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Lung Radioprotection by WR-2721 at Low X-Ray Doses per Fraction*

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Abstract—*The response of mouse lungs to single doses and ten fractionated irradiations has been tested using breathing rate and lethality as assays for damage. The radioprotective effect of* 300 mg/kg *WR-2721 has been determined for mice breathing air or* 10% *oxygen. The protection factor was assessed from dose response curves obtained at monthly intervals from* 24 *to* 48 weeks. *A low protection factor* (1.2–1.4) *was observed for single doses in air or* 10% *oxygen and also for ten fractions in air. Considerably more protection was seen with ten fractions in mice breathing the reduced oxygen concentration (protection factors of* $PF = 1.5–1.7$). *It is postulated that the low PF values normally reported for lung are due to the naturally high oxygen concentration in all cells in this tissue. A fraction of the cells becomes sufficiently hypoxic in* 10% *oxygen to be susceptible to WR-2721 radioprotection. This subpopulation can then be detected with small X-ray fractions* (≤5 Gy) *but not with large single doses.*

INTRODUCTION

A RANGE of radioprotection factors has been observed in different normal tissues with the phosphorothioate drug WR-2721. High protection factors (PF)‡ have been shown in bone marrow and parotid ($PF = 2.5–3.0$), whereas low PF values have been measured in kidney, lung and CNS ($PF = 1.3–0.5$) [1]. The cause of this variability needs to be investigated if WR-2721 or similar radioprotective thiols are to be safely used in the clinic to specifically spare normal tissue damage. The factors already identified as important in modifying the action of WR-2721 are: (1) drug concentration in each tissue; (2) endogenous levels of sulphydryl compounds; (3) the level of dephosphorylating enzymes; (4) the precise level of oxygenation; and (5) the X-ray dose at which the effect is measured.

There is little information for most of these factors as they relate to lung radiosensitivity and the limited ability of WR-2721 to protect the lung. We recently investigated the possibility that the low radioprotection by this drug in lung was due to the high natural oxygen level overwhelming the proton donation from the thiol [2]. We observed significantly greater radioprotection by WR-2721 when the level of oxygen in the lung was reduced to 7% at the time of irradiation relative to air or 100% oxygen. A similar effect has recently been reported by Down and Steel [3]. These results support the idea that competition exists between oxygen and thiols in the process of fixation and repair of the initial radiochemical lesion.

The clinical usefulness of WR-2721, however, depends on the radioprotection that will be observed in air-breathing patients at the low X-ray doses per fraction which are used in fractionated radiotherapy, and with the drug dose that can be tolerated in repeated administrations. The suggestion that radioprotection in various normal tissues increases as the X-ray dose per fraction is reduced [4] has prompted us to investigate radioprotection with large single doses and with ten small fractions of X-rays. The experiment was performed using mice breathing either air or a lower oxygen concentration during each irradiation. Ten percent oxygen was chosen as the lowest level that can be safely used in combination with repeated restraint of the mice and administration of WR-2721, using X-rays at a low dose rate. Because of cumulative drug toxicity

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‡PF = X-ray dose with protector divided by dose without protector to cause the same biological effect.

[5] we restricted the drug dose administered to 300 mg/kg for each fraction and also for single doses.

MATERIALS AND METHODS

A total of 216 CBA/HtfBSVS male mice were used in this experiment (54 dose groups of four mice). They were 8–10 weeks old and weighed approximately 25 g at irradiation. The irradiation procedures have been reported in detail elsewhere [6]. Briefly, the unanaesthetised mice were irradiated to the whole thorax, while restrained in Perspex jigs; 240 KVcp X-rays were used at a dose rate of 1.8 Gy/min (HVL 1.3 mm Cu). For irradiation at reduced oxygen tension the mice were enclosed in a Perspex box through which 10% oxygen at 22°C flowed at 20 l/min for 45 sec before and also during the irradiation.

WR-2721 was kindly provided by Dr. V. Narayanam of the Drug Investigation Division, N.C.I.; it was freshly made up in distilled water shortly before each experiment and was injected i.p. 30 min before irradiation. The time in solution before injection ranged from 0.5 to 1.5 hr.

Lung damage was assessed at monthly intervals from 4 months after irradiation by monitoring the breathing rate [6]. The breathing rate (breaths/min) was measured in a whole-body plethysmograph; the rate was recorded as pressure changes using a microphone diaphragm as the transducer. The non-invasive breathing rate assay can be used to follow the progression of lung damage from acute pneumonitis to late fibrosis [7]. Dose–response curves were constructed for changes in breathing rate and for the more severe lung damage that led to pulmonary death.

