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The effect of cis-platinum alone or in combination with radiation on mouse lung.

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In memory of my father,
Charles Philip Duffett
and dedicated to my mother,
Yvonne Duffett
who together, laid the
foundation on which any
progress I make is built.

With thanks to my wife,
Alisa Mary,
for her loving
encouragement.

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Abstract

Cis-platinum is a widely used cytotoxic agent with known radiosensitising properties. It is used in the treatment of various types of lung cancer that may include radiation to the lung as part of the treatment protocol. There is little evidence and some conflict as to whether it sensitises pulmonary tissue to the effects of radiation treatment. This project investigates the effect of cis-platinum alone or in combination with radiation on mouse lung.

Four end points were used to evaluate treatments. They were: the release of pulmonary surfactant, changes in breathing rate, a histology based score of damage and changes in TGF-β - a cytokine important in the development of fibrosis. Single doses of either cis-platinum or radiation, cis-platinum given immediately before a single dose of radiation, cis-platinum given immediately before the first of two fractions of radiation and cis-platinum given at various times before and after a single dose of radiation were investigated.

Cis-platinum alone was observed to cause an increase in the phospholipid content of lavaged surfactant. Cis-platinum was observed to cause an early release in surfactant and a trend existed for it to induce an early increase in breathing rates as compared to that induced by radiation alone. Cis-platinum was observed to increase radiation damage as assessed using a histology based scoring system. Higher TGF-β levels in lavaged surfactant were observed in C57/Bl mice as compared to Balb/C. No difference in TGF-β levels was seen in homogenised lung between the strains. Cis-platinum may cause changes in TGF-β in C57/Bl mice but further work is necessary to confirm this.

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Chapter 1. Overview

This project was designed to test the hypothesis that cis-platinum causes an adverse increase in pulmonary radiation toxicity.

Cis-platinum, while used primarily as a cytotoxic agent is a potential radiation sensitiser. It is a desirable component in treatment of cancer in combination with radiation therapy of the thoracic region. There is, however, scant and conflicting evidence of its role in lung radiation damage.

Three indicators of lung damage were chosen for testing the hypothesis that cis-platinum causes an adverse increase in radiation pulmonary toxicity. They were measurement of pulmonary surfactant in broncho-alveolar lavage: a breathing rate assay and histological evaluation of lung sections.

It is of interest to consider what is meant by 'radiation damage', as in different circumstances the term will have different significance. In a study with direct clinical implications, such as this one, it is important to relate the term back to end points of clinical interest. Treatment-induced life shortening is of obvious concern and relates directly to LD50 type studies in experimental models. Undesirable side effects caused by treatment may, however, become important at much lower doses or dose volumes than would be necessary to induce death in humans. Functional or mechanistic assays in experimental models may thus be used either relating in a direct way to a clinical endpoint – e.g. increased breathing rate in rodents as a model of shortness of breath in patients – or as predictors of later responses. Breathing rate has also been used in this way i.e. as a predictor of radiation-induced mortality.

The possibility of schedule dependence was tested by administering cis-platinum at different times relative to a single dose of radiation. The endpoints used were the breathing rate assay and the surfactant assays. The role of cis-platinum in fractionated radiation protocols was investigated by determining its effect on a split dose of radiation, again using the breathing rate and release of surfactant as endpoints.

Tumour Growth Factor β (TGF-β) is a cytokine that may be involved in the induction of pulmonary fibrosis after radiation (Rubin et al. 1990, Rodemann et al. 1995 and

Johnston et al. 1995). Further it has been proposed that TGF-β is a mediator of drug-induced pulmonary fibrosis (Hoyt and Lazo 1988, 1990). This common mechanism may be the way in which drugs such as bleomycin and cyclophosphamide interact with radiation to cause enhanced radiation toxicity. Experiments were performed to investigate whether cis-platinum causes an increase in TGF-β either alone or by enhancing the release caused by radiation.

Chapter 2. Introduction to radiation effects on the lung

The lung is the dose-limiting organ in radiation treatment involving the thorax (Rubin et al. 1968). Tolerance is dependent on total dose, fractionation and irradiated volume with the "critical lesion" leading to impaired ventilation and perfusion. This may exhibit as shortness of breath in varying degrees and if severe, death. What is not clear is what specific events are critical in the pathway to pulmonary insufficiency.

A simple explanation for the development of radio-pathology attributes development of a lesion to loss of an underlying cell population. It has been a tenet of radiobiology that cell death occurs when the cell attempts to divide. Tissues have been divided into acutely responding and late responding, according to the time and nature of response. Acutely responding tissues usually have an identifiable critical cell population with a short cell cycle and rapid turnover. For example in the gastro-intestinal system survival of the stem cell population of the jejunal crypts will determine the development of radiation induced pathology (Scherer et al. 1991). Late responding tissues may have identifiable critical cell populations with long cell cycle times and slow turnover. For example in the central nervous system development of radiation pathology has been attributed to glial cells (Scherer et al. 1991).

The case is not so clear for the development of lung pathology. With over 40 cell types with widely varying population kinetics the lung is a complex organ functioning both as an interface for gas exchange and an important biochemical processing plant. While the critical lesion from a clinical point of view (determined by dose) occurs late, the development of its radio-pathology is characterised by a number of preceding events that occur relatively early. As yet, no single cell population has been clearly identified as responsible for this pathology.

A number of candidates have been proposed including vascular endothelial cells and type II pneumocytes. An emerging hypothesis is that the radiation response of the lung is likely to be due to a complex cascade of biochemical signals (such as those involving cytokines) triggered by radiation. In this model the development of radiation pathology (fibrosis) is seen as a continuum with an inflammatory response lead-

ing to the expression and maintenance of a cascade of inter-cellular and intra-cellular messages. Both the debate over vasculature vs. interstitium and the importance of molecular messengers is not unique to the lung.

It is clearly vital that an understanding of these complex processes be gained if more effective treatments are to be devised and undesirable side effects of radiation treatment of the lung avoided. A central theme of radiobiological research into the development of lung pathology is thus the elucidation of the underlying radiation lesions. Some of the research investigating this and key aspects of radiation induced lung pathology are outlined in the subsequent chapters.

The experimental work described in this thesis started as an empirical investigation of the response of the lung to combined treatment with cis-platinum and radiation. Three endpoints were used in this investigation. These were measurement of the release of surfactant after treatment, measurement of breathing rates after treatment and a simple histological scoring technique. Cis-platinum, its interaction with radiation and previously published research where damage to the lung was assessed is reviewed together with literature relevant to the experimental methods used.

The experimental work was performed using a mouse model. Strain dependent differences in the response of mouse lung have been recorded prompting the use of two strains. The background for this is discussed below.

The empirical approach is, however, of limited benefit and contributes little to a general understanding of the processes involved. Although far from complete, an attempt was made to investigate possible mechanisms of interaction between cis-platinum and radiation by examining the effect of radiation alone or in combination with cis-platinum on TGF-β - a cytokine implicated in the fibrotic process. The role of TGF-β in the development of radiation induced lung fibrosis is discussed below.

Chapter 3. Lung Pathology induced by Ionising Radiation

The radiation response of the lung may be divided into three phases: an immediate reaction, an early response and a late reaction. These have been labelled as the *latent phase* (no gross histological changes); the *pneumonitic phase* and the *fibrotic phase*. The appearance and extent of each phase is dependent on the total dose administered and the fractionation of the dose. The overall clinical response will also depend on the volume of lung irradiated (Coggle 1986).

The latent phase

The latent phase, seen after moderate doses (greater than ~10-15Gy) is characterised by features also seen in other tissues soon after treatment with radiation - fluid leakage in the interstitium, cell swelling, and membrane effects. This phase may be non-existent at doses less than 10Gy. In the case of the lung an important consequence of this type of reaction is the release of surface active material (surfactant) into the alveolar space within hours after 30Gy ^{137}Cs gamma rays administered to mouse lung (Rubin et al. 1980). Early changes in fine structure (using electron microscopy) of mouse lung have also been noted and include increased oedema, fibrin deposition, histiocytic invasion and fibrosis, which were observed to occur, usually in the noted order within 24 h of exposure to 10 to 30Gy of 280kVp x-rays (Penny et al. 1977). After single doses at both 1 h and 24 h post treatment pronounced depletion of lamellar bodies in Type II pneumocytes was observed. This decrement was not specifically dose related and was usually followed by cellular hypertrophy and increased numbers of lamellated structures by 7 days after treatment.

Some controversy exists about endothelial changes observed after radiation. In the period up to one month after treatment Penny et al. (1977) did not observe significant changes in endothelial cells (10 to 30Gy of 280KvP x-rays). They did observe microvilli projecting into the capillary lumen.

This can be contrasted with the observations of Adamson et al. (1970) who noted the primary site of injury to the lung as being the capillary with the earliest evidence of

injury exhibited at 2 days after treatment with 11Gy, with the appearance of intracellular vacuolation leading to occlusion of capillary lumina. After a few days vacuolated cells were seen to rupture and strip off the basement membrane. The observed lesions reached a maximum at day 10 after 11Gy. No damage was observed in type II pneumocytes. Injury to endothelial cells and not to epithelium was also reported by Phillips (1966) and again by Phillips and Margolis in 1974.

The pneumonitic phase

Radiation pneumonitis follows the latent phase and may prove lethal if the dose is sufficiently high. The most significant changes in mouse lung are observed in the period from 2 to 6 months (8 to 24 weeks) post irradiation (Molls and Van Beuningen 1991). The characteristic features of radiation pneumonitis are interstitial oedema, oedema into the alveoli, infiltration by inflammatory cells and desquamation of epithelial cells from the alveolar walls. Alveolar septa were noted to be thickened. During this phase changes have also been noted in capillaries.

Physiological changes noted during the pneumonitic phase include a decreased arterial PO_2 and pulmonary hypertension (Tillman et al. 1989), both of which were noted first during the 2 days immediately post treatment after which they returned to normal and then persisting when radiation pneumonitis started.

The type II pneumocyte in the pneumonitic phase

Interest has focused on the type II pneumocyte both because of its relatively active division, its role as a stem cell for the type I pneumocyte and because of its surfactant producing status. The labelling index of type II pneumocytes after radiation has been examined by various authors. Gross et al. (1988) showed that after single doses of 19Gy to the thorax of CF female mice the labelling index increased 3 fold relative to controls at 18-22 weeks post treatment. The increase in type II replicative activity was seen as a protective response to radiation that was enhanced by the administration of corticosteroids. Corticosteroids increased type II replicative activity. The authors suggest that their results are evidence that the type II pneumocyte is a target cell in the radiation response of the lung.

A 50-70% decrease in total numbers of type I, type II pneumocytes and capillary endothelial cells of rats irradiated unilaterally to the thorax with 30Gy was observed at 12 weeks after treatment (Vergara et al. 1987). The increased replication rate and decreased cell number would indicate an increased turnover and reduced mean survival time for the type II pneumocytes. In work by Coultas et al. (1981) proliferation of type II pneumocytes in mice was observed to initially decrease for the first six days after 10Gy x-rays or 7.7Gy neutrons. Three to five weeks after treatment a gradual increase relative to controls was observed.

The radiation response after selective stimulation of type II pneumocytes in Balb/C mouse lungs by butylated hydroxytoluene (BHT) showed an increased radiosensitivity as assessed by $LD_{50/180}$ (Ullrich et al. 1982). Both 300 kVp x-rays and fission neutrons were used to treat the mice. The $LD_{50/180}$ ranged from 2.69Gy with BHT given two days before irradiation with x-rays to 14.45Gy for BHT given 6 days before irradiation. The $LD_{50/180}$ was 9.59Gy without BHT. Similar differences were seen after neutrons but with $LD_{50/180}$ values ranging from 0.98Gy to 5.75Gy. These results may indicate that the type II pneumocytes represent the target cell responsible for the radiation response of the lung.

No differential radioprotection of either type II pneumocytes or endothelial cells (mouse lung) was noted when assessed relative to lethality after the radioprotective compound WR2721, was administered before treatment with single doses of 250 kVp x-rays (Travis et al. 1987). Endothelial cell function as assessed by angiotensin converting enzyme levels and type II pneumocyte response was determined by lavaged phospolipids and proteins. This work suggested that neither the type II nor the endothelial cell could be accepted or rejected as the target cell responsible for radiation pneumonitis.

It has been suggested by Gross et al. (1988) that the response of type II pneumocytes may be a protective response initiated against radiation damage occurring in other target cells. This could be due to changes in surfactant mechanics, for example as a result of protein leakage, signalling the type II pneumocyte to compensate (Gross 1980). The administration of corticosteroids 10-11 weeks after radiation (19Gy ^{60}Co) caused an increase in type II pneumocyte replicative activity, an increase in surfactant production and release and a concomitant decrease in mortality. This indicated that the

replicative ability of the type II pneumocytes was intact after radiation. Corticosteroids did not appear to cause any decrease in the amount of serum protein leaked from the capillaries (Gross 1980, Gross and Narine 1988).

The replicative activity of type II pneumocytes in the lungs of CF#1 mice after treatment with 18Gy (250kVp X-rays) was examined by Gross et al. in 1987. No significant changes in type II replicative activity as assessed by tritiated thymidine labelling were observed until 11 weeks post treatment after which the labelling index rose to between four and six times that of controls. At this time the breathing frequency was also noted to be elevated to approximately 1.5 times that of controls and histological examination showed pneumonitis.

Strain dependent differences in the pneumonitic phase

Strain differences in the radiation response of mice during the pneumonitic phase have been noted (Sharplin and Franko 1989a). These include differences in the protein content of oedema, absence or presence of hyaline membranes and absence or presence of fibrosis. Nine strains were tested and could be grouped into three categories based on histological appearance. Group 1 included strains of C57/Bl origin and were characterised by protein rich oedema containing fibrin, hyaline membranes and fibrosis. Group 2 contained Balb/C and SWR/J strains and was characterised by both protein rich and protein poor oedema, hyaline membrane and small foci of fibrosis. The third group comprised C3H and CBA strains and showed protein poor oedema, wispy deposits in alveoli and no signs of fibrosis.

Obstruction of 70% of acini (the respiratory bronchiole, alveolar duct and alveolar sac) in the mouse lung was noted as being sufficient to cause respiratory distress in all strains observed (Sharplin and Franko 1989a). All acini appeared to be perfused in the one strain (Balb/C) examined after infusion with carbon particles. Patent capillaries were also seen in oedematous and fibrosing alveolar walls of rat lungs (Travis et al. 1977). The perfusion of non-ventilated regions of lung is a key factor in the development of radiation pathology of the lung as blood flow through such regions will not result in gas exchange and will therefore increase pulmonary insufficiency.

Strain differences in the expression of radiation damage to the lung were also noted when comparing CBA with C57Bl mice (Down and Steel 1983). C57Bl mice did not show a pneumonitic response at doses causing a significant reaction in CBA mice . The $LD_{50/180}$ for C57/Bl mice was observed to be greater than 20Gy whereas for CBA mice it was between 12 and 15Gy.

This differences in radiation response by different mouse strains was investigated further by Ward et al. (1989) who observed lung angiotensin converting enzyme (ACE) activity, plasminogen activator (PLA) activity and hydroxyproline content in four strains of mice – C57Bl/6J, C57Bl/10J, CBA/J and C3H/HeJ. The C57Bl strains, which exhibit late fibrosis, showed lower levels of ACE and PLA than the other strains and these did not reduce in response to irradiation (14Gy 300-kV X rays). The levels measured in CBA and C3H/HeJ mice - strains not prone to fibrosis - were by contrast higher and showed a radiation induced reduction.

The role of macrophages in the pneumonitic phase

Radiation has been observed to produce a reduction in the clearance of inhaled bacteria which is thought to be due to the direct killing effect of radiation on the pulmonary macrophage population (Lungren and Hahn 1979). A dose dependent decrease in numbers of lavaged macrophages, with subsequent recovery to greater than control numbers has been observed after thoracic radiation of mice (Peel and Coggle 1980). There appears to be a proliferating, radiosensitive population of pulmonary macrophages present in mouse lung (Gross 1977, Coggle and Tarling 1982). Steinberg et al. (1992) observed an initial reduction in the total cell content in broncho-alveolar lavage (BAL) from mice two days after irradiation with 10Gy. The number of cells subsequently increased significantly above controls to a maximum at day 56. A strong significant increase in number of bi-nucleated macrophages and in micronuclei content was also seen. The phagocytic activity was increased shortly after irradiation and decreased later. A highly significant threefold increase in alveolar macrophage number lavaged from mice at 4 months after treatment with 24Gy in two fractions was noted by Gross (1978).

Alveolar macrophages from both lungs of unilaterally irradiated sheep exhibited an impaired ability to generate superoxide on stimulation with phorbol myristate (Till-

man et al. 1989). Increased numbers of neutrophils were also noted in the irradiated lung indicating an acute inflammatory response.

The capillary endothelium in the pneumonitic phase

The response of the endothelium to radiation has been examined both *in-vivo* and *in-vitro* (Kwock et al. 1987). A single dose of 30Gy resulted in a 70% decrease in perfusion at 2-3 weeks post treatment in the irradiated right lung of rats. Pulmonary radiographs after barium sulphate perfusion showed widespread loss of capillary filling. Histological examination of the treated lung showed intact capillaries and thus perfusion deficiencies were thought to be due to alterations at the precapillary level. Arterioles were observed to contain areas denuded of endothelium and regions where endothelial cells appeared to be bulging into the lumen. The *in-vitro* work using endothelial cells growing as mono-layers showed morphological changes and loss of attachment after treatment. Delay in the onset of changes after fractionated treatment was observed in both systems.

The fibrotic phase

The fibrotic phase in the lung describes the phase in which gross evidence of fibrosis is evident on examination by light microscopy of suitably stained sections. Although its onset occurs gradually it is typically evident during the latter period of the development of radiopathology – from about 32 weeks after radiation.

As discussed below there is considerable variation in the fibrotic response of different mouse strains and this must be considered when using the mouse as a model for radiation induced pulmonary injury. The dose limiting reaction in therapeutic treatment regimes is usually that of pneumonitis. Although fibrosis is a common consequence of radiation treatment of the lung it may only result in mild deterioration in lung function (Gross 1977).

Changes observed in the development of fibrosis include alveolar wall thickening, accumulation of hyaline, deposition of fibrin in the alveolar space and occlusion of capillaries by fibrin (Molls and Van Beuningen 1991).

Strain dependent differences in the fibrotic phase

There appears to be considerable variation in the type and extent of fibrotic response seen in different mouse strains (Sharplin and Franko 1989b). Different strains of mice can be divided into three groups according to their response - no fibrosis, foci of fibrosis and extensive contracted fibrosis. Mice strain dependent differences observed in the pneumonitic phase as described above (Sharplin and Franko 1989a) are observed in the fibrotic phase in that those strains that do not show indications of fibrosis in the pneumonitic phase do not develop later chronic fibrosis.

Changes in perfusion during the fibrotic phase appear to be an important factor in pulmonary insufficiency. Right ventricular hypertrophy was observed in all mouse strains (three C57 strains, SWR, A, Balb/C, CBA and two C3H strains) tested after 11 and 13Gy (Sharplin and Franko 1989b). In addition non-perfused areas were observed in all strains. Thickening of vessel walls, perivascular fibrosis and narrowed lumina were all seen.

Lung plasminogen activator and angiotensin converting enzyme (both markers for pulmonary endothelial function) activities were reduced to lower levels in mouse strains that showed fibrosis during the early radiation response than in non-fibrosing strains (Franko et al. 1991). The authors found the fibrosing phenotype to be autosomal recessive by examining the response of hybrid strains.

An increase in breathing rate was seen in CBA mice treated with 13Gy 230kV X rays at approximately 16 weeks after treatment. A second increase was seen at around 36 weeks. The first increase coincided with histological changes typical of radiation pneumonitis. The second increase in CBA mice corresponded histologically with pleural effusions. Little evidence of fibrosis was present. C57/Bl mice did not show the early functional or histological reaction however a similar response was seen at 36 weeks after treatment.

Vascular effects and the fibrotic phase

The ratio of blood flow in irradiated left lung relative to unirradiated right lung of rats was determined using ^{99m}Tc techniques after single doses (Peterson et al. 1992a) and after fractionated doses (Peterson et al. 1992b). A significant dose dependent reduc-

tion in blood flow in the irradiated lung was seen for both single doses (11-21Gy ^{137}Cs) and fractionated doses (daily fractions of either 3 or 4Gy for 4 weeks). The reduction was followed by recovery to near normal levels five weeks after lower doses (11-13.5Gy). A lesser degree of recovery was seen after doses from 15 to 18Gy and almost no recovery was seen after 21Gy for the duration of measurement – 50 weeks.

The fibrotic response may be as a result of changes in endothelial structure and function. This has been investigated by several authors. Law (1985) looked at the connection between vascular permeability and late fibrosis by examining the accumulation of albumin and fibrinogen after unilateral lung irradiation of mice. Leakage and deposition of fibrinogen was hypothesised to result in its subsequent organisation increasing the amount of extravascular connective tissue in the irradiated lung. It was found that the extravascular content of both albumin and fibrinogen was increased from 1 - 12 weeks after radiation. Subsequently protein levels returned to normal after 10Gy, remained elevated after 15Gy and increased after 20 and 25Gy. The collagen content of the lung was assessed by measuring the amount of hydroxyproline present. The hydroxyproline content per gram of dry weight lung tissue was increased at 18 weeks after doses of radiation (15-25Gy) sufficient to cause prolonged increase in extravascular albumin and fibrinogen. Increases were however small and did not appear to progress into the late stage. No evidence for increases in fibrosis corresponding to the prolonged elevation in protein levels was seen.

Adamson et al. (1988) investigated the relationship between alveolar epithelial injury and endothelial injury on the induction of pulmonary fibrosis using hyperoxia to induce injury. They showed that the induction of fibrosis, as determined by hydroxyproline content per mg dry weight of cultured mouse lung explants, after exposure to 95% oxygen, of the donor animals required significant denudation of the epithelial surface before hydroxyproline levels were increased. Explants cultured when the lung showed only endothelial injury were not different from air exposed controls. As alveolar damage increased, particularly to type I epithelial cells so did the amount of protein in lavage fluid increase and the overall rate of DNA synthesis in explants decrease. Injury to endothelium only was not associated with fibrosis. It should be noted that oxygen toxicity produces acute functional and structural changes unlike radiation damage, which is normally only expressed at the first mitotic division.

Law et al. (1989) investigated the causal relationship between exudation of vascular protein into the alveolar walls and into the air spaces during pneumonitis and the later development of fibrosis. Leakage into the alveolar wall was used as a measure of endothelial damage and into the air space as a measure of epithelial damage. CFLP mice were irradiated with either 250kVp X rays or neutrons from 16MeV deuterons on Be. During the first 6 weeks lavaged surfactant and protein levels were elevated after both X rays and neutrons (RBE=1). During pneumonitis at 12 to 14 weeks after irradiation, surfactant levels were normal, pulmonary blood volume was decreased and alveolar and interstitial albumin increased. At late times (42 to 64 weeks) alveolar albumin returned to normal while interstitial albumin remained elevated. RBE values of approximately 1.4 for changes in pulmonary blood volume and interstitial and alveolar albumin at 15 and at 46 weeks were comparable to the RBE of 1.5 for survival at 26 weeks (180 days). The time course for release of albumin did not correlate with increases in collagen biosynthesis.

Hazard

The hazard (where hazard is the rate of death at a specified time and represents the risk of death from pneumonitis in mice still surviving at that time) function for radiation pneumonitis in mice was investigated by Tucker and Travis (1992) and seemed to show multiple peaks corresponding to waves of death separated by about 33 days. The times of peak hazard were dose dependent with higher doses giving rise to earlier peak risks. As would be expected higher doses increased the risk of dying as indicated by a higher peak hazard value. A better understanding of the hazard function may shed light on underlying causes of death when correlated with biological events.

Chapter 4. Biochemical Changes in the lung after radiation

The importance of biochemical changes in the lung has been emphasised by Rubin et al. (1992) who proposed that the release of trophic factors that act through autocrine or paracrine pathways resulted in the development of the radiation induced late effect. It was proposed that macrophages, type II pneumocytes and endothelial cells release factors such as tumour necrosis factor (TNF), Interleukin 1 (IL-1), platelet derived growth factor (PDGF) transforming growth factors α and β (TGF-α, TGF-β), prostaglandin E2 (PGE_2) and mononucleocyte derived growth factor (MDGF). These factors act either directly or via other cells on the fibroblasts to stimulate the fibrotic process. Thus control of proliferation of pulmonary fibroblasts and of the synthesis and turnover of extracellular matrix components is effected by the production of locally produced factors which may be perturbed by radiation.

Plasminogen activator and fibrinolysis

Plasminogen activator (PLA, a key factor in the tissue fibrinolytic system) activity in lavage fluid and in alveolar macrophages from rats was examined by Ts'ao and Ward (1985) who found that PLA activity was decreased in the irradiated lung after unilateral irradiation. The decrease, measured at 2 and 6 months after irradiation was dose dependent in the range 10 - 30Gy, however the levels measured at all doses at 6 months were 10 - 15% higher than those measured at 2 months. The number of lavaged macrophages was increased in the irradiated lung. The PLA activity of macrophages was also reduced and their fibrinolytic inhibitor activity was increased but this appeared to be a result of the increase in the number of macrophages. The fibrinolytic activity as measured using a fibrin plate assay, of irradiated rat lung receiving 25Gy to the right hemithorax was also examined by Ts'ao et al. (1983). It was found that the fibrinolytic activity of the irradiated lung was about half that of the controls which was thought to be due to reduced plasminogen activator activity rather than increased fibrinolytic activity.

Angiotensin converting enzyme

Changes in the level of angiotensin converting enzyme (ACE) after radiation were studied to investigate the role of the endothelium in the response of the lung to radiation (Ward et al. 1983). ACE resides on the lumenal surface of the vascular endothelium where it catalyses angiotensin II synthesis and bradykinin inactivation, and was used as an indicator of endothelial functional status. Angiotensin II is an important vasoconstrictor. Rats received a single dose of 35Gy to the right hemithorax. The right lung was noted to be significantly more perfused than controls at up to 14 days post treatment after which the perfusion decreased until 90 days after which a plateau was maintained. ACE activity showed no statistically significant changes for the first fourteen days after which a progressive decline was noted until reaching a plateau at 90 days. No change was observed in ACE activity in the shielded left lung. The reduction in ACE activity may be as a result of endothelial cell loss. Ultrastructural changes noted were perivascular and endothelial oedema at up to 30 days. From 30 days onward endothelial blebbing, degeneration and fragmentation, an increase in capillary basement membrane, increases in interstitial mast cells, fibroblasts, plasma cells and alveolar macrophages were observed. From 90 to 150 days interstitial collagen deposition and gradual obliteration of capillaries by fibrotic reaction were noted (Ward et al. 1983).

Prostacyclin

In a set of experiments the production of prostacyclin (PGI_2) was examined after unilateral irradiation of rat lung by Ts'ao et al. (1983). The vascular endothelium in the lung is a major source of PGI_2 which functions as an inhibitor of platelet aggregation and as a vaso-dilator. PGI_2 showed a significant decrease during the first day post treatment after which it returned to control levels until day 14 after which it showed a steady increase until the experiment was terminated at 6 months. The shielded lung did not show any changes in PGI_2 production. An inverse relationship between PGI_2 production and perfusion was noted when the quantities were expressed in terms of the ratio of irradiated lung values to unirradiated lung values. Increased production of the prostacyclin may be a compensatory response to the hypoperfusion observed. It

was thus not possible to discern from the results presented whether the increase in PGI_2 is pathologic or compensatory (Ts'ao et al. 1983).

Serum proteins in lavage

Gurley et al. (1993) examined the biochemical composition of broncho-alveolar lavage fluids from irradiated rat lung. Complex changes in the protein composition of the lavage fluid were observed after doses of 7.5 and 15Gy x-rays with dose dependent differences in magnitude, kinetic course and qualitative nature being noted. It was found that the more traditional indexes of hyperpermeability in the lung, namely lung wet weight and total protein were not reliable indicators of the x-ray induced hyperpermeability response as underlying changes in the protein compositions were noted before any changes in wet weight or total protein content could be measured. In addition to changes in the plasma proteins found in the lavage fluid, the authors also observed changes in non-plasma constituents. Of the proteins identified albumin, transferrin and immunoglobulin were elevated by 15 fold, 10 fold and 5 fold respectively at 5 weeks after treatment. By week 9 all components were normal except for transferrin and albumin which remained elevated and an uncharacterised component (fraction 6) which was decreased relative to controls. The authors suggested that the progressive development of fibrosis may be caused by an increase in the lung permeability and associated protein leakage.

Effects of ß-interferon

A study was undertaken by McDonald et al. (1993) to investigate the radiosensitising properties of ß-interferon in pulmonary damage in mice. The authors showed an increase in early lung damage after 12.5Gy but a protection against radiation induced fibrosis that was hypothesised to be due to an alteration of immune mechanisms. Morphological changes observed after interferon alone showed Type I pneumocyte lysis, but no apparent changes in Type II pneumocytes or in endothelial cells. Septal wall thickening was reduced after interferon and 12.5Gy when compared to 12.5Gy alone.

Independent mechanisms for the early pneumonitic response and the late fibrotic response were proposed with the late response being seen as not simply replacement of

damaged cells but the result of a complex biomolecular process initiated at the time of radiation. The increased early toxicity was postulated as being due to additive toxicity of the interferon on the type I pneumocytes and radiation mediated toxicity in the type II pneumocytes. Lavaged procollagen at 7 days after irradiation with 12.5 or 15Gy was reduced in mice treated with interferon (McDonald et al. 1993).

Biochemical effects on alveolar macrophages

The role of the alveolar macrophage (AM) in this model was examined by measuring the effect of conditioned medium (from the culture flasks of alveolar macrophages extracted from irradiated rabbit lungs) on rabbit fibroblasts (Rubin et al. 1992). Radiation (5, 10 or 17.5Gy) was shown to reduce the number of AM's in the lavage from the 1st week after treatment. Regeneration of AM's occurred from week 2 and by 4 weeks the number of AM's lavaged was twice that of controls. A stimulation of fibroblast proliferation was seen after all doses, but was most pronounced after 17.5Gy, with a maximum at 2 weeks after treatment. An increase in both TGF-ß and TGF-α was measured, with a maximum level attained by 2 weeks and sustained for the 4 weeks of measurement.

Transforming Growth Factor - β

TGF-α has been shown to be a strong stimulus for fibroblast proliferation (Coff et al. 1987.) TGF-ß is a strong signal for extracellular collagen matrix production (O'Conner-McCourt and Wakefield 1987.)

Rubin et al. (1992) further investigated the cascade of biochemical changes in the lung with the measurement of transforming growth factor ß 1,2 and 3 ($TGFß_{1,2\ \&\ 3}$) mRNA expression, which was observed to be initially depleted at 1 day after treatment and then raised at 14 days after treatment (Finkelstein et al. 1994). Collagen types I & III mRNA levels were found to be raised at 14 days after treatment. The collagen I:collagen III ratio was found to be increased at 14 days. Fibronectin and collagen IV mRNA levels were also increased at 14 days. The authors propose an afferent / efferent phase model of radiation induced injury and response. During the afferent phase radiation induced damage in the cell triggers the release of cytokines

such as TGF-ß. These factors act in an autocrine or paracrine fashion to initiate or control a cellular function such as fibroblast proliferation or collagen production. This response forms the efferent phase in the authors' model and may lead to late complications such as pulmonary fibrosis.

The multicellular interactions of epithelial cells, endothelial cells, macrophages, and fibroblasts, mediated by cytokines following radiation injury was reviewed by Rodemann and Bamberg (1995). The life cycle of the fibrocyte from stem cell, to fibroblast, to mature fibrocyte responsible for the synthesis of interstitial collagens, fibronectin, proteoglycan and other extra cellular matrix components was presented in this review. The complex interaction of cell types via growth factors was stressed. It was proposed by the authors that after irradiation, specific cell types (e.g. alveolar macrophages and type II pneumocytes) may be stimulated to produce altered amounts of growth factors such as interferon-α, IL-1, PDGF, TGF-α and TGF-β. These factors could then alter the proliferation, differentiation and matrix gene expression of the lung parenchymal fibroblasts leading to pulmonary fibrosis.

Three actions of TGF-β were proposed to be important in fibrosis. Firstly, TGF-β can directly affect the gene expression of extracellular matrix molecules in stromal cells to induce synthesis of collagen and inhibit the formation of collagenase. Secondly, TGF-β can induce fibroblast proliferation by stimulating the production of PDGF that in turn acts on the fibroblasts to promote fibrogenesis. Thirdly, TGF-β can establish a state of autocrine stimulation in fibroblasts resulting in chronic activation and differentiation to the fibrotic phenotype (Rodemann and Bamberg, 1995).

The role of TGF-β_1 in radiation fibrosis has been further reported on by Burger et al. (personal communication, 1994a) and Burger et al. (1994b). Irradiation of primary cultures of type II pneumocytes from rats was shown to produce an immediate synthesis of TGF-β_1. This was then shown, using a co-culture system, to produce a significant increase in the DNA synthesis, proliferation and clonogenic activity of fibroblasts. The irradiated type II pneumocytes were shown to cause an increase in the fraction of post mitotic cells in the fibroblast co-culture. This experiment is an elegant demonstration of radiation effects in one lung cell type causing the release of factors that initiate a response in another cell type. In this case the type II pneumo-

cytes are irradiated in isolation and then grown in co-culture with fibroblasts where they initiate changes indicative of a progression to a fibrotic state.

TGF-ß has also been proposed as being involved in the fibrotic response of the lung after treatment with drugs such as bleomycin and cyclophosphamide (Hoyt and Lazo 1990). Levels for TGF-β mRNA were increased 1 week after treatment with bleomycin in C57Bl/6 mice (a strain which shows pulmonary fibrosis after treatment with Bleomycin) whereas the TGF-β mRNA levels did not increase in Balb/C mice (a strain resistant to fibrosis) (Hoyt and Lazo 1988). Strikingly similar results were found in C57BL/6 (fibrosis sensitive) and C3H/HeJ (fibrosis resistant) mice after treatment with ionising radiation by Johnston et al. (1995). TGF-β_1 mRNA and TGF-β_3 mRNA were increased twofold at 8 weeks in the C57/Bl mice while TGF-β_1 remained stable in the C3H/HeJ mice. TGF-β_3 was increased slightly in the resistant strain at 8 weeks.

Chapter 5. Pulmonary surfactant after radiation

What is surfactant?

Surfactant is responsible for preventing the collapse of the small airways in the lung. It accomplishes this by reducing the surface tension in the alveoli. The surface tension in any particular alveolus is inversely proportional to the concentration of surfactant per unit area of alveolar surface. Should the alveolus tend to collapse the concentration of surfactant per unit area will increase and thus reduce the surface tension further, preventing collapse. The reduction in surface tension produced by the surface active properties of surfactant also facilitates breathing by reducing the amount of effort required to re-inflate the lungs at end expiration.

Synthesis of surfactant is thought to take place in the endoplasmic reticulum before passing through the Golgi bodies to be stored in the lamellar body of the type II pneumocyte (Rooney et al. 1975.) The lamellar bodies migrate to the apical surface of the cell and are extruded into the alveolar space where they are thought to pass through a tubular myelin form before forming a mono-layer (Goerke, 1974.)

The composition of surfactant is given in Table 1. The largest fraction comprises the phospholipids of which phosphatidylcholine-dipalmitate is the largest single component.

Table 1. Percentage Compositions of Pulmonary Surfactant. Data from King et al. (1972).

Chemical Components		Lipid Fraction		Phosphatidylcholine derivatives	
Lipid	85	Phosphatidylcholine	75	Palmitate	71
Protein	13	Neutral Lipid	9.1	Myristate	6.1
Hexose	<1.7	Cholesterol Phosphatidyl-ethanolamine	6.3	Stearate	3.6
Nucleic Acid	<0.7	Sphingomyelin	2.1	Palmitoleate	11
Hexosamine	<0.5	Lysolecithin	0.9	Oleate	3.9
				Unident.	3.6

Radiation effects on pulmonary surfactant

An increase in the amount of disaturated phosphatidyl choline (DPC) in both the lung and alveolar surface lining layer was reported by Gross in 1978 after irradiation of mouse lung with 250kVp x-rays. Mice were irradiated with 2 fractions of 6Gy separated by 48h. This increase was observed to begin at 7 days post treatment and to have returned to control levels by 12 weeks.

An increase in lavaged phospholipid from mouse lung was also observed by Rubin et al. in 1980 at 1 h, 24 h and 1 week following irradiation. LAF_1/J mice were irradiated with 30Gy ^{137}Cs gamma rays.

Sequential changes in the pulmonary surfactant system after irradiation with 13Gy and 19.5Gy ^{137}Cs gamma rays included: increases in lavaged surfactant from as early as 24 h after treatment persisting to 4 weeks and returning to normal by 18 weeks; morphologic changes indicative of lamellar bodies in the type II cells discharging their content into the alveolar lumen during the first week post treatment; increases in lamellar body number by 18 weeks; elevated protein levels and some evidence of collagen formation by 18 weeks (Shapiro et al. 1982). No changes were seen after 6.5Gy. This increase was linked in a later paper by the same group to the pneumonitic phase of pulmonary toxicity (Rubin et al. 1983). An increase was observed to occur in both the surfactant radiation dose response curve and in the dose response curve for lethality with a threshold dose of 12Gy in both cases. This was taken to indicate that the early release of alveolar surfactant may serve as a marker for later radiation damage.

The release of surfactant after irradiation by type II pneumocytes taken from rabbits and cultured *in-vitro* has also been shown, with a threshold dose of between 10 and 15Gy (Shapiro et al. 1984). The response appeared to be via triggering of a membrane receptor. In addition radiation appeared to abolish the ability of the pneumocytes to respond to stimulation by terbutaline – a beta adrenergic agonist that normally initiates a release of surfactant by type II pneumocytes. The *in-vitro* work suggest to the authors that the release of surfactant is a direct effect of radiation on the type II pneumocyte.

Surfactant function was shown to be impaired during radiation pneumonitis after unilateral irradiation of sheep lung (Tillman et al. 1989). The ability of lavaged surfactant to reduce surface tension was greatly reduced (producing a minimum surface tension twice that of controls) in lavage fluid from irradiated lungs. The protein content of lavage fluid from irradiated lungs was also increased compared to controls and it was suggested that this was responsible for reducing the surface activity of the surfactant.

Increases in surfactant levels do not appear to be merely related to a radiation induced release of stored surfactant as changes in both turnover and synthesis of the components of surfactant have been measured. A reduction in the rate of radioactivity loss from ^{3}H-choline labelled disaturated phosphatidylcholine from the lung of mice was observed 2-6 weeks after irradiation with 10Gy 250kVp x-rays (Coultas et al. 1987). This loss indicated a reduction in phosphatidylcholine turnover and occurred during the period when lavaged surfactant levels were elevated. At 3 weeks after irradiation an increase in ^{3}H-choline incorporation into disaturated phosphatidylcholine was noted as indicative of increased synthesis. It was therefore proposed that there are at least two different responses leading to increased levels of surfactant - the initial one possibly due to release of stored surfactant and its reduced removal and a slightly later one due to an increased rate of synthesis.

In addition to measuring surfactant release by determining the amount of lavaged phospholipid, the protein content of the lavage fluid may also be measured. Anderson et al. (1985) examined protein levels in lavage fluid from mouse lung after neutrons or x-rays and found a biphasic response. In the period up to 6 weeks protein levels increased to 4 times control levels but did not appear to show a radiation dose response (in contrast to phospholipid release) nor an RBE relative to neutrons greater than 1. Protein level was therefore thought to be unrelated to cell death. The second phase of protein release with a maximum at about 15 weeks and with levels of 20-30 times control corresponded with times of animal death. This second phase was dose dependent for both neutrons and x-rays. The authors could not determine the RBE for the endpoint of protein release during the second phase due to the range of doses used.

