiments from age-matched control mice, which showed no difference in LD₅₀, and have been combined for analysis. Again, the dose-response curves from mice given cyclophosphamide at all three times prior to radiation are displaced to lower total doses than those for mice given radiation alone. However, there is little time effect for the treatments; i.e., the dose-response curves at 1, 3, or 6 months cannot be resolved.

Dose-response curves of mortality for those animals surviving up to 1 year after single doses or fractionated doses of radiation are shown in the bottom parts of Figs. 3 (bottom) and 4 (bottom), respectively. Only three time intervals allowed this analysis, 1 month after single doses and 1 and 3 months after fractionated doses. As observed at the earlier assay time, cyclophosphamide given prior to radiation caused a significant reduction in the LD₅₀ for this later wave of damage.

Isoeffect Data

Radiation isoeffect data have been obtained at the LD₅₀ level from the logit fits to the lethality data shown in Figs. 3 and 4. These values with 95% confidence limits are given in Tables 1 and 2, as well as the DEF for the cyclophosphamide-treated mice.

Hematological Changes

Transient decreases in blood leukocyte counts were observed in the first month after either single or fractionated doses of cyclophosphamide. Thus, at the time of all irradiations, leukocyte counts in all Cy-treated mice were within control values. Single doses of radiation alone did not change leukocyte counts at any time, indicating that any deaths in this arm of the study were not due to sepsis. Fractionated irradiation caused a transient depression in leukocyte counts (50% of controls) by the second week after treatment which returned to normal by 2 months. Overall, peripheral leukocyte counts were not altered in any mice when death occurred. This finding, combined with histological confirmation of severe lung damage, rules out sepsis as a cause of death for mice in these experiments.

Except for an initial transient decrease in hemoglobin levels, blood hemoglobin levels in all treated mice were similar to those in untreated control mice, indicating that anemia did not account for any deaths during the experimental periods. Even mice with tooth growth abnormalities maintained on wetted feed did not exhibit anemia, suggesting nutritional status was not grossly inadequate.

Tooth Abnormalities

A few deaths (<1%) were due to loss of one or more sets of incisors, as described previously (21–23). Loss of incisors oc-
The combination of soft food and weekly clippings occurred about 60 days after the single dose of Cy, but not until upper incisors had to be clipped weekly to prevent excessive elongation. The objective of these studies was to quantify the response of the lung to irradiation at long intervals after treatment with cyclophosphamide, a widely used chemotherapeutic agent with well-defined pulmonary toxicity. The doses of drug chosen, although higher than those used in other studies (2, 3, 24-26), represented the MTD in our mice. The morphological changes induced in the lung by cyclophosphamide alone, radiation alone, or both agents given at various time intervals were the same qualitatively. These changes appeared sooner after cyclophosphamide than after radiation, because few mice given the drug before irradiation died after 26 weeks, all iso-effect data were compared at this time, the standard assay time for death from radiation pneumonitis. Analyses at earlier times would not have changed the conclusions.

The data presented here clearly show that cyclophosphamide given either as a single dose or in a fractionated regimen significantly reduces the radiation dose that can be given to the lung for as long as 6 months later, i.e., some of the initial drug damage is “remembered” by the tissue. After a single dose of cyclophosphamide, the amount of drug damage remembered appears to increase up to 3 months (i.e., the radiation LD50 is lower at 3 months than at 1 month) with some recovery by 6 months (the radiation LD50 is now higher although the 95% confidence limit overlap). Even at this late retreatment time (6 months) lungs treated previously with cyclophosphamide have not returned to the sensitivity of normal (i.e., non-cyclophosphamide-treated) lungs.

Fractionated doses of the drug also caused a decrease in the isoeffect dose of the lung to subsequent irradiation, but there was less enhancement as compared with the drug given as a single dose, even though both drug doses represented the MTD for each regimen. In addition, there was little change in radiation isoeffect dose at any of the three times tested. These data indicate that the drug, when given as the MTD in a fractionated schedule, caused less damage than a single dose alone and that this damage neither progressed nor recovered for as long as 6 months later.

If it is accepted that depletion of a critical population of cells, “target cells,” to a threshold level is necessary before overt tissue damage and dysfunction occurs and that changes in the radiation isoeffect dose (LD50 in these studies) reflect relative tissue damage and dysfunction occurs and that changes in the time course of depletion and repopulation of the putative target cells in the lung for radiation. A reduction in the isoeffect radiation LD50 would represent cell killing and subsequent regeneration of the tissue; an increase would reflect repopulation and subsequent repopulation by these cells.