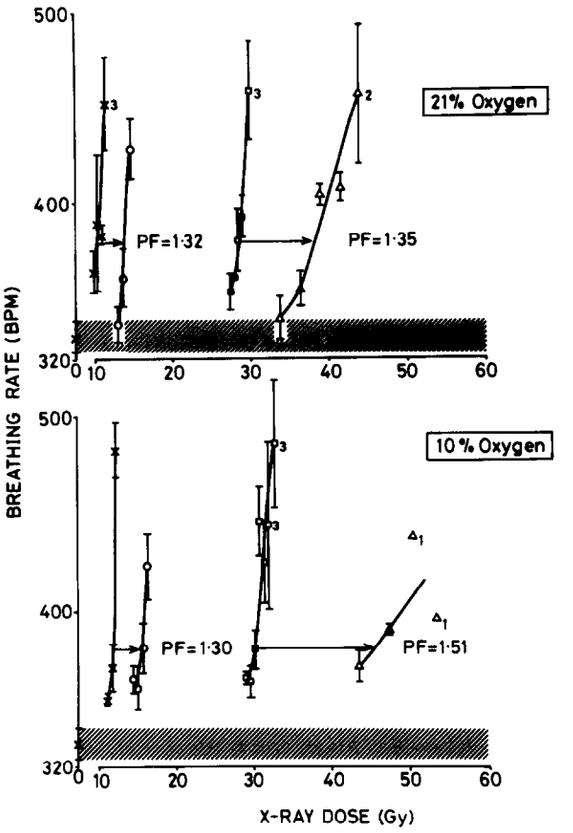

Fig. 1. Dose–response curves for animals tested 32 weeks after localised irradiation in air (upper panel) or 10% oxygen (lower panel). Each point represents the mean of 4 mice (unless otherwise indicated) ± 1 S.E.M. The breathing rate increased from 332 ± 8 breaths/min to about 480 breaths/min in severely damaged lungs. The more severe damage led to death and some of the upper dose groups are therefore depleted to the indicated numbers. The protection assessed was remarkably constant with single doses (in air or 10% oxygen) or with 10 fractions in air. More protection was seen with 10 small fractions in mice breathing 10% oxygen. X = single dose X-rays; ○ = single dose X-rays with WR-2721; □ = 10 fraction X-rays; △ = 10 fraction X-rays + WR-2721.

RESULTS

No change in lung function due to drug toxicity was observed after WR-2721 alone, whether it was given in 1 or 10 doses. An example of the data for irradiated mice is presented as dose–response curves in Fig. 1, for an assay performed at 32 weeks after irradiation. Each panel shows two pairs of dose–response curves for mice treated with X-rays alone or at 30 min after administration of 300 mg/kg WR-2721. The top panels are for animals breathing air, the bottom for mice breathing 10% oxygen. The vertical error bars represent 1 S.E.M. and the number of mice contributing to each point is indicated if it is less than four. Radioprotection by WR-2721 is apparent in all four regimes, and this effect can be quantified as a protection factor (ratio of doses to produce any chosen isoeffect level). PF values are indicated at 380 breaths/min. Similar values were determined at other test times and these are summarised for tests made at 24–48 weeks in Table 1. The uncertainty estimate on the PF value is obtained from the envelope drawn through the vertical standard error bars on breathing rates, translated graphically into a horizontal dose uncertainty (approximately 1 S.E.M.).

An alternative analysis of these breathing rate data can be made by assessing the dose required to cause a significant level of damage in 50% of the mice. Such an ED_{50} analysis has also been made of the data obtained at various intervals. Those for the 32-week assay are shown in Fig. 2, fitted by logit analysis. For most schedules the dose range gave rise to some groups with no responders and other groups with 100% responders at this time. The PF values from this method of analysis are

Table 1. Protection factors for single dose or ten fractions with WR-2721 in air- or 10% oxygen-breathing mice