In rats receiving either 7.5 or 15Gy to the thorax, changes in lung weight and volume, lavaged alveolar macrophage number, interstitial mast cell number, lavaged phospholipid levels, lavaged protein levels and lavaged histamine levels were followed with

time for up to 13 weeks after treatment (Lehnert et al. 1991). In the 15Gy dose group, alveolar macrophages, mast cells, lavaged phospholipid, lavaged protein and lung weight were all increased by 5 weeks after treatment. These changes were not seen in the lower dose group at this time. By week 7 and 9 phospholipid levels had subsided in the 15Gy group as had hyperpermeability levels as indicated by lavaged protein or increases in lung wet or dry weight. The ratio of mast cells remained elevated and an increase in histamine levels was noted. In the 7.5Gy dose group lung weights and protein levels were significantly increased by week 13. An increase in phospholipid levels preceded that of protein levels and was thought to be independent of changes in the permeability of the lung. Changes in permeability were thought to be independent of mast cell status and histamine levels. The authors suggested that elevations in mast cell number may contribute to radiation induced fibrosis by mitogenic stimulation of fibroblasts (Lehnert et al. 1991).

Surfactant composition after irradiation

Gross (1979) reported that the phospholipid composition of the surfactant from irradiated mouse lung appeared to be normal. In a later paper (Gross 1991) he reported different subtypes discernible when the surfactant was fractionated on continuous sucrose density gradients. Analysis was performed when the breathing rate of mice exceeded 300 breaths per minute indicative of pneumonitis. The proportion of high buoyant density subtype was increased about twofold in surfactant from irradiated mice while the low buoyant density subtype was greatly reduced or absent. In irradiated mice the high buoyant density subtype appeared to contain electron dense material on examination by electron microscopy. This disappeared if the fraction was repurified and the appearance of the subtype matched that of controls. The high buoyant density subtype of irradiated mice had abnormal surface activity which returned to normal on repurification. The phospholipid composition of all subtypes was the same in both irradiated and control mice.

A possible explanation for the increase in high buoyant density subtype is offered by work showing inhibition of serine protease (convertase) activity which is thought to be responsible for converting the high buoyant density subtype to the low buoyant density subtype (Gross 1991). It was found that the alveolar lavage fluid from mice

with radiation pneumonitis caused an inhibition of convertase with an 18-fold excess of antiprotease activity.

Does surfactant predict later radiation effects?

The response of the alveolar surfactant system in mouse lung after treatment with either 7.5 MeV neutrons or 250 kVp x-rays was examined (Ahier et al. 1985). An increase in lavaged surfactant, with a maximum at 3 weeks after irradiation was seen. No differences in response after neutrons or x-rays was observed with regard to the time course of surfactant release. The RBE for surfactant release was ~1 which did not correlate with the RBE for death.

This work was used to support the contention that the release of surfactant is merely co-incidental to lethality and not part of the pathway leading to pneumonitic death. This point of view was placed before the editors of the International Journal of Radiation, Oncology, Biology and Physics in a letter published by Down et al. in 1988.

Three arguments supporting the contention that lavaged surfactant was not a marker for later radiation damage were presented. Firstly, the threshold doses for lethality and surfactant release differed in the rabbit (Rubin et al. 1986). Secondly, that there were mouse strain specific differences with regard to the time course of release of surfactant. Thirdly, that the RBE for x-rays relative to neutrons, calculated using the endpoint of surfactant release was different to that calculated using death as the endpoint.

In their response Rubin et al. (1988) agree that the mechanism for surfactant release and cell death (presuming that cell death were the true underlying radiation lesion in pulmonary injury) may be separate pathways but that this should not necessarily invalidate the usefulness of surfactant release as a predictor for pneumonitic lethality.

Rubin et al.'s acknowledgement that surfactant release and later pulmonary damage may be on different pathways places the measurement of surfactant as an indicator of late radiation damage on the level of empirical coincidence. This would obviate its inclusion as a reliable marker for all but the protocols in which it had been measured as useful.

A different and possibly clinically more useful approach to early markers of pulmonary damage has been reported by Rubin et al. (1989) who examined serum levels of surfactant apoprotein in rabbits. Serum apoprotein was found to be increased from day 1 after treatment with lethal doses (14 - 16Gy ^{60}Co) of radiation, decreasing to near normal by day 21 after which it again increased. The dose response of the measured levels of protein in the serum paralleled that of lethality. Surfactant apoprotein was not measured in the serum after stimulation of surfactant release by terbutaline supporting the hypothesis that damage to endothelium and basement membranes was essential to release of apoprotein.

A decrease in the compliance or elasticity of the lung during pneumonitis is thought to be due to a decrease in the compliance of the alveolar surface lining layer. This decrease may be associated with the leakage of proteins into the alveolar space causing a decrease in the surface tension reducing activity of surfactant (Gross 1978). The minimum surface tension of surfactant from irradiated mice was higher than that of controls. The same author (Gross 1981) found a decrease in lung compliance during the pneumonitic phase that may be attributable to changes in the surface layer mechanics. At nine months after treatment a low compliance was noted but this could not be attributed to changes in the surface layer and was ascribed to loss of compliance of the tissue element as manifested by the appearance of alveolar wall fibrosis (Gross 1981).

Chapter 6. Breathing rates after radiation

Travis et al. (1979) described a technique for measuring mouse breathing rates as a determinant of pulmonary function after irradiation. An apparatus was constructed consisting of a small airtight perspex chamber fitted at each end with a door. One of the doors contained a microphone. The signal from the microphone was fed through a series of amplifiers and filters culminating in a rate meter which produced a voltage proportional to the breathing rate of the mouse in the chamber. This signal was recorded on chart recorder as was a second signal proportional to the amplitude of the breathing the mouse's breathing.

The frequency of breathing was recorded as a function of radiation dose. Statistically significant changes in mean breathing rate at 16 weeks after irradiation were recorded at a dose of 15Gy with a threshold of about 10Gy for the dose response. This increase preceded histological evidence of fibrosis and occurred before deaths from pulmonary insufficiency. It was concluded that the technique was a viable alternative for assessing radiation lung damage in small animals and had the advantages of being non-invasive, sensitive to lower doses than LD_{50}, and allowed sequential changes to be assessed (Travis et al. 1979). Since then, numerous authors have used the technique to assess pulmonary damage after radiation.

The amplitude signal was not calibrated in their experiments and was subject to more technical difficulties than the breathing rate measurements. Nonetheless, a dose dependent reduction in amplitude was seen at 16 weeks post treatment.

It was suggested (Travis et al. 1979) that factors affecting amplitude and breathing rate would include: airway resistance, ventilation, lung volume, shape of breathing cycle and changes in lung compliance. Pulmonary fibrosis would cause a change in lung compliance. However the changes in breathing rate noted by the authors were observed to occur before the onset of fibrosis noted in previous experiments (Travis et al. 1978.) The changes in breathing rate at 16 weeks were thus ascribed to a change in lung compliance due to oedema typical of radiation pneumonitis.

It was concluded that the technical difficulties in consistently determining the amplitude of breathing made breathing rate the measurement of choice. Breathing rate

measurements were seen to provide a quantitative, non-invasive method for assessing radiation lung damage. The method allowed changes to be recorded with time after treatment and was sensitive to lower doses than LD50.

A number of factors were identified by Travis et al. (1981) as affecting the breathing rate of mice used for studies of radiation damage to the lung. These include age, acclimatisation to the measuring process, movement, mouse strain, and sex (in CBA mice). The importance of controlling measuring conditions and maintaining uniformity within experiments was stressed.

Since the publication of the work (Travis et al. 1979) the breathing rate technique has been widely adopted as a method of assessing the response of the lung to radiation.

Breathing rates and radiation repair

The repair capacity of mouse lung as assessed by a reduction in the breathing rate compared to that produced by a single dose of radiation has been investigated by a number of authors. These include Travis et al. 1983a; Travis et al. 1983b; Parkins et al 1985; Down et al. 1986; von Rongen et al. 1995. In each case the technique has produced results comparable to that obtained using other methods, such as LD_{50} studies, for assessing pulmonary damage. It has also proved sensitive to changes induced by small doses per fraction. Von Rongen et al. (1995) showed a repair half time ($t_{½}$) of between 0.25 and 0.75 h for mice irradiated with six equal dose of 2.09Gy given at intervals of 0 to 45 minutes and topped up 24 h later with a range of single doses. The $t_{½}$ values obtained were similar for both the breathing rate endpoint and for death due to radiation pneumonitis. Parkins et al. (1985) used breathing rate measurements to determine repair parameters at up to 17 months after irradiation with either 1, 10, 20 or 40 equal fractions of fast neutrons or x-rays. The data obtained could be well fitted using the linear-quadratic model of radiation damage.

Down et al. (1986) used the technique to determine dose recovery factors (DRF) after treatment with progressively lower dose rates. A continuous increase in tolerance for early radiation pneumonitis reaching a DRF of 2.6 at 2cGy/min was observed.

Terry et al. (1988) assessed the amount of residual damage in lung after a range of radiation doses was administered. A second dose of radiation administered at between

1 and 6 months after initial treatment was used to determine the amount of damage still "remaining" in the lung as a result of the first dose. Breathing rate data were used to show that the amount of residual damage was highest at 1 month after treatment and lowest at 3 months. This partial recovery was followed by an increase in the amount of damage remembered – i.e. a reduction in the amount of second dose that could be delivered for the same effect. It was hypothesised that the data showed an early target cell depletion with regeneration up to 3 months.

Breathing rates, radiation and chemical modifiers

Breathing rates have also been used as an end point for assessing radiation treatment regimes including sensitizers and protectors.

Travis et al. (1987) and Parkins at al. (1984) investigated lung radioprotection by WR-2721 after single dose and fractionated doses respectively. The single dose work by Travis et al. showed dose modifying factors of 1.2 to 1.4 for pneumonitis and 1.5 to 1.6 for the later occurring fibrosis. These dose modifying factors for pneumonitis and fibrosis were significantly different. It is interesting to speculate that this difference may be attributable to different mechanisms of injury involved in the two endpoints. If pneumonitis were an interim step on the path to fibrosis then one might expect the DMF values for pneumonitis to be similar to those obtained for fibrosis. It may be that cell populations with different susceptibility to protection by thiols are involved in the aetiology of damage for the different endpoints.

A low protection factor (DMF = 1.2 – 1.4) was observed for WR2721 administered before a single dose in air, a single dose in 10% oxygen or before 10 fractions in air (Parkins et al. 1984.) Considerable more protection (DMF = 1.5 – 1.7) was observed for 10 fractions in 10% oxygen. It was postulated that in 10% oxygen a sufficient proportion of the normally well-oxygenated cells in the lung became sufficiently hypoxic to benefit from WR-2721 protection. Oxygen dependent protection of radiation lung damage in mice was also demonstrated by Down et al. in 1984 again using breathing rate as an endpoint for determining radiation effect.

Protection by Interleukin 1 against lung toxicity after treatment with cyclophosphamide and radiation was observed by Dorie et al. (1991.) Interleukin 1 significantly

reduced the breathing rates for mice treated with high cyclophosphamide doses and radiation.

Breathing rates, radiation and cytotoxic agents

In 1986 Hill et al. investigated the effect of Actinomycin D in combination with radiation on mouse lung. No effect on breathing rate was observed after combinations where drug was administered 4 weeks before, immediately after or 16 weeks after single doses of 240kV x-rays.

Lung damage, that is pneumonitis as assessed by breathing rates, was seen to occur earlier and to be more severe after maximally tolerated doses of cyclophosphamide followed by single doses of gamma rays at either 1, 3 or 6 months (Travis et al. 1990). This may indicate that the lung would be sensitive to re-treatment with radiation after full tolerance doses of cyclophosphamide.

A number of different drug treatments were used to modify the dose dependent rise in breathing rate observed at 16 weeks after irradiation of mice hemi-thoraxes (Lockart et al. 1992). Cyclophosphamide was seen to accentuate and accelerate the rise in breathing rate. BCNU delayed the onset of the increase in breathing rates. Doxorubicin, Carboplatin, vindesine and vinblastine all had no substantial effect on breathing rate. Busulphan in DMSO was seen to be radio-protective, an effect later attributed to the DMSO.

Kallman (1994) reviewed a number of experiments in which the breathing rate technique was used to assess the therapeutic effectiveness of treatments in which various chemotherapeutic agents and radiation were combined. For every schedule, therapeutic gain factors were determined as the ratio of effectiveness for tumour growth inhibition to each of three normal tissue endpoints. The normal tissue endpoints used were: pneumonitis (breathing rate at 5 months); pulmonary fibrosis (breathing rate at 10 months) and duodenal crypt cell survival. For every combination tested there was at least one schedule seen to be therapeutically superior. Substances tested included cis-platinum, 5FU, cyclophosphamide and etoposide. The cis-platinum work is commented upon in more detail below.

Breathing rates and irradiated volume

Herrmann et al. (1997) showed that the induction of structural lung damage as assessed by histological evaluation and hydroxyproline content was independent of irradiated volume. However functional damage as assessed by breathing rates depended on the volume irradiated. The lack of volume effect at the structural level would indicate that damage is dependent only on dose deposited within the volume being assessed and is not influenced by dose deposited outside the volume being assessed. A contrasting scenario might be that where factors released in adjacent irradiated tissue modified the response of the volume under consideration.

A heterogeneous response to irradiation of partial lung volumes has been shown by Liao et al. (1995) and Travis et al. (1997.) Higher doses were needed to obtain equivalent response when smaller volumes of lung were irradiated. Increases in breathing rate correlated with lethality when irradiated volume was greater than 50% of a reference volume. For irradiated volumes less than 40% of the reference volume breathing rate increases were not accompanied by increases in lethality. It was also noted that for a given volume irradiated the iso-effective dose was always lower for treatment areas in the base of the lung than for areas treated in the apex of the lung. The authors suggested that the heterogeneity of response is a results from the anatomy of the tracheo-bronchial tree. The distribution of non-gas exchanging airways in the irradiated volume will influence the dose response. It was also noted that histological evidence of damage was always confined to the irradiated volume only. This is in contrast to results reported by Morgan et al. (1995) who showed bilateral lymphocytic alveolitis of activated T lymphocytes and a diffuse increase in gallium uptake after unilateral lung irradiation.

Inter-strain differences in breathing rate

Different strains of mice have been shown to respond differently to radiation treatment of the lung. These strain dependent differences could be used to provide models for mechanistic studies of the radiation response of the lung.

Down et al. (1983) using breathing rate and lethality as end points contrasted the response of CBA mice with that of C57Bl mice. It was found that two distinct phases of

damage could be observed in CBA mice as an early pneumonitic phase and a later phase associated with pleural effusions. C57Bl mice failed to show the pneumonitic phase. The authors speculated that the difference suggested damage to separate tissue compartments as being responsible for the acute and chronic phases of radiation injury.

Down et al. (1986) refined this work using CBA, C57Bl mice and their F1 hybrid cross (CBBF1). CBA mice showed a peak response at 16 weeks after which the breathing rates declined. The response of C57Bl mice was considerably delayed in comparison and that of the CBBF1 mice was intermediate between the parent strains.

Chapter 7. Cis-platinum (cis-dichlorodiammineplatinum II)

What is cis-platinum?

The biological activity of platinum co-ordination complexes was first noted in experiments in which bacteria were cultured in the presence of platinum electrodes and media containing ammonium chloride where an inhibition of bacterial growth surrounding the platinum electrode was recorded (Rosenberg et al. 1965). Rosenberg (1969) soon noted the potential for the clinical use of platinum co-ordination complexes in the treatment of malignancies.

Cis-dichlorodiammineplatinum II (cis-platinum) was the original complex generated in Rosenberg's experiments and has since proved to be one of the more effective clinical agents in the treatment of cancer (Muggia 1991, Comis 1994). It is the compound of interest in this study. Its structure and chemistry have been extensively reviewed (Calvert et al. 1993).

Cis-platinum is thought to exert its biological effect by bidentate binding of the cis-platinum to DNA. It binds to all bases except thymine with a marked preference for guanine (Munchausen and Rahn 1979). Denaturation of DNA is caused at very low doses of cis-platinum. Inactivation of translation (1 molecule of cis-platinum per 100 bases) is observed before interstrand cross linkage occurs (20 molecules of cis-platinum per 1000 bases) (Munchausen and Rahn 1979).

Work published by Sorensen et al. (1988) has failed to show a correlation between inhibition of DNA synthesis and lethality. Repair deficient CHO cells were observed to die at doses of cis-platinum which failed to cause inhibition of synthesis while repair proficient CHO cells survived doses which inhibited synthesis and arrested the cells in S-phase (Sorensen and Eastman 1988). It was suggested that toxicity may occur via induction of apoptosis. Both Sorensen et al. (1990) and Barry et al. (1990) have shown that cell death following administration of cis-platinum was accompanied by fragmentation of DNA into multimers of about 180 base pairs consistent with internucleosomal cleavage by an endonuclease, followed by loss of membrane integrity

and cell shrinkage, all features which have been reported as characteristic of apoptosis (Wyllie et al. 1980).

Cells are capable of repairing cis-platinum lesions. This was demonstrated when normal human fibroblasts were allowed to remain confluent after treatment with cis-platinum and survival was seen to increase with time (Pera et al. 1981). The number of platinum-DNA residues decreased as a first order process with a half-life of 2.5 days. This is in agreement with work by Van den Berg et al. (1975) that showed that damage to DNA arising from cis-platinum treatment could be repaired by a caffeine sensitive post replication repair process.

A number of proteins have been observed to bind to cis-platinum adducts (Chu 1994). These include XPE-BF and ERCC1, which are involved in repair of cis-platinum lesions. HMG1, HMG2, SSRP1 and human upstream binding factor have also been observed to bind to cis-platinum adducts. Cellular resistance or sensitivity to cis-platinum may be viewed as a dynamic competition among proteins which either repair or interfere with repair or which signal for cell death via apoptosis (Chu 1994).

Pharmacokinetics

In-vivo, cis-platinum is cleared from the plasma by tissue uptake, urinary excretion and by irreversible binding to plasma proteins and metabolites (Calvert et al. 1993). Therefore, total platinum is made up of protein bound and ultrafilterable (unbound) species. The unbound platinum is thought to be responsible for anti-tumour activity and toxicity. Renal toxicity, when comparing platinum analogues with different ligands has been seen to correlate with the stability of the compound in aqueous solutions, with the distribution half-life and with the cumulative 24 h urinary excretion (Calvert et al. 1993). In addition toxicity has been observed to correlate with platinum retention in the kidneys of nude mice.

Cis-platinum plasma levels have been observed to decay following a biphasic pattern with a short initial distribution half-life of the order of minutes followed by a longer elimination phase with a half-life of the order of days (Litterst et al. 1979). Seventy nine percent of an i.p. dose of cis-platinum was excreted in mouse urine on the first day after administration (Lange et al. 1972). Hydration before and after cis-platinum

(i.v.) administration was observed not to affect the levels of cis-platinum measured in plasma in mice, rats, dogs or sharks (Litterst et al. 1979). The same authors observed an initial distribution to nearly all organs in the body followed by an accumulation in kidney, liver, muscle and skin. The general distribution was independent of species studied. Tissue:plasma ratios of greater than 1 were maintained for at least 1 week after treatment and in kidney, liver, skin and lung may have been elevated for 2-4 weeks after treatment Litterst et al. 1979).

A $t_{½\alpha}$ of 0.61 h and $t_{½\beta}$ of 1.28 h was measured in lung for cis-platinum given i.v. (maximally tolerated dose of 4mg/kg) to mice with the highest concentration of 1.9μg/g wet weight measured at the earliest time interval of 10mins after injection (Siddik et al. 1988). The initial rapid clearance of drug from the plasma is represented by $t_{½\alpha}$, and $t_{½\beta}$ is the half life for the longer elimination phase. The plasma $t_{½\alpha}$ was determined to be 0.27 h with a $t_{½\beta}$ of 36.5 h (Siddik et al. 1988). Tissue to plasma ratios for lung were seen to increase from 0.41 at 10 min to 12.3 at 6 days, indicating retention of drug in tissue.

Maximum plasma concentration after an i.p. injection of 6mg/kg was seen at 5 minutes in C3Hf/Sed mice (Fu et al. 1988). The α half-life, after i.p. injection, of ultrafilterable platinum in plasma was determined to be 0.31 h and the β half-life was found to be 27.7 h.

It may thus be hypothesised that maximum lung concentrations of cis-platinum occur within minutes of i.p. injection. However, these concentrations were measured by atomic absorption spectroscopy and thus represent the total tissue or plasma platinum. This does not necessarily reflect the amount of active drug in the sample. Cis-platinum may be irreversibly bound to material not important in causing cell death (Melvik et al. 1992).

The i.v. and i.p routes of administration gave comparable levels of drug in the lung at both 4 and 8 days after injection in dogs (Pretorius et al. 1981). The mean serum concentration of cis-platinum reached a maximum 4 h after i.p. injection.

Circadian variation in toxicity

Several authors have noted a pronounced circadian variation in toxicity of cis-platinum. Shakil et al. (1994) recorded that 80% of mice treated with 16mg cis-platinum/kg died when cis-platinum was given 3 h after lights on compared to a 10% death rate when cis-platinum was given 9 h after lights on.

Similar findings were noted by Boughattas et al. (1990) who reported that cortical tubular necrosis in mice was half as severe when cis-platinum was administered 16 h after lights on than when given at 0 or 8 h after lights on.

The lowest renal toxicity in rats was measured when cis-platinum was administered near to the normal circadian maximum urinary volume (Levi et al. 1982).

A 3- to 8-fold difference in the time to 50% mortality was noted in rats after administration of cis-platinum. The lowest toxicity was observed when cis-platinum was given close to the mid activity span (Hrushesky et al. 1982).

Interaction with radiation

It is evident that cis-platinum is capable of increasing the severity of radiation damage in a number of systems.

Cis-platinum was noted to interact with radiation by Zak et al. (1971) who observed an increased lethality in mice given cis-platinum i.p. and treated with single doses of x-rays. Enhanced survival was seen in mice inoculated with P388 lymphocytic leukaemia cells when they were treated with both cis-platinum and radiation as opposed to either agent alone indicating possible radiosensitisation by cis-platinum (Wodinsky et al. 1974).

Increased radiosensitivity was measured in bacterial spores after cis-platinum was administered before radiation (Richmond et al. 1977) and in *E.coli* (Richmond et al. 1977b).

Cis-platinum may increase sensitivity to radiation by interfering with repair of radiation damage. Cis-platinum was observed to remove the shoulder in the radiation survival curve of TC40 mammalian cells (Alvarez et al. 1978). Cis-platinum, at mini-

mally toxic doses, was seen to inhibit repair of radiation damage in split dose experiments using V-79 cells (Dritschilo et al. 1979). Minimal inhibition of repair was seen after single dose, delayed plating type repair experiments. Cis-platinum (even with non-toxic doses of 1μmol dm^{-3}) was observed to increase the number of hypoxic V-79 cells killed, especially at low (0-4Gy) doses of radiation (Korbelik et al. 1989).

A trend toward greater than additive delay in tumour growth was observed in RIF-1 and KHT tumours after combined cis-platinum and radiation although this was not seen in the EMT6 tumour (Twentyman et al. 1979). An enhanced response was seen after combined treatment with radiation and cis-platinum of mouse mammary tumours and an intracerebral rat brain tumour (Douple et al. 1977). Supra-additivity was seen in the regrowth response of the SCVII and RIF-1 murine tumours to combined cis-platinum and radiation treatment (Kallman et al. 1991).

Sensitisation after treatment with both radiation and cis-platinum has also been observed in normal tissue systems.

In mice an enhanced reaction was seen in bladder for both early and late reactions by Lundbeck et al. (1992). An early (30 days after treatment) change in bladder reservoir function was seen in animals treated with 20Gy plus 6mg/kg cis-platinum at 24 h before, 15 min before and 4 h, 72 h and 336 h after radiation. A late (110 days after treatment) reduction in bladder reservoir function was seen after treatment with cis-platinum administered at a range of times between 168 h before radiation and 72 h after radiation.

In mouse kidney significant renal toxicity was seen when cis-platinum was combined with radiation with the toxicity of combined treatments always more severe than the single agent treatments. The α/β ratio (from the linear quadratic survival model) was not changed by the presence of cis-platinum indicating that increased renal damage was most likely additive (Stewart et al. 1989). In intestinal crypts from mouse duodenum cis-platinum caused a significant increase in cell death when combined with radiation (Dewitt et al. 1985). Enhanced gut damage assessed using the intestinal crypt assay was seen for cis-platinum given up to 6 h before radiation but not at longer time intervals before or when given after (2 to 24 h) irradiation (Von der Maase 1984). In-

creased damage has been observed in rat skin for cis-platinum given 2 h or immediately before radiation (Douple et al. 1979).

Mechanism of interaction

Cis-platinum may interact with radiation in one of two ways. Studies in bacterial systems (Richmond 1984) have indicated that cis-platinum can act as a reactive free radical, via radiation induced reactive Pt(I) intermediates and / or in part by interaction of Pt(II) species with DNA. This direct pathway has been termed sensitisation and although dependent on free radical chemistry, other mechanisms such as sulfhydryl depletion, radiolytic toxic product formation and cell cycle perturbation may be important (Douple 1988).

The second pathway of interaction is mediated not by direct interaction of radicals but rather by biomolecular processes that occur after radiation and result in the inhibition of repair. This has been termed potentiation (Douple 1988). Coughlin and Richmond (1989) hypothesised that in mammalian systems the relatively low concentrations of cis-platinum obtainable in the cell favours the biomolecular pathway rather than the more directly mediated free radical or radiolytic pathway. The initial sensitisation may depend on the presence of free drug whereas that of potentiation depends on bound drug.

Chapter 8. Cis-platinum and radiation damage to the lung

Experimental studies

Cis-platinum was reported by Von der Maase et al. (1986) as having no effect on lung damage of $C_3D_2F_1$/Bom mice (C_3H/Tif α x DBA/2) as assessed by breathing rate and lethality when drug was given 15 minutes before graded doses of radiation. The authors state, but do not show data, that cis-platinum did not modify the radiation response when given at different intervals from 28 days before to 28 days after radiation. Cis-platinum was administered at a maximally tolerated dose defined as that dose which would kill 1% of the mice within 150 days.

Dose enhancement factors (DEF) of greater than 1 were found for lung damage in C_3H/Km mice after a variety of different cis-platinum / radiation dose combinations (Tanabe et al. 1987). Cis-platinum was given i.p. at 4, 8, or 12 mg/kg or as 5 fractions of 0.8, 1.6 or 2.4 mg/kg when 5 daily fractions were given. Only the thorax was irradiated. The end point assessed was the breathing rate at 5 and at 10 months after treatment. In general it was found that the highest DEFs were obtained for schedules in which 5 daily drug doses were followed by 5 daily irradiations (1.15 - 1.47). However, it was also found that DEFs of greater than 1 were obtained where a single dose of cis-platinum was administered before a set of 5 daily irradiations and where cis-platinum was given before or after each fraction. The authors noted (but did not show data) that a significantly depressed breathing rate was measured in mice after cis-platinum alone at times associated with the early phase of radiation damage (5 months). This had returned to normal by the time that late phase radiation damage would have been expressed (10 months). Breathing rates of combined treatment groups were elevated during both the early and late phases indicating that the breathing rate changes reflected real interactions between drug and radiation and not merely general physiological effects caused by the drug.

The greatest effects were observed when cis-platinum was given as 5 fractions daily during the week preceding 5 daily fractions of 4Gy each. The lowest DEFs for lung damage appeared to be achieved after simultaneous administration of cis-platinum

and radiation - contributing to a high therapeutic gain factor (TGF) where the TGF was defined as the ratio of the DEF for tumour to the DEF for normal tissue. A TGF of greater than 1 would signify a that the tumour tissue was being sensitised more than normal tissue.

There did however appear to be a large variation between some experiments (See Table 2. presenting data from Tanabe et al. 1987). For 1.6 mg/kg given daily immediately before irradiation a DEF of 1.22 ± 0.06 was obtained at five months after treatment. For both the lower dose of 0.8 mg/kg and the higher dose of 2.4 mg/kg cis-platinum daily significantly lower DEFS of 0.8 ± 0.06 and 1.02 ± 0.07 respectively at 5 months after treatment.

In addition the DEF does not always correlate consistently with dose as evidenced in the group receiving a single cis-platinum dose 24 h before daily irradiation. In this case the DEF dropped from 1.20 ± 0.05 to 0.94 ± 0.09 for the 5 month endpoint and from 1.13 ± 0.03 to 1.03 ± 0.03 for the 10 month endpoint when the cis-platinum dose was increased from 4 mg/kg to 12 mg/kg. This would indicate that in these cases more cis-platinum does not result in a higher DEF. In contrast, for cis-platinum given 24 h after a set of 5 daily irradiations the DEF for the five month endpoint drops from 1.22 to 0.98 as the dose increase from 4 mg/kg to 12 mg/kg while showing no significant change for the 10 month endpoint.

Table 2. Dose Effect Factors for breathing rates after treatment with cis-platinum and radiation: Data from Tanabe et al. 1987.

Treatment	Time between cis-platinum and radiation (h)	Daily dose of cis-platinum. (mg/kg)	Dose Effect Factor (5 months)	Dose Effect Factor (10 months)
♦↑♦↑♦↑♦↑♦↑	0	0.8	0.80 ± 0.06	1.00 ± 0.03
		1.6	1.22 ± 0.06	1.14 ± 0.08
		2.4	1.02 ± 0.05	1.07 ± 0.04
♦↑♦↑♦↑♦↑♦↑	2	0.8	1.10 ± 0.07	1.07 ± 0.04
		1.6	1.10 ± 0.05	1.19 ± 0.04
		2.4	1.12 ± 0.09	1.23 ± 0.06
↑♦↑♦↑♦↑♦↑♦	6	0.8	1.20 ± 0.05	1.21 ± 0.05
		1.6	1.25 ± 0.06	1.16 ± 0.05
		2.4	1.27 ± 0.11	1.30 ± 0.08
♦↑↑↑↑↑	24	4	1.20 ± 0.05	1.13 ± 0.03
		8	1.11 ± 0.06	1.08 ± 0.02
		12	0.94 ± 0.09	1.03 ± 0.03
↑↑↑↑↑♦	24	4	1.22 ± 0.06	1.08 ± 0.03
		8	1.12 ± 0.06	1.08 ± 0.04
		12	0.98 ± 0.06	1.14 ± 0.07
↑↑↑↑↑♦♦♦♦♦	72	0.8	1.15 ± 0.06	1.14 ± 0.03
		1.6	1.23 ± 0.07	1.17 ± 0.03
		2.4	1.25 ± 0.07	1.23 ± 0.05
♦♦♦♦♦↑↑↑↑↑	72	0.8	1.15 ± 0.05	1.09 ± 0.03
		1.6	1.47 ± 0.09	1.23 ± 0.04
		2.4	1.28 ± 0.08	1.31 ± 0.05

♦ = cis-platinum injection
↑ = radiation fraction

There are thus two sets of interesting results on cis-platinum enhancement of radiation damage to the lung: Von der Maase et al. (1986) present data showing no effect of the drug on radiation whereas Tanabe et al. (1987) show schedule dependent increases in breathing rates after treatment with both agents. The primary difference between these studies is that Tanabe et al. (1987) used fractionated doses of radiation in combination with either single or fractionated doses of drug, whereas Von der Maase et al. (1986) describe results obtained using single doses of both agents. Since Tanabe et al. (1987) obtained some enhancement when cis-platinum was given as a single dose before or after 5 fractions of radiation it may be hypothesised that it is the division of the radiation dose into fractions which may be important, possibly through cis-platinum influencing repair between fractions. Work by Peckham and Collis (1981) supports this hypothesis as no effect of cis-platinum on radiation induced toxicity was observed.

Clinical observations

An anecdotal case of possible cis-platinum radiosensitisation of lung in a patient was reported by Golding and van Zanten (1983). A 41-year-old man was treated for a poorly differentiated squamous cell tumour in the upper left lobe. He was treated with

55Gy in 44 1.25Gy fractions. Two fractions were given per day with a three hour inter fraction time. Four mg of cis-platinum per m^2 body surface area was given 15 minutes after each irradiation. Two months after the end of treatment he was diagnosed as having necrosis of the lung within the irradiated field. No micro-organism could be cultured in samples from the lung. A second infiltrative lesion in the peripherary of the radiation field was noted at four months after treatment. At both times there was no evidence of tumour. The authors suggest that the toxicity is a result of the combination of super-fractionated radiation and cis-platinum.

In a study reporting on combined cis-platinum and radiation treatment of non-small cell lung cancer 331 patients were randomised into one of 3 arms (Schaake-Koning et al. 1994.) Radiation was administered as 10 by 3Gy fractions followed by a 3 week rest period then 10 by 2.5Gy fractions. Cis-platinum was given either as $30mg/m^2$ on the first day of each treatment week or as $6mg/m^2$ given daily before radiotherapy. Survival was significantly improved ($p=0.009$) in the radiotherapy plus daily cis-platinum group compared to the radiotherapy only group. The radiotherapy plus weekly cis-platinum group showed an intermediate survival that was not different to either the radiation alone group or the radiation plus daily cis-platinum. While cis-platinum did induce significant nausea and vomiting an increase of late radiation lung damage was not observed.

Chapter 9. Methods

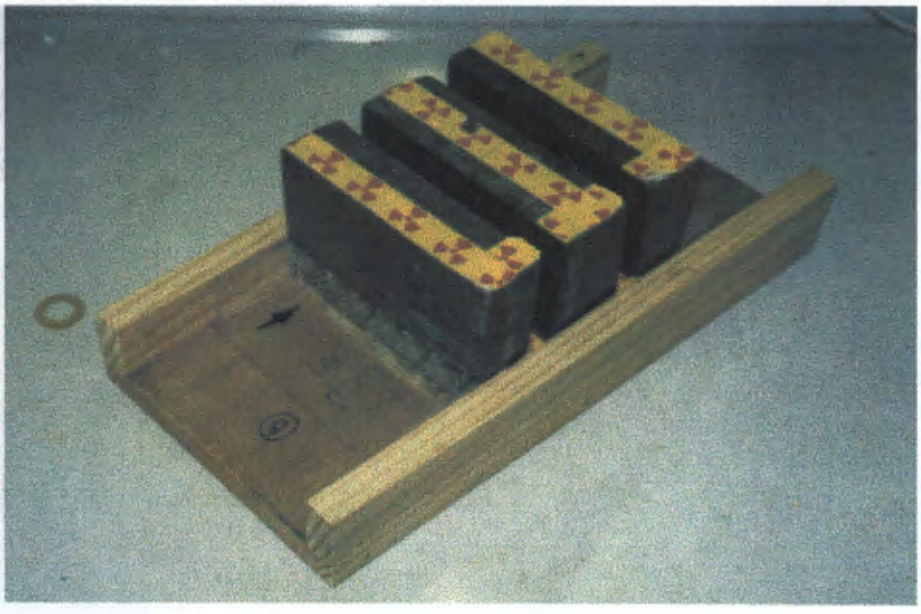
Mice

Specific pathogen free female Balb/C and female C57/Bl mice of age 4 - 6 months and weighing 20±2g were used in the experimental work. They were housed in standard mouse cages with bedding of wood shavings. They were fed mouse cubes (Specialist Animal Feeds, Cape Town) and water ad lib. A 12-h light/dark cycle was in effect, with lights on at 6:00 am.

Irradiations

A Perspex jig was constructed for irradiating mice. It comprised two rows of six chambers each just big enough to hold one mouse. Lead blocks (8cm thick) were constructed to shield the head and abdomen of the mice. Beam divergence was accounted for in the construction of the jig and shielding. Mice were introduced into the jig without anaesthesia for early experiments. D. Hirst (personal communication 1994) advised that anaesthetising the mice prior to placing in the chambers and for the duration of the irradiation would

Figure 1. Radiation jig and shielding. The jig comprises two rows of six perspex chambers sized hold one mouse each. The shielding was milled to account for beam divergence at an SSD of 80cm.



result in better dosimetry and would be of equivalent stress to jigging without anaesthesia). Thus, mice were anaesthetised for the latter experiments. The jig and shielding are illustrated in Figure 1.

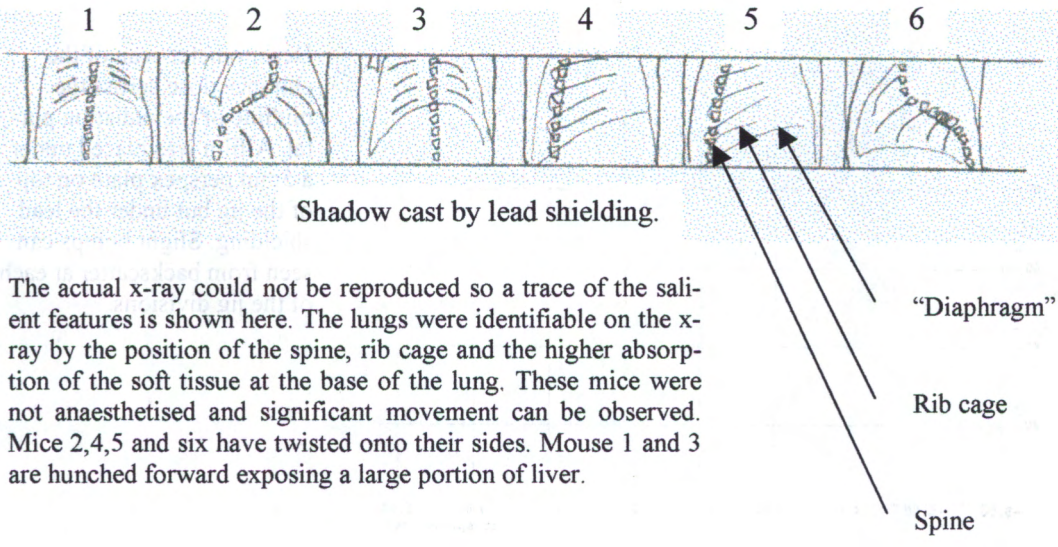
The early experiments were performed using an Eldorado 6 ^{60}Co unit. Dosimetry measurements described below revealed an unacceptable variation in dose at different positions in the jig. For this reason the remaining experiments were done using a Theratron 2 ^{60}Co treatment unit.

The Eldorado 6 unit is dedicated for use in radiobiology and has a fixed head with the beam directed downwards. The Theratron 2 is a standard treatment unit in daily use in the Dept. of Radiation Oncology.

Dosimetry was performed by placing an ionisation chamber in the position of one of the mice. A build up cap was used to ensure full dose build up and wax phantom mice were present in each of the jig positions to ensure that full scatter was present. The dose rate in the jig was found to be 0.8 times that of the open field dose rate.

Figure 2. Close up of jig with one mouse in position. Shielding has been removed for the photo.

Figure 3. Trace of an X-ray of mice showing position in jig..