Fig. 5 shows the LD50 levels for radiation pneumonitis as a function of time after cyclophosphamide treatment from only the single dose data. First, although the single dose of cyclophosphamide used in this study (300 mg/kg) was sublethal for cervical lymph nodes would not have changed the conclusions.
animal survival, on its own it clearly killed some cells; i.e., the LDL for whole thorax irradiation was always less in the cyclophosphamide-treated mice than in control mice (non-drug treated) (Fig. 5, hatched area). The fluctuations in the LDL levels for radiation pneumonitis with time between the two treatments, although not significant, suggest that the number of target cells for radiation did not remain constant but continued to decrease up to 3 months, with an indication of proliferation by 6 months after single doses of cyclophosphamide.

In addition, the response of the lung to both treatments occurred well before the response to radiation alone (see Figs. 1 and 2) and was more consistent with the time the response was observed after cyclophosphamide alone. This finding, combined with the consistently lower LDL levels, indicates that the lungs of mice previously treated with cyclophosphamide contained fewer target cells at the time of irradiation than the lungs of normal, non-drug-treated mice. Therefore, not only did a lower radiation dose deplete the cells to a critical level, but it also took less time to achieve that level, because the tissue was nearer to the critical threshold level of cell depletion at the time of exposure.

This hypothesis suggests that radiation and cyclophosphamide share the same target cells. However, the cellular etiology and pathogenesis of both radiation- and cyclophosphamide-induced lung damage is unknown and controversial (28–31). The type II epithelial cell has been implicated as the target cell for radiation-induced lung damage, at least for the early-appearing pneumonitis (32–34). The target cell in the lung for cyclophosphamide is even less apparent, but labeling studies suggest that the type II cell is involved (3, 26). Thus the type II cell may be the target for both cytotoxic insults.

The differences in latent times for lung damage after radiation alone or drug treatment alone further support the suggestion that the type II cell is the common target. Whereas lung damage

is not overtly expressed (as either increased BPM or death) until at least 3 months, even after a radiation dose that is lethal to 100% of the mice, cyclophosphamide-induced lung damage is expressed within the first 2 months after the dose is administered. These differences in latent times are consistent with labeling studies which indicate that proliferation of type II cells occurs within the first week after cyclophosphamide administration (26), whereas type II cell proliferation occurs much later (between 1 and 3 months) after irradiation (32). The damage would therefore occur later after radiation exposure than after cyclophosphamide treatment.

Whereas we suggest that changes in the radiation dose for iso-effect simply reflect stem cell depletion, Rubin (30) would argue that the mechanism of action of these two cytotoxic insults is different and that they affect either (a) different target cells or (b) the same target cells but via different pathophysiological mechanisms. However, the consistently lower LDL levels for radiation pneumonitis in mice previously treated with cyclophosphamide suggests that these two cytotoxic agents share a common target cell even if they each have more than one.

Although neither the single dose nor fractionated doses of cyclophosphamide alone killed any mice, both caused some lung damage, which was expressed as an increase in breathing rates significantly above that of age-matched control mice. These changes in BPM were accompanied by progressive histological changes similar to those described previously, although after lower cyclophosphamide doses. The drug appeared to produce a chronic, irreversible effect on the lung, similar to that described by Siemann et al. (3) and Morse et al. (35).

It is interesting that these morphological changes, which appeared to be progressive, were not accompanied by progressive changes in lung function or by a corresponding progressive increase in radiation sensitivity of the lung, even after a high single dose of the drug. Rather, lung sensitivity (at the worst) remained the same between 3 and 6 months after cyclophosphamide treatment, a time in which the pathology changed from an alveolitis to an alveolitis accompanied by fibrosis. This apparent progression in the pathology did not represent a progression to a more severe lesion. In addition, unlike the mice given radiation alone, which continued to die for up to 1 year after single or fractionated doses, only a few mice given both cyclophosphamide and radiation died after 26 weeks in those three studies available for analysis. Thus, the enhancement of this later wave of injury seems to be a result not of chronic cyclophosphamide damage but rather of chronic radiation-induced lung failure.

Considering the implications of these data for the clinical treatment of patients with recurrent malignancies, it is surprising that few data are available in the literature on this topic. Only two other studies have assessed the radiation response of other normal tissues, bone marrow (36) and bladder (37), after cyclophosphamide treatment. Although both of these studies used lower drug doses than those used in the present study, both reported a “significant” reduction in the radiation tolerance of these two tissues for up to 9 months after cyclophosphamide treatment, in agreement with our conclusions.

In summary, prior treatment with cyclophosphamide reduces the radiation dose that can be given to the lung for as long as 6 months after drug treatment. Although the MTD of cyclophosphamide given as either a single dose or multiple doses enhanced the sensitivity of the lung to subsequent irradiation, fractionated doses caused less enhancement. In addition, lung damage occurred sooner when drug administration preceded
irradiation. These data indicate that the lung will be sensitive
to retreatment with radiation when a full “tolerance” dose of
cyclophosphamide has preceded irradiation.

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