Inspired gas	Regime	24 weeks	28 weeks	32 weeks	36 weeks	40 weeks	48 weeks
				Breathing rate: at 380 breaths/min			
Air	s/d	1.23 (1.20–1.27)	1.26 (1.18–1.30)	1.32 (1.25–1.36)	1.34 (1.24–1.41)	1.33 (1.24–1.38)	1.38 (1.26–1.48)
	10 fractions	1.31 (1.26–1.38)	1.34 (1.30–1.40)	1.35 (1.29–1.39)	1.35 (1.28–1.44)	1.28 (1.24–1.36)	1.32 (1.22–1.38)
$10\%\ O_2$	s/d	1.29 (1.20–1.33)	1.30 (1.20–1.34)	1.30 (1.23–1.34)	1.36 (1.31–1.40)	1.38 (1.31–1.43)	1.31 (1.20–1.39)
	10 fractions	1.57 (1.50–1.67)	1.56 (1.49–1.70)	1.51 (1.45–1.60)	1.57 (1.51–1.65)	1.51 (1.44–1.70)	1.54 (1.42–1.75)
				ED_{50}: To exceed 1.2 × control breathing rate			
Air	s/d	<1.15	1.27 (1.19–1.35)	1.29 (1.10–1.51)	1.32 (1.29–1.36)	1.31 (1.11–1.53)	1.31 (1.23–1.39)
	10 fractions	1.35 (1.29–1.41)	1.36 (1.33–1.39)	1.35 (1.32–1.38)	1.41 (1.37–1.44)	1.43 (1.38–1.49)	1.28 (1.27–1.29)
$10\%\ O_2$	s/d	1.30 (1.27–1.34)	1.30 (1.27–1.34)	1.34 (1.25–1.44)	1.40 (1.37–1.44)	1.40 (1.36–1.44)	1.30 (0.94–1.81)
	10 fractions	1.67 (1.49–1.85)	1.67 (1.49–1.86)	1.56 (1.52–1.60)	1.72 (1.57–1.88)	1.70 (1.56–1.86)	1.73 (1.53–1.95)
				Lethality: LD_{20}			
Air	s/d	<1.32	<1.32	1.32 (1.22–1.42)	1.33 (1.23–1.43)	1.27 (1.24–1.31)	1.33 (1.29–1.38)
	10 fractions	<1.45	<1.45	1.45 (1.44–1.48)	1.44 (1.40–1.49)	1.43 (1.39–1.47)	1.47 (1.31–1.65)
$10\%\ O_2$	s/d	-	-	-	>1.31	>1.32	1.35 (1.21–1.50)
	10 fractions	<1.47	<1.47	1.49 (1.44–1.54)	1.44 (1.36–1.52)	1.57 (1.52–1.61)	1.58 (1.54–1.63)

very similar to those from the raw breathing rate data, but the logit fit to the data provides computed instead of graphical confidence limits on the ED_{50} value so that a standard error on the protection factor can more readily be derived. These generally, but not always, give a somewhat narrower range than those obtained from the graphical envelope of errors on the raw breathing rate data (Table 1). They allow an objectively stricter comparison of schedules.

Figures 1 and 2 demonstrate that the protection obtained with large single doses in air or 10% oxygen did not differ significantly from that obtained with ten small fractions, each of 3.4–4.4 Gy, when the mice were breathing air. By contrast, the mice breathing 10% oxygen showed a much greater radioprotective action of WR-2721 when the fractionated schedule was used than with single doses.

If an individual pair of dose-response curves for single doses, either for breathing rate or ED_{50}, is compared and PF values are calculated at different isoeffect levels, the PF values appear to be increasing at low levels of damage, i.e. at lower X-ray doses. However, this trend does not extend over a wide dose range since the PF for 10×3.4 Gy is similar to that for 1×13 Gy in air. A significant increase with decreasing dose per fraction is, however, apparent with the lower oxygen concentration.

Figure 3 shows the data obtained incidentally from lethality due to excessive pulmonary damage in the high dose groups. Data are shown for the final testing time of 48 weeks. The same pattern is seen as for the other endpoints, although an increased PF is now detectable with fractionation of the dose in air, as well as in 10% oxygen.

A detailed study of Table 1 shows that the higher PF is seen with ten fractions in 10% oxygen at all testing times and with all three methods of analysis. Figure 4 illustrates this effect graphically:

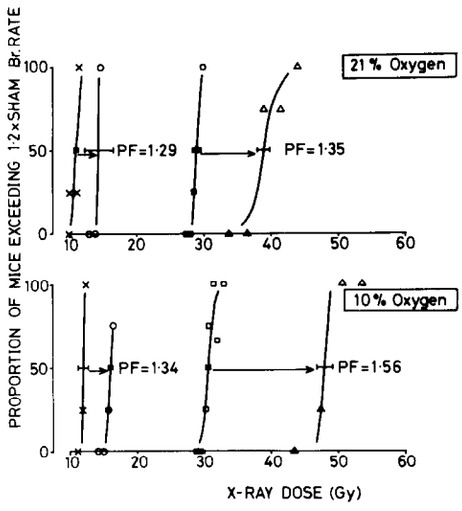

Fig. 2. Dose–response curves obtained from the raw data in Fig. 1 by assessing what proportion of animals in each dose group exceeded a threshold level of damage (20% increase in breathing rate relative to controls). Upper panel: irradiated in air. Lower panel: irradiated in 10% O_2. All protection factors are similar except those for mice irradiated with small fractions in 10% oxygen.