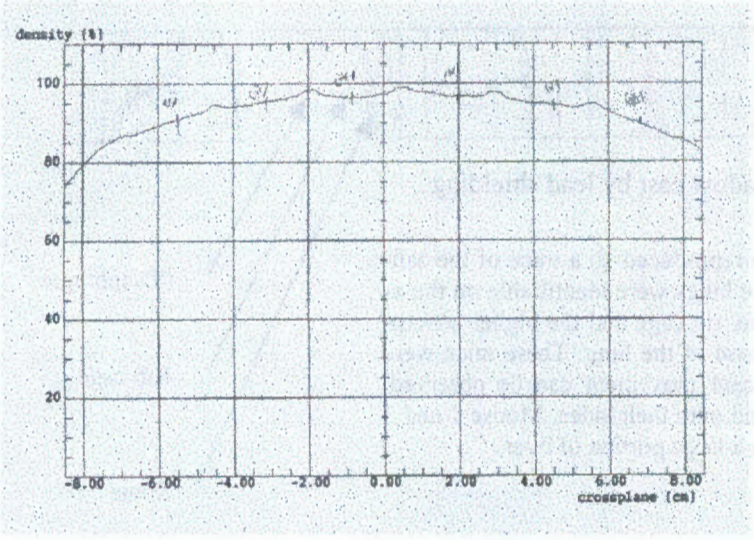
The actual x-ray could not be reproduced so a trace of the salient features is shown here. The lungs were identifiable on the x-ray by the position of the spine, rib cage and the higher absorption of the soft tissue at the base of the lung. These mice were not anaesthetised and significant movement can be observed. Mice 2,4,5 and six have twisted onto their sides. Mouse 1 and 3 are hunched forward exposing a large portion of liver.

An X-ray was made by duplicating the treatment set-up, including mice, on a diagnostic x-ray machine and exposing an x-ray film placed under the treatment jig. The film showed that on average only 85% of the lung was included in the field. It also revealed that the mice had too large a degree of movement available to them. The spinal columns of the mice are clearly visible and show a great variation in conformation. The film is shown in Figure 3,

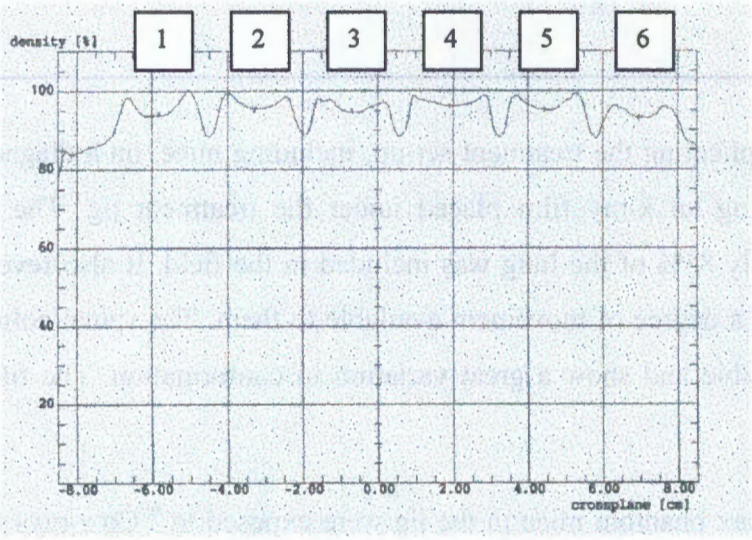
Further check films with wax phantom mice in the jig were exposed to ^{60}Co γ-rays from the Eldorado 6 unit and scanned on a densitometer to investigate the uniformity of the dose distribution (Figure 4). An arbitrary spot on the film, selected by eye as being in the darkest region, was chosen to set the 100% reading. The results are presented in Chapter 10.

The incident dose was determined using a film placed on top of the jig without build-up. The exit dose was determined using a film placed under the jig. The figures obtained using the densitometer and the check films should be regarded as relative and not absolute and were used to determine the dose distribution in the radiation set-up. The 16% difference in incident dose rates between rows within the jig was deemed unacceptable and resulted in a revision of the radiation set up.

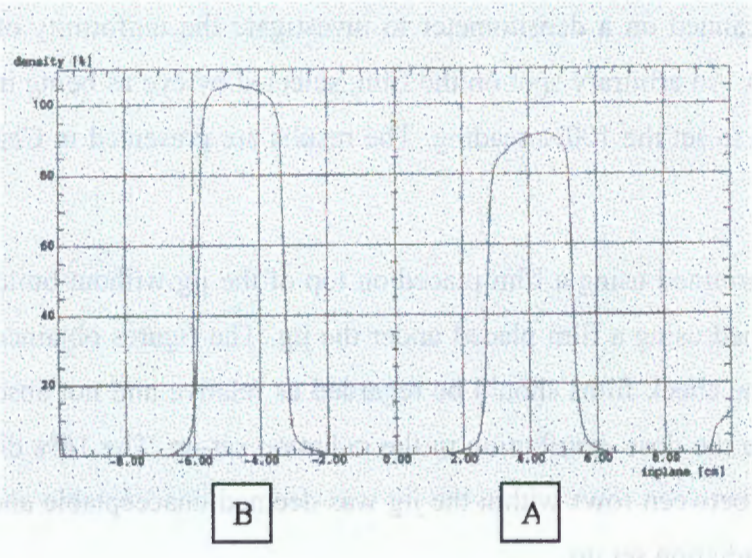
Figure 4. Sample densitometer scans from check films of the treatment jig irradiated using the Eldorado 6 unit.



Densitometer scan of the entrance dose across the midline of the radiation portal. A film was placed under a 5mm perspex plate on top of the jig but under the lead shielding. Slight bumps can seen from backscatter at each of the jig divisions.



Densitometer scan of the entrance dose across the midline of the radiation portal. A film was placed underneath a 5mm perspex sheet on top of the jig but below the lead shielding. Wax phantom mice were in position. Small peaks from tangential scatter can be seen at each of the jig divisions. Valleys caused by absorption can be seen in each division.



Densitometer scan of the exit dose made perpendicular to the above two scans through position 3. The uneven dose distribution is clearly evident.

1. The jig was modified by moving the head shielding to increase the amount of irradiated lung. A Computer Assisted Tomographic scan of mice in the jig with markers indicating the boundaries of the irradiation field showed that 100% of the lung was now included in the field. (Due to technical reasons at the time of the scan, it was not possible to obtain a hard copy and evaluation was performed on screen using the CAT software.)

2. A Theratron 2 ^{60}Co therapy unit was used for most of the experiments. This unit had the benefit of proper collimation, a higher dose rate and larger field size. A field size of 30cm by 30cm could be selected to improve the flatness of the field over the jig.

3. Dosimetry was performed in each irradiation position within the jig using a PTW-UNIDOS dosimeter and a Baldwin-Farmer ionisation chamber with full build up.

4. Mice were anaesthetised prior to being placed in the jig. A 1:4 mixture of 20mg/ml Xylazine: 100mg/ml Ketamine was prepared on the morning of irradiation. Each mouse was injected with 1.5µl/g (Balb/C) or 1.2µl/g (C57/Bl) of the mixture, which provided approximately 50 minutes of anaesthesia. This resulted in a more consistent positioning of the mice and a more uniform dose distribution. Under anaesthesia, no movement apart from breathing occurred during irradiation.

Cis-platinum

Cis-platinum was obtained in 10mg vials of lyophilised powder from Lennon Ltd. A fresh vial was prepared on the day of use by dissolving the lyophilised powder in 20ml of sterile saline (0.9%) to give a final drug concentration of 0.5mg/ml cis-platinum. Cis-platinum was given by intra-peritoneal (i.p.) injection. Control mice not due to receive drug were given an i.p. injection of an equivalent volume of saline as a placebo.

Mice were hydrated 2 h before and 1 h after administering cis-platinum by i.p. injection of 1ml 0.5% Glucose in 0.45% saline. The hydration mixture was sterilised by filtration through a 0.22µm filter.

Control treatments

All experiments included sham treated control groups. For those groups acting as controls for drug treated groups the sham treatment consisted of the hydration procedure and an equivalent amount of saline injected in place of cis-platinum. For those groups acting as controls for radiation treated groups mice were placed in treatment jigs and placed under the treatment unit for an equivalent time to that of the irradiated mice.

Controls for combined treatment groups received both sham treatments.

Plethysmography

Description of unit

A mouse sized whole body plethysmograph was constructed for use in investigating the breathing rates of mice after combined cis-platinum and radiation.

A plastic tube of internal diameter 57 mm and length 165 mm was mounted on a base and fitted at both ends with removable doors sealed with rubber O-rings. The volume of air in the chamber was determined to be 200 cm^3 by filling the chamber with water and then measuring the volume of water using a measuring cylinder. A condenser microphone was

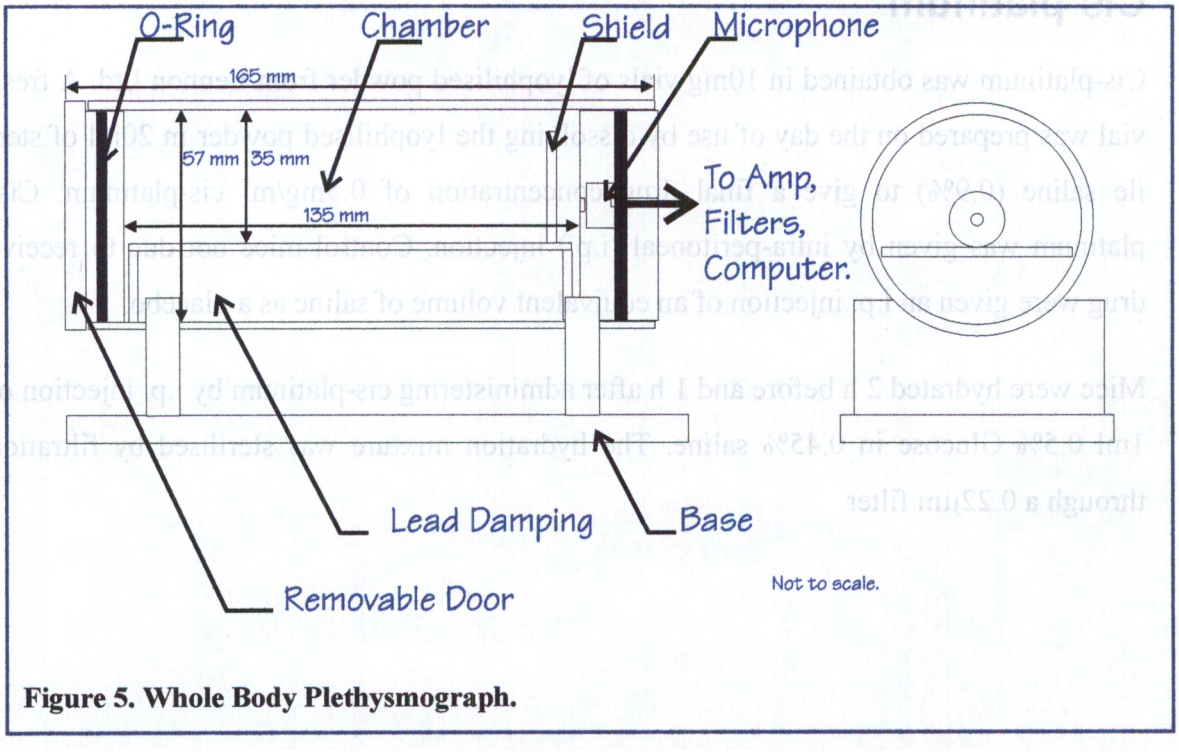


Figure 5. Whole Body Plethysmograph.

mounted in the centre of one of the doors. Approximately one third of the chamber was filled with lead to reduce extraneous vibrations. The lead was separated from the chamber by a Perspex floor. The chamber is depicted in Figure 5.

The signal from the microphone was fed to a purpose built amplifier and low pass filter from whence it was passed to an analogue to digital converter (PC-30B, Eagle Electric, Cape Town) for recording and analysis on an IBM-compatible PC. Data was collected using the software package supplied with the analogue to digital converter (STATUS 30 V2.00 Data Acquisition Software, Eagle Electric, Cape Town.) This package allows data to be stored on disk and to be analysed by Fast Fourier Transformation. The circuit diagram for the amplifier and filter is given in Figure 6.

The amplifier design was based on four LF351 wide bandwidth JFET input operational amplifiers. This chip offers high input impedance, high slew rate, low input bias current as well as wide bandwidth. It also has very low noise. The initial stage comprises an emitter-follower that provides a high impedance load for the electret microphone. The signal from this stage is passed to an inverting amplifier with variable gain. The following stage is a low pass filter with a desired -3dB point at 15 Hz and roll-off of ~18db per octave.

The frequency response of the electronic side of the system was determined by feeding

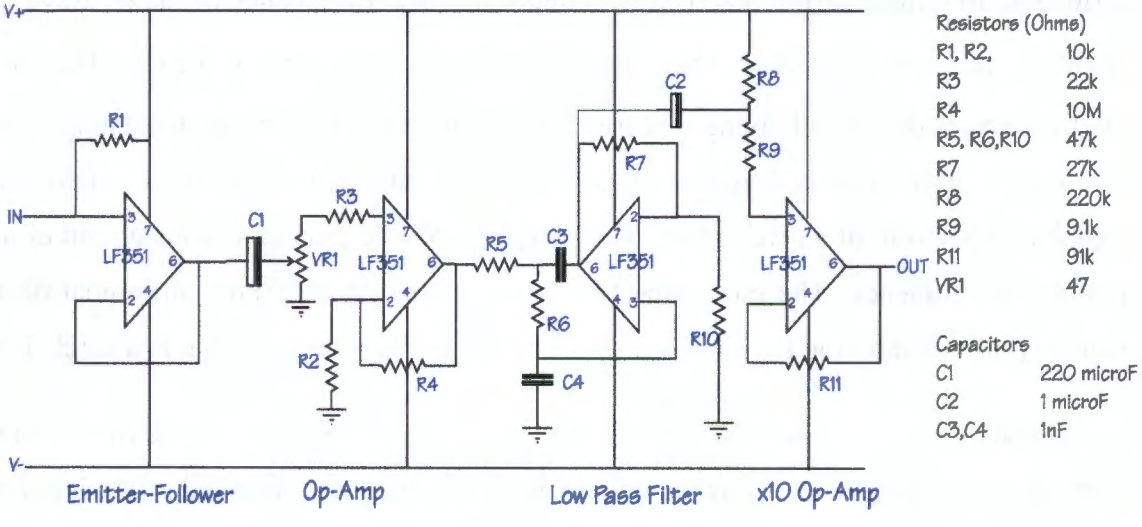


Figure 6. Circuit diagram of amplifier and filter for plethysmograph.

the signal from a signal generator into the input of the amplifier and determining the frequency at both input and output using an oscilloscope. This was then compared to the frequency obtained by collecting the same data on the PC and processing by Fourier Transformation.

Only data from the third week of measurement was included for analysis. When a clear peak could not be discerned in the Fourier Transform the mouse was returned to the chamber and the measuring cycle repeated.

It was not possible to calibrate the plethysmographic chamber itself as apparatus capable of generating pressure changes of known frequency within the range 2 - 20 Hz was not available.

Principles of Fourier analysis

In 1807 J.B. Fourier showed that any function could be described as the sum of a series, which for a two dimensional wave (x,y) can be given as:-

$$y(x) = \sum_{n=1}^{\infty} B_n \sin\left(\frac{n\pi x}{L}\right)$$

with $B_n = A_n \cos(\omega_n t_0 - \delta_n)$ (French, 1982.) Thus a two dimensional wave can be described as an infinite series of sine waves where ω_n is the frequency of the n^{th} wave, δ_n its phase and A_n its amplitude. The frequency spectrum of the wave is then a collection of the frequencies of the underlying sine waves (harmonics) and their associated amplitudes. The Fast Fourier Transformation is a numerical technique that can be used to derive the frequency spectrum of a given wave. The spectrum may be plotted as a histogram of amplitude vs. frequency. The more closely a wave approximates a pure sinusoidal disturbance of infinite duration the narrower its spectrum will be - i.e. converge to a single line.

The characteristic frequency of a complicated wave can be considered to be the harmonic with the largest amplitude. Thus the breathing rate of mice was recorded as the harmonic frequency at the centre of the highest peak in the frequency spectrum obtained by Fast Fourier Transformation of the recorded breathing wave.

Recording technique

The breathing rates were sampled at a frequency of 50Hz. 4096 samples were taken each time thus the duration of measurement was 81.92 seconds.

Mice were placed in the chamber for 1 minute to acclimatise before recording started. After the initial measurement, they showed no signs of agitation at being in the chamber and willingly entered it. Measurements were performed weekly at approximately the same time each day. Measurements were performed sequentially across groups within an experiment rather than sequentially within a particular group. This minimised the difference in time of measurement between different groups within an experiment.

Surfactant analysis

Introduction

Two techniques were used to determine the phospholipid content of surfactant extracted by broncho-alveolar lavage (BAL). High Pressure Liquid Chromatography was used to determine the amount of phosphatidylcholine as an indicator of the total amount of surfactant. A colorimetric assay based on an enzymatic cleavage of phosphate was used as an indicator of total surfactant phospholipid content.

Dissection and lavage procedure

Mice were killed before broncho-alveolar lavage by cervical dislocation. They were then taped, dorsal side down, to a perspex jig and exsanguinated. The peritoneal cavity was opened and a small incision made in the peritoneum. The tissue surrounding the trachea was carefully excised and the trachea was cut above the level of the larynx. An 18G catheter was inserted in the trachea. A thread of cotton was tied around the trachea to prevent the catheter slipping out. 0.8 ml of ice cold saline was slowly injected into the lungs and withdrawn to lavage the surfactant from the lung. Three aliquots were collected from each mouse. The catheter was left in place until the last aliquot after which it was removed and any fluid retained in it added to the lavage.

Samples were stored in liquid nitrogen until analysis.

HPLC

The lipid content of a sample was extracted for analysis by HPLC using a modification of the method of Bligh and Dyer (1959). 3ml of methanol:chloroform (2:1) followed by 1ml chloroform and 1ml Millipore filtered water was added to 0.8ml of lavage fluid. This was vortexed vigorously for 2 minutes and then spun at 1000g for 10 minutes. The lower chloroform layer was drawn off using a Pasteur pipette. 2ml chloroform was added to the supernatant and the sample was vortexed and centrifuged. The lower layer was drawn off and added to the previous chloroform extract.

The chloroform was evaporated off at 37°C under nitrogen. The lipid fraction was then left as a thin layer on the bottom of the tube. This was re-dissolved in 100µl spectrographic grade ethanol (Merck).

The lipids were analysed by HPLC using a method published by Jungawala et al. (1975). An LKB variable wavelength monitor set at 203nm was used to detect peaks. A 25cm * 4.6mm Spherisorb silica column with average particle size 5µm was used. A flow rate of 1ml/minute acetonitrile:methanol:water in the ratio 65:21:14 was used as the mobile phase. 10µl of sample was injected for each run.

Integration was carried out using a dedicated Apple IIe computer running Chromatochart from Interactive Microware Inc. A sample trace is show in Figure 7. The area under the peak, in arbitrary units, eluting at approximately 13 minutes was used as a relative measure of lavaged surfactant.

The column was regenerated by running 20-30 column volumes of each of the following: methanol; dichloromethane; iso-propyl alcohol; hexane and then in the reverse order through the column. In addition, a 40µm guard column was fitted to increase column life. This did not significantly affect retention time or peak width of the chromatogram. After about 150 injections the back pressure and retention time of the column had increased and a new column was fitted.

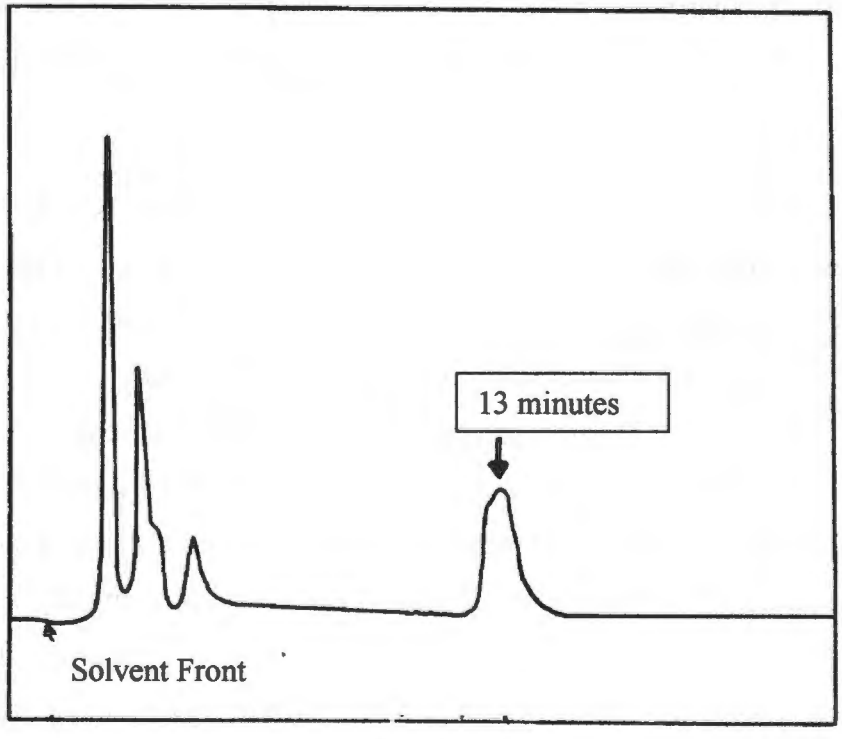
Enzymatic assay

A total phospholipid assay kit (Boehringer Mannheim No 691844, Mannheim, Germany) was adapted to enable numerous small samples to be processed. The kit as supplied consists of a buffer, enzymes and a standard. The composition of these is shown in Table 3. An additional buffer was prepared comprising 1mg/ml Triton X in 6mM $CaCl_2$ (Takayama et.al. 1977.) The enzyme solution was prepared for storage by adding 2 ml of the manufacturer's buffer to the enzyme mixture and gently agitating until the enzymes were dissolved. The resulting solution was divided into 10 by 200µl aliquots and stored in liquid nitrogen.

When required an aliquot was thawed and dissolved in a 50/50 mix of the manufacturer's buffer and the Triton X buffer. Total volume of the resulting solution was 48ml.

The solution was then divided into 1ml aliquots for use in the assay. To each aliquot

Figure 7. Sample trace of the phospholipid component from broncho-alveolar lavage fluid extracted from Balb/C female mice and analysed using HPLC. The area of the peak labelled with an arrow was used as arelative measure of surfactant.



100µl of extracted surfactant was added. The samples were incubated at 37°C for 25 min before being read in a Phillips SP8-400 spectrophotometer at 500nm.

Each group of samples read included a control at the start and finish of the run, as well as standards for construction of a standard curve. The amount of phospholipid per 100µl extracted lavage was interpolated from the standard curve and the amount of lavaged phospholipid per mouse lung was then calculated.

Table 3. Composition of enzyme assay kit, as supplied by the manufacturer.

Buffer	
Tris hydroxymethyl aminomethane	50mmol/l pH 8
Phenol	20mmol/l
Enzymes & Reagents	
Phospholipase D	≥1000 U/l
Choline oxidase	≥1400 U/l
Peroxidase	≥ 800 U/l
4-aminophenazone	8mmol/l
Standard	
Choline chloride	54.1mg/100ml

Histology

Mice were killed by cervical dislocation at 120 days after treatment. Four groups of mice were treated. A control group received placebo. Groups were treated with 6mg cis-platinum/kg, 12Gy ^{60}Co γ-rays and with 6mg cis-platinum/kg immediately before 12Gy ^{60}Co γ-rays.

The ventral chest wall was opened and the trachea freed from surrounding tissue. A catheter was inserted into the trachea and tied in place with a length of thread. The lungs and heart were then removed en-bloc and suspended in 10% phosphate buffered formalin. 0.8ml of 10% phosphate buffered formalin was used to gently inflate the lung. The catheter was removed and the trachea tied closed with the thread. The tissue was then left in formalin for at least 4 weeks to fix before being processed for sectioning. Dehydration was carried out by successive immersion in increasing concentrations of alcohol before a final xylene bath and then immersion in wax. Sections of approximately 5µm thick were cut parallel to the thoracic midline and from about midway through the right lung. Sections were stained with haematoxylin and eosin (H&E) and with Von Giesons stain for collagen.

The reaction was qualitatively evaluated as well as scored using a modification of the reaction scoring system described by Travis (1980). A grid of 100 blocks in a 20x objective and a 10x eyepiece was used to evaluate sections stained with H&E. A block was scored as positive if it contained one or more of the following :-

- macrophage infiltration in air spaces
- mononuclear inflammatory cells in alveolar walls
- oedema in alveolar wall or air spaces
- fibrin in air spaces.

Three, randomly selected grids not including airways or blood vessels were counted for each lung section. The percentage of lesion occupying the section was then calculated as the mean percentage of positive blocks.

TGF-β Analysis

Principle of assay

The Quantikine™ human TGF-β1 immunoassay kit from R&D Systems (R&D Systems, Increase, Minneapolis, USA) was used to measure TGF-β1 in broncho-alveolar lavage fluid and homogenised lung tissue from mouse lungs. This kit is suitable for the quantitative determination of active transforming growth factor beta 1 concentrations in cell culture supernate, serum, plasma and other biological fluids. It is a sandwich enzyme immunoassay technique.

The microtiter plate provided in the kit is coated with TGF-β soluble receptor type II that binds TGF-β1. Standards and samples are pipetted into the wells of the plate. The immobilised receptor then captures any active TGF-β present in the solutions. After washing, a polyclonal antibody specific for TGF-β1 is added. This binds to the immobilised TGF-β forming a *sandwich* comprising the receptor, the growth factor and the antibody. After washing, a substrate solution is added which, develops colour in proportion to the amount of sandwiched TGF-β1. This can be quantitated using a multi-well plate reader.

Method

Mice were killed by cervical dislocation and the lungs dissected and lavaged with ice-cold saline as described earlier in the methods section. The lavage fluid was centrifuged at 1000 x g for 10 minutes to remove any cells from suspension. The supernatant was drawn off and stored in liquid nitrogen for analysis once all samples had been collected.

The samples were thawed before analysis. An activation procedure was followed to ensure that any TGF-β existing in the samples in latent form was converted to the active form required for recognition in the assay. The activation procedure followed was slightly different from that in the kit and is described below.

A solution of 2.5 N acetic acid/10 M urea was made by slowly adding 35.9ml of glacial acetic acid to 100ml of de-ionised water. 150.2g of urea was added to the well-mixed solution. This was mixed until the urea had dissolved and then made up to 250ml using de-ionised water. A solution of 2.7 N NaOH/1.0 M HEPES was made by slowly dissolving 27g NaOH in 150ml de-ionised water. This was mixed and then 59.5g HEPES was added. The solution was mixed and the volume brought to 250ml with de-ionised water.

0.5ml of 2.5 N acetic acid/10 M urea was added to 0.5ml of lavage fluid. This was mixed and then incubated for 10 minutes at room temperature. 0.5ml of 2.7 N NaOH/1.0 M HEPES was added to each to neutralise the acidified sample. The pH was checked to confirm a reading of between 7.2 and 7.6. The activation procedure resulted in a 1:3 dilution of the original sample.

A dilution of 1:4 - sample:RD6M diluent as recommended by the manufacturer for platelet poor plasma was made by adding 4.5ml RD6M to each activated sample.

The wash buffer was prepared from the concentrate supplied in the kit by diluting 20ml of supplied concentrate to a final volume 500ml with de-ionised water.

The TGF-β standard was reconstituted with 2ml of RD6M to give a resultant solution of concentration 2000 pg/ml TGF-β1. This was left to stand for 15 minutes with gentle agitation before making a dilution series with concentrations of 2000pg/mL, 1000pg/mL, 500pg/mL, 250pg/mL, 125pg/mL, 62.5pg/mL, 31.25pg/mL and 0pg/mL.

200μL of sample or of standard was added to each well in the multi-well plate. Each sample and each standard was analysed in duplicate. The wells were covered with the adhesive strips provided in the kit and left to incubate for 3 h at room temperature. Each well was then aspirated and washed by the addition of 400μL wash buffer for a total of four washes. At each wash step, care was taken to remove all of the wash buffer. The plate was inverted and blotted dry after the last wash.

200μL of TGF-β1 conjugate was added to each well and the wells covered with a fresh adhesive strip. The plate was then incubated at room temperature for 1.5 h after which the wash process as described above was repeated.

200μL of Substrate Solution was then added to each well, followed by an incubation of 20 minutes at room temperature. After this period, 50μL of Stop Solution was added to each well. The plate was gently tapped to ensure thorough mixing of the solution.

The optical density at 450nm was read on an Anthos Labtec H2 multi-well plate reader with wavelength correction at 540nm.

A standard curve was constructed by plotting the mean absorbance vs. concentration of TGF-β standard. A linear regression was performed on the data using the regression module in STATISTICA (Statsoft, Inc.) The resulting equation representing the best fit to the standard curve data was then used to calculate the concentration of TGF-β in the sample. The measured concentration was then multiplied by a factor of 12 to account for the dilution occurring during the extraction and activation procedure.

Analysis of TGF-β in lung tissue

A modification of the above technique for analysing surfactant was used to analyse the amount of TGF-β in lung tissue.

Mice in which TGF-β was to be measured in lung tissue were treated with radiation and / or cis-platinum as described in Chapter 10, page 117. At 1 week and one month after treatment, mice were killed by cervical dislocation and their lungs excised and stored in liquid nitrogen until the time of assay.

Lungs were removed from liquid nitrogen, allowed to thaw before being blotted on tissue paper, and weighed. The samples were homogenised with 4ml of 2.5 N acetic acid/10 M urea per gram of tissue. The resulting homogenate was transferred to polypropylene tubes and allowed to incubate for 10 minutes at room temperature. The samples were then spun at 10000 x g for 10 minutes. The centrifuge temperature was maintained at 4°C. The supernatant was transferred to clean tubes and 4ml 2.7 N NaOH/1.0 M HEPES per gram of original tissue was added. The samples were vortexed and then spun for a further 10 minutes at 10000g.

200µl of the homogenised lung sample or standard was added to the wells in a multi-well plate and the analysis completed as described above.

The measured concentration was multiplied by a dilution factor of 9 to account for the dilution occurring during the homogenisation and activation procedure.

Protein determination

The Bio-Rad Protein Assay (Bio-Rad Cat No. 500-0002) based on the Bradford dye-binding procedure (Bradford 1976) is a simple colorimetric assay for measuring total protein concentration. The standard assay is used with samples having protein concentrations between 200 µg/ml and 1400 µg/ml (20-140µg total). The assay is based on the colour change of Coomasie brilliant blue G-250 dye in response to various concentrations of protein. The dye binds to primarily basic (especially arginine) and aromatic amino acid residues. The assay is useful for measuring proteins and polypeptides with MW greater than 3000-5000, depending on the charged groups.

The lung homogenate used in the TGF-β analysis was also analysed using the Bio-Rad protein Assay for total protein.

The samples as prepared for the TGF-β assay were used for total protein analysis. As described previously the samples were homogenised with 4ml 2.5 N acetic acid/10 M urea per gram of tissue. The resulting homogenate was transferred to polypropylene tubes and allowed to incubate for 10 minutes at room temperature. The samples were then spun at 10000 x g for 10 minutes. The supernatant was transferred to clean tubes and 4ml 2.7 N

NaOH/1.0 M HEPES per gram of original tissue was added. The samples were vortexed and then spun for a further 10 minutes at 10000 x g.

A standard curve was constructed using 0,2,4,6,8 and 10μg albumin / 1.6 ml water, to which 0.4ml Bio-Rad protein dye reagent was added. All samples were read at 595nm in the spectrophotometer. The absorbance of the homogenate samples, diluted 1:50 was measured and the amount of protein determined.

Statistics

Statistica from Statsoft Inc. was used for all statistical tests. All error bars shown in the figures are standard deviations unless otherwise stated. In some instances not all error bars are shown in a figure to enhance clarity.

Student's t-test was used to assess significance of difference between groups.

Kaplan-Meier product limit survival analysis techniques (Kaplan et al. 1958) were used to analyse survival of mice. The technique was also explored as a means of comparing breathing rate data from different treatment groups by defining a "response breathing rate". Mice that exhibited breathing rates above this response breathing rate were assessed as complete or "dead". Those not responding were included in the analysis as censored. Non-parametric techniques could then be used to compare groups and test for statistical significance.

Chapter 10. Experimental work – Results

The experimental work is described in five sections:

- Calibration and dosimetry
- Surfactant analysis
- Breathing rate analysis
- Histological analysis
- TGF-β analysis.

Calibration and dosimetery

Densitometry

The results of the densitometer scan of the check films made of the jig with wax phantom mice in position is given in Table 4. The densitometer readings showed a variation from an average reading of 110% for positions in row B to 94% in row A in the incident dose rate. The exit dose rate averaged 95% in both row A and row B.

Within row A, a maximum density produced by the incident beam of 97% was recorded for the central positions while a minimum of 90% was seen for the outside positions. Likewise, for row B a value of 113% was recorded for the central positions and 105% for the outside positions.

Table 4. Densitometer readings expressed as a percentage relative to a control reading on the exposed film.

	Row A		Row B	
	Incident	Exit	Incident	Exit
1	91	95	106	94
2	96	95	112	95
3	97	95	113	95
4	97	96	113	96
5	94	96	110	95
6	90	93	105	93
Average	94	95	110	95

The results of dosimetry performed using the Baldwin-Farmer ionisation chamber at the various positions in the mouse restraining jig is given in Table 5. The lowest dose rate was found to be 10% lower than the highest. In order to correct this imbalance, positions

that were contained within a spread of 5% were grouped. Thus, A 2-5 (Table 5) and B 1 - 5 were grouped to give a mean dose rate of 2.13Gy/min on 6th of December 1995. Mice treated in the remaining positions (mean dose rate of 2.01Gy/min on the 6th of December 1995) were given an additional exposure to bring them up to the required total dose.

Table 5. Exposure values for irradiation set-up on the Theratron 2 as measured on 6 December 1995.

Position	Row A (Gantry Side) (Gy/min)	Row B (Target Side) (Gy/min)
1 (rightmost looking at the gantry)	1.97	2.11
2	2.15	2.13
3	2.15	2.09
4	2.17	2.13
5	2.13	2.09
6 (leftmost looking at the gantry)	2.05	1.99

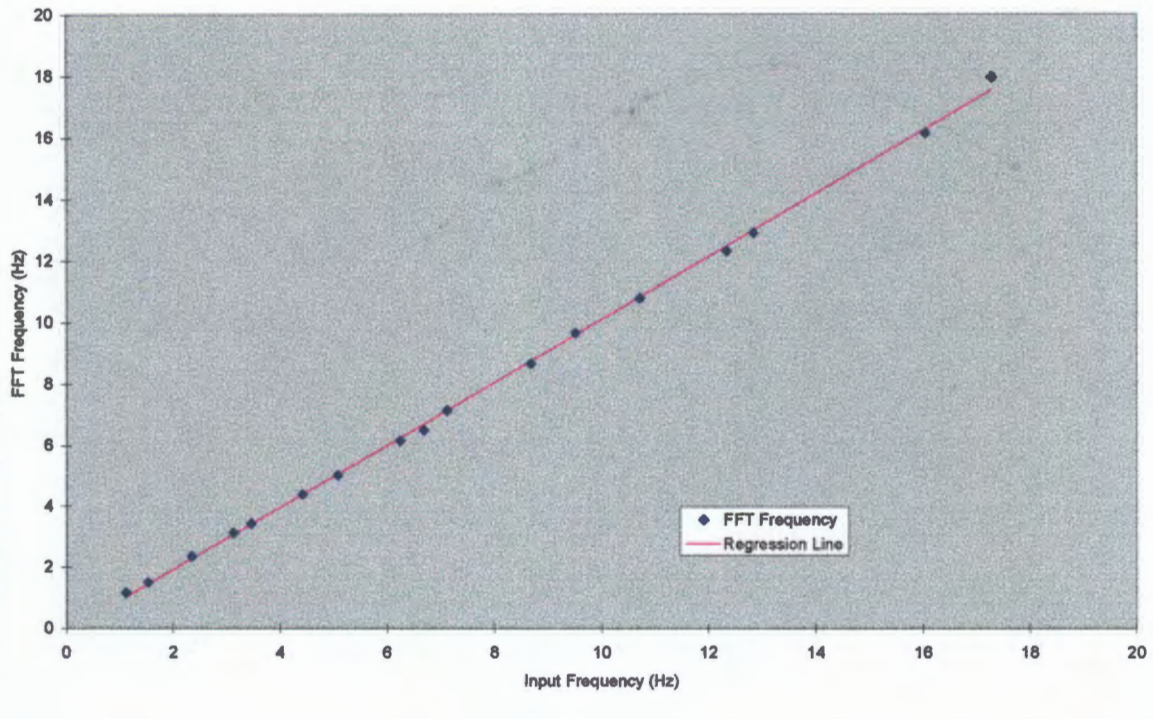
Calibration of plethysmograph

The results of the calibration procedure as described in Chapter 9 using a signal generator are presented in Figure 8 and Figure 9. A linear regression of data collected at a sampling rate of 50 Hz vs. the input frequency gave a correlation coefficient of $R^2 = 0.999$, an intercept of -0.132 ± 0.066 and a slope of 1.022 ± 0.0074 (Figure 8).

A graph of log relative amplitude vs. log frequency is shown in Figure 9. It can be seen that the curve is not very flat over the region of interest (4-10 Hz). While this did not affect the frequency response of the system as seen in Figure 8, it did make it impossible to compare the amplitude of breathing rate data obtained from mice.

An example of recorded waves and their associated Fast Fourier Transforms is shown in Figure 10. As can be seen the pure sine wave produced by a signal-generator results in extremely sharp FFT's.

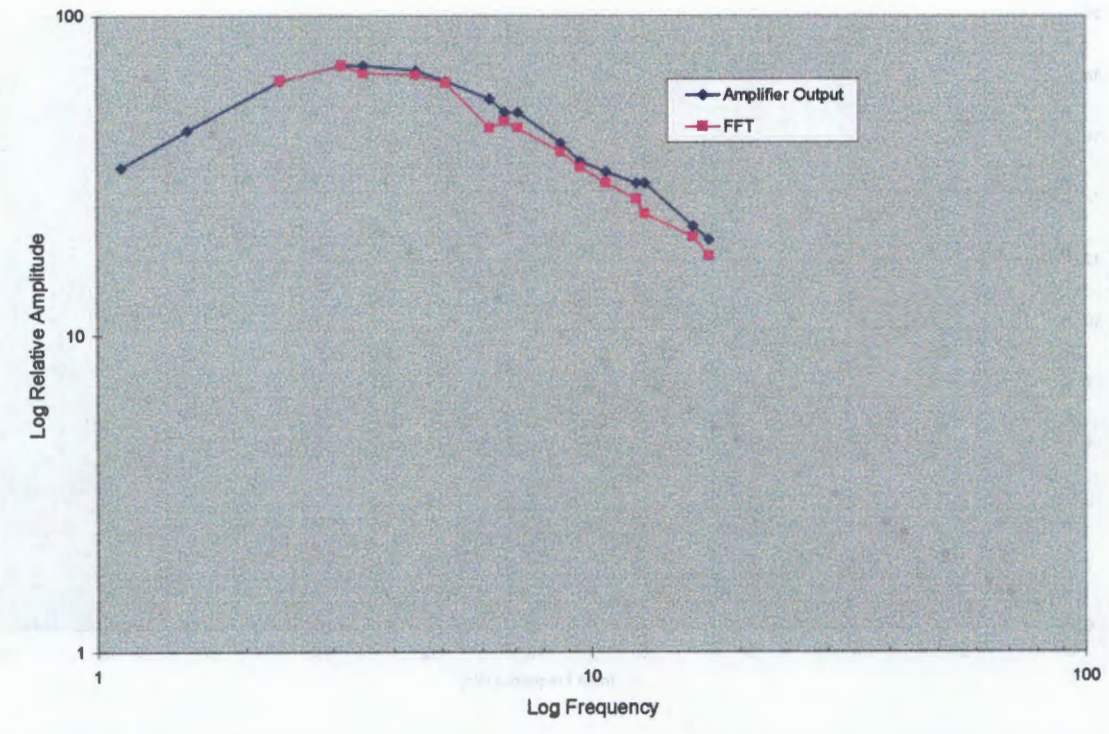
Figure 8. FFT frequency vs. input frequency for calibration of plethysmograph.