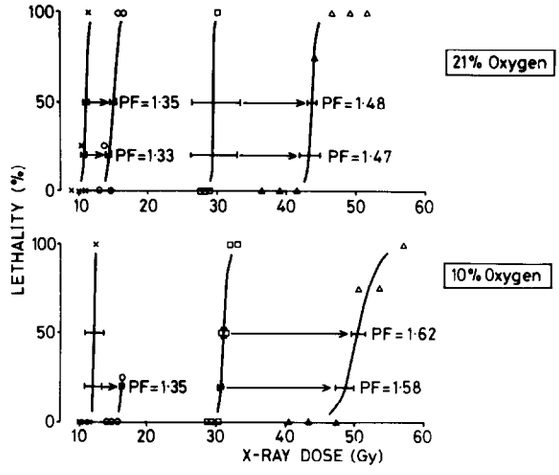

Fig. 3. Lethality dose–response curves for animals at 48 weeks after irradiation. PF values shown at LD_{20} and LD_{50} levels. Upper panel: irradiated in air. Lower panel: irradiated in 10% oxygen. An increase in PF values is observed with fractionation in both gas phases.

the three panels show PF values for ED_{50}, raw breathing rate data and lethality respectively. The upper two panels show that the increase in PF when ten fractions are used instead of one is significant for the mice breathing a low oxygen tension but not for those breathing air. Significance is judged by whether *all* the ten fraction points for *all* the assay times fall outside the range for the single dose values. The lowest panel, for lethality, appears to show a significant increase in both air and 10% oxygen, although some of the points have upward or downward arrows, indicating they are minimum or maximum values. These points, of course, carry less weight. Figure 4B also contains the values published by Parkins *et al.* [2], Travis *et al.* [8] and Down and Steel [3]. Travis used the same drug dose for mice irradiated breathing air (i.e. 300 mg/kg) and reported PF values on the low side of those from the present experiments. Using a higher drug dose (400 mg/kg) the other two studies showed slightly higher PF values both in air and in 10% oxygen.

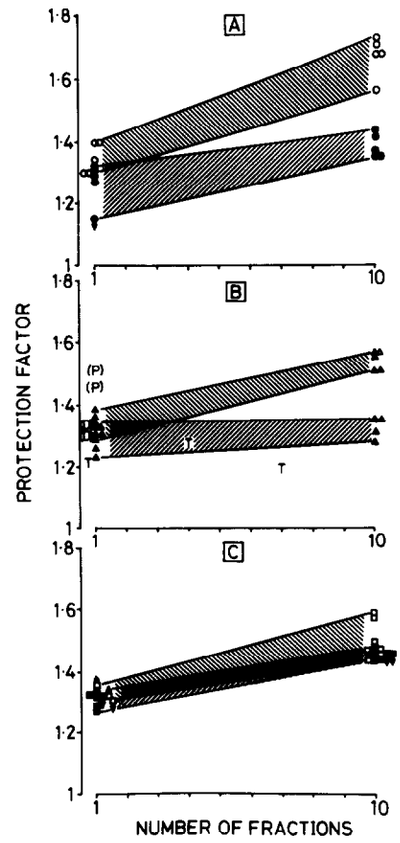

Fig. 4. Protection factors for lung damage in the present work assessed at testing times from 28 to 48 weeks using three different endpoints. Panel A, ED_{50} dose for mice exceeding 20% above control breathing rate. Panel B, 380 breaths/min. Panel C, lethality at the LD_{20} level. Open symbols represent PF values from mice breathing 10% oxygen; closed symbols represent mice breathing air. A consistent increase in PF is seen with 10 fractions given to mice breathing 10% oxygen, but not in those breathing air. Panel B also contains published data. T = Travis et al. [8]; *D = Down and Steel* [3]; *and P = Parkins* et al. [2].