The heterogeneous samples obtained from mice did not produce such sharp peaks in the Fourier transformation of the data. In general, the data from untreated control mice showed the most variability. In particular the data from mice measured for the first time gave very broad Fast Fourier Transformations. This was most likely due to the stress that the mouse experienced on being placed in the measuring chamber.

FFT's from mice are shown in Figure 11.

Figure 9. Log amplitude of the FFT peak vs. log frequency for calibration of plethysmograph.



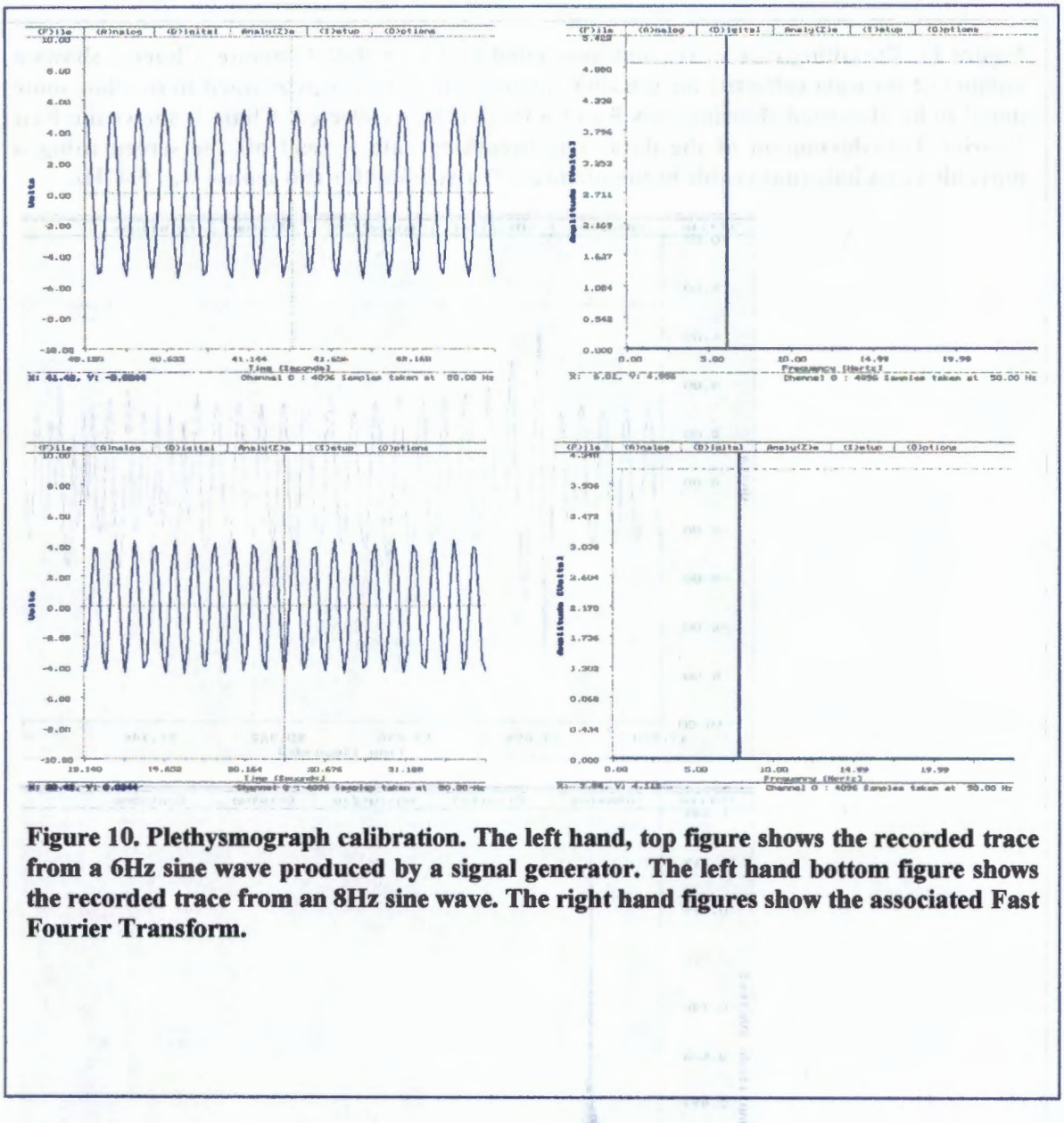
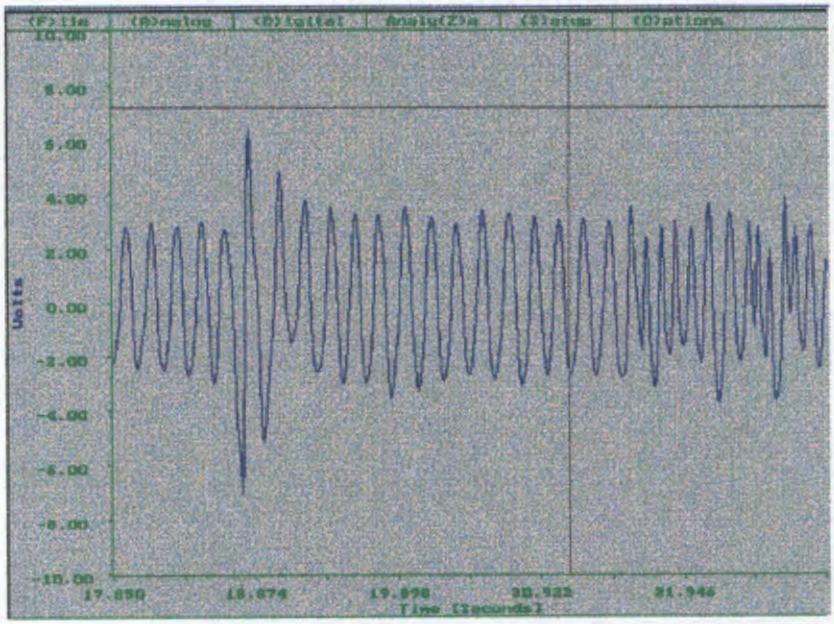


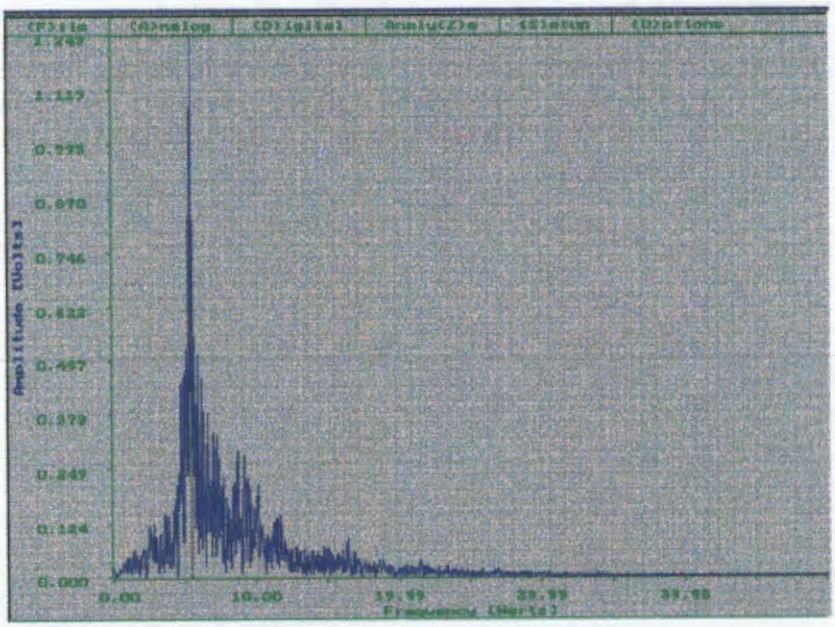
Figure 10. Plethysmograph calibration. The left hand, top figure shows the recorded trace from a 6Hz sine wave produced by a signal generator. The left hand bottom figure shows the recorded trace from an 8Hz sine wave. The right hand figures show the associated Fast Fourier Transform.

Figure 11. Breathing rate waves and associated FFT in a Balb/C mouse. Chart A shows a portion of the data collected for a Balb/C mouse. The screen was zoomed in to allow some detail to be discerned showing only 5 s of a total of 80 s collected. Chart B shows the Fast Fourier Transformation of the data. The breathing rate is read off the screen using a movable cross hair (not visible in the picture). The reading for this mouse was 5.47Hz.

A



B



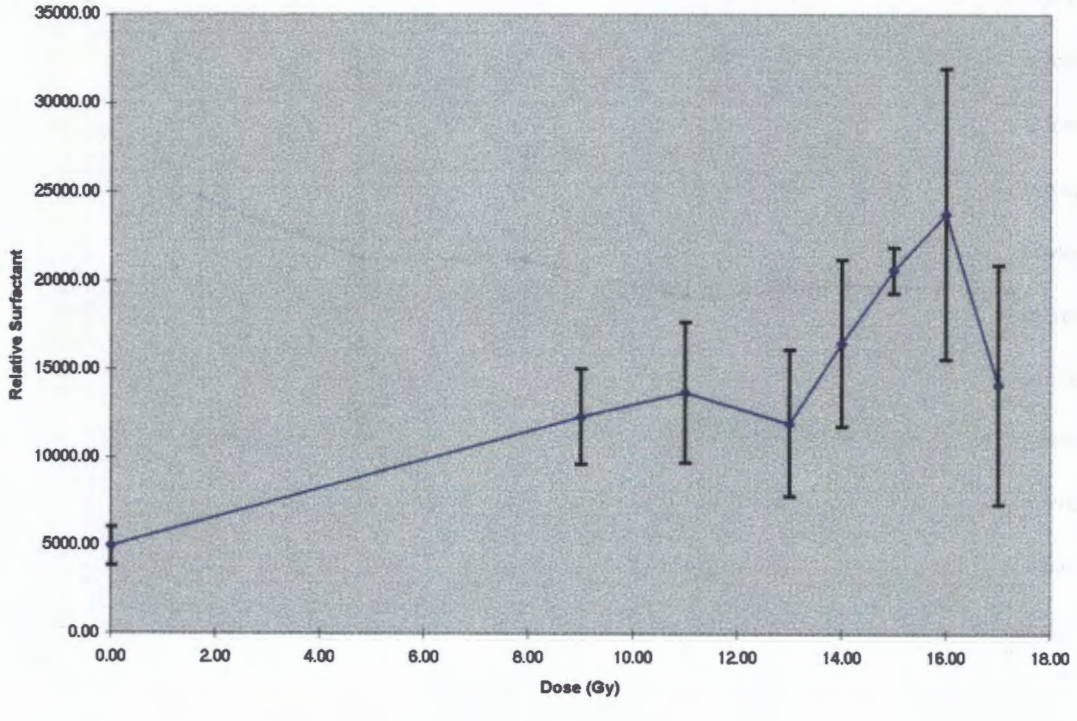
Surfactant analysis

In the initial work surfactant was measured at 28 days after treatment with either cis-platinum, radiation or a combination of cis-platinum and radiation using the HPLC techniques described in Chapter 9. These first experiments were performed to verify that the techniques used to measure surfactant produced similar results to those of Rubin et al. (1984). Once this had been established the effect of timing, of cis-platinum administration relative to radiation, on surfactant levels at 28 days after treatment was investigated using the HPLC technique and then the level of surfactant with time after treatment was investigated using an enzymatic assay for phospholipids as described in Chapter 9.

Surfactant after single doses of radiation

The effect of single doses of ^{60}Co radiation on surfactant levels was measured at 28 days after 0, 9, 11, 12, 13, 14, 15, 16, 17Gy. Four mice were irradiated at each dose, using the Eldorado 6 unit. The data are displayed in Figure 12.

Figure 12. Relative level of surfactant at 28 days after treatment with single doses of ^{60}Co γ-rays. Surfactant was determined using HPLC. Error bars show SD.



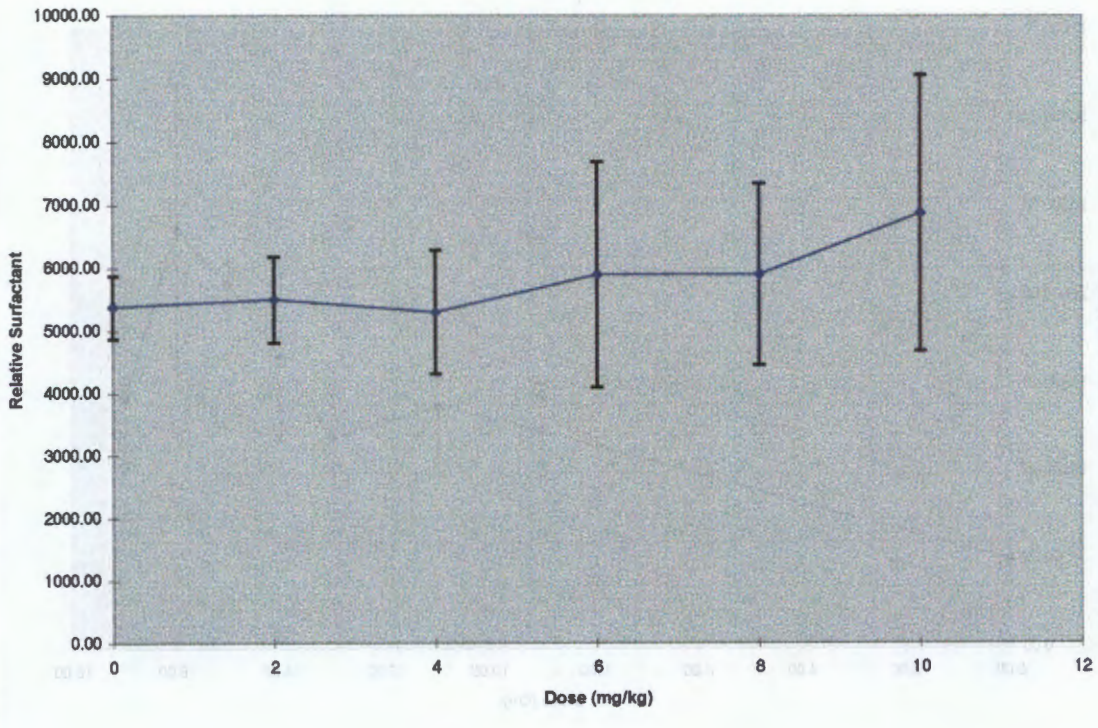
The relative level of surfactant present in broncho-alveolar lavage fluid increased with dose after about 13Gy. Points at 9, 14, 15 and 16Gy were all significantly greater than the control ($p<0.05$). Only the 15Gy point was significantly greater than the 9, 11 and 13Gy points ($p<0.05$).

Surfactant after single doses of cis-platinum

The relative level of surfactant after cis-platinum alone was determined at 28 days after 0, 2, 4, 6, 8 and 10mg/kg cis-platinum. Four female Balb/C mice weighing 20g were treated by i.p. injection of cis-platinum at each dose as described in the methods section. No increase in lavaged phospholipid content as determined relative to controls using the HPLC technique was seen with increasing doses of cis-platinum (Figure 13). 10mg/kg was estimated as being the maximally tolerated dose (MTD) for Balb/C mice.

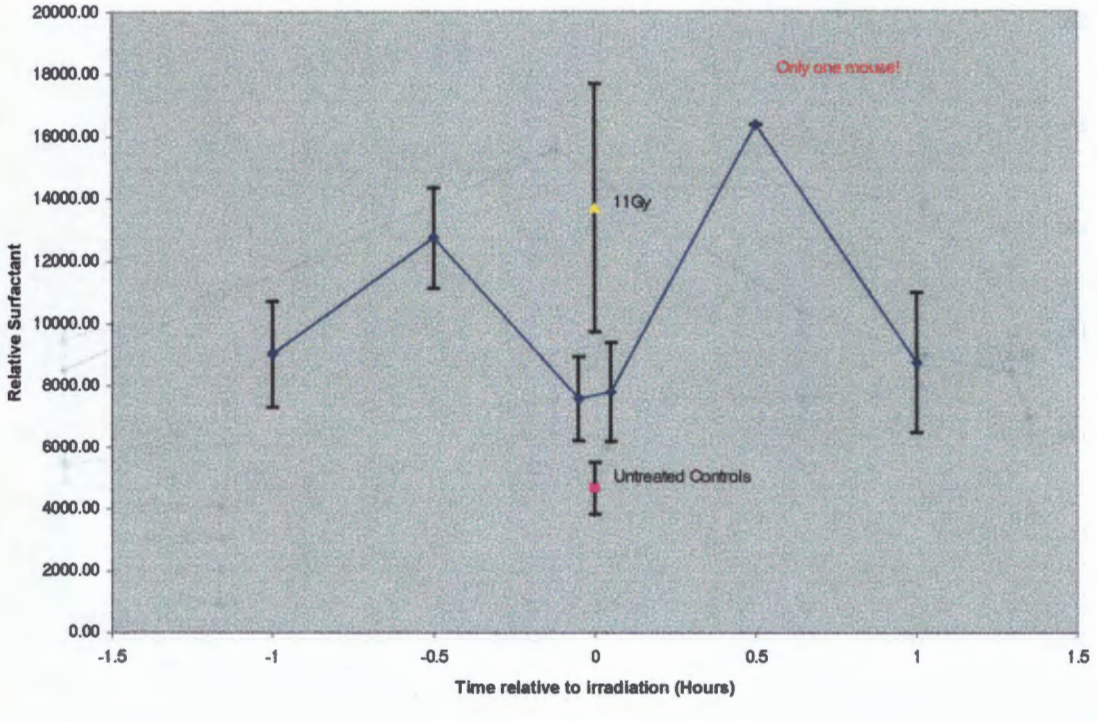
Surfactant after cis-platinum at different times relative to radiation

Figure 13. Relative level of surfactant vs. dose of cis-platinum. Surfactant was measured using HPLC. Cis-platinum was administered by i.p. injection. Error bars show SD.



The effect of cis-platinum on the surfactant system at different times relative to a single dose of radiation was examined by administering 8mg/kg cis-platinum at 1 h, half an hour and immediately before and after 11Gy ^{60}Co. The Eldorado 6 unit was used for irradiation. The cis-platinum dose of 8mg/kg was chosen as being 80% of the MTD in order to maximise potential drug effect without any drug induced lethality. Four mice were treated in each group and killed by cervical dislocation at 28days after treatment. The toxicity experienced during this experiment was higher than expected with the number of mice surviving in each point being very low. Only 1 out of 4 mice treated survived when cis-platinum was administered 0.5 h after 11Gy. The data is displayed in Figure 14. An increase in the level of surfactant relative to untreated control mice was seen for all times of cis-platinum treatment. This increase was generally lower, but not statistically significant at the 5% level, than that seen for 11Gy irradiation only as determined in the radiation only group.

Figure 14. Relative level of surfactant lavaged from Balb/C female mice at 28 days after treatment with 8mg/kg cis-platinum administered at different times relative to a single dose of 11Gy ^{60}Co γ-rays. Error bars show SD.



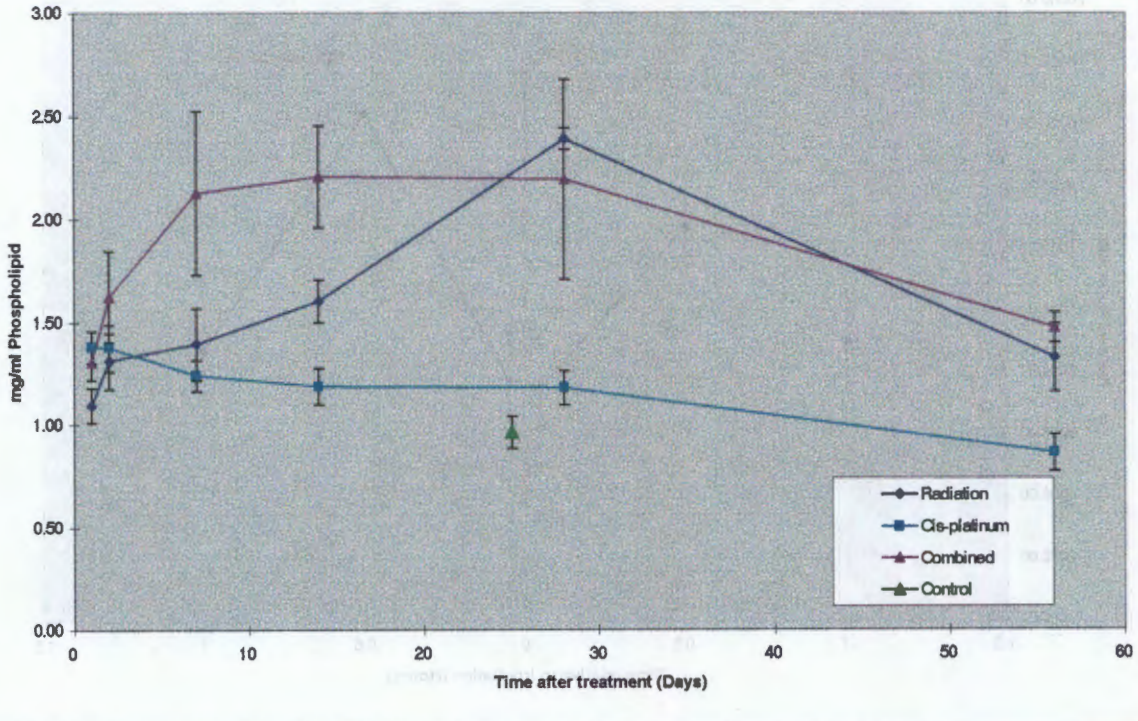
Time course of surfactant release

The time course of the release of surfactant was measured using the enzymatic assay of total phospholipid described in the methods section. 24 mice per group were treated as described in the methods with either 13Gy ^{60}Co alone, 8mg/kg cis-platinum alone or 8mg/kg cis-platinum given immediately before 13Gy ^{60}Co. All irradiations were performed using the Eldorado 6 unit. A control group was treated with placebo. The dose of cis-platinum was chosen as being 80% of the MTD. The dose of radiation was increased from 11 to 13Gy to bring it closer to the threshold dose required to produce an increase in surfactant (as was seen in the experiment to determine surfactant levels after single doses of irradiation described above).

Mice were killed, at were 1, 2, 7, 14, 28 and 56 days after treatment, in groups of four selected randomly from the pool of 24 mice in each treatment arm.

The data show a slight increase at 1 day after treatment with cis-platinum alone, a large increase at 28 days after treatment with radiation alone and a large early increase peaking

Figure 15. Changes in lavaged phospholipid with time after treatment. Balb/C female mice were treated with either 8mg/kg cis-platinum, 13Gy ^{60}Co or 8mg/kg cis-platinum immediately before 13Gy. Error bars show SD.



at 7 days after treatment with cis-platinum and radiation. This is illustrated in Figure 15.

The level of surfactant after cis-platinum alone is significantly higher than controls at 1, 2, 7, 14 and 28 days with $p < 0.0001$ in each case.

The different time course of release of surfactant after combined treatment is strikingly evident with significant differences between the radiation only and combined treatment groups being noted at 2, 7 and 14 days after treatment ($p = 0.014$, 0.0025, and 0.0015 respectively).

A second, large group (300) of mice were prepared and treated to investigate the release of surfactant after 6mg cis-platinum/kg given at different times relative to 12Gy ^{60}Co irradiation. This was intended to be a repeat of the earlier experiment examining the effect of the timing of cis-platinum relative to radiation. The experiment was abandoned when nearly half of the mice had died within the first two weeks after treatment. This early toxicity was presumed due to circadian variations in susceptibility to cis-platinum. Treatment of this group took place in the early morning at approximately 7a.m., which is about one hour after lights on for the mouse house. It is possible that because of circadian variation in toxicity as discussed in Chapter 7, page 50, administration of the drug at 7a.m. resulted in greater nephrotoxicity.

Assessment of breathing rates

The experiments described in this section made use of a mouse plethysmograph and Fast Fourier analysis to assess the effect of cis-platinum, radiation and cis-platinum in combination with radiation on mouse breathing rate. Initial experiments in this section investigated the effect of single dose of cis-platinum and radiation alone or in combination. The breathing rate of two strains of mice, Balb/C and C57/Bl, were compared. The effect of timing of cis-platinum administration relative to radiation was investigated to determine if a particular time of administration produced a more severe normal tissue reaction. Possible effects of cis-platinum on repair were investigated by means of split dose experiments.

Single radiation doses

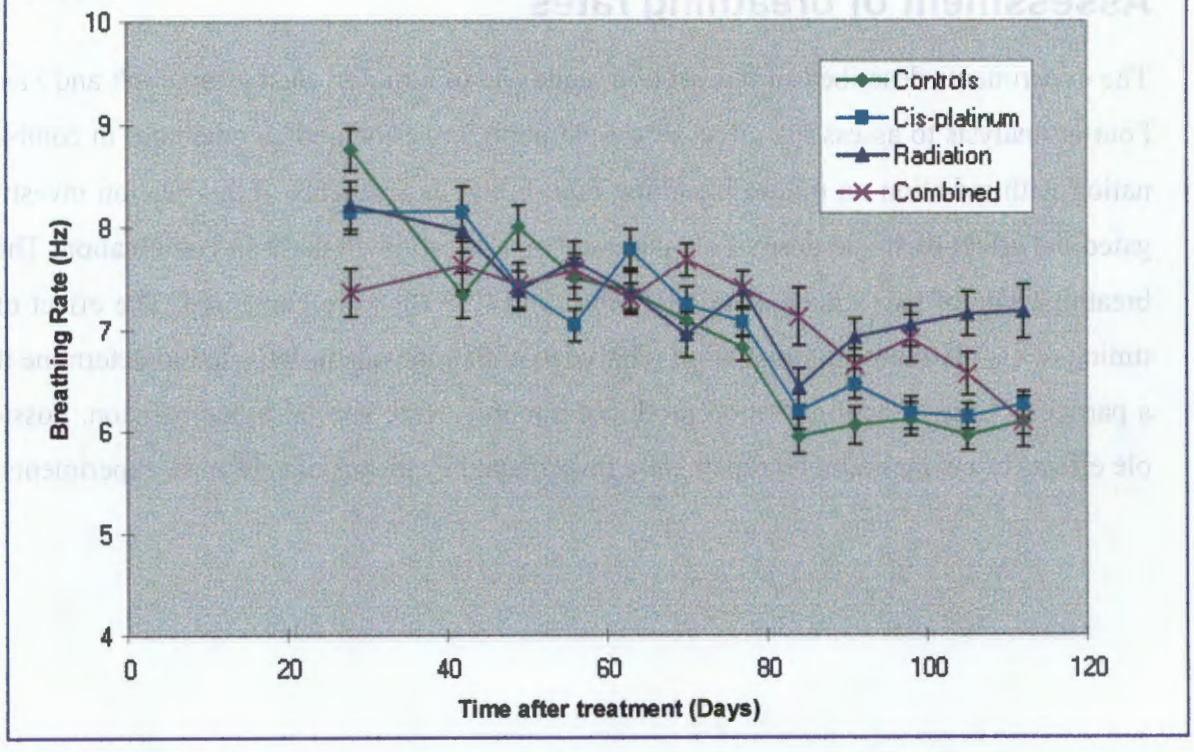
Breathing rates after 8mg cis-platinum/kg and 13Gy

The breathing rate of mice was determined in controls and after treatment with either, 13Gy alone, 8mg/kg cis-platinum alone, 8mg/kg cis-platinum immediately before 13Gy. The Eldorado 6 was used for irradiation. Mice were selected on the basis of weight (20 ± 2g) from stock that were 6 to 8 weeks old.

Data was recorded weekly from 4 to 16 weeks after treatment. Each mouse was sampled in triplicate at 500Hz for 4096 samples. The sampling technique was changed at 12 weeks to sampling once at 50Hz for 4096 samples because no significant difference could be detected between the 500Hz sampling technique and the 50Hz sampling technique. The 50Hz sampling technique allowed one measurement of longer duration to be made with less disturbance to the mouse. This resulted in far fewer readings having to be discarded because no clear peak could be discerned in the FFT.

An age dependent decrease in the breathing rate was observed in all groups until about 12

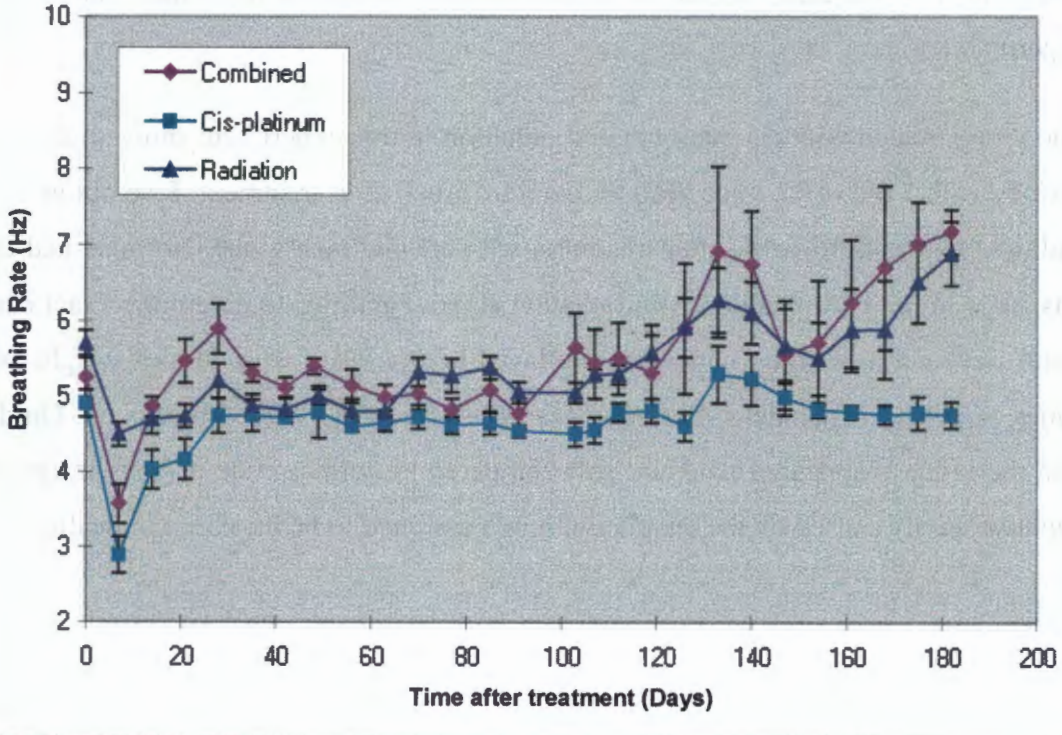
Figure 16. Changes in breathing rates with time after treatment. Balb/C female mice were treated with either 8mg/kg cis-platinum, a single dose of 13Gy ^{60}Co or with 8mg/kg cis-platinum immediately before 13Gy. Control mice received placebo treatments. Error bars show SD.



weeks after measurement started (Figure 16). Thereafter the breathing rate in the group treated with radiation rose from about 6.2Hz to about 7Hz. The control group showed a mean breathing rate of 6.03±0.08Hz during the period 12 to 16 weeks. The breathing rate of the cis-platinum treated group was not significantly different from the controls except at 4 and 13 weeks after measurement started. These differences were most likely due to random experimental error. A trend toward higher breathing rates in the cis-platinum treated group may be evident from 12 weeks onward.

The combined treatment group showed a significantly higher breathing rate from 10 weeks after treatment, after which it dropped to the level of controls at 16 weeks. It should also be noted that the group treated with cis-platinum and radiation started out with an initially lower breathing rate when measurements commenced at 4 weeks after treatment. The breathing rate in this group did not show the same age dependent decrease, but remained constant until 10 weeks after treatment. The breathing rate then dropped as described above. It is possible that cis-platinum changes the time course of radiation damage to the lung causing the breathing rates to be increased earlier than for radiation alone.

Figure 17. Changes in breathing rates with time after treatment. Balb/C female mice were treated with either 7mg/kg cis-platinum, a single dose of 12Gy ^{60}Co or with 7mg/kg cis-platinum immediately before 12Gy. Error bars show SD.



The radiation alone group showed an increase in breathing rates, compared to controls, from week 12 onwards which correlates with literature values and confirms that our technique was sensitive to radiation induced changes in breathing rates.

Breathing rates after 7mg cis-platinum/kg and 12Gy

The breathing rate of mice was determined for up to 180 days after treatment with 7mg/kg cis-platinum, 12Gy ^{60}Co radiation from the Eldorado 6 unit or 7mg/kg cis-platinum administered immediately before 12Gy ^{60}Co. Twelve mice were treated in each group. Five-month-old mice weighing 20±2g were used.

An elevation in breathing rates was evident at the time of the first measurement (Figure 17). This was followed by a decrease, at 7 days after treatment, for mice receiving cis-platinum and is most likely due to systemic effects caused by acute drug toxicity. Mice were observed to be hunched and fluffed up at this stage, generally sitting quietly. The cis-platinum plus radiation group showed an increase, starting at 14 days after treatment and persisting to approximately 60 days after treatment, in breathing rate above the level of either the radiation alone or cis-platinum alone groups. From 70 days onward the error bars overlapped except for one point at 77 days with a trend for the combined treatment group to have a higher breathing rate than the radiation alone group. An increase in the breathing rate of radiation treated groups was noted between about 120 and 140 days after treatment. This increase was seen to occur at about the same time as that seen in the next experiment, Figure 18.

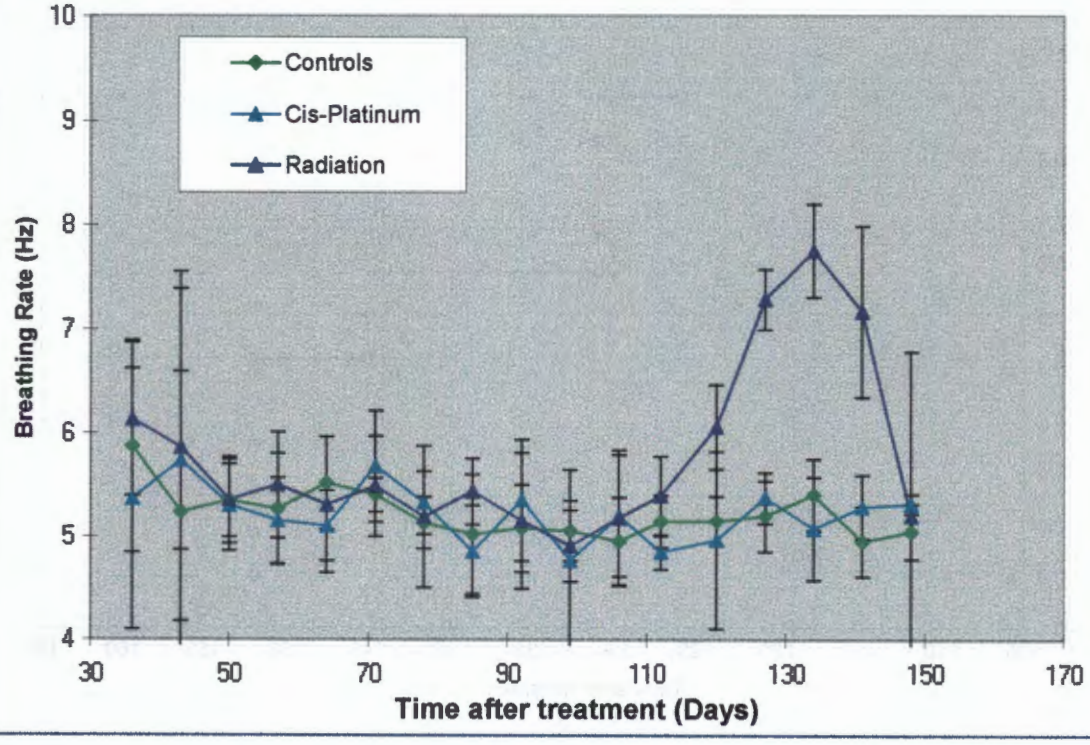
The group treated with cis-platinum and radiation in this experiment showed severe early toxicity with 8 out of 12 mice dead by the third week after treatment. Five out of 12 mice had died by the third week after treatment with cis-platinum alone. No mice had died at this stage in the group treated with radiation alone. Facilities to determine exact cause of death were not available. Symptoms displayed before death were loss of weight and appetite, pinched in abdomens, fluffed appearance and quiet, isolated behaviour. On dissection the kidneys appeared pale and soft compared to normal mice of similar age. Acute nephro-toxicity caused by the cis-platinum was assumed to be the cause of death.

Breathing rates after 6mg cis-platinum/kg and 12Gy

Mice were treated with 6mg cis-platinum/kg in an attempt to reduce the acute early toxicity seen in the previous experiment. In addition to the cis-platinum alone group, groups were treated with 12Gy ^{60}Co γ-rays (Eldorado 6) and 6mg cis-platinum/kg immediately before 12Gy ^{60}Co γ-rays. Six mice were treated in each group. No early deaths were observed in the control group or in the radiation alone group. One mouse in the cis-platinum alone group died during the second week after treatment. All mice in the combined treatment group died during the first week after treatment.

The breathing rates as recorded for the control group, the cis-platinum alone group and the radiation alone group are shown in Figure 18. The control group did not show any significant change in breathing rate during the period observed, although a slight trend to lower breathing rate may be evident. The cis-platinum treated group showed an increased variability when compared to the controls but was not significantly different from the controls at any stage. The radiation alone group was not significantly different from controls until 112 days after treatment at which stage a sharp increase in breathing rates oc-

Figure 18. Changes in breathing rates in Balb/C female mice with time after treatment with 6mg cis-platinum/kg or a single dose of 12Gy ^{60}Co. (All mice receiving combined treatment in this experiment died within 1 week of treatment). Error bars show SD. Control mice received placebo treatments.

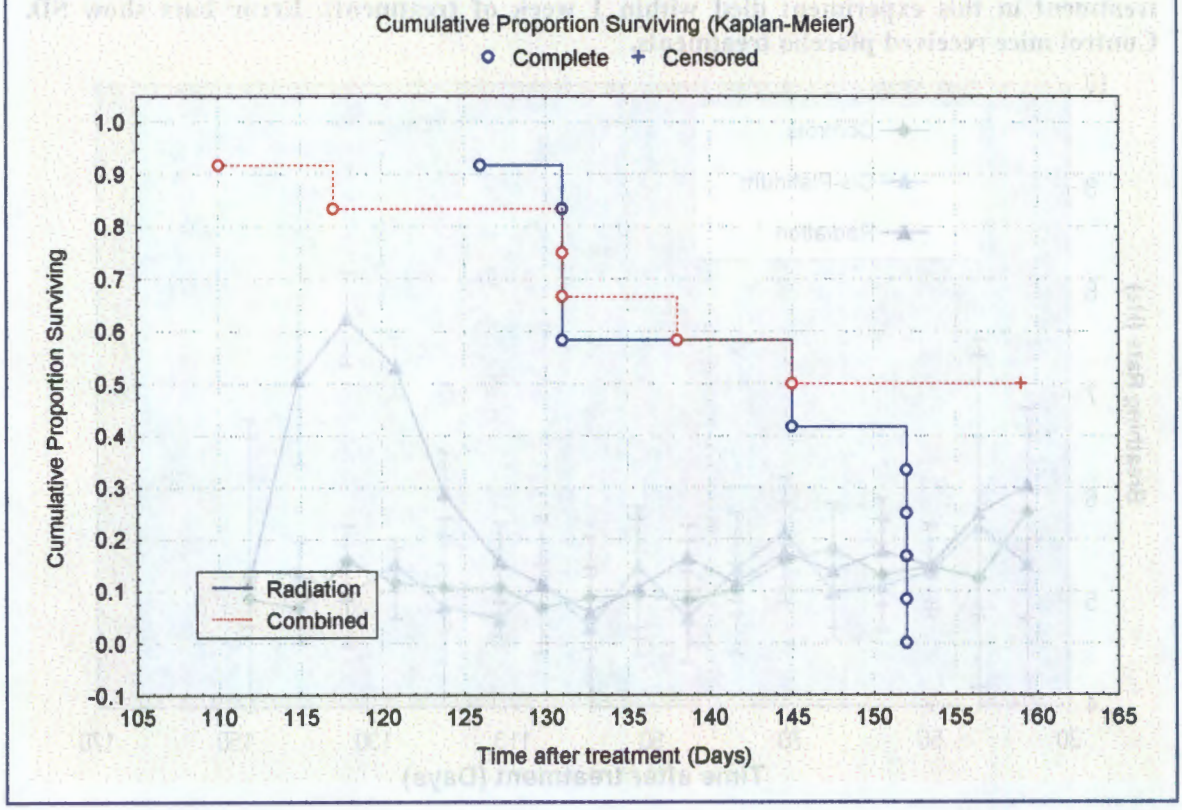


curred until 134 days after treatment. At this stage the mean breathing rate dropped rapidly. It is important to note however that two of the mice died at this stage leaving only three mice of six mice alive, which may have affected the mean breathing rate.

Comparison between Balb/C mice and C57/Bl mice after treatment with 6mg cis-platinum/kg and 12Gy

Groups of 12 mice, either C57/Bl or Balb/C females were treated with 6mg cis-platinum/kg alone, 12Gy ^{60}Co γ-rays alone (Theratron 2) or 6mg/kg given immediately before 12Gy. 12 Mice in each strain received placebo injections of saline and mock radiation treatments. The treatments all took place at the same time of day (approximately 6pm) to minimise circadian variation in toxicity of cis-platinum. The breathing rate of each mouse was recorded once a week at the same time of day each week. Measurements were recorded across the groups as described in the methods section to minimise variation

Figure 19. Cumulative proportion of Balb/c female mice surviving after treatment with 12Gy ^{60}Co γ-rays or 6mg cis-platinum/kg immediately before 12Gy.



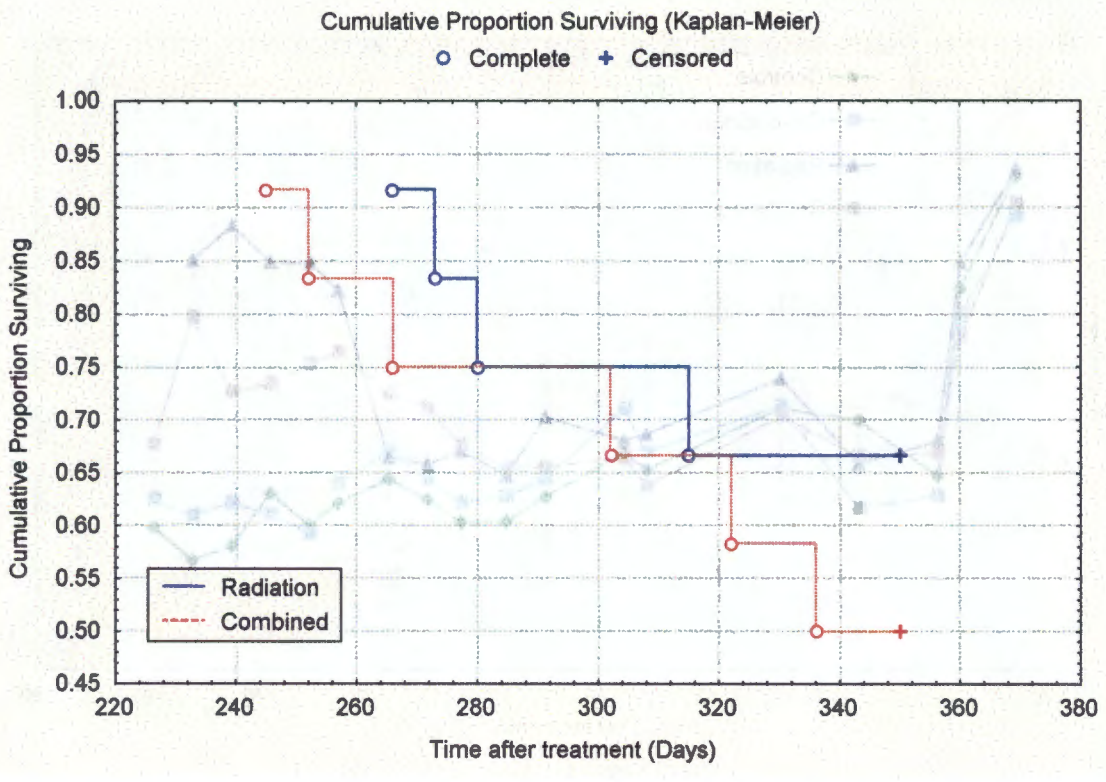
between groups.

No early toxicity was observed. All mice survived more than 10 weeks post treatment with treatment related deaths starting at 110 days post treatment in the Balb/C groups and at 242 days post treatment in the C57/Bl mice. In the C57/Bl cis-platinum only treatment group 4 of 12 mice were dead by 350 days after treatment. No mice died in either the Balb/C or the C57/Bl control group.

Survival time - Balb/C and C57/Bl mice

Figure 19 shows the results of a Kaplan-Meier survival analysis on the Balb/C group. No mice died in the control or cis-platinum only groups. No tests of statistical significance were applied as 12 mice per group was considered to small for this type of analysis. A sample size of 30 or more is considered the minimum required for an accurate estimate of median survival, hazard rate or probability density in the Kaplan-Meier analysis. (Statsoft

Figure 20. Cumulative proportion of C57/Bl female mice surviving after treatment with 12Gy ^{60}Co γ-rays or 6mg cis-platinum /kg immediately before 12Gy.



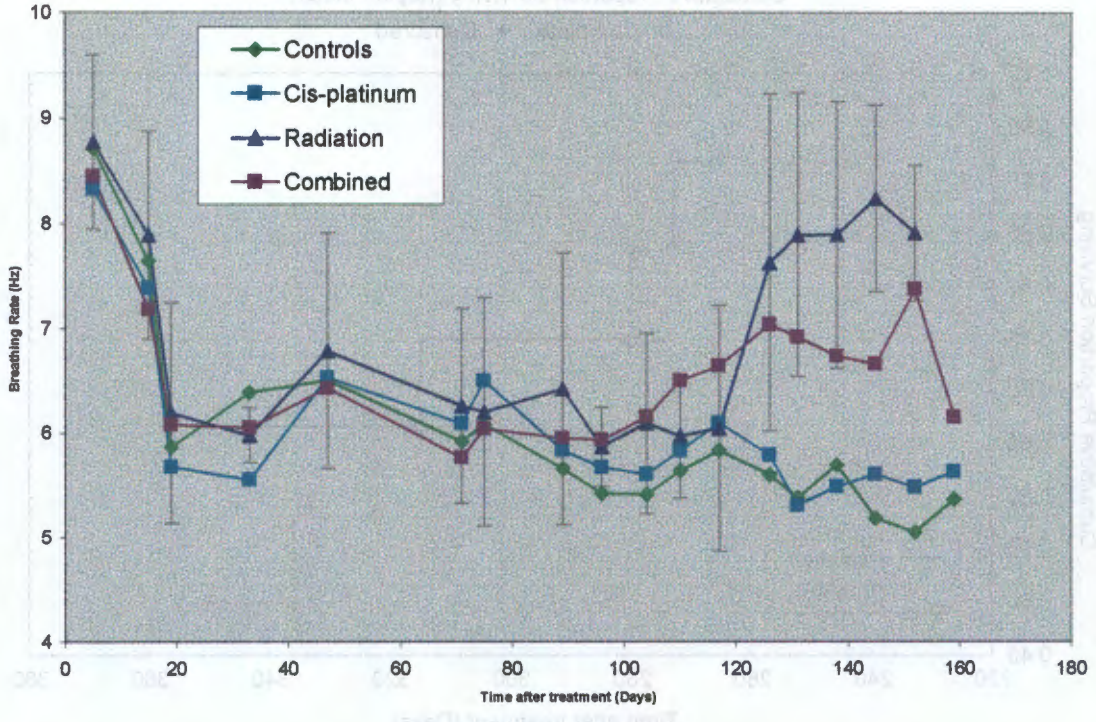
Inc. 1997.) The Cox F-test may be used as a two-sample test for comparing groups with n of less than 50 per group. However no trend to difference could be observed as illustrated in the figure and in view of the lack of reliability in the estimate of median survival at an n of 12 per group a test of significance was not applied.

The same observations may be made with regard to C57/Bl mice although the time of death is markedly different with the first mice dying at 245 days after treatment (Figure 20). As for the Balb/C no difference in survival was seen for the combined treatment group as compared with the radiation only group.

Breathing rates – Balb/C and C57/Bl mice

The breathing rate of Balb/C mice after treatment with 6mg/kg alone, 12Gy ^{60}Co or 6mg cis-platinum/kg immediately before 12Gy ^{60}Co irradiation is shown in Figure 21. All groups showed an initially high breathing rate ascribed to the stress of the first measure-

Figure 21. Changes in breathing rate in Balb/C female mice with time after treatment. Mice were treated with 6mg cis-platinum/kg, a single dose of 12Gy ^{60}Co γ-rays or with 6mg/kg immediately before 12Gy. Error bars show SD and have been omitted from some groups for clarity. Controls received placebo treatment.

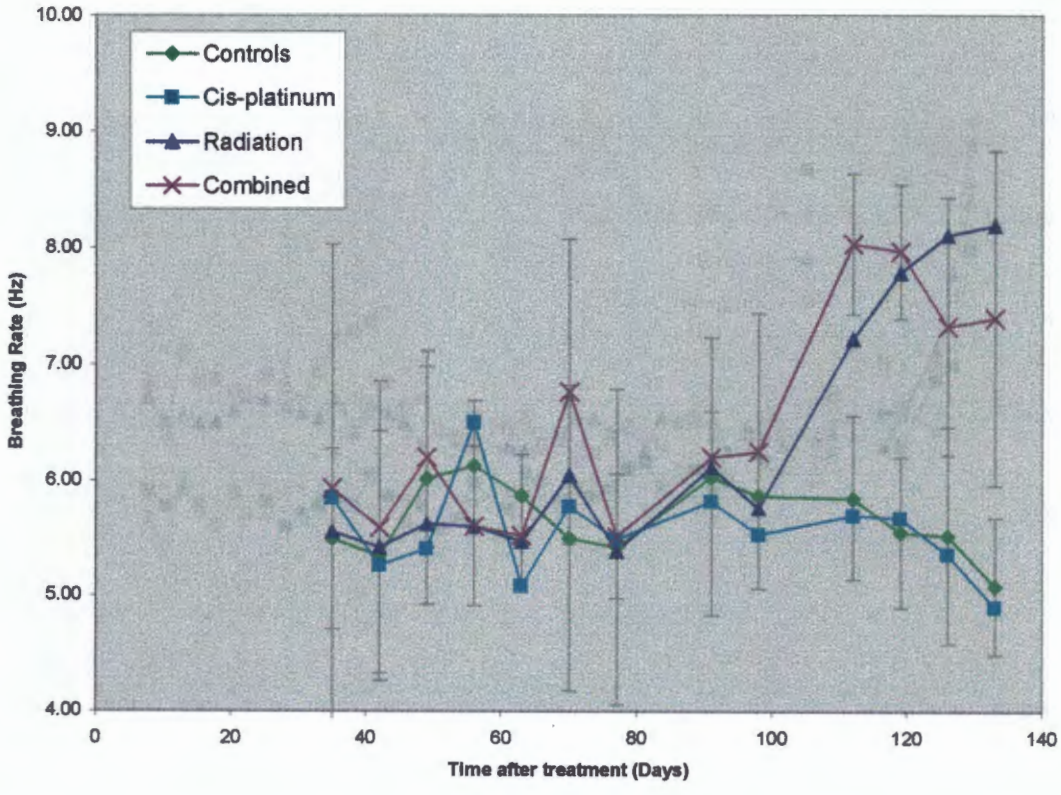


ment. The breathing rate dropped until the third measurement and then "stabilised" for all groups until approximately 100 days after treatment when the radiation treated groups showed an increase in mean breathing rate as compared to both the control and cis-platinum only groups. No significant difference could be discerned in the breathing rates of the cis-platinum treated group when compared to the control group although a trend toward higher breathing rate may be apparent.

Similarly no differences could be detected in the radiation only vs. combined treatment groups. In this experiment the combined treatment group showed a trend to start increasing earlier at about 100 days after treatment but this did not continue and after about 120 days the radiation only group showed a higher mean breathing rate.

The same observations can be made of the data collected during a repeat of the Balb/C experiment as illustrated in Figure 22. Once again groups of six mice were treated as

Figure 22. Changes in breathing rate in Balb/C female mice with time after treatment. Mice were treated with 6mg cis-platinum/kg, a single dose of 12Gy ^{60}Co γ-rays or with 6mg/kg immediately before 12Gy. Error bars show SD and have been omitted from some groups for clarity. Controls received placebo treatments. (Repeat experiment).

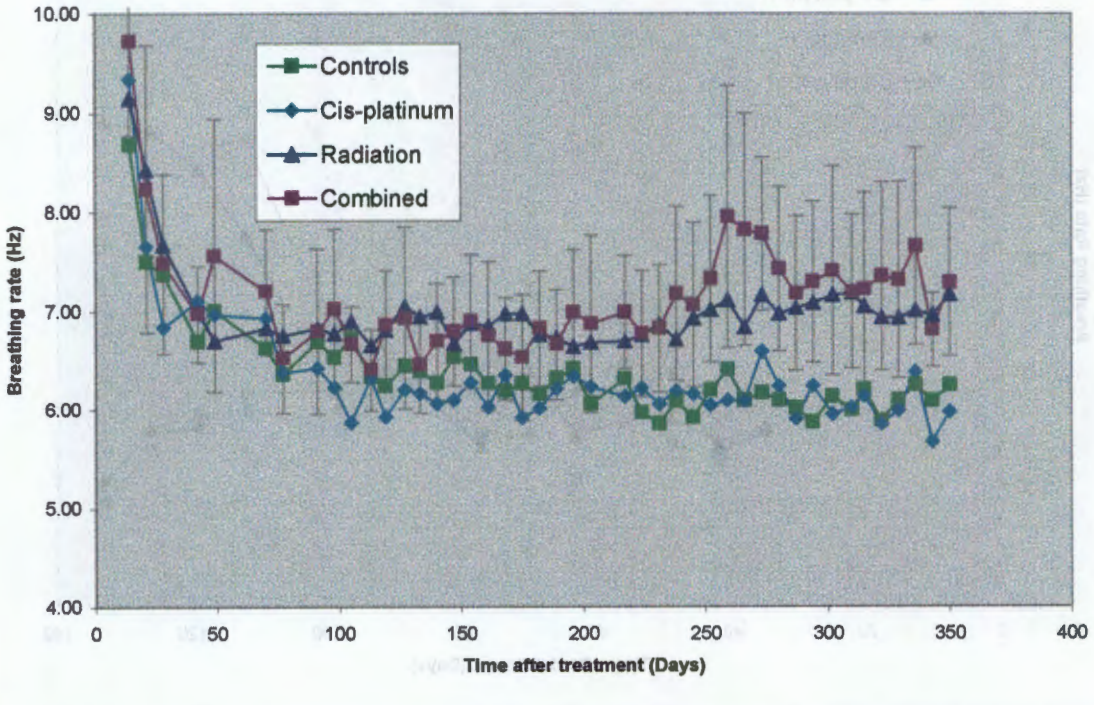


controls, with 6mg cis-platinum/kg alone, 12Gy ^{60}Co γ-rays alone (Theratron 2) and 6mg cis-platinum/kg immediately before ^{60}Co γ-rays. In this case, however, there is a trend for the combined treatment group to exhibit a higher breathing rate than the radiation only group from 100 to 120 days after treatment.

It should be noted that mice in both these groups started dying at about this time and by 140 days at least 5 mice had died in each group that had received radiation as part of the treatment protocol.

The data from treatment of C57/Bl female mice are shown in Figure 23. The C57/Bl mice show a much higher initial breathing rate that is consistent with their more excitable temperament as compared to the Balb/C mice. This increase rapidly declines and by the third measurement appeared to be levelling off. There may be an age-related decrease in breathing rate apparent from about 42 to about 110 days after treatment. After this the

Figure 23. Changes in breathing rate in C57/Bl female mice with time after treatment. Mice were treated with 6mg cis-platinum/kg, a single dose of 12Gy ^{60}Co γ-rays or with 6mg/kg immediately before 12Gy. Error bars show SD and have been omitted from some groups for clarity. Controls received placebo treatments.



breathing rate of the control and cis-platinum only groups levelled off at just above 6Hz.

Groups receiving irradiation showed a gradual increase in breathing rates from approximately 100 days after treatment until the experiment was terminated at 350 days by which time the breathing rate was about 7Hz. The combined treatment group showed a trend to higher breathing rates than the radiation alone group but the difference was never significant. The maximum average breathing rate for Balb/C mice receiving irradiation reached approximately 8.1Hz whereas for the C57/Bl mice it reached 7.25Hz. The maximum breathing rate for Balb/C mice receiving both cis-platinum and irradiation reached about 8Hz. For C57/Bl mice the breathing rate after combined treatment also reached 8Hz but was not significantly different from the radiation only group (approx. 7.11Hz) at that time - 259 days.

The most striking difference between the strains of mice was the time scale of changes. The Balb/C strain showed a marked response to radiation at 100 days after treatment with a steep increase in breathing rates that peaked at about 130 to 140 days by which time significant numbers of mice were dead (about 50% in each group that received 12Gy ^{60}Co irradiation.) At 100 days after treatment the C57/Bl's showed a gradual increase in breathing rate of mice receiving irradiation. While the age effects complicated interpretation of the results it appeared as is if the increase levelled out after about 140 days. However, there was an indication of a second increase occurring in the combined treatment group, starting at about 245 days after treatment.

For C57's 4 out of 12 mice died in the radiation only group between 266 and 315 days after treatment after which no further deaths occurred until the experiment was terminated at 350 days. 6 out of 12 mice died in the combined treatment group between 245 and 322 days after treatment.

Breathing rates after cis-platinum administered at different times relative to radiation

Cis-platinum has been shown to produce a varied change in the radiation response of different systems when administered at different times relative to the time of radiation treatment (Muggia at al. 1979, Douple 1988.). A possible variation in the levels of surfactant present in lavage fluid with the time of cis-platinum treatment relative to radiation was

seen in the present work and is illustrated in Figure 14. The experiment described in this section investigated the effect of administering cis-platinum at different times relative to radiation on breathing rate.

Groups of six mice were treated with 6mg cis-platinum/kg administered either 1 h, 0.5 h, immediately before irradiation with 12Gy ^{60}Co γ-rays from the Eldorado 6 and immediately, 0.5 h and 1 h after irradiation. Treatments were administered as described previously in Chapter 9.

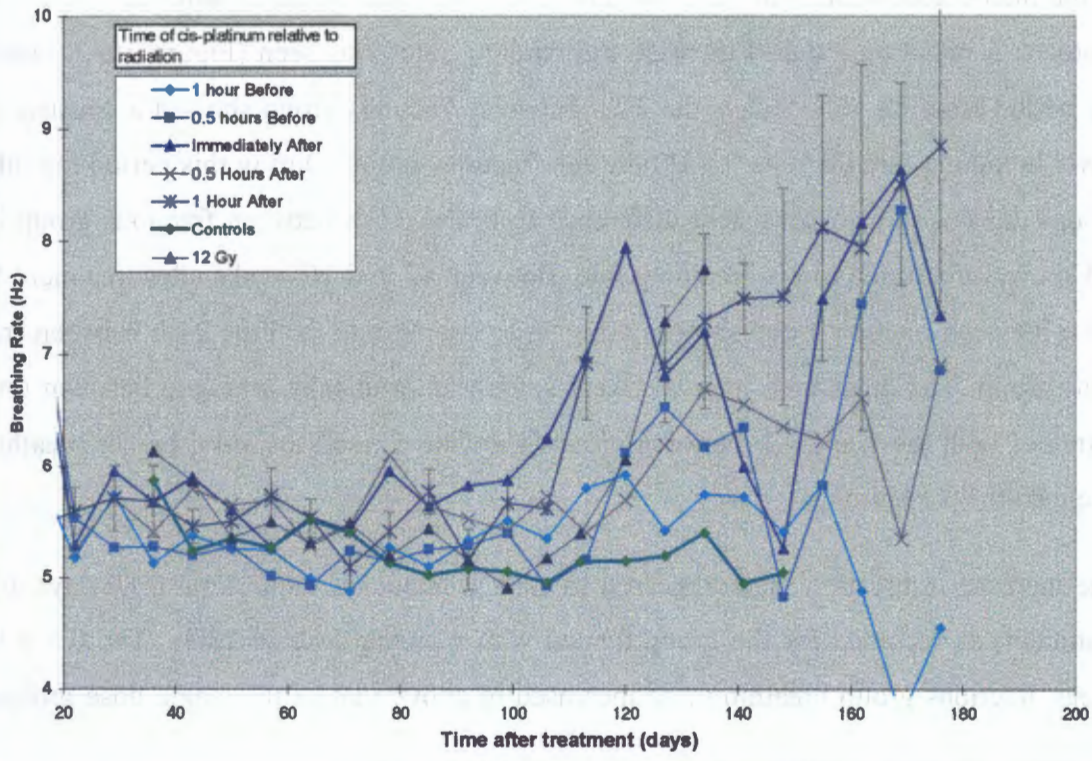
The response is shown in Figure 24. Both the group in which cis-platinum was given immediately after treatment and group in which cis-platinum was given 1 h after treatment showed an earlier increase in breathing rates than the radiation alone group. However, the group that received radiation 0.5 h after cis-platinum did not follow this pattern and instead the breathing rate of mice treated in this group increased at approximately the same time as that of the radiation only group. In each of these groups mice started dying at about 120 days and by 141 days there were only two mice left in the 1 h after and immediately after groups. The 0.5 h after radiation group had 4 mice still alive at 162 days after treatment.

When cis-platinum was given 1 h before radiation the breathing rates were greater than for control mice from about 90 days but the error bars (standard deviations) overlapped. At 169 days after treatment the breathing rate in this group dropped to below mean control breathing rates although it should be noted that there were only two mice alive at this time. Mice receiving cis-platinum 0.5 h before irradiation showed an increase in breathing rates at about the same time as mice treated with radiation alone. There was a trend for this group to show a lower breathing rate than for the group treated with 12Gy radiation only.

A sharp dip was seen in the mean breathing rate at 148 days after treatment of the group given cis-platinum immediately after radiation, the group given cis-platinum 0.5 h before radiation and the radiation only treatment group. In each of these a significant portion of the mice in the group had died by that time. It is not clear whether this dip is a result of a selection of breathing rates that occurs with death or if it is perhaps an experimental artefact occurring in measurement at that time. Of the two groups that did not show this dip the 1 h after group had 2 mice remaining alive and the 0.5 h after had 3 mice remaining alive at that time.

In this series of experiments all 6 mice treated with cis-platinum immediately before radiation died within 7 days of treatment.

Figure 24. Changes in breathing rates of Balb/C mice after treatment with 6mg cis-platinum /kg at different times relative to 12Gy ^{60}Co γ-rays. Error bars show SD and have been ommitted from all but one group for clarity. Control received placebo treatments.



Split radiation doses

8mg cis-platinum/kg and 2 fractions of 6.5Gy ^{60}Co γ-rays

The breathing rate of mice treated with a split dose of radiation, with and without cis-platinum was examined to investigate the effect of cis-platinum on repair of radiation damage. Radiation was given as 2 fractions of 6.5Gy separated by 0.5, 1, 2, 4, 8, or 24 h. The Eldorado 6 ^{60}Co unit was used in this experiment. Cis-platinum (8mg/kg i.p.) was given immediately before the first dose of radiation. Significant toxicity was experienced in the cis-platinum treated mice with only the groups with 0.5, 2, 4 and 8 h between fractions completing treatment with sufficient mice to enable analysis.

Five-month-old mice weighing 20±2g were used to avoid the age related changes seen in earlier single dose experiments. Twelve mice were treated in each group. The radiation alone groups received an i.p. injection of saline of volume equivalent to that required for 8mg/kg cis-platinum. Mice in the combined group were injected i.p. with 8mg/kg cis-platinum.

Radiation only

There did not appear to be any significant difference in breathing rate in mice, irrespective of the inter-fraction intervals, until 84 days after treatment when, as with the single dose protocol, a radiation induced increase in breathing rates was seen (Figure 25A). During the period from 84 to 112 days the 24 h between fractions group showed a consistently lower breathing rate than the 0.5 H between fractions group. During this period the other groups did not show a consistent difference from the 0.5 h between fractions group but did show a trend to a lower breathing rate. Between 12 and 16 weeks after treatment the 0.5 h between fraction group showed a higher breathing rate than the 24 h between fractions group. The other time intervals were somewhat erratically arranged between these extremes with the 1 and 4 h between groups showing a trend toward a higher breathing rate than the 0.5 h group.

The increase in breathing rate appeared to start at about the same time (~80 days after treatment) as recorded for the group treated with a single dose of 13Gy. The 0.5 h between fractions group breathing rate increased to above that of the single dose group at

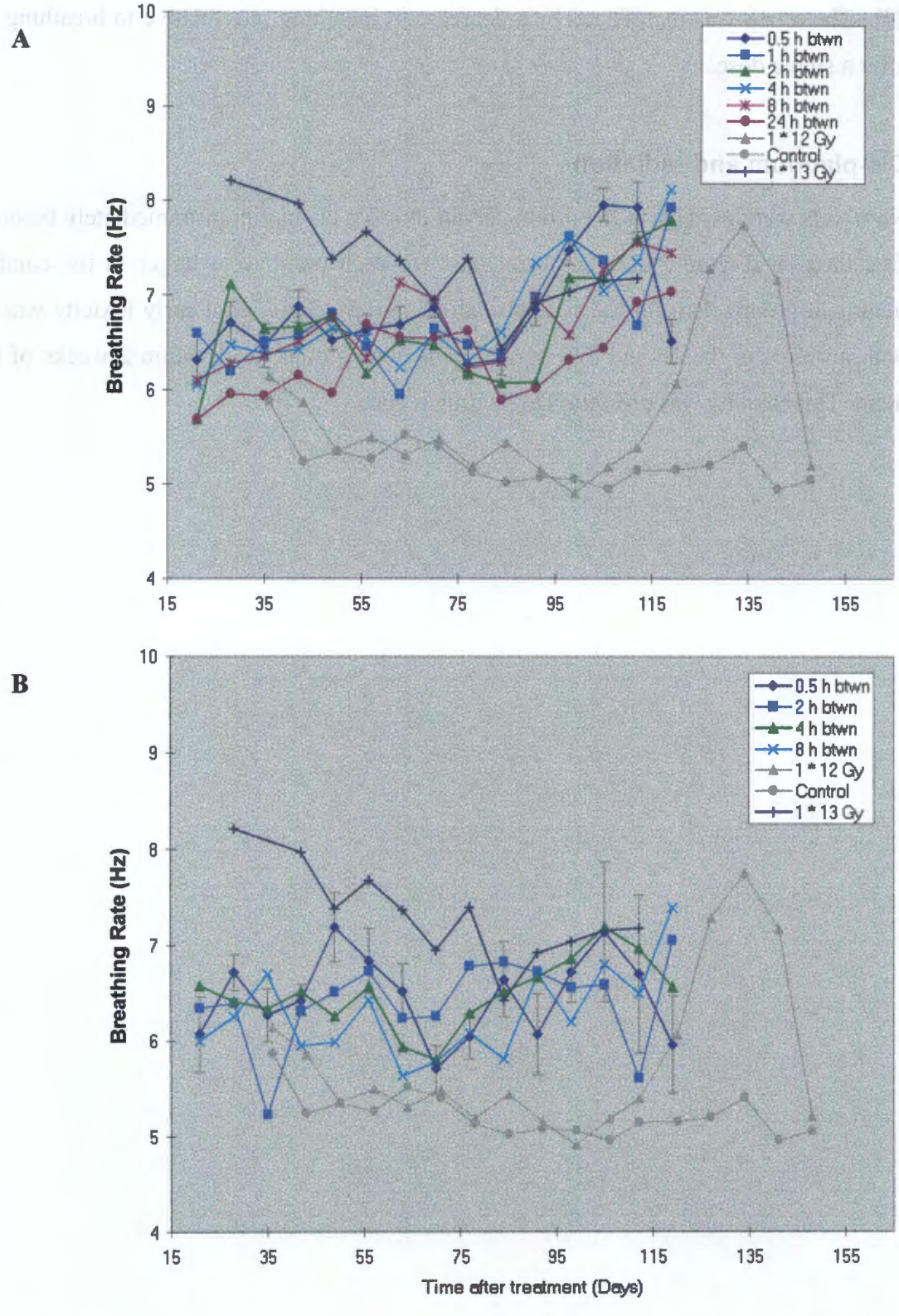
about 100 days after treatment. The longer time intervals between fractions groups showed lower breathing rates than the single dose group for all times after about 80 days.

Although it was not possible to demonstrate clearly for all time intervals between two fractions of radiation, there was an indication of repair when fractions were separated by 24h. The repair was manifested by a decrease in breathing rate relative to breathing rates after a single dose.

Cis-platinum and radiation

No trends were evident in the groups given 8mg/kg cis-platinum immediately before the first fraction (Figure 25B). The error bars for each point were larger in the combined treatment groups than for the radiation alone groups. Significant early toxicity was seen with all mice in the 1h and 24h between fractions groups dead within 2 weeks of treatment. This toxicity was presumed to be drug related.

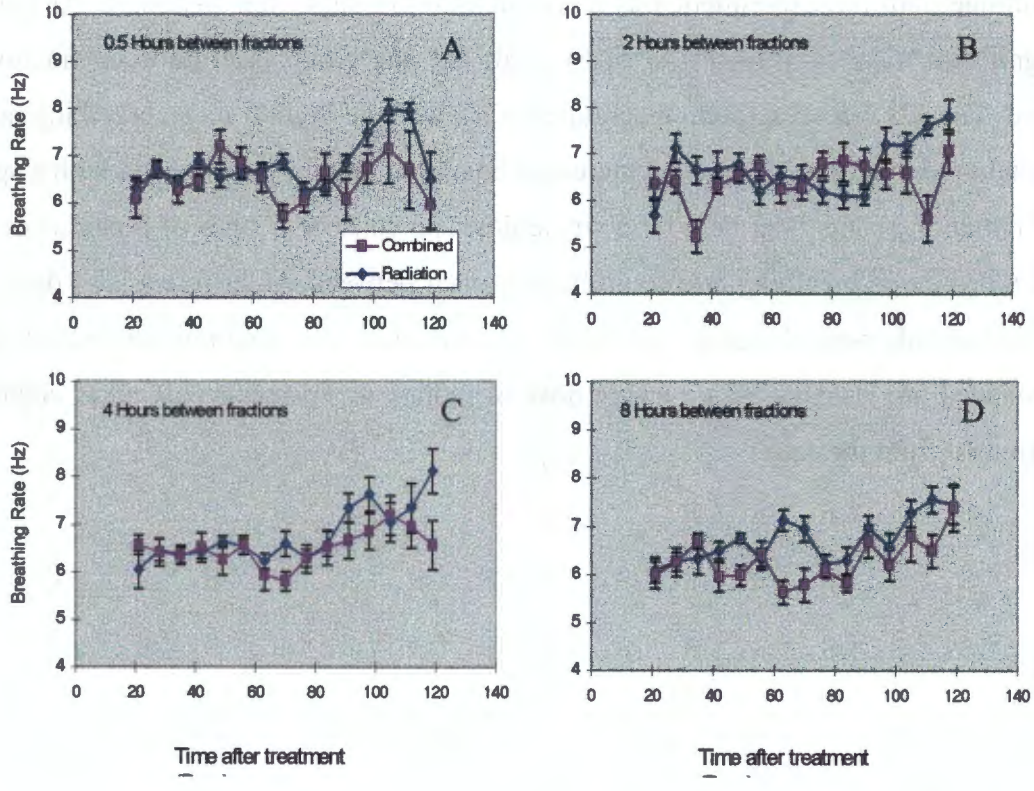
Figure 25. Changes in breathing rates of Balb/C female mice after treatment with 2 fractions of 6.5Gy ^{60}Co γ-rays separated by various times. Chart A shows the breathing rates when radiation only was administered and chart B shows the breathing rate when 8mg cis-platinum/kg was given immediately before the first fraction of radiation. The control and radiation only (12Gy) breathing rates from the subsequent split dose experiment are shown in grey. The legend shows time between fractions. Error bars show SD and have been omitted from some groups for clarity.



Breathing rates in the groups treated with radiation only and with cis-platinum and radiation but where radiation fractions were separated by the same time interval were directly – compared, and the data is shown in Figure 26 (data from Figure 25). There was a trend for the groups treated with cis-platinum immediately before radiation to show a reduced breathing rate over the time period 11 to 15 weeks when compared against the groups treated without cis-platinum at the same inter-fraction interval. The breathing rate also showed a trend to be reduced relative to the single dose group (Figure 25B).

Figure 26A, shows the results for the 0.5 hr inter-fraction interval. For this inter-fraction interval the breathing rates are the same in both the cis-platinum treated and radiation alone groups until 90 days after treatment. After this the cis-platinum treated mice show a breathing rate approximately 10% below that of the radiation treated group. The same trend was seen in 2, 4 and 8 hr inter-fraction intervals but with only isolated points being

Figure 26. Changes in breathing rate with time after 2 fractions of 6.5Gy ^{60}Co γ-rays separated by various times. Each chart compares the breathing rate with time for a group treated with radiation alone to that of a group treated with 8mg cis-platinum/kg immediately before the first fraction of radiation. Error bars show SD.



different (Figure 26B, C and D).

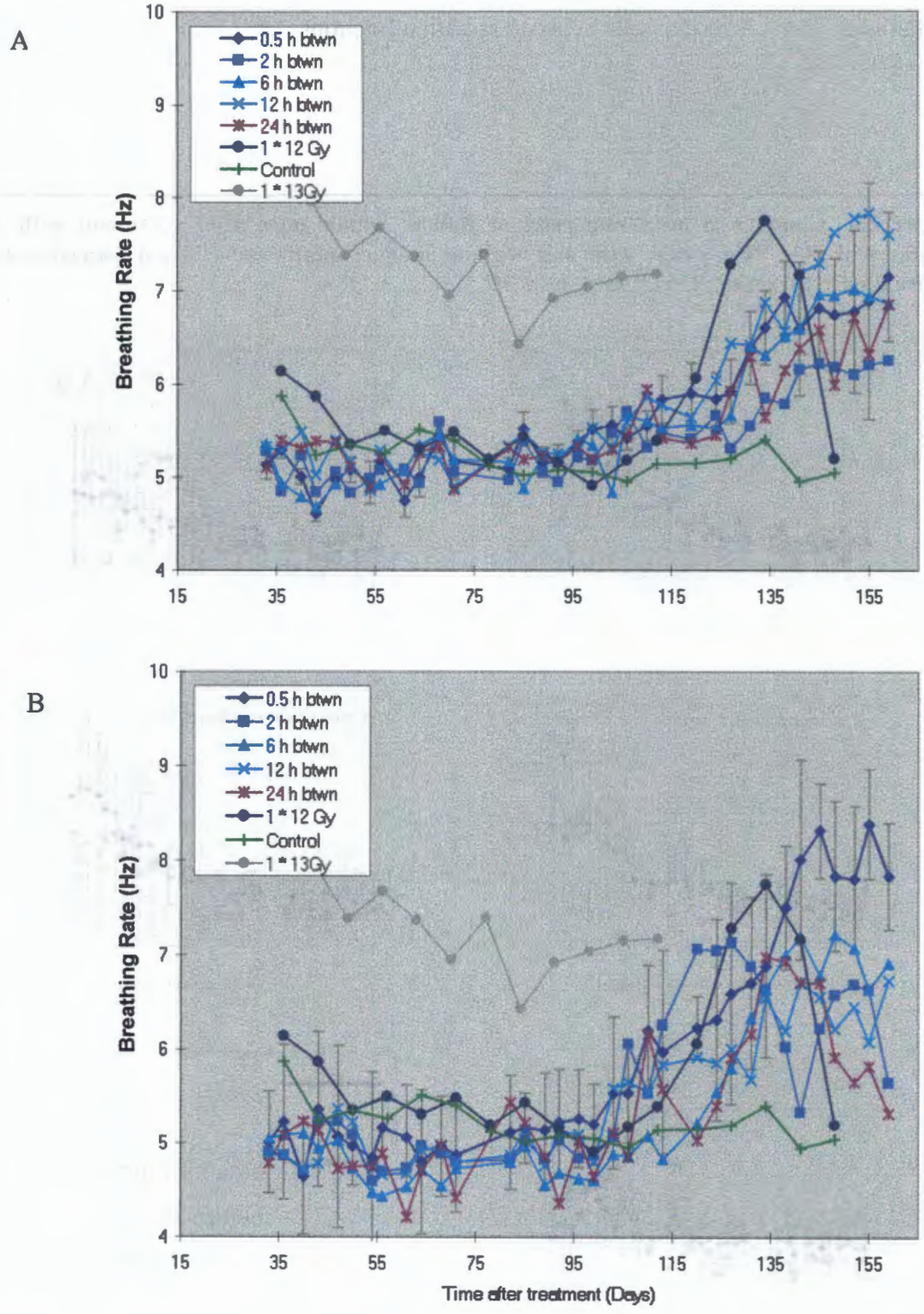
6mg cis-platinum/kg and 2 fractions of 6Gy ^{60}Co γ-rays

The split dose experiment was repeated using lower doses of both cis-platinum and radiation and using the Theratron 2 rather than the Eldorado 6 to irradiate the mice. Six Balb/C female mice, weighing 20±2 g and at least 5 months old were used per group. All groups were treated in the late afternoon to minimise early drug related deaths. In this experiment only 1 mouse of 30 receiving cis-platinum died within the first two weeks. The rest survived until at least 131 days after treatment when the first deaths that may be ascribed to radiation pneumonitis occurred. Mice receiving cis-platinum were treated and placed in the jig at the same time as their radiation only counterparts.

Radiation only

The breathing rates after split doses of radiation are shown in Figure 27A. The mean breathing rate of all groups is not different from control mice until about 124 days when a gradual increase can be seen. The increase shows a trend to occur at a later time after treatment than seen for mice receiving a single dose of 12Gy. The increase appears to continue until the experiment was terminated at 159 days after treatment. At this time a significant number of mice (>3 of 6) in all but one group (12h between fractions) had died. The 12h between fractions group also showed the highest mean breathing rate of all radiation only groups. While the increased breathing rate for mice treated with a split dose of radiation occurs later than for a single dose, no consistent signs of repair as evidenced by a decreased breathing rate could be discerned between the different split dose groups. It is thus only possible to say, on the basis of this data, that splitting the dose of radiation produced less damage than a single dose of radiation. No repair half times could be determined from the data.