DISCUSSION

A survey of normal tissue protection factors [1] led to the observation that tissues which express their radiation reactions at low X-ray doses tend to have large PF values, whereas those that respond at very high X-ray doses show less protection. Furthermore, within some systems, including lung, the PF values derived at different isoeffect levels along a pair of dose–response curves show a

similar trend of elevation at low X-ray doses [4, 9]. It was therefore considered worthwhile to look in more detail at the effect of X-ray dose level by giving a variety of fractionation schemes in which the same dose of drug was given with different sizes of X-ray dose per fraction. In some of the published studies it is difficult to make an analysis of protection factors as a function of X-ray dose per fraction because less drug is often used with each irradiation in the more fractionated schedules than with single doses. In the present experiments, and in those of Travis *et al.* [8], no significant change in PF was observed for mice breathing air over a wide range of doses per fraction. However, a significantly higher PF was observed at low doses in mice breathing 10% oxygen in the present experiments.

Utley *et al.* [10] showed no change in PF if WR-2721 was given in one or two fractions and the survival of intestinal crypts measured. However, a slight increase in PF relative to single doses was seen with ten fractionated treatments when skin damage was studied. Stewart and Rojas [5] showed no change in PF for skin reactions when using one or five fractions and Echols and Yuhas [11] reported the reverse effect, i.e. reduced protection with ten fractions compared with large single doses. Meistrich *et al.* [12] also saw a reduced PF for testis with fractionated schedules, but this was attributed to a cytotoxic effect of repeated drug exposure on the spermatocytes. Thus no clear picture emerges about the X-ray dose dependence of radioprotection by WR-2721.

A dose-dependent protection factor would be expected in two circumstances: (a) if there was a different degree of protection of damage caused by single-hit events (which dominate at low X-ray doses) and of multi-hit events (which dominate at higher radiation doses); or (b) if the oxygen concentration in the tissue were heterogeneous, so that a mixture of protection factors would be observed for different subpopulations, and these would influence the response at different dose levels.

Studies with fast neutrons and α particles (reviewed by Rojas *et al.* [13]) indicate that the protective effect of thiols against high LET radiation is less than against X-rays. This indicates that a smaller radioprotective action should be observed with the single-hit component of damage, since this predominates in high LET damage. This would therefore not provide a rational explanation for increased PF values at low X-ray doses.

The data presented here may provide support for the idea that heterogeneity of oxygenation is more important, even in normal tissues. It has been shown, both *in vitro* and *in vivo*, that cells are most susceptible to radioprotection by thiols when their oxygen tension is just above the K value [14–17]. At this concentration barely enough oxygen is available to make the cells sensitive to radiation, and any competitive interaction of thiols and oxygen is therefore maximally effective. At *either* higher or lower oxygen concentrations the radioprotection is reduced. In skin the maximum protection is observed with WR-2721 in mice breathing air or 50% oxygen. However, for lung protection by WR-2721 the PF increases at lower oxygen tensions, at least down to 7% oxygen in the inspired gas [2, 3]. Indeed, Hornsey *et al.* [18] have indicated that the radiobiological K value for lung is around 1% oxygen in the inspired gas. Experiments are in progress to test whether WR-2721 would be more effective on lung if the oxygen tension in the inspired gas was reduced further, towards 1%. High dose rate electron irradiations are required for these experiments in order to avoid asphyxia.

Within any tissue there is a distribution of oxygen tensions amongst cells, at different distances from blood vessels and depending on whether they have an arterial or venous supply of blood [19, 20]. However, the cells in the lung are at a higher oxygen tension than cells anywhere else in the body, many of them being directly in contact with approximately 13% oxygen in the alveolar spaces, when the animals breathe air [21]. It therefore seems likely that in lung all the cells are naturally too well oxygenated to be very susceptible to sulphydryl radioprotection. When the oxygen in the inspired gas is reduced to 10%, however, it seems quite feasible that a few lung cells will find themselves at a much reduced oxygen concentration, almost low enough in itself to make them radioresistant. If even 90% of the cells were close to the radiobiological K value they would be difficult to detect radiobiologically since they would only influence the response at low doses per fraction, e.g. 3–4 Gy, and would have little effect at high doses of 10–15 Gy. It seems likely to us that this may be the explanation of the present results. We would emphasize that different subpopulations of cells will dominate the response at different dose levels, even in normal tissue.

From the present set of data it would seem unlikely that any sulphydryl reagent will give marked protection of the lung, either in mouse or man, unless it is combined with a reduced oxygen tension. Then, although the reduced oxygenation (e.g. 10% oxygen) and the sulphydryl (e.g. low dose WR-2721) would each be relatively ineffective protectors on their own, a larger overall protection against lung damage might be

expected from the combination at clinically relevant X-ray dose levels.

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