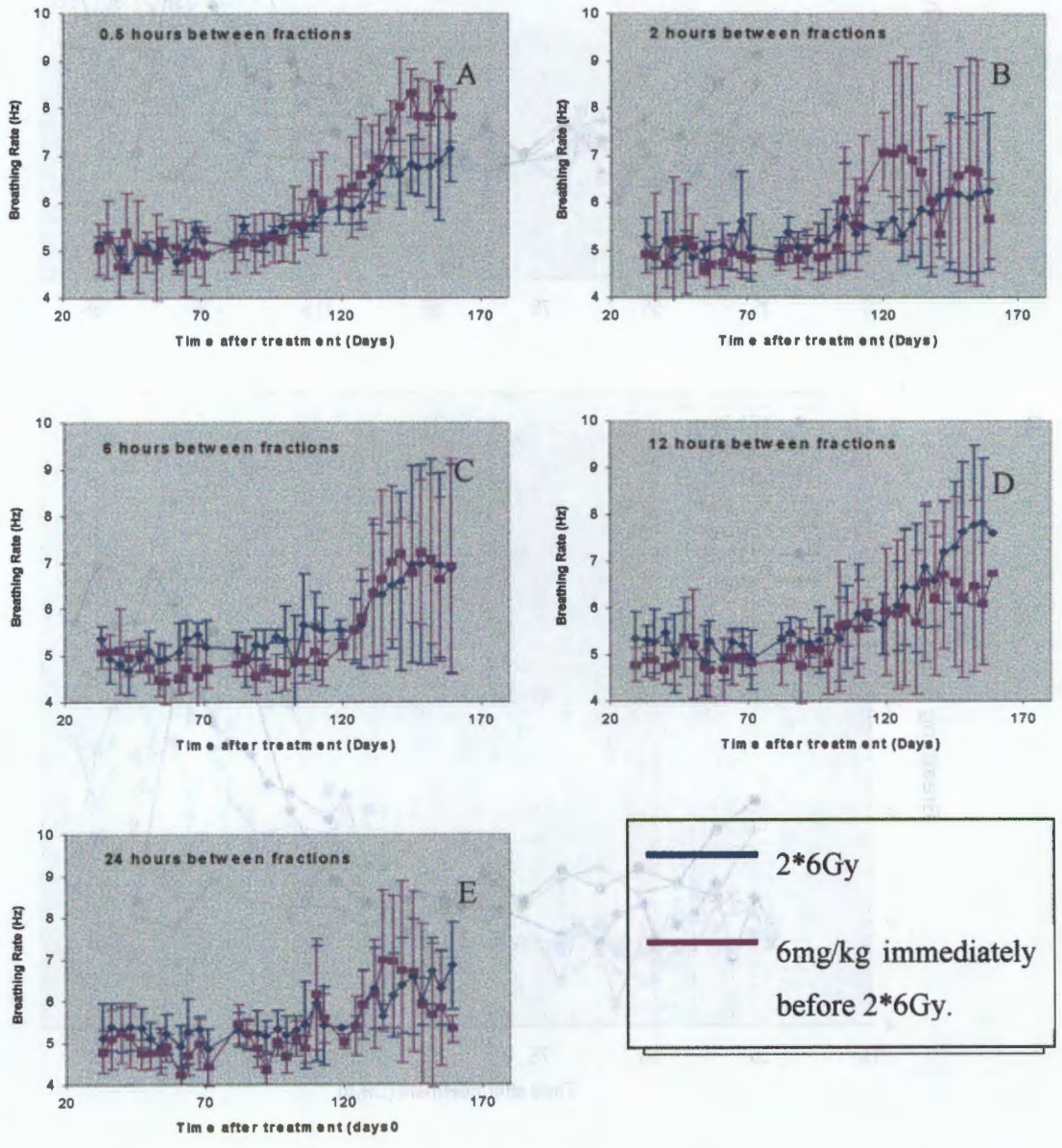
Figure 27. Changes in breathing rates of Balb/C female mice after treatment with 2 fractions of 6Gy ^{60}Co γ-rays separated by various times. Chart B shows the breathing rate when 6mg cis-platinum/kg was given immediately before the first fraction of radiation. The breathing rate of mice treated with a single dose of 13Gy from a previous experiment is shown in grey. The legend shows time between fractions. Error bars show SD and have omitted from some groups for clarity.



Cis-platinum and radiation

Figure 27B shows the results obtained when 6mg/kg of cis-platinum was given before the first fraction of radiation. There was a trend for the increase in breathing rate to occur at the same time as or before (~100 days) the increase in breathing rate of the single dose (12Gy), radiation only group, but there was no consistent difference between the groups with the different time intervals between radiation fractions.

Figure 28. Changes in breathing rates of Balb/C female mice after treatment with 2 fractions of 6Gy ^{60}Co γ-rays with and without 6mg cis-platinum/kg given immediately before the first fraction. Error bars show SD.



A comparison between mice treated with cis-platinum and their corresponding radiation only group showed that for the shorter inter-fraction intervals there was a trend to higher breathing rates in the groups that received cis-platinum. In some isolated points at greater than 120 days this was significant (Figure 28A and B). Groups with longer inter-fraction intervals showed very similar breathing rates for the duration of measurement (Figure 28C, D and E).

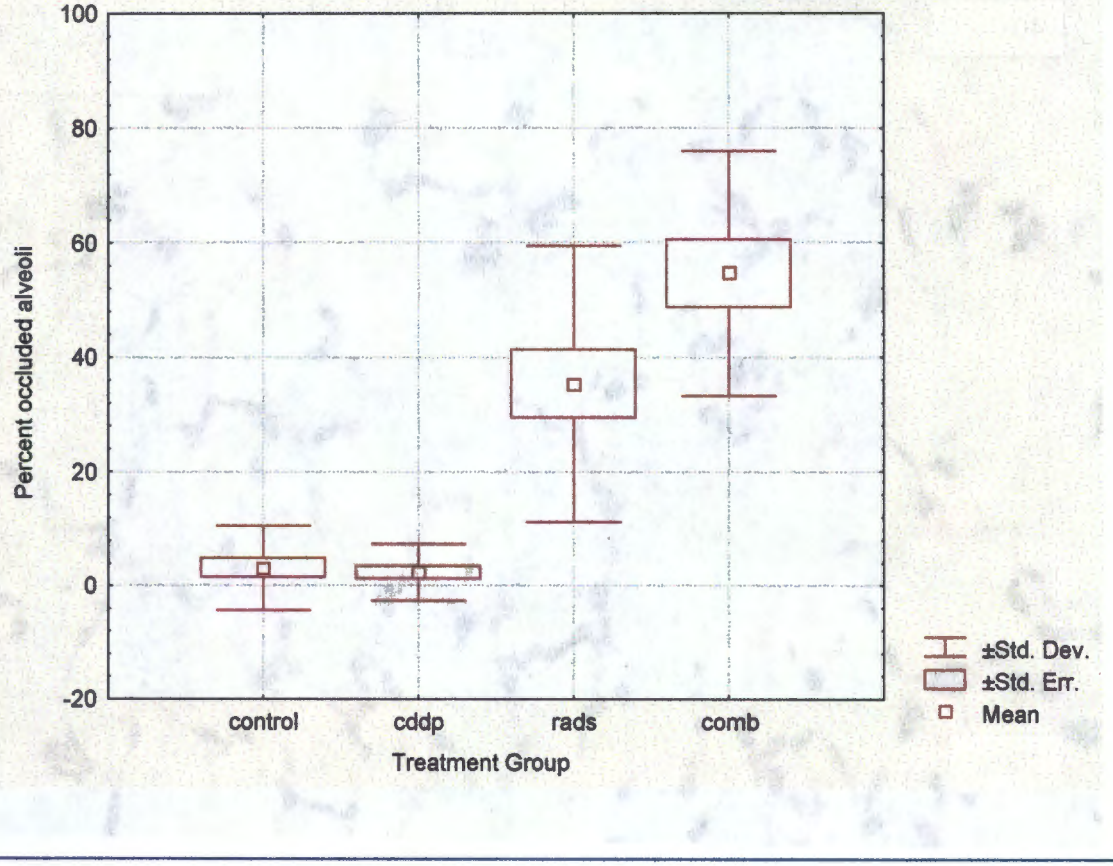
Histology

Lung sections from control mice and mice treated with cis-platinum only, showed very few occluded alveoli. Mean scores of 3% and 2% were seen in the control group and the group treated with cis-platinum alone respectively (Figure 29).

The radiation treated group showed a significantly higher score, with respect to the control group, of 35% ($p<<0.0001$). The score (55%) for the combined treatment group was significantly greater than that of the radiation only group ($p=0.033$)

Sample sections from normal lung, stained with haemotoxylin and eosin are shown in Figure 30.

Figure 29. Box and whisker plot showing mean score, standard error and standard deviation for lung sections from mice killed at 120 days after treatment with 6mg cis-platinum/kg (cddp), 12Gy ^{60}Co γ-rays (rads) or 6mg cis-platinum/kg immediately before 12Gy ^{60}Co γ-rays (comb). Controls received placebo treatments.



The section shows an open latticework of alveoli interspersed with bronchioles and blood vessels. Little or no evidence of alveolar obstruction was seen. The occurrence of alveolar macrophages was extremely rare. In most normal sections none were observed.

Figure 30. Sections from normal lung stained with haemotoxylin and eosin.

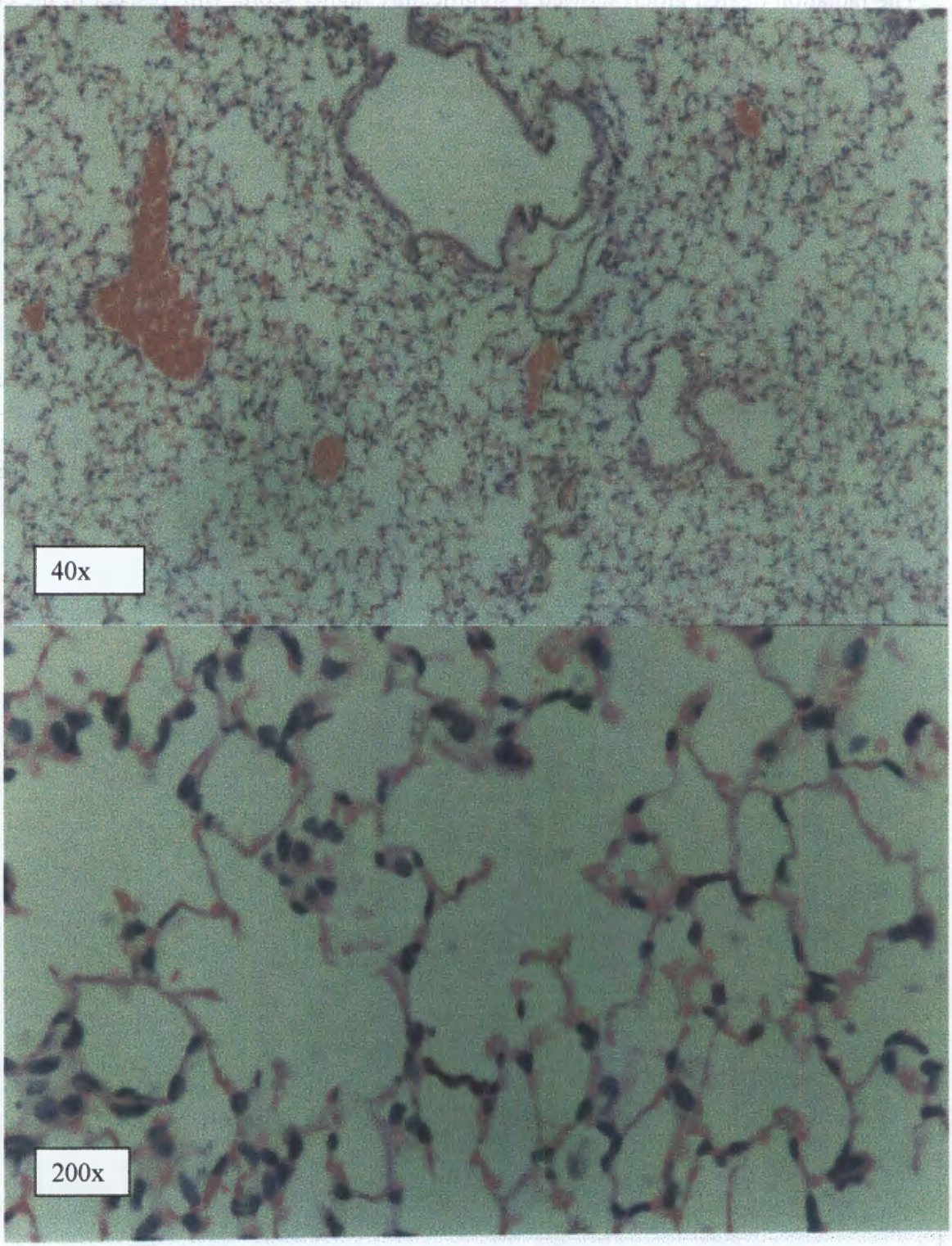
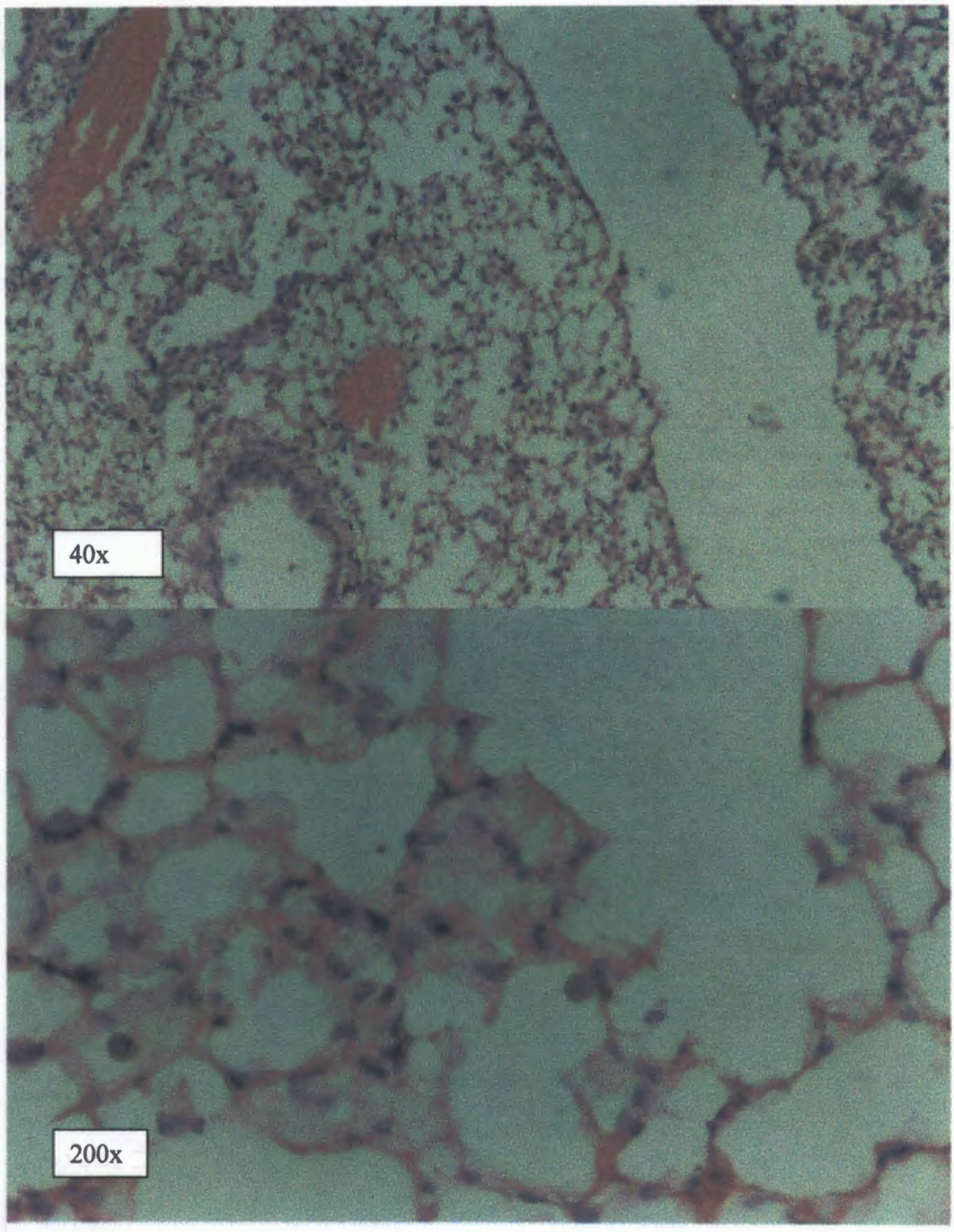


Figure 31 shows a sample section from an irradiated lung, stained with haemotoxylin and eosin. In contrast to normal lung many alveoli were obstructed and a large number of alveolar macrophages were observed.

Figure 31. Sections from irradiated lung stained with haemotoxylin and eosin.

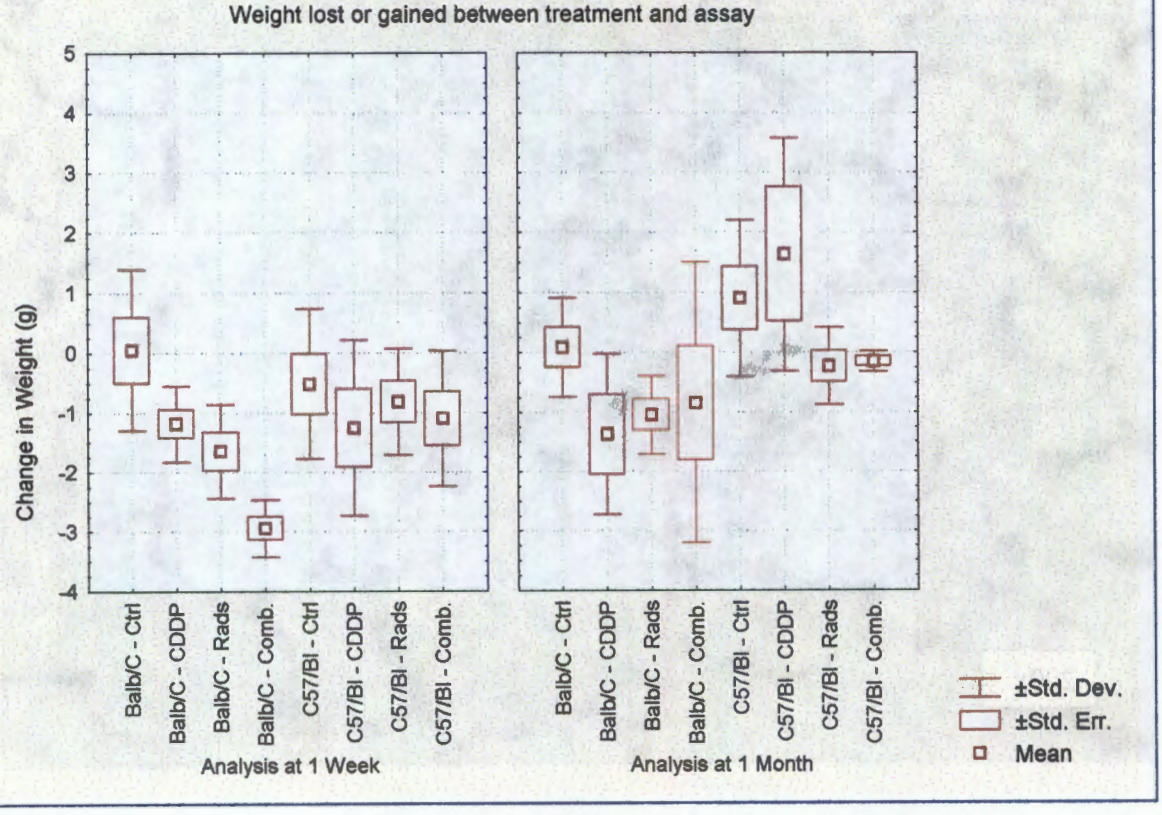


TGF-β analysis

TGF-β has been implicated in the aetiology of radiation fibrosis, and therefore levels were determined with radiation and with cis-platinum and radiation. Two strains of mice – the weakly fibrosing Balb/C's and the fibrosis prone C57/Bl strain – were treated with the standard treatments used thus far.

12 mice per group were treated with 6mg cis-platinum/kg, 12Gy ^{60}Co γ-rays from the Theratron 2 or 6mg cis-platinum/kg immediately before 12Gy ^{60}Co γ-rays as described in Chapter 9. Control groups (12 mice per group for each strain) received placebo treatments. Balb/C female mice and C57/Bl female mice were treated. There were thus 4 groups of each strain treated – in each case a control group and three treatment groups. Care was taken to treat each group at the same time of day, with all irradiation taking

Figure 32. Weight lost or gained at one week and at one month by Balb/C female mice and C57/Bl female mice after treatment with 6mg cis-platinum/kg (CDDP), 12Gy ^{60}Co (Rads) or 6mg cis-platinum/kg immediately before 12Gy (Comb.). Controls received placebo treatment.



place between 17h30 and 18h30.

Half of the mice in each group were killed at 1 week after treatment and the lungs lavaged. Both the lung tissue and lavage were placed in cryotubes immediately after extraction and immersed in liquid nitrogen. The remaining mice were killed at 1 month after treatment and the lungs lavaged as described in the methods section. Again, both lavage and lung tissue were place in cryotubes and immersed in liquid nitrogen immediately after extraction. The weight of each mouse was recorded at treatment and at time of death.

Once all samples had been collected they were prepared for analysis of TGF-β using the sandwich enzyme immuno-assay as described in Chapter 9. The lung homogenate samples were also processed for total protein content as described in Chapter 9.

The amount of TGF-β was calculated per lavage sample, per lung, per g lung wet weight and per mg total protein.

Changes in mouse weight

The weight change induced by the various treatments is shown in Figure 32. Balb/C mice showed a significant weight loss within the first week of treatment. The radiation only group lost approximately 1.65g which was significantly different from the mean weight change of the control group ($p=0.024$). The cis-platinum plus radiation groups weight loss of 3g was significantly greater than that of the radiation only group ($p=0.0062$). The cis-platinum only group showed a mean weight loss of 1.18g that was not significantly different from the control group's weight change.

C57/Bl groups receiving either cis-platinum, radiation or cis-platinum plus radiation showed a slight weight loss at one week after treatment. However, none of the treated group's weight loss was significantly different from each other nor significantly different from the control group's weight change.

Three of six mice in the C57/Bl group assayed at 1 week after treatment died before the week elapsed and were excluded from further analysis.

At one month after treatment the Balb/C mice continued to show an overall weight loss. The combined treatment group showed an increase in weight from that recorded at 1 week that was not significant at the 5% level. Only the radiation only group was significantly

different to controls (p=0.026) at one month after treatment. All mice treated survived till time of assay.

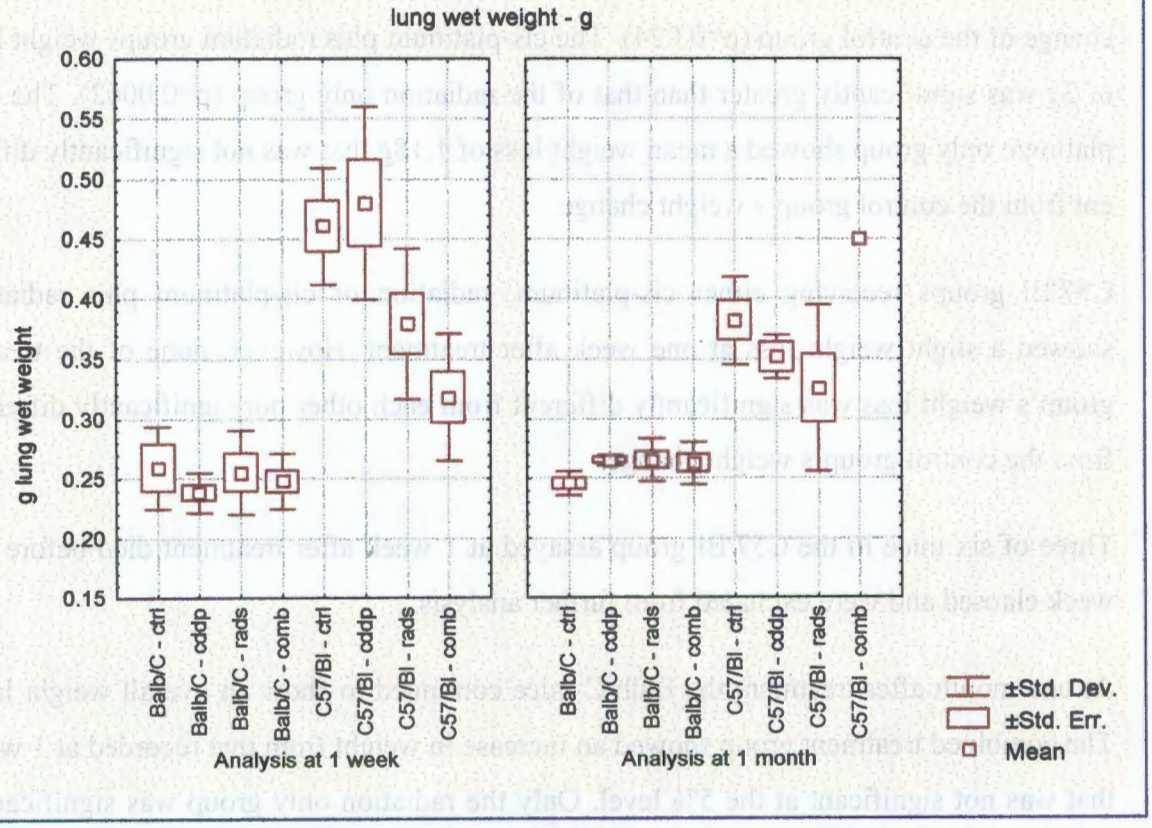
All C57/Bl groups showed an increase in weight at one month after treatment as compared to one week after treatment. While the radiation only group and the cis-platinum and radiation groups showed less weight gain than their control and cis-platinum counterparts this was not significant at the 5% level.

Both Balb/C and C57/Bl mice treated with either radiation or cis-platinum and radiation showed a trend to weight loss at 1 month after treatment, relative to the respective control groups.

Changes in lung wet weight

None of the Balb/C groups at either 1 week or 1 month showed any significant changes in

Figure 33. Changes in lung wet weight (g) at one week and at one month after treatment with 6mg cis-platinum/kg (cddp), 12Gy ^{60}Co γ-rays (rads) or 6mg cis-platinum/kg immediately before 12Gy (comb). Controls received placebo treatment.



lung wet weight.

The C57/Bl radiation only group at 1 week after treatment showed a lung wet weight that was significantly different less than controls ($p=0.042$) as did the group which received 6mg cis-platinum/kg immediately before 12Gy ^{60}Co γ-rays ($p=0.0013$). The lung wet weight in this group was lower than the radiation only group but the difference was not significant.

The C57/Bl control groups lung wet weight was significantly less at one month after treatment than at 1 week after treatment ($p=0.019$). The C57/Bl cis-platinum treated group and the C57/Bl radiation treated group showed a trend to lower lung wet weight than controls at 1 month after treatment that was not significant. The C57/Bl combined treatment group only contained one mouse at one month.

Changes in lung protein

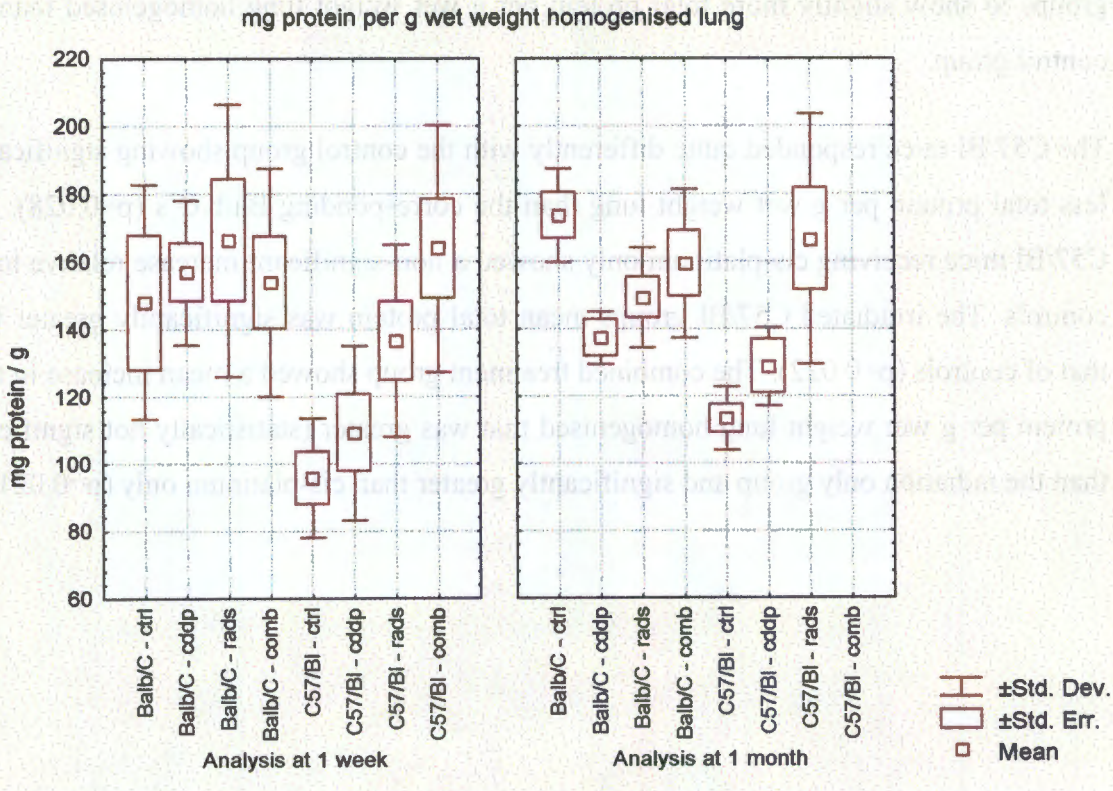
Protein changes after treatment with cis-platinum alone, radiation alone or cis-platinum immediately before radiation are shown in Figure 34. The Balb/C groups did not show a significant increase in total protein at 1 week after treatment with cis-platinum, radiation or cis-platinum immediately before radiation. There was a very slight trend for the treated groups to show slightly more total protein per g wet weight lung homogenised than the control group.

The C57/Bl mice responded quite differently with the control group showing significantly less total protein per g wet weight lung than the corresponding Balb/C's ($p=0.028$). The C57/Bl mice receiving cis-platinum only showed a non-significant increase relative to the controls. The irradiated C57/Bl groups mean total protein was significantly greater than that of controls ($p=0.022$). The combined treatment group showed a mean increase in total protein per g wet weight lung homogenised that was greater (statistically not significant) than the radiation only group and significantly greater than cis-platinum only ($p=0.021$).

At one month after treatment the total protein per gram wet weight lung for Balb/C control mice was increased slightly – a mean at one week of 148mg protein/g tissue compared with a mean of 173mg protein /g tissue – but this was not statistically significant. The cis-platinum treated group and the radiation only treated group showed significantly less protein than the control group with p values of 0.029 and 0.031 respectively. The cis-platinum and radiation treatment group also showed a decrease in total protein per g wet weight homogenised lung that was statistically not significant when compared to the control group. C57/Bl mice showed an increase in total protein for both the cis-platinum treated group and the radiation only treated group. This was significant for the radiation treated group (p=0.013).

The total protein per g wet weight extracted at one month in all C57/Bl groups appeared to be increased relative to that at one week but this increase was not significant at the 5% level.

Figure 34. mg protein per g homogenised lung at one week and at one month in Balb/C female mice and C57/Bl female mice after treatment with 6mg cis-platinum/kg (cddp), 12Gy ^{60}Co (rads) or 6mg cis-platinum/kg immediately before 12Gy (comb). Controls (ctrl) received placebo treatments.

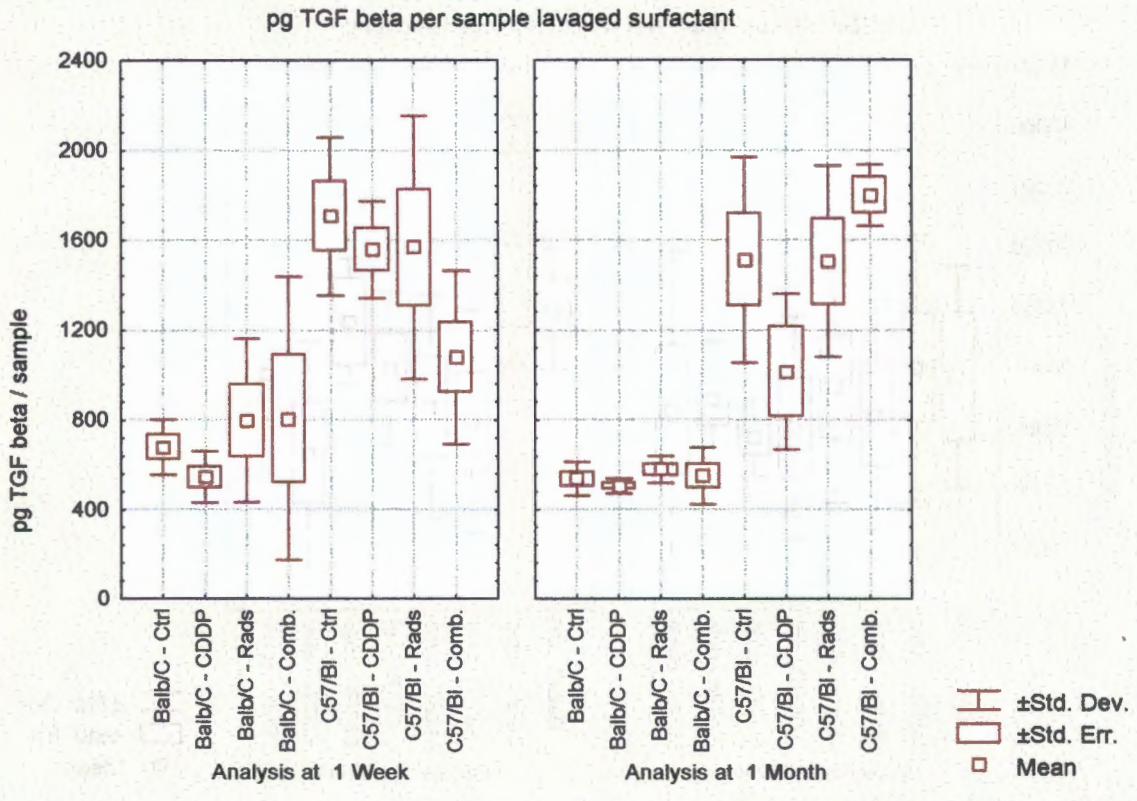


The protein content per g wet weight lung was significantly lower in control (placebo treated) C57/Bl mice both at one week and one month after treatment compared with Balb/C mice ($p=0.027$ and $p=0.00012$ respectively).

TGF-β per sample lavaged surfactant

A pronounced strain dependent difference in TGF-β in pulmonary lavage was seen between the Balb/C groups and the C57/Bl groups (Figure 35). The mean TGF-β value in the control C57/Bl group at one week after treatment was significantly greater than that of the Balb/C control group by a factor of approximately 2.5 ($p=0.00027$). At one month after treatment the C57/Bl control group showed approximately three times more TGF-β than the Balb/C control group. This difference was also highly significantly ($p=0.0016$). Both the Balb/C and the C57/Bl control groups showed a slight decrease (non-significant)

Figure 35. TGF-β per sample lavaged surfactant (pg) at one week and at one month in Balb/C female mice and C57/Bl female mice after treatment with 6mg cis-platinum/kg (CDDP), 12Gy ^{60}Co (Rads) or 6mg cis-platinum/kg immediately before 12Gy (Comb.). Control groups received placebo treatments.

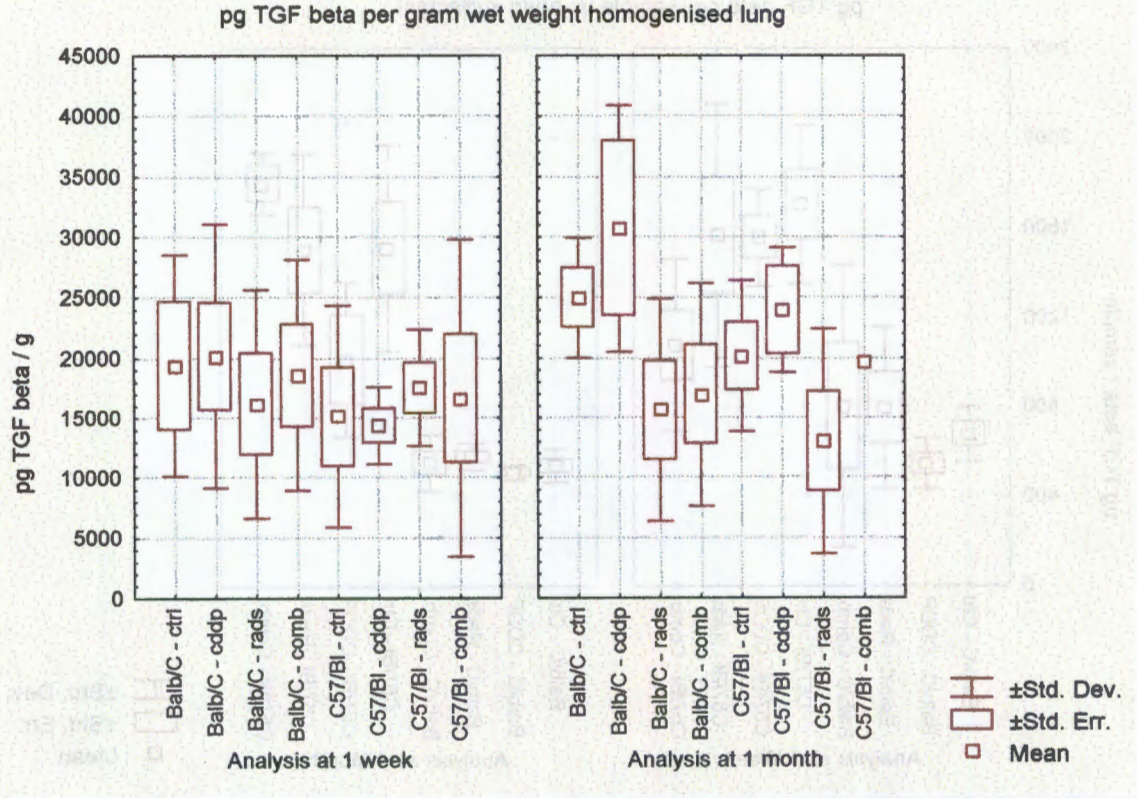


in the mean TGF-β levels at one month compared to the levels at one week.

No significant changes were recorded in TGF-β in lavage fluid at one month after treatment as compared to one week after treatment for any of the Balb/C groups.

Within the C57/Bl strain at one week after treatment a significant decrease (as compared to the control group) in TGF-β was seen in the cis-platinum immediately before radiation group (p=0.021). At one month after treatment the cis-platinum only group showed a non-significant decrease and the combined cis-platinum and radiation group showed a non-significant increase relative to the control group. The TGF-β values for both these groups were significantly different to their 1 week counterparts (cis-platinum only p=0.031, combined p=0.018). The radiation only group did not appear to change.

Figure 36. TGF-β (pg) per g wet weight homogenised lung at one week and at one month in Balb/C female mice and C57/Bl female mice after treatment with 6mg cis-platinum/kg (cddp), 12Gy ^{60}Co (rads) or 6mg cis-platinum/kg immediately before 12Gy (comb). Controls received placebo treatments.

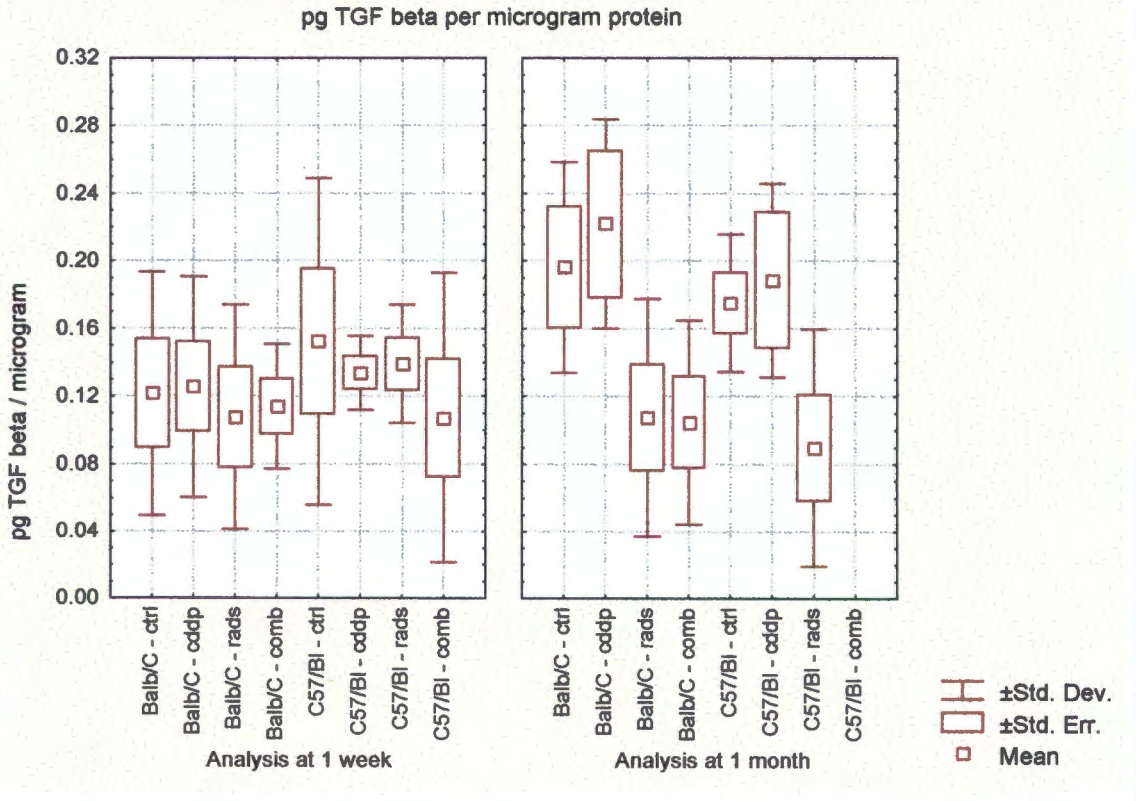


TGF-β in homogenised lung

For Balb/C mice the amount of TGF-β in pg per g wet weight lung tissue did not show any significant difference for any of the treatment groups as compared to controls at one week after treatment (Figure 36). At one month after treatment both the control and the cis-platinum treated groups showed a trend to higher TGF-β levels than at one week but this was not significant. The radiation and combined treatment groups at one month showed TGF-β levels lower than controls but this was not significant.

The amount of TGF-β per g wet weight lung tissue in C57/Bl mice did not appear to change for any of the treatment groups at either 1 week or 1 month after treatment (Figure 36). Although the radiation treated group at one month after treatment showed a lower level of TGF-β than the controls this was not significant. Only one sample could be analysed for pg TGF-β per gram wet weight homogenised lung for cis-platinum plus radia-

Figure 37. Picograms TGF-β per μg protein lung at one week and at one month after treatment in Balb/C female mice and C57/Bl female mice after treatment with 6mg cis-platinum/kg (cddp), 12Gy ^{60}Co (rads) or 6mg cis-platinum/kg immediately before 12Gy (comb).



tion group at one month after treatment (Figure 36). This single value was well within one std. deviation of the mean value for the control group at one month after treatment.

None of, either the Balb/C nor the C57/Bl groups at one week after treatment showed any change in pg TGF-β per μg protein (Figure 37).

At one month after treatment the Balb/C control group and the Balb/C group treated with cis-platinum appeared to be increased relative to the control group at one week after treatment but this was not significant. Similarly the C57/Bl control and cis-platinum group appeared to show elevated levels of TGF-β compared to the C57/Bl control at 1 week but this was not significant. The Balb/C radiation and combined groups at one month after treatment showed TGF-β levels lower than controls but this was not significant (Figure 37).

The level of TGF-β in the C57/Bl radiation treated group was less than that of the control group. This difference was significant ($p=0.046$). No samples could be analysed for pg TGF-β per μg protein per g lung for the cis-platinum plus radiation group (Figure 37).

Chapter 11. Discussion

Introduction

It is vital to consider the response of the lung (or any normal tissue system) as a complex and dynamic interaction of factors that act to produce an endpoint. Changes in any one of these factors may produce a change in outcome, which may be clinically beneficial or could lead to an enhanced normal tissue toxicity. Thus in the lung it would be naive to consider radiation induced pulmonary toxicity merely as the result of radiation mediated cell loss in any particular cellular compartment within the lung.

Toxicity in the lung appears to be related to either the early pneumonitic toxicity characterised by inflammation and oedema or to be due to the later fibrotic reaction. Both of these pathological states may be viewed as evolutionary processes.

In pneumonitis or fibrosis, radiation events would certainly include cell loss in one or more critical populations but may also involve direct effects not related to mitotic death of cells. Thus the almost immediate observation of surfactant release and associated changes in lamellar bodies of the type II cell could be the initial events in a physiological and biomolecular cascade which lead to the toxic effect. Release of biologically active molecules (cytokines, icams (inter cellular adhesion molecules), growth factors or chemotaxins) may recruit other cell populations which in term will play an active role in the final clinical outcome.

The final fibrotic state may be an inappropriately large response to damage caused by fibroblasts responding not only to actual cell loss but also to a selection of the many factors present in the lung during the course of its response to radiation.

It has also been hypothesised that injury to other systems in the thoracic region may contribute to lung damage. Watanabe et al. (1974) suggested that fibrosis may be a consequence of radiation induced blockage in the mediastinal lymphatic channels leading to an accumulation of mast cells within the lung. While this hypothesis has not been proved it is an example of the systemic approach to lung damage under discussion.

Within this framework it is not meaningful to ascribe the response of the lung to a single cell population. There may be times at which a group of cells predominates - for example type II pneumocytes during the pneumonitic phase. It should be noted that the type II pneumocytes might in turn be responding at that time to stimulus from another group of cells.

The usefulness of a model that assumes a single target cell would lie in devising strategies that result in reducing the radiation effect on that cell. This may then allow a therapeutic gain to be achieved.

The usefulness of a systemic (holistic) approach lies in developing strategies that modify one or more pathways within the process thus producing a reduction in the toxic response. A reduction in pulmonary toxicity after radiation may reduce undesired side effects of therapy and may enable higher doses to be administered thus achieving better tumour control. Where radiation sensitising drugs are present in treatment protocols a reduction in the radiosensitivity of the lung may allow higher doses of either drug or radiation to be used again with better tumour control.

Understanding and applying a systemic model of radiation effect on a normal tissue is potentially liberating as, while it may not be possible to protect a specific cell population from radiation induced mitotic death, it may be possible to change the biochemical environment within which the reaction is occurring and hence modify the outcome.

For example corticosteroids which have multiple regulatory effects have been observed to protect against radiation induced pulmonary lethality (Phillips et al. 1975). Gross et al. (1988) noted both stimulation of surfactant release and of type II proliferation after administering corticosteroids. Thus this observation may be used to provide evidence either for a single target cell model or for a systemic model. TGF-β has been implicated as important in causing fibrosis after radiation (Rodeman et al. 1995). If this is so, then control of TGF-β synthesis and release after radiation may facilitate control of radiation induced fibrosis in spite of underlying cell death.

Pulmonary surfactant

Effects of single doses of radiation

Rubin et al. 1980 and 1983 proposed that surfactant release might be a predictor of later occurring radiation damage to the lung after demonstrating the correlation between surfactant released at 7 and at 28 days after radiation with death during radiation pneumonitis. A part of this project was based on the hypothesis that this marker effect could be used to assess pulmonary damage due to combinations of chemotherapy drugs and radiation.

The first experiments presented in this work show that the HPLC assay used was able to duplicate the results obtained by Rubin et al. (1979, 1983) after treatment with radiation only (Figure 12, page 81). The technique used to determine the level of surfactant in lavaged fluid was different to that used by Rubin et al. (1979, 1983) who made use of thin layer chromatography followed by two chemical assays to determine total phosphorus and disaturated phosphatidyl choline. The HPLC technique presented here only examined the relative levels of the phosphatidyl choline peak.

The hypothesis that surfactant release could be used as a marker for radiation damage may be naïve and has come under criticism (Down et al. 1988). For release of surfactant to predict later radiation damage the release would need to be part of the mechanistic pathway leading to the later effect. The specific origin of surfactant release does not appear to have been clarified yet. It may result from increased synthesis and turnover as a result of some signal – perhaps part of the response to DNA damage. It may result from the release of stored surfactant from damaged and / or dying cells. In either case, until the precise mechanism of release is better understood as well as its relationship to later occurring damage, its value as a predictor must be established empirically for every new pulmonary challenge. The wide variety of treatment combinations and possible variations in response make this extremely difficult. As discussed in Chapter 5, Ahier et al. (1985) showed no difference between release of surfactant after neutrons or after x-rays. This did not correlate with the difference seen in later responses (LD_{50}).

It may be that the release of surfactant after treatments including cis-platinum would not correlate with later effects. If surfactant were to be used as a measure of the pulmonary

toxicity of treatments including cis-platinum and radiation its reliability as a predictor of effect under these treatment conditions would need to be established.

The release of surfactant is however of interest in its own right as surfactant levels are an important part of the homeostatic balance in the lung. In addition, surfactant may play a role in minimising the effect of radiation damage. It is thus important to identify agents which on their own or in combination with radiation cause changes in surfactant levels.

Single doses of cis-platinum

While no significant increase (apart from a slight trend) could be seen when surfactant was measured by HPLC after cis-platinum alone, changes were observed using the enzymatic technique. The level of surfactant at 2 days after treatment with cis-platinum only was approximately 1.5 times that observed at 56 days after treatment (Figure 15, page 84) and was significantly different to controls. This increase after cis-platinum would appear to be a novel finding.

It may be that the HPLC technique while measuring changes in one component is missing changes that are detected by the enzymatic analysis, which measures changes in all phospholipids. In addition loss of surfactant can occur in the HPLC analysis during the extraction of the phospholipid component. The enzymatic analysis does not require an extraction procedure.

The release may be a manifestation of the pulmonary response to damage. Cis-platinum as a DNA active agent may induce damage response mechanisms that result in the release of surfactant. It also may be a more direct response with DNA-protein links directly affecting the surfactant release mechanism.

Cis-platinum at different times relative to radiation

There is some conflicting evidence in the literature as to whether cis-platinum causes an enhanced reaction in the lung when administered with radiation. It is possible that this controversy arises as a result of schedule dependent differences in the way that lung tissue responds after treatment with the combined agents. Von der Maase et al. (1986) presented data showing no effect of the drug on radiation whereas Tanabe et al. (1987) showed schedule dependent increases in breathing rates after treatment with both agents. In the

experiments presented here cis-platinum was given either before or after radiation at times chosen to be within or close to the maximum (hypothesised) concentration of cis-platinum in the lung. This was based on measured concentrations in the lung after i.v. injection showed an initial rapid appearance and subsequent clearance with a $t_{½\alpha}$ of 0.61 h and $t_{½\beta}$ of 1.28 h. The peak concentration occurred at 10mins after injection (Siddik et al. 1988). Maximum plasma concentration after an i.p. injection of 6mg/kg was seen at 5 minutes in C3Hf/Sed mice (Fu et al. 1988).

As indicated in Chapter 10 (Figure 14, page 83) the amount of surfactant measured after cis-platinum was administered at different times relative to a single dose (11Gy) of radiation was not statistically different to that after radiation alone. Although the number of mice treated in this experiment was small, surfactant released after cis-platinum at between 1 h before and 1 h after radiation as measured by HPLC does not appear to change the release after radiation alone.

Time course of surfactant release

A statistically significant early release in surfactant after combined treatment with cis-platinum and radiation was shown in the experiment examining the time course of surfactant release after treatment with cis-platinum alone or in combination with radiation (Figure 15, page 84). This result is of particular interest as it may be indicative of a drug – radiation interaction. Drug-radiation interactions in general are complex and subject to debate. It is often difficult to discern when a true synergistic interaction is taking place.

The cumulative effect, with time, on the surfactant system may be represented by calculating the area under the curve of surfactant level against time (Figure 15, page 84) up to a particular time for each treatment minus the corresponding control area. This would hypothetically represent the total amount of surfactant released during that time interval by the treatment. Then, if the sum of the individual areas up to a particular time is less than the area under the combined treatment curve for that time it may be suggested that the two agents are acting synergistically to give a greater than additive effect. Table 6 shows the calculated areas.

Table 6. Area under the curve for surfactant level against time after treatment with cis-platinum, radiation alone or cis-platinum immediately before radiation.

Treatment	Time interval (days)					
	1 to 2	2 to 7	7 to 14	14 to 28	28 to 56	TOTAL
Radiation alone	0.2	1.9	3.8	14.5	25.1	45.5
Cis-platinum alone	0.4	1.7	1.8	3.1	3.0	10.0
Cis-platinum immediately before radiation	0.5	4.6	8.4	17.3	24.4	55.2
Areas added separately	0.7	3.7	5.5	17.6	28.1	55.5
Ratio of areas	0.8	1.2	1.5	1.0	0.9	1.0

The areas for the intervals from 2 to 7 days and 7 to 14 days for the combined treatment appear to be greater than the sum of the individual treatment areas and may thus indicate a greater than additive effect during this period. The ratio of the area under the combined treatment curve to the sum of the individual areas in these cases is greater than 1. However, the ratio of areas for the overall measured period (56 days) is close to 1 indicating that the same amount of surfactant in total over this period was released after combined treatment as after the individual treatments summed.

The early release in surfactant may be a result of release of stored surfactant, of increased synthesis of surfactant or a combination of release and synthesis. The same is true for the release of surfactant after treatment with radiation alone where precursor studies have shown an increase in synthesis and turnover (Coultas et al. 1987). Increased release, without compensatory synthesis, may result in depletion of surfactant at later times after radiation treatment. This may have a deleterious effect on the adaptive response of the lung to damage. Furthermore, re-treatment during any period of depletion may result in additional inability of the lung to maintain function during this time.

An alternate hypothesis is also possible. The late radiation response of the lung may be a result of excessive early release of biologically active substances during the pneumonitic phase. Re-treatment during a time of depletion of these substances may increase lung tolerance to radiation.

HPLC and enzymatic assay for surfactant

The HPLC technique was developed because of the potential that it might offer in analysing any changes that might have been visible in the different components of surfactant. The dominant peak in the chromatogram was presumed to be phosphatidylcholine from

its correspondence with values in the literature for elution time (Jungawala et al. 1975) and because phosphatidylcholine is the predominant phospholipid in surfactant (King et al. 1972). At the time of these experiments a phosphatidylcholine standard was not available.

It was found that the HPLC technique was not well adapted to processing multiple samples as each sample had to be run in series. Periodic column regeneration was needed. The gradient elution technique used added greatly to the time required per sample as the column had to be returned to the starting solvent composition between samples and a stable baseline ensured. At best speed, a single sample required nearly an hour to process. Columns and HPLC grade solvents used added to the expense of the technique. Its use is thus more appropriate if specific use were made of the technique's ability to separate a sample into constituent components.

In contrast to the HPLC technique the enzymatic assay was well suited to multiple small samples once the kit had been adapted as described in the methods section (Chapter 9, page 67). Samples could be prepared in parallel and readings taken rapidly and easily using a spectrophotometer. The modifications to the kit make it highly cost effective assay. The enzymatic assay measures changes in total phospholipid thus giving an overall picture of total surfactant release following treatment.

Because of the lack of clarity on surfactant as a marker in combined treatment protocols and the lack of any mechanistic hypothesis for its role in radiation damage, this line of investigation was not persued

Comments on broncho-alveolar lavage

The lavage procedure in these experiments, in which surfactant is extracted from the lung, is an extremely delicate procedure. Between 8 and 10% of all extraction attempts failed for reasons such as the trachea slipping off the catheter during extraction and spilling lavage fluid. When this happened the sample was not included in the experiment. Variability in lavage efficiency may have a pronounced effect on the level of surfactant recorded.

Breathing rates

Single dose studies

The following single dose breathing rate experiments were performed:

- Breathing rates after 8mg/kg and 13Gy
- Breathing rates after 7mg and 12Gy
- Breathing Rates after 6mg and 12Gy

In comparison with placebo treated controls, in each case no change was seen after cis-platinum alone, an increase in breathing rate was seen at some time after radiation and a similar increase was seen after combined cis-platinum and radiation. No consistent trend or obvious differences could be seen between the radiation only groups and the cis-platinum plus radiation groups. The experiments all exhibited a high degree of variability.

A mouse's breathing rate will change depending on numerous factors. These include normal physiological factors such as response to stress, temperature or degree of activity. The magnitude of these responses may be at least as great or greater than changes induced by radiation. Changes of up to 40% were observed within the first two weeks after treatment in control mice as the mice adapted to the unfamiliar and possibly stressful environment of the plethysmograph. This took the form of a rapid decrease in the measured breathing rate. After this, a week to week variation of 5 to 15% for the control groups was observed. Age related changes were evident in control groups. Generally a small decrease was noted but for the first breathing rate experiment this was as large as 52% (Figure 16, page 86).

Variability in breathing rate measurements

Every effort was made to minimise variability in the measuring technique. Steps taken included: measurements taken at the same time each day in the same environment; measurements made across groups rather than completing groups in series, waiting for mice to settle, discarding results which did not show a sharp Fast Fourier transform and using mice matched for age and weight. In spite of this it may be that the endpoint is not sufficiently discerning to allow small changes to be detected.

Travis et al. (1979) showed that for an increase in dose from 13 to 16Gy CBA male mice showed an increase in breathing rate of approximately 10% at 16 weeks after treatment. For an increase in radiation dose from 8 to 13Gy they showed an increase of approximately 5%. No increase in breathing rate was seen when the dose was increased from 0 to 8Gy. The data presented here show an increase at 16 weeks of between 5 and 8% from 0 to 12Gy and of 17% between 0 and 13Gy. The mean breathing rate of untreated mice in Travis et al's work was reported as approximately 340 breaths per minute or 5.6Hz with a standard error of the mean of approximately 5 to 15 b.p.m. or 0.08 to 0.25Hz. In comparison the mean breathing rate of control mice in the data presented here ranged from approximately 4.5 to 5.5Hz with standard deviations of ±0.25 to ±1.0Hz with occasional points having a standard deviation as high as 2.5Hz. The errors presented by Travis et al. appear smaller but it should be emphasised that these authors used standard errors of the mean with only 10 mice in each group. In this work, the errors are expressed as standard deviations, as this is more appropriate for the small groups (n= 6 to 12).

Down and Steel (1983) reported on breathing rates of C57Bl and CBA male mice which showed a similar variability and error as described above for both control mice and mice treated with 13Gy. Likewise, results from Travis et al. (1980) showed a similar variability and error for controls, mice treated with 12Gy and mice treated with 13Gy (male CBA/Ht mice). Higher doses (15 and 20Gy) did not appear to show as great a variability, instead showing steep dose response curves at earlier times. Mice in these groups were all dead within 25 weeks.

The work presented here would thus appear to be qualitatively similar to results reported in the literature and the measuring technique neither substantially better nor worse.

The data from the first experiment in the breathing rate series (Figure 16, page 86) was complicated by the age dependent decrease in the breathing rate of the controls. Older mice were used in later experiments and this decrease was no longer evident.

Control mice in different experiments showed substantial variation. While a possible explanation exists for the higher breathing rate of all the mice in the first breathing rate experiment – they were treated at a younger age – it is less clear what may have caused the variation in later experiments. Possible explanations could include variations in measuring conditions For example ambient temperature differences between summer and winter.

Different times of day for measurement of one group as compared to the next may also contribute. An additional factor may be small age differences between groups at the time of treatment.

Cis-platinum toxicity

Despite the hydration protocol used when administering cis-platinum considerable and often unpredictable early toxicity was seen in groups of mice receiving cis-platinum. Initial attempts to reduce the toxicity included reducing the cis-platinum dose from 8mg/kg to 6mg/kg and reducing the radiation dose from 13Gy to 12Gy.

The initial group of mice treated with 8mg/kg cis-platinum (page 86) did not show early drug-related toxicity. Subsequent attempts to repeat experiments at this dose of cis-platinum were abandoned because of high levels of toxicity and the dose of cis-platinum was reduced to 7mg/kg and then to 6mg/kg. The reason for the lack of toxicity in the initial group is not clear.

The variation in cis-platinum toxicity between experiments may be due to circadian variability in the tolerance of mice to cis-platinum as recorded by Shakil et al. 1993. (see Chapter 7, page 50). In these experiments time of injection for any particular group of mice was determined by logistical factors such as beam availability and number of groups treated per day. In retrospect, time at which cis-platinum was administered should have been a controlled aspect of the experimental design and indeed was factored into the later breathing rate experiments (See Figure 21, Figure 22, Figure 23, page 92).

Effect of death on mean breathing rate

Death of mice in the breathing rate analysis may cause significant changes in the mean breathing rate for a particular group as each death will remove a value from the group. Such data censoring may lead to anomalies in interpreting the results that could be significant with small numbers of mice in each group – or with small numbers remaining at the end of a measurement period. The longer a treatment is followed the 'better' the breathing rate data might appear as only the stronger mice remain in the experiment. If a mouse with a high breathing rate dies then the mean breathing rate of the remaining mice will be reduced. Conversely if a mouse with a low breathing rate dies then the mean will

shift to a higher value. A possible means of accounting for this is discussed below in the split dose experiments by using a 'Kaplan-Meier" survival type analysis.

Comparison between Balb/C mice and C57/Bl mice

It has previously been shown in the literature that C57/Bl mice do not show pneumonitis for doses of radiation causing late radiation fibrosis (Down and Steel 1983). C57/Bl mice have also been observed to show signs of fibrosis during the acute phase of the radiation response. This progresses to extensive contracted fibrosis during the late phase. The Balb/C strain shows small foci of fibrosis during the acute phase of the radiation response that progresses to foci of contracted fibrosis during the late phase (Sharplin and Franko, 1989a and 1989b).

The steep increase to approximately 7Hz (420 BPM) in breathing rates seen (page 86, Figure 16) in the Balb/C strain from 90 to 115 days (13 to 16 weeks) after irradiation with 13Gy are consistent with pulmonary insufficiency caused by radiation pneumonitis. A slightly lower dose of 12Gy (Figure 17, page 87 and Figure 18, page 89) caused a similar increase but occurring slightly later at about 125 to 140 days (17 to 20 weeks) after treatment.

Similar results were recorded for CBA by Down and Steel (1983) who observed a peak of approximately 420 BPM at 14 weeks after treatment with 13Gy. Travis et al. (1980) did not see the same peak at 14 weeks after treatment of CBA mice with 13Gy. Instead the breathing rate was observed to gradually increase to and then fluctuate between 360 and 380 BPM from 16 to 52 weeks after treatment. They did however see a steep increase with a maximum breathing rate of 450 occurring at about 22 weeks after treatment with 14Gy.

The less severe and more gradual increase in breathing rate seen in the C57/Bl strain (page 94, Figure 23) may be consistent with the onset of radiation fibrosis without a significant pneumonitic reaction. Down and Steel (1983) observed a similar gradual increase in the breathing rate of C57Bl mice after treatment with 13Gy that reached a maximum of 450 BPM at 36 weeks after treatment. They were however not able to correlate this with histological observations of fibrosis and instead noted the presence of pleural effusions.

While cis-platinum did not have a measurable effect on the breathing rate of either strain in these experiments there was a trend for the combined treatment groups to exhibit a higher breathing rate. This may be due to the rise in breathing rate starting slightly earlier after treatment with both cis-platinum and radiation than after treatment with radiation only. The trend was most persistently observed in the C57/Bl group that showed a higher breathing rate for most times between 180 and 350 days after treatment (Figure 23, page 94).

If the breathing rate in fact increased earlier after treatment with both cis-platinum and radiation then this would correlate with the early release of surfactant seen after combined treatment (Figure 15, page 84).

Breathing rates after cis-platinum administered at different times relative to radiation

The variability seen towards the end of the experiment, with few mice remaining in the groups prevents conclusion being made about dose-time effects of cis-platinum on radiation. Once again, a trend toward an early increase in breathing rate is visible with the groups given cis-platinum one hour and half an hour after radiation showing increased breathing rates occurring before those treated with radiation only. This trend however is not evident in the groups where cis-platinum is given before radiation.

While all mice in the group in which cis-platinum was given immediately before radiation died at one week after treatment in this experiment, other experiments where cis-platinum was given immediately before radiation showed a trend toward an early increase (Figure 22, page 93 and Figure 21, page 92). It is thus unlikely that the trend for breathing rates to occur earlier after combined treatment is a schedule dependent effect.

The 1h before treatment group did not show any substantial increases in breathing rates typifying the breathing rate of other groups that received radiation. The 0.5h before group showed a trend to a lesser increase in breathing rates than that observed in other experiments where cis-platinum was given immediately before radiation. The increase in breathing rate was also less where cis-platinum was given after radiation in this same experiment. This lack of increase in breathing rate may be an effect of combined treatment on the lung but may also be due to the effect of these combined treatment schedules on

other tissues within the field. It should be noted that in the 1h before group 3 of 6 mice had died by 134 days and in the 0.5h before group 3 of 6 mice had died by 148 days.

Split doses of radiation

Two split dose experiments – the first with 8mg/kg cis-platinum and 2 fractions of 6.5Gy and the second with 6mg cis-platinum/kg and two fractions of 6Gy – were performed. The second set with lower doses was performed to try and reduce the toxicity seen after higher doses and thus to produce more consistent results. (This was also done with the single dose studies in which the dose of cis-platinum was reduced from 8mg/kg to 6/mg/kg and the dose of radiation from 13Gy to 12Gy).

Substantial change in control breathing rates was noted between the two sets of experiments. In the first set the breathing rate of the control group settled at about 6Hz (360 BPM) after age related changes had stabilised. In the second set of experiments performed 24 months later and starting with slightly older mice, the breathing rate of control mice was about 5Hz (300 BPM). It is not clear what the cause of this difference was.

There is some contrast between the two experiments. The first set, where groups were treated with cis-platinum immediately before 2 fractions of 6.5Gy, a trend for the breathing rate to be lower than the radiation only groups was seen. The second set, where groups were treated with cis-platinum immediately before 2 fractions of 6Gy, a trend for the breathing rate to be higher at shorter inter-fraction intervals than the corresponding radiation only groups was seen. The second set was performed with the revised treatment set up on the Theratron 2. Treatment also occurred at a consistent time for each group (approximately 6pm) resulting in no early toxicity.

While the control groups in the two sets of experiments showed differing breathing rates the higher radiation doses (13Gy or 2 fractions of 6.5Gy) appeared to cause an earlier increase in breathing rate for both single and split doses than the lower radiation doses (12gy and 2 fractions of 6Gy).

Kaplan-Meier type analysis of breathing rates

An alternative analysis for breathing rate data makes use of Kaplan-Meier type survival analysis that accounts for censoring. In this analysis 'survival' is replaced by a defined 're-

sponse' breathing rate. Mice which exceed the response breathing rate are scored as having responded while those that do not achieve the response breathing rate within the period of the experiment or were removed from the experiment before completion are scored as censored. This type of analysis has not been reported in the literature. A similar analysis that allows ordinal data to be transformed into a quantal response and analysed using probit or logit techniques has been reported (Travis et al. 1986, Van Rongen et al. 1995).

A difficulty in designing this kind of experiment lies in choosing the 'response' breathing rate. The response breathing rate should be low enough to be sensitive and yet must be high enough to measure a real response i.e. it must be high enough such that the control group does not show a response. The level chosen should not be so high such that all points are censored. It should also be consistent between experiments.

Von Rongen et al. (1995) chose levels of 20, 25 and 30% above that of controls to investigate repair half-times in lung after clinically relevant doses per fraction. They did not justify this choice but found that values for repair half-time (0.2 to 0.6 h) were not inconsistent with those obtained using mortality (~0.3 h).

There are at least three potential difficulties in designing an analysis using the Kaplan-Meier technique:

1. Variability in control breathing rate may mean that at some time after treatment individual control mice will exceed the response.

2. The choice of breathing rate is not related to an underlying mechanism i.e. arbitrarily sliding the response level up and down could vary the result significantly.

3. Number of mice required is high. Kaplan-Meier type survival analyses require at least 30 subjects in each treatment group for reliable estimates of median 'survival'. (StatSoft, Inc. 1997.)

A suitable study in which groups of at least 30 mice were treated with increasing doses of radiation and their breathing rates recorded may allow a suitable response breathing rate to be chosen such that the median response rate showed a dose response comparable to that obtained using other techniques. Although none of the groups in this project con-

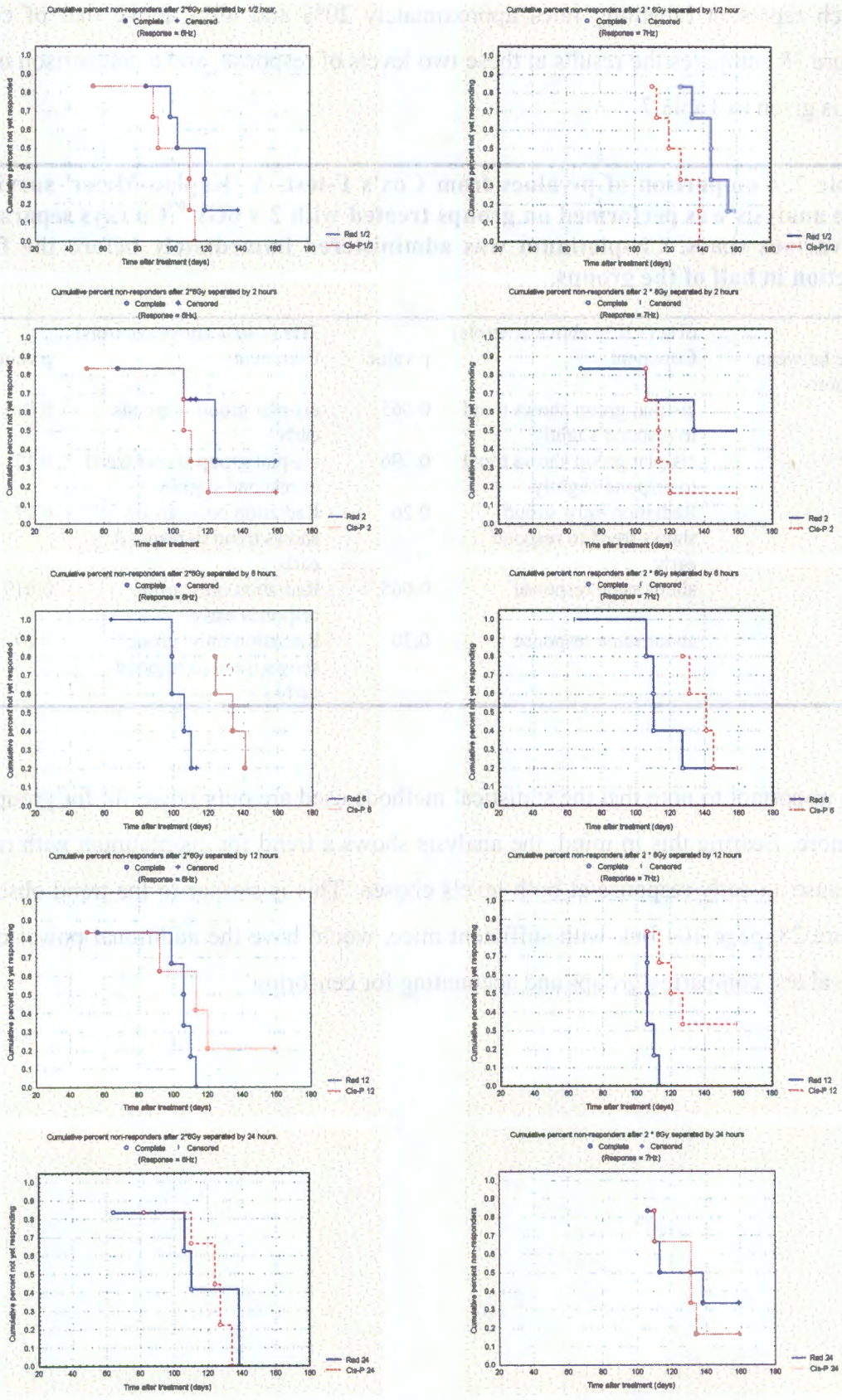
tained more than 30 mice the analysis was performed with the results from the experiment in which mice were treated with 2 fractions of 6Gy ^{60}Co γ-rays with and without cis-platinum preceding the first fraction (Figure 38). Response levels of 6 and 7Hz were used which represent breathing rates approximately 20% and 40% above that of controls. Figure 38 compares the results at these two levels of response, and a comparison of p values is given in Table 7

Table 7. Comparison of p-values from Cox's F-test. A 'Kaplan-Meier' survival type analysis was performed on groups treated with 2 x 6Gy ^{60}Co rays separated by various times. Cis-platinum was administered immediately before the first fraction in half of the groups.

	6Hz (~20% above controls)		7Hz (~40% above controls)	
Time between fractions	Comment	p value	Comment	p value
0.5	cis-plat group shows trend to respond slightly	0.065	cis-plat group responds early	0.015
2	cis-plat group shows trend to respond slightly	0.096	cis-plat group shows trend to respond slightly	0.12
6	Radiation only group shows trend to respond early	0.26	Radiation only group shows trend to respond early	0.27
12	about same response	0.065	Radiation only group responds early	0.019
24	about same response	0.30	Radiation only group shows trend to respond early	0.29

It is important to note that the statistical methods used are only powerful for groups of 30 or more. Bearing this in mind, the analysis shows a trend for cis-platinum with radiation to cause an early response at both levels chosen. This is similar to the trend observed in Figure 28, page 104 but, with sufficient mice, would have the additional power of a statistical test comparing groups and accounting for censoring.

Figure 38. Cumulative percent non-responders for response breathing rates of 6Hz (left) and 7Hz (right) after treatment with 286Gy with and without cis-platinum.



Overall comments on breathing rate experiments

In summary, considerable variability was seen in the breathing rate experiments that may be masking fine changes. Some evidence of repair as shown by a decrease in breathing rate after a split dose of radiation was observed but repair half times could not be determined. Although a trend for cis-platinum treated groups to show an early increase in breathing rate was observed in both single radiation dose and split dose experiments it could not be statistically verified and was not observed in all experiments.

Histology

The pneumonitic phase of radiation injury to the lung is characterised by interstitial oedema, fluid leakage into the alveoli, an infiltration of inflammatory cells and possible desquamation of epithelial cells from the alveoli walls. Travis (1995) used these lesions as the basis for a scoring system to quantitatively evaluate lung sections after radiation treatment. Mild lesions involving less than a third of the section were scored as 1, one third to two thirds a value of 2 and more than two thirds a value of 3. Normal sections were assigned a value of 0.

Initially Travis (1995) used an ocular grid to count the percentage of lesions occupying a section and this number converted to the appropriate score. After a large number of sections had been counted it was found that the severity of injury could be estimated using the scoring system. T-tests were used to compare treatment groups. As only relatively few sections were to be evaluated in the work presented here the results were not converted to a reaction score but were evaluated using the actual percentages of lesion for each section.

While the result is statistically significant the experiment may be criticised in that only one section from each mouse was evaluated. This was taken parallel to the thoracic midline and approximately through the midline of the left half lung. In contrast Travis (1995) evaluated two sections from each lobe. (There are 5 lobes in a mouse lung – one in the left lung and four in the right lung).

In evaluating the sections the four different lesions are not equally easy to observe. Macrophages are generally obvious. Oedema in alveolar walls or air spaces is somewhat subjective and on H&E stained sections is difficult to distinguish from fibrin in the air spaces. In practice this was evaluated simply as matter occluding the air space.

TGF-β

If TGF-β is involved in the development of radiation fibrosis in the lung it may be expected that changes could be determined in the amount of TGF-β present in either lung tissue or broncho-alveolar lavage fluid after radiation treatment. Further it may also be expected that there would be strain dependent differences in the levels of TGF-β that would correlate with the different fibrotic response shown by suitably chosen strains. Finally if cis-platinum were to sensitise the lung to late radiation damage with the underlying reaction being an increase in lung fibrosis then treatment with cis-platinum might produce changes in TGF-β after treatment with both cis-platinum and radiation. It is conceivable that cis-platinum might cause changes in the fibrotic response by some pathway which does not include TGF-β.

Changes in mouse weight

Weight changes after treatment were used in these experiments as an indicator of possible systemic effects of treatment. The results indicate that Balb/C mice are more sensitive to the acute effects of treatment at the dose of both cis-platinum and radiation used as they showed a greater initial weight loss than the C57/Bl mice. This trend was continued at one month with the Balb/C mice not showing similar weight gains as the C57/Bl mice.

Changes in lung wet weight

The decrease in lung wet weight noted in the C57/Bl strain after treatment with radiation and after treatment with cis-platinum immediately before radiation at one week and at one month after treatment may be an experimental artefact due to the low numbers of mice contributing to each point. As this was an invasive assay lungs from a different group of mice contributed to the mean weight of control mice at one month as opposed to that at one week after treatment. It should also be noted that lung weight changes after treatment have been noted as being variable (Coggle et al. 1986).

Changes in lung protein

The results indicate that radiation caused an early increase in total protein in lung in C57/Bl mice but not in Balb/C mice (Figure 34, page 114). Since the protein content is

measured with respect to lung wet weight it may be that Balb/C mice are showing a greater relative increase in lung wet weight from non-protein components than C57/Bl mice. The documented strain differences indicate that the Balb/C mice show a typical early pneumonitis characterised by pulmonary oedema that may be masking increases in total protein. The increase in total protein shown in the C57/Bl's may include components responsible for the later fibrotic response shown by the strain.

At one week after treatment, lung protein levels in the C57/Bl group treated with both cis-platinum and radiation showed a trend to increase more than for the group treated with radiation only. The protein samples for the group treated with cis-platinum and radiation were lost during the extraction and analysis process. It was thus not possible to determine if this trend persisted at one month after treatment.

The differences in protein response between strains may be a contributing factor in the differences in fibrotic response. It has previously been suggested that the increase in lung permeability and associated protein leakage may be part of the progressive development of fibrosis (Gurley et al. 1993). The increased protein levels may be due to greater loss of endothelial function in the C57/Bl strain. As discussed in Chapter 3, page 25, Franko et al. (1991) showed lower activities for factors important in angio-genesis in those strains that showed fibrosis, including C57/Bl.

TGF-β per sample lavaged surfactant

If TGF-β is part of the pathway to pulmonary fibrosis then it might be expected that the Balb/C strain would have less TGF-β in lavage as the Balb/C only shows foci of fibrosis while the C57/Bl shows extensive contracted fibrosis. The results did show no significant changes for the Balb/C mice and various significant differences between points for the C57/Bl groups. However, these followed a somewhat random pattern of change and although some significant differences between points were evident they were not part of a properly constructed hypothesis. It would not be valid to draw conclusions from them without supporting evidence from repeat experiments. Indeed, it may be that the changes in TGF-β observed for the C57/Bl mice are artefacts resulting from experimental error or small numbers of mice.

The experimental design must be criticised on two counts. In the first place, the TGF-β is recorded per lavage sample. While every effort was made to perform the lavage consistently different amounts of lavage fluid were retrieved from each mouse both because of physical differences between mice and because of variation in extraction efficiency. An improvement in the experiment may be to record the amount of TGF-β per mg lavage fluid extracted.

In the second place, the sample is processed after lavage by the addition of two aliquots of buffer and centrifuged twice with the supernatant being the component of interest. Different samples may be processed with differing efficiencies. Clearly an internal control, consisting of a known amount of TGF-β added before processing may help to assess and correct for this.

TGF-β in homogenised lung

While no obvious trends could be observed in TGF-β in homogenised lung – one isolated point where TGF-β was measured in C57/Bl mice at one month after radiation was significantly lower than controls – it is of interest to note the strain difference in TGF-β levels in lavaged surfactant at both one week and one month after treatment. This difference is not apparent in TGF-β in homogenised lung. The C57/Bl and Balb/C strains have been observed to have different fibrotic responses to radiation (Sharplin and Franko 1989b). The C57/Bl strain shows diffuse fibrosis after radiation whereas the Balb/C only shows foci of fibrosis.

Future direction

In many of the experiments the inconclusive results may be clarified by using larger numbers of mice. Of the endpoints investigated the histology scoring system showed a significant increase in damage after combined treatment. The ability of the assay to discern this change should be verified by establishing a radiation dose response curve and then repeating the combined treatment with at a fixed dose of cis-platinum preceding at least three different radiation doses.

Analysis of breathing rates using the Kaplan-Meier 'survival' type analysis should be investigated to ascertain if it would improve the ability of the breathing rate assay to investigate small changes. One possible way to do this would be to treat three groups of at least 30 mice with small increments in radiation dose e.g. 12Gy, 12.25Gy and 12.75Gy. An estimate of the resolving power of the assay could then be obtained in terms of radiation dose. If the technique proved sensitive then it could be used to test for schedule dependent effects of cis-platinum administered at different times relative to radiation. It is important that this be established for two reasons. Firstly, in the terms of the clinical use of combined cis-platinum and radiation adverse schedules must be identified and avoided. Secondly, in order to investigate adverse effects of cis-platinum in combination with radiation on such factors as repair of radiation damage a schedule that produces a measurable adverse effect must be established. In a negative sense, it is important that the 'optimum' time of administration of cis-platinum should be used in further investigations of its effect on radiation pulmonary toxicity.

The experiment investigating schedule dependent effects of cis-platinum on surfactant should be repeated using the enzymatic assay and extending it to additional time points both within the 56 day period repeated here and to times beyond 56 days.

If the early release of surfactant after combined treatment with cis-platinum and radiation *is* a real result then it would be of interest to investigate the mechanism of action. It would also be of interest to see if other, known pulmonary toxic drugs such as Bleomycin produced a similar result. If so, this would be relevant information for consideration in the debate regarding the role of surfactant as a marker for later pulmonary damage. Surfactant release data from treatment protocols including radiation that were know to cause increased pulmonary damage could be compared to protocols, such as those including cis-

platinum, that were not know to cause increased pulmonary damage. A positive correlation between surfactant release and the inclusion of pulmonary toxic drugs may indicate that the surfactant system is predictive for later damage. A negative correlation may indicate that it was not involved. However, without a proper mechanistic understanding neither would be conclusive.

It would also be of interest to see if these drugs, used on their own, produced a release of surfactant as was shown for cis-platinum.

An *in-vitro* system may be useful as a model to further investigate the release after treatment with cis-platinum alone and after combined treatment. (In-vitro models have been developed using cultured type II pneumocytes e.g. Shapiro et al. 1984, Burger et al. 1994a and Burger et al. 1994b).

Differences in TGF-β in lavaged surfactant between Balb/C mice and C57/Bl mice should be confirmed using larger groups of mice in each point. It would also be of interest to extend this work to a mouse strain in the third group identified by Sharplin and Franko (1989b) that showed no signs of fibrosis. A suitable candidate might be the CBA mouse. The TGF-β results obtained for homogenised lung should also be reinvestigated with larger groups of mice. It is extremely difficult in this kind of experiment where the levels of the substance being investigated are likely to change with time to decide on time points for investigation. The times chosen in this work were based on the time points used in investigating surfactant. In the first instance reliable values for untreated controls should be obtained.

Conclusions

In conclusion the work presented here was unable to identify a consistent, substantive effect of cis-platinum on the radiation response of the lung as assessed using the methods described. The breathing rate assay, the focus of much of the work presented, does not appear to be sensitive enough to detect small changes between groups of mice treated with different protocols.

A trend may exist for cis-platinum to cause an early release in surfactant and an early increase in breathing rates as compared to that induced by radiation alone. Cis-platinum alone may cause a release of surfactant.

Cis-platinum was observed to increase radiation damage assessed using a histology based scoring system.

The level of TGF-β in lavaged surfactant was greater for the C57/Bl strain as compared to the Balb/C strain. Cis-platinum may cause a decrease in TGF-β in lavaged surfactant from C57/Bl mice at 1 week after treatment but further work is necessary to confirm this. The level of TGF-β in homogenised lung was not different for C57/Bl mice as compared with Balb/C mice. No changes in TGF-β in homogenised lung could be observed in either strain at one week and at one month after treatment with cis-platinum, radiation or cis-platinum immediately before radiation.

References

Adamson I.Y.R., Young L, Drummond D.H., Relationship of alveolar epithelial injury and repair to the induction of pulmonary fibrosis., *Am. J. Path.*, 130(2), 377-383, 1988.

Adamson, I.Y.R., Bowden, D.H., Wyatt, J.P., A pathway to pulmonary fibrosis: An ultrastructural study of mouse and rat following radiation to the whole body and hemithorax., *Am. J. Pathology.*, 58(3), 481-487, 1970.

Ahier R.G., Anderson R.L., Coultas P.G., Responses of mouse lung to irradiation: 1. Alterations in alveolar surfactant after neutrons and X-rays., *Rad. Onc.*, 3, 61-68, 1985.

Alvarez V., Cobreros G., Heras A., Lopez Zumel C., Studies on cis-dichlorodiammineplatinum(II) as a radiosensitiser., *Br. J. Cancer.*, 37, Suppl III, 68-72, 1978.

Anderson R.L., Ahier R.G., Coultas P.G., Responses of mouse lung to irradiation: 2. Levels of alveolar protein in lung lavage fluid following neutrons or X-rays., *Rad. Onc.*, 4, 167-174, 1985.

Andrews PA, Murphy MB, Howell SM., Metallothionein-mediated cisplatin resistance in human ovarian carcinoma cells., *Canc. Chemo. Pharm.*, 19, 149-154, 1987.

Barry M.A., Behnke C.A., Eastman A., Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia., *Biochem. Pharmacol.*, 40, 2353-2362, 1990.

Bhoughattas N.A., Levi F., Fournier C., Hecquet B., Lemaigre G., Roulon A., Mathe G., Reinberg A., Stable circadian mechanisms of toxicity of two platinum analogs (cisplatin and carboplatin) despite repeated dosages in mice., *J. Pharmacol Exp Ther.*, 255(2), 672-679, 1990.

Bligh E.G., Dyer W.J., A rapid method of total lipid extraction and purification., *Can. J. Biochem. Physiol.*, Vol 37, 911-917, 1959.

Bradford M., *Anal. Biochem.*, 72., 248., 1976.

Burger A., Bamberg M., Rodemann H.P., Radiation induced synthesis of the fibrogenic cytokine TGF-β by type II pneumocytes., *personal communication* 1994a

Burger A., Güven N., Hämmerle H., Bamberg M., Rodemann H.P.: Co-culture systems of alveolar type-II-pneumocytes and lung fibroblasts as a model for radiation induced lung fibrosis., *Eur. J. Cell Biol.*, 63, 113- 1994b.

Calvert H., Judson I., Van der Vijgh W., Platinum Complexes in cancer Medicine: Pharmacokinetics and Pharmodynamics in relation to toxicity and therapeutic activity. *Cancer Surveys.*, 17. 189-217, 1993.

Calvert H., Judson I., Van der Vijgh W.J.F.: Platinum complexes in cancer medicine: Pharmacokinetics and pharmacodynamics in relation to toxicity and therapeutic activity., *Cancer Surveys.*, 17, 189-217, 1993.

Chu G.: Cellular responses to Cisplatin. The roles of DNA-binding proteins and DNA repair., *J. Biol. Chem.*, 269(2), 787-790, 1994.

Chung-Hsin Ts'ao, Ward, W.F., Port, C.D., Radiation injury in the lung I. Prostacyclin (PGI_2) production, arterial perfusion, and ultrastructure., *Rad. Res.*, 96, 284-293, 1983.

Chung-Hsin Ts'ao, Ward, W.F., Port, C.D.: Radiation injury in rat lung III. Plasminogen activator and fibrinolytic activities., *Rad. Res.*, 96, 301-308, 1983.

Chung-hsin Ts'ao., Ward, W.F., Plasminogen activator activity in lung and alveolar macrophages of rats exposed to graded single doses of γ rays to the right hemithorax., *Radiation Research.*, 103, 393-402, 1985.

Coff R.J. Jr, Derynk R., Wilcox J.J., Bringman T.S., Goustin A.S., Moses H.L., Pittelkow M.R., Production and auto-induction of transforming growth factor alpha in human keratinocytes., *Nature.*, 328, 817-820, 1987.

Coggle, J.E., Tarling, J.D., Cell Kinetics of pulmonary alveolar macrophage in the mouse., *Cell & Tiss. Kinet.*, 15(2), 139-143, 1982.

Comis R.L., Cisplatin: The Future., *Seminars in Oncology.*, 21(5 Suppl 12), 109-113, 1994.

Coughlin C.T., Richmond R.C., Biologic and clinical developments of cisplatin combined with radiation: Concepts, Utility, Projections for New Trials, and the emergence of Carboplatin., *Seminars in Oncology.*, 16(4) Sup 6, 31-43, 1989.

Coultas P.G., Ahier R.G., Anderson R.L., Altered turnover and synthesis rates of lung surfactant following thoracic irradiation., *Int. J. Radiat. Oncol. Biol. Phys.*, 13, 233-237, 1987.

Coultas, P.G., Ahier, R.G., Field, S.B., Effects of neutron and x-irradiation on cell proliferation in mouse lung., *Radiation Research.*, 85, 516-528, 1981.

Dewit L., Begg A.C., Köhler Y., Stewart F.A., Bartelink H., Influence of cis-diamminedichloroplatinum(II) on mouse duodenal crypt stem cell survival after multifraction x ray treatment., *Int. J. Radiat. Oncol. Biol. Phys.*, 11, 1809-1816, 1985.

Dorie M.J., Bedarida G., Kallman R.F., Protection by interleukin 1 against lung toxicity caused by cyclophosphamide and irradiation., *Rad. Res.*, 128(3), 316-319, 1991.

Douple E.B., Eaton W.L., Tulloh M.E., Skin radiosensitisation studies using combined cis-dichlorodiammineplatinum(II) and radiation., *Int. J. Radiat. Oncol. Biol. Phys.*, 5, 1383-1385, 1979.

Douple E.B., Keynote address: Platinum-Radiation Interactions., *NCI Monographs.*, 6, 315-319, 1988.

Douple E.B., Richmond R.C., Logan M.E., Therapeutic potentiation in a mouse mammary tumor and an intracerebral rat brain tumor by combined treatment with cis-dichlorodiammineplatinum(II)., *J. Clin. Hematol. Oncol.*, 7, 585-603, 1977.

Down J.D., Coultas P.G., Field S.B., Letter to the editors: Is surfactant release a reliable predictor of radiation pneumonytis., *Int. J. Radiat. Oncol. Biol. Phys.*, 14(1), 211-212, 1988.

Down J.D., Easton D.F., Steel G.G., Repair in the mouse lung during low dose-rate irradiation., *Radiother. Oncol.*, 6(1), 29-42, 1986.

Down J.D., Laurent G.J., McAnulty R.J., Steel G.G., Oxygen-dependent protection of radiation lung damage in mice by WR-2721., *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.*, 46(5), 597-607, 1984.

Down J.D., Nicholas D., Steel G.G., Lung damage after hemithoracic irradiation: dependence on mouse strain., *Radiother. Oncol.*, 6(1), 43-50, 1986.

Down J.D., Steel G.G., The expression of early and late damage after thoracic irradiation: a comparison between CBA and C57Bl mice., *Rad. Res.*, 96(3), 603-610, 1983.

Down, J.D., Steel, G.G., The expression of early and late damage after thoracic irradiation: A comparision between CBA and C57Bl mice., *Radiation Research.*, 96, 603-610, 1983.

Dritschilo A., Piro A.J., Kelman A.D., The effect of cis-platinum on hte repair of radiation damage in plateau phase chinese hamster (V-79) cells., *Int. J. Radiat. Oncol. Biol. Phys.*, 5., 1345-1349, 1979.

Field S.B., Hornsey S., Slow repair after X rays and fast neutrons., *Br. J. Radiol.*, 50., 600-601., 1977.

Finkelstein, J.N., Johnston, M.S., Baggs, R., Rubin, P., Early alterations in extracellular matrix and transforming growth factor ß gene expression in mouse lung indicative of late radiation fibrosis., *Int. J. Radiat. Oncol. Biol. Phys.*, 28(3), 621-631, 1994.

Franko, A.J., Sharplin, J., Ward, W.F., Hinz, J.M., The genetic basis of strain-dependent differences in the early phase of radiation injury in mouse lung., *Radiation Research.*, 126, 349-356, 1991.

French A.P., Vibrations and Waves. M.I.T. Introductory Physics Series., *Van Nostrand Reinhold (UK) Co. Ltd.*, p189, 1982.

Fu K.K., DeGregorio M.W., Phillips J.W., Plasma and tumour concentrations of Cisplatin following intraperitoneal infusion or bolus injection with or without continuous low dose rate irradiation., *NCI Monographs.*, 6, 123-127, 1988.

Goerke J., Lung Surfactant., *Biochem et Biophys. Acta.*, Vol 344., 241-261., 1974.

Golding R.P., Van Zanten T.E.G., Lung destruction after cis-platinum radiosensitisation. Correspondence., *Br. J. Radiology.*, 56, 281-281, 1983.

Gross N.J., Early physiologic and biochemical effects of thoracic X-irradiation on the pulmonary surfactant system., *J. Lab. Clin. Med.*, 91(3), 537-544. 1978.

Gross N.J., Experimental Radiation pneumonitis. III. Phospholipid studies on the lungs., *J. Lab. Clin. Med.*, 93, 627-637, 1979.

Gross N.J., Experimental radiation pneumonitis: changes in physiology of the alveolar surface., *J. Lab. Clin. Med.*, 92, 991-1001, 1978.

Gross N.J., Inhibition of surfactant subtype convertase in radiation model of adult respiratory distress syndrome., *Am. J. Physiol.*, 260, L311-L317, 1991.

Gross N.J., Mechanical properties of mouse lungs: effects of degassing on normal, hyperoxic, and irradiated lungs., *J. Appl. Physiol.*, 51(2), 391-398, 1981.

Gross N.J., Pulmonary effects of radiation therapy., *Ann. Inter. Med.*, 86., 81-92., 1977.

Gross N.J., Surfactant subtypes in experimental lung damage: radiation pneumonitis., *Am. J. Physiol.*, 260, L302-L310, 1991.

Gross, N.J., Alveolar macrophage number: An index of the effect of radiation on the lung., *Radiation Research.*, 72, 325-332, 1977.

Gross, N.J., Narine, K.R., Couletti-Squinto, L., Replicative activity of lung type 2 cells following X irradiation., *Radiation Research.*, 111, 143-150, 1987.

Gross, N.J., Narine, K.R., Experimental Radiation Pneumonitis, Corticosteroids increase the replicative activity of alveolar type 2 cells., *Radiation Research.*, 115, 543-549, 1988.

Gross, N.J., Radiation pneumonitis in mice: Some effects of corticosteroids on mortality and pulmonary physiology., *J. Clin. Invest.*, 66, 504-510, 1980.

Gurley L.R., London J.E., Tietjen G.L., van der Kogel A.J., Dethloff L.A., Lehnert B.E.: Lung Hyperpermeability and changes in biochemical constituents in bronchoalveolar lavage fluids following X irradiation of the thorax., *Rad. Res.*, 134, 151-159, 1993.

Haston C.K., Hill R.P., Newcomb C.H., Van Dyk J., Radiation-induced lung damage in rats: the influence of fraction spacing on effect per fraction., *Int. J. Radiat. Oncol. Biol. Phys.*, 28(3), 633-640, 1994.

Herrmann T., Baumann m., Voigtmann L., Knorr A., Effect of irradiated volume on lung damage in pigs., *Radiother Oncol.*, 44(1), 35-40, 1997.

Hill S.A., Travis E.L., Denekamp J., Actinomycin D and radiation: effects on mouse lung., *Eur J Cancer Clin Oncol.*, 22(5), 577-582, 1986.

Hoyt D.G., Lazo J.S., Alterations in pulmonary mRNA encoding procollagens, fibronectin and transforming growth factor-β precede bleomycin-induced pulmonary fibrosis in mice., *J. Pharmacol. Exp. Ther.*, 246, 765-771, 1988.

Hoyt D.G., Lazo J.S., Bleomycin and cyclophosphamide increase pulmonary type IV procollagen mRNA in mice., *Am. J. Physiol.*, 259, L47-L52, 1990.

Hrushesky W.J., Levi F.A., Halberg F., Kennedy B.J., Circadian stage dependence of cis-diamminedichloroplatinum lethal toxicity in rats., *Cancer Research.*, 42(3), 945-949, 1982.

Ishikawa T., Ali-Osman F., Glutathione-associated cis-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells. Molecular characterization of glutathione-platinum complex and its biological significance., *J. Biol. Chem.*, 268, 20116-20125, 1993.

Johnston C.J., Piedboeuf B., Baggs R.B., Rubin P., Finkelstein J.N., Differences in correlation of mRNA gene expression in mice sensitive and resistant to radiation-induced pulmonary fibrosis., *Rad. Res.*, 142, 197-203, 1995.

Jungawala F.B., Evans E.E., McCluer R.H., High Performance Liquid Chromatography of Phasphatidycholine and Sphingomyelin with detection in the region of 200nm., *Biochem J.*, Vol 155., 55-60, 1975.

Kallman R.F., Rapacchietta D., Zaghloul M.S., Schedule dependent therapeutic gain from the combination of fractionated irradiation plus c-DDP and 5-FU or plus c-DDP and cyclophosphamide in c3H/Km mouse model systems., *Int. J. Radiat. Oncol. Biol. Phys.*, 20, 227-232, 1991.

Kallman R.F., The importance of schedule and drug dose intensity in combinations of modalities., *Int. J. Radiat. Oncol. Biol. Phys.*, 28(3), 761-771, 1994.

Kaplan E.L., Meier P., Non-parametric estimation from incomplete Observations., *Journal of the American Statistical Association.*, 53, 457-481, 1958.

King R.J., Clements J.A., Surface Active materials from dog lung. II. Composition and physiological correlations., *Am. J. Physiol.*, Vol 223, 715-726, 1972.

Korbelik M., Skov K.A., Inactivation of Hypoxic cells by Cisplatin and radiation at clinically relevant doses., *Radiation Research.*, 119, 145-156, 1989.

Kwock L., Davenport W.C., Clark R.L., Zarembra J., Lingle B., Chaney E.L., Friedman M., The effects of ionizing radiation on the pulmonary vasculature of intact rats and isolated pulmonary endothelium., *Radiation Research.*, 111, 276-291, 1987.

Lange R.C., Spencer R.P., Harder HC., Synthesis and distribution of Pt cis-dichlorodiammineplatinum(II)., *Journal of Nuclear Medicine.*, 13, 328-330, 1972.

Law, M.P., Vascular permeability and Late Radiation fibrosis in mouse lung., *Radiation Research.*, 103, 60-76, 1985.

Law, M.P., Ahier R.G., Vascular and Epithelial damage in the lung of the mouse after X-rays or neutrons., *Radiation Research.*, 117., 128-144., 1989.

Lehnert B.E., Dethloff L.A., Finkelstein J.N., Van der Kogel., Temporal sequence of early alterations in rat lung following thoracic X-irradiation., *Int. J. Radiat. Biol.*, 60(4), 657-675, 1991.

Liao Z.X., Travis E.L., Tucker S.L., Damage and morbidity from pneumonitis after irradiation of partial volumes of mouse lung., *Int. J. Radiat. Oncol. Biol. Phys.*, 32(5) 1359-1370, 1995.

Litterst CL., LeRoy AF., Guarino AM., Disposition and distribution of platinum following parenteral administration cis-Dichloro diammineplatinum(II) to animals., *Cancer Treatment Reports.*, 63(9-10) 1485-1492, 1979.

Lockart S.P., Down J.D., Steel G.G., Mouse hemithoracic irradiation and its interaction with cytotoxic drugs., *Radiother Oncol.*, 24(3), 177-185, 1992.

Lundbeck F., Overgaard J., Early and late changes in the normal mouse bladder reservoir function due to irradiation and cis-DDP., *Br. J. Cancer.*, 66, 99-105, 1992.

Lundgren, D.L., Hahn, F.F., Suppression of pulmonary clearance of staphylococcus aureus in mice that had inhaled either CeO_2 or PuO_2., *Radiation Research.*, 77, 361-376, 1979.

McDonald, S., Rubin, P., Chang, A.Y.C., Penney, D.P., Finkelstein, J.N., Grossberg, S., Feins, R., Gregory, P.K., Pulmonary changes induced by combined mouse ß-interferon (rMuIFN-ß) and irradiation in normal mice - toxic versus protecive effects., *Rad. Onc.*, 26, 212-218, 1993.

Melvik J.E., Dornish J.M., Pettersen E.O., The binding of cis-dichlorodiammineplatinum(II) to extracellular and intracellular compounds in relation to drug uptake and cytotoxicity in vitro., *Br. J. Cancer.*, 66, 260-265, 1992.

Meyer, K.R., Ullrich, R.L., Effects of x-rays and fission neutrons on an induced proliferative response in lung type II epithelial cells., *Radiation Research.*, 85, 380-389, 1981.

Molls, M., Van Beuningen, D., Radiation Injury of the Lung: Experimental Studies, Observations after Radiotherapy and Total Body Irradiation prior to bone marrow transplantation., *In: Radiopathology of Organs and Tissues.*, Edited by Scherer, E., Streffer, C., Rüdiger Trott, K., Springer-Verlag, Berlin Heidelberg, 1991.

Morgan G.W., Breit S.N., Radiation and the lung: A re-evaluation of the mechanisms of mediating pulmonary injury., *Int. J. Radiat. Oncol. Biol. Phys.*, 31(2), 361-369, 1995.

Muggia F.M., Glatstein E., Summary of investigations on platinum compounds and radiation interactions., *Int. J. Radiat. Oncol. Biol. Phys.*, Vol 5, 1407-1409, 1979.

Muggia F.M.: Cisplatin Update., *Seminars in Oncology.*, 18(1 Suppl 3), 1-4, 1994.

Munchausen L.L., Rahn R.O., Biologic and chemical effects of cis-dichlorodiammineplatinum (II) (NSC-119875) on DNA., *Canc. Chem. Rep.*, 59, 643-646, 1979.

O'Conner-McCourt M.D., Wakefield L.M., latent transforming growth factor-b in serum., *J. Biol. Chem.*, 262, 14090-14099, 1987.

Parkins C.S., Fowler J.F., Denekamp J., Lung radioprotection by WR-2721 at low X-ray doses per fraction., *Eur. J. Cancer Clin. Oncol.*, 20(11), 1445-1450, 1984.

Parkins C.S., Fowler J.F., Repair in mouse lung of multifraction X rays and neutrons: extension to 40 fractions., *Br. J. Radiol.*, 58(695), 1097-1103, 1985.

Peckham M.J., Collis C.H., Clinical objectives and normal tissue responses in combined chemotherapy and radiotherapy., *Bull cancer.*, 68(2), 132-141, 1981.

Peel, D.M., Coggle, J.E., The effect of x-irradiation on alveolar macrophages in mice., *Radiation Research.*, 81, 10-19, 1980.

Penny, D.P., Rubin, P.R., Specific early fine structural changes in the lung following irradiation., *Int. J. Radiat. Oncol. Biol. Phys.*, 2, 1123-1132, 1977.

Pera M.F., Rawlings C.J., Roberts J.J., The role of DNA repair in the recovery of human cells from cisplatin toxicity., *Chem.-Biol. Interactions.*, 37, 245-261, 1981.

Peterson L.M., Evans L.M., Thomas K.L., Graham M.M., Vascular response to fractionated irradiation in the rat lung., *Rad. Res.*, 131, 224-226, 1992b.

Peterson L.M., Evans M.L., Graham M.M., Eary J.F., Dahlen D.D., Vascular response to radiation injury in the rat lung., *Rad. Res.*, 129, 139-148, 1992a.

Peto J., The calculation and interpretation of survival curves., *Cancer clinical trials - Methods and Practice.*, Buyse et al. Oxford Medical Publishers., p362-380.

Phillips T.L., Wharam M.D., Margolis L.W., Modification of radiation injury to normal tissues by chemptherapeutic agents., *Cancer.*, 35, 1678-1684, 1975.

Phillips, T.L., An ultrastructural study of the development of radiation injury in the lung., *Radiology.*, 87, 49-54, 1966.

Phillips, T.L., Margolis, L., Radiation pathology and the clinical response of lung and oesophagus., *Front Radiat Ther Oncol.*, 6, 254-273, 1974.

Pretorius R.G., Petrilli E.S., Kean C., Ford L.C., Hoeschele J.D., Lagasse L.D., Comparision of the Iv and Ip routes of administration of Cisplatin in Dogs., *Cancer Treatment Reports.*, 65, 1055-1062, 1981.

Richmond R.C., Powers E.L., Radiation sensitization of a bacterial spore by cis-dichlorodiammineplatinum(II) and other metals., *J. Clin. Hematol. Oncol.*, 7, 580-584, 1977.

Richmond R.C., Toxic variability and radiation sensitization by dichlorodiammine-platinum(II) complexes in Salmonella typhimurium cells., *Radiation Research.*, 99, 596-608, 1984.

Richmond R.C., Zimbrick J.D., Hykes D.L., Radiation-induced DNA damage and lethality in *E coli* as modified by the antitumor agent cis-dichlorodiammineplatinum(II)., *Radiation Research.*, 71, 447-460, 1977b.

Rodemann H.P., Bamberg M., Cellular Basis of radiation-induced fibrosis., *Rad. Onc.*, 35, 83-90, 1995.

Rooney S.A., Page-Roberts B.A., Motoyama E.K., Role of lamellar inclusions in surfactant production: studies on phospholipid composition and biosynthesis in rat and rabbit lung subcellular fractions., *J. Lipid Res.*, Vol. 16, 418-425., 1975.

Rosenberg B., Van Camp L., Krigas T., Inhibition of cell division in Escerichia Coli by electrolysis products from a platinum electrode., *Nature.*, 205, 698-699, 1965.

Rosenberg B., Van Camp L., Trosco J.E., Platinum compounds: A new class of potent antitumour agents., *Nature.*, 222, 385-386. 1969.

Rubin P., Casarett G.W., Respiratory System. In: Clinical Radiation Pathology., Vol1., Saunders, Philadelphia, pp 423-470, 1968.

Rubin P., Finkelstein J., Shapiro D., Molecular biology mechanisms in the radiation induction of pulmonary injury syndromes: Interrelationship between the alveolar macrophage and the septal fibroblast., *Int. J. Radiat. Oncol. Biol. Phys.*, 24, 93-101, 1992.

Rubin P., Finkelstein J.N, Siemann D.W., Shapiro D.L., Van Houtte P., Penney D.P., Predictive biochemical assays for late radiation effects., *Int. J. Radiat. Oncol. Biol. Phys.*, 12, 469-476, 1986.

Rubin P.R., McDonald S, Maasilta P, Finkelstein J.N., Shapiro D.L., Penney D., Gregory P.K., Serum markers for prediction of pulmonary radiation syndromes. Part I: Surfactant Apoprotein., *Int. J. Radiat. Oncol. Biol. Phys.*, 17, 553-558, 1989.

Rubin P.R., Shapiro D.L., Finkelstein J.N., Penney D.P., The early release of surfactant following lung irradiation of alveolar type II cells., *Int. J. Radiat. Oncol. Biol. Phys.*, 6, 75-77, 1980.

Rubin P.R., Siemann D.W., Shapiro D.L., Finkelstein J.N., Penney D.P., Surfactant release as an early measure of radiation pneumonitis., *Int. J. Radiat. Oncol. Biol. Phys.*, 9, 1669-1673, 1983.

Rubin R., Finkelstein J.N., Prophets that don't predict., *Int. J. Radiat. Oncol. Biol. Phys.*, 14(1), 212, 1988.

Rubin, P., Shapiro, D.L., Finkelstein, J.N., Penney, D.P., The early release of surfactant following lung irradiation of alveolar type II cells., *Int. J. Radiat. Oncol. Biol. Phys.*, 6, 75-77, 1980.

Schaake-Koning C, van den Bogaert W, Dalesio O, Festen J, Hoogenhout J, van Houtte P, Kirkpatrick A, Koolen M, Maat B, Nijs A, et al., Radiosensitization by cy-

totoxic drugs. The EORTC experience by the Radiotherapy and Lung Cancer Cooperative Groups., *Lung Cancer,* 10, Suppl 1:S263-70, 1994.

Scherer, E., Streffer, C., Rüdiger Trott, K., *Radiopathology of Organs and Tissues.*, Springer-Verlag, Berlin Heidelberg, 1991

Shakil A., Hirabayashi N., Toge T., Circadian variation of 5-flourouracil and cis-platinum toxicity in mice., *Hiroshima Journal of Medical Sciences.*, 42(4), 147-154, 1993.

Shapiro D.L., Finkelstein J.N., Penney D.P., Siemann D.W., Rubin P., Sequential effects of irradiation on the pulmonary surfactant system., *Int. J. Radiat. Oncol. Biol. Phys.*, 8, 879-882, 1982.

Shapiro D.L., Finkelstein J.N., Rubin P.R., Renney D.P., Siemann D.W., Radiation induced secretion of surfactant from cell cultures of type II pneumocytes: An *in vitro* model of radiation toxicity., *Int. J. Radiat. Oncol. Biol. Phys.*, 10, 375-378, 1984.

Sharplin, J., Franko, A.J., A qauntitative histological study of strain dependent differences in the effects of irradiation on mouse lung during the intermediate and late phases., *Radiation Research.*, 119, 15-31, 1989b.

Sharplin, J., Franko, A.J., A quantitative histological study of strain-dependent differences in the effects of irradiation on mouse lung during the early phase., *Radiation Research.*, 119, 1-14, 1989a.

Siddik Z.H., Jones M., Boxall F.E., Harrap K.R., Comparative distribution and excretion of carboplatin and cisplatin in mice., *Cancer Chemotherapy and Pharmacology.*, 21, 19-24, 1988.

Sorenson C.M., Barry M.A., Eastman A., Analysis of events associated with cell cycle arrest at G2 phase and cell death induced by cisplatin., *J. Natl. Canc. Inst.*, 82, 749-755, 1990.

Sorenson C.M., Eastman A., Influence of cis-diamminedichloroplatinum(II) on DNA synthesis and cell cycle progression in excision repair proficient and deficient Chinese hamster ovary cells., *Cancer Res.*, 48, 6703-6707, 1988.

StatSoft, Inc. Electronic Statistics Textbook. Tulsa, OK: StatSoft. WEB: http://www.statsoft.com/textbook/stathome.html. 1997.

Steinberg F., Rehn B., Kraus R., Quabeck K., Bruch J., Beelen DW., Schaefer UW., Streffer C., Activity testing of alveolar macrophages and changes in surfactant phospholipids after irradiation in broncho-alveolar lavage: experimental and clinical data., *Environmental Health Perspectives.*, 97, 171-5, 1992.

Stewart F.A., Oussoren Y., Bartelink H., The influence of cisplatin on the response of mouse kidneys to multifraction irradiation., *Radiotherapy and Oncology.*, 15, 93-102, 1989.

Takayama M., *Clinica Chimica Acta.*, Vol 79, pp93, 1977.

Tanabe M., Godat D., Kallman R.F., Effects of fractionated schedules of irradiation combined with cis-diamminedichloroplatinum(II) on the SCCVII/ST tumor and normal tissues of the C3H/KM mouse., *Int. J. Radiat. Oncol. Biol. Phys.*, 13, 1523, 1987.

Terry N.H., Tucker S.L., Travis E.L., Residual radiation damage in murin lung assessed by pneumonitis., *Int. J. Radiat. Oncol. Biol. Phys.*, 14(5), 929-938, 1988.

Tillman B.F., Loyd J.E., Malcolm A.W., Holm B.A., Brigham K.L., Unilateral radiation pneumonytis in sheep: physiological changes and broncho-alveolar lavage., *J. Appl. Physiol.*, 66(3), 1273-1279, 1989.

Travis E.L., Bucci L., Fang M.Z., Residual damage in mouse lungs at long intervals after cyclophosphamide treatment., *Cancer Res.*, 50(7), 2139-2145, 1990.

Travis E.L., Down J.D., Hall L., Vojnovic B., Holmes S.J., Factors affecting the breathing rate of mice as used for studies of radiation damage to lungs., *Br. J. Radiol.*, 54(637), 50-53, 1981.

Travis E.L., Liao Z.X., Tucker S.L., Spatial heterogeneity of the volume effect for radiation pneumonitis in mouse lung., *Int. J. Radiat. Oncol. Biol. Phys.*, 38(5), 1045-1054, 1997.

Travis E.L., Newman R.A., Helbing S.J. WR 2721 modification of type II cell and endothelial cell function in mouse lung after single doses of radiation., *Int. J. Radiat. Oncol. Biol. Phys.*, 13, 1355-1359, 1987.

Travis E.L., Parkins C.S., Down J.D., Fowler J.F., Maughan R.L., Is there a loss of repair capacity in mouse lungs with increasing numbers of dose fractions?, *Int. J. Radiat. Oncol. Biol. Phys.*, 9(5), 691-699, 1983.

Travis E.L., Parkins C.S., Down J.D., Fowler J.F., Thames H.D. Jr., Repair in mouse lung between multiple small doses of X rays., *Radiat. Res.*, 94(2), 326-339, 1983.

Travis E.L., Parkins C.S., Holmes S.J., Down J.D., Fowler J.F., WR-2721 protection of pneumonitis and fibrosis in mouse lung after single doses of x rays., *Int. J. Radiat. Oncol. Biol. Phys.*, 10(2), 243-251, 1984.

Travis E.L., The sequence of histological changes in mouse lungs after single doses of X-rays., *Int. J. Radiat. Oncol. Biol. Phys.*, Vol 6, 345-347, 1980.

Travis E.L., Vojnovic B, Davies E.E., Hirst D.G., A plethysmographic method for measuring function in locally irradiated mouse lung., *Br. J. Radiol.*, 52, 67-74, 1979.

Travis E.L., Vojnovic B., Davies E.E., Hirst D.G., A plethysmographic method for measuring function in locally irradiated mouse lung., *Br. J. Radiology.*, 52, 67-74, 1979.

Travis E.L., Vovnovic B., Hirst D.G., Hill S.A., Quantitative changes in rodent lungs after irradiation (Abstract)., *Br. J. Radiol.*, 51, 239, 1978.

Travis, E.L., Harley, R.A., Fenn, C.O., Klobukowski C.J., Hargrove H.B., Pathologic changes in the lung following single and multifraction irradiation., *Int. J. Radiat. Oncol. Biol. Phys.*, 2, 475-490, 1977.

Travis E.L., Tucker S.L., The relationship between functional assays of radiation response in the lung and target cell depletion., *Br. J. Cancer.*, 53., Suppl VII., 304-319., 1986.

Tucker S.L., Travis E.L., Time course for the hazard of radiation-induced pneumonitis death in mice., *Int. J. Radiat. Biol.*, 62(5), 627-639, 1992.

Twentyman P.R., Kallman R.F., Brown J.M., The effect of time between x-irradiation and chemotherapy on the growth of three solid mouse tumors. III. cis-diamminedichloroplatinum., *Int. J. Radiat. Oncol. Biol. Phys.*, 5, 1365-1367, 1979.

Ullrich R.L., Meyer K.R., The influence of Butylated Hydroxytoluene-Induced Cell Proliferation on Mouse Lung Damage after X Rays or Fission Netrons., *Rad. Res.*, 89., 428-432., 1982.

Van den Berg H.W., Roberts J.J., Post-replication repair of DNA in chinese hamster cells treated with cis-platinum (II) diammine dichloride. Enhancement of toxicity and chromosome damage by caffeine., *Mutation Research.*, 33, 279-284, 1975.

Vergara, J.A., Thet, R.U., Thet, L.A., Changes in Lung morphology and cell number in radiation pneumonitis and fibrosis: A quantitative ultrastructural study., *Int. J. Radiat. Oncol. Biol. Phys.*, 13, 723-732, 1987.

Von der Maase H., Interactions of radiation and adriamycin, bleomycin, mitomycin C or cis-diamminedichloroplatinum(II) in intestinal crypt cells., *Br. J. Cancer.*, 42, 779-786, 1984.

Von der Maase H., Overgaard J., Vaeth M., Effect of cancer chemotherapeutic drugs on radiation-induced lung damage in mice., *Rad. Onc.*, 5, 245-257, 1986.

Von Rongen E., Travis E.L., Thames H.D. Jr., Repair rate in mouse lung after clinically relevant radiation doses per fraction., *Rad. Res.*, 141(1), 74-78, 1995.

Ward, W.F., Sharplin, J., Franko, A.J., Hinz, J.M., Radiation induced pulmonary endothelial dysfunction and hydroxyproline accumulation in four strains of mice., *Radiation Research.*, 120, 113-120, 1989.

Ward, W.F., Solliday, N.H., Molteni, A., Port C.D., Radiation Injury in rat lung II. Angiotensin converting enzyme activity., *Radiation Research.*, 96, 294-300, 1983.

Watanabe S., Watanabe K., Ohishi T., Aiba M., Kageyama K., Mast cells in the rat alveolar septa undergoing fibrosis after ionizing radiation., *Lab. Invest.*, 31, 555-567, 1974.

Wodinsky I., Swiniarski J., Kensler C.J., Venditti J.M., Combination radiotherapy and chemotherapy for P388 lymphocytic leukemia in vivo., *Canc. Treat. Rep.*, 4, 73, 1974.

Wyllie A.H., Kerr J.F.R., Currie A.R.: Cell death: the significance of apoptosis., *Int. Rev. Cytol.*, 68, 251-306, 1980.

Zak M., Drobnik J., Effect of cis-dichlorodiammineplatinum (II) on the post-irradiation lethality in mice after irradiations with X-rays., *Strahlentherapie.*, 142, 112-115, 1971.

Appendix A. Index of figures