Oxygen Toxicity and Radiation Injury to the Pulmonary System

A Thesis submitted for the Degree of Doctor of Philosophy

BY

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ABBREVIATIONS

ABBREV	NAME	ABBREV	NAME
PAM	PULMONARY ALVEOLAR MACROPHAGE	**LT_{50}**	LENGTH OF TIME TO 50% DEATH
BAL	BRONCHOALVEOLAR LAVAGE	**TEM**	TRANSMISSION ELECTRON MICROSCOPY
SOD	SUPEROXIDE DISMUTASE	**CO_2**	CARBON DIOXIDE
ATP	ADENOSINE TRIPHOSPHATE	**ADCC**	ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY
O_2^-	SUPEROXIDE ANION	**MEM**	MODIFIED EAGLES MEDIUM
H_20_2	HYDROGEN PEROXIDE	**N Na OH**	NORMAL SODIUM HYDROXIDE
$HO^\bullet$	HYDROXYL RADICAL	**EDTA**	DISODIUM ETHYLENEDIAMINETETRA ACETATE
ODFR	OXYGEN DERIVED FREE RADICAL	**DETAPAC**	DIETHYLENE-TRIEINEPENTAACETIC ACID
PMN	POLYMORPHONUCLEAR NEUTROPHIL	**PBS**	PHOSPHATE-BUFFERED SALINE
DNA	DESOXYRIBONUCLEIC ACID	**Fe++**	FERRIC
Mn	MANGANESE	**Fe+++**	FERROUS
Cu/Zn	COPPER/ZINC	**ARDS**	ADULT RESPIRATORY DISTRESS SYNDROME
H_20	WATER	**$Fi0_2$**	INSPIRED OXYGEN PERCENTAGE
TLC	TOTAL LUNG CAPACITY	**IPF**	IDIOPATHIC PULMONARY FIBROSIS
IGM	IMMUNOGLOBULIN M	**$^\bullet NO$**	NITRIC OXIDE
$ONOO^-$	PEROXYNITRITE RADICAL	**$Pa0_2$**	PARTIAL PRESSURE ARTERIAL OXYGEN
Hg	HAEMOGLOBIN	**$P(A\text{-}a)0_2$**	ARTERIAL-OXYGEN GRADIENTS
$paC0_2$	PARTIAL PRESSURE ARTERIAL CARBON DIOXIDE		

FOREWORD

These studies have taken a number of years to complete, but the findings remain relevant.

This work commenced almost 20 years ago, when I left Australia to work at the University of Iowa in full-time bench research. At that stage I had completed my FRACP, and had completed clinical training in pulmonary diseases. After two years at the University of Iowa, I returned to South Australia to a full time staff physician post in Thoracic Medicine at the Royal Adelaide Hospital. Research was discouraged within the Department of Thoracic Medicine, with clinical work being given absolute priority. Nevertheless, I wrote two seed grants that were funded by external bodies, and continued to pursue some of the unfinished work from Iowa. Mr. Grantley Gill, Reader in Surgery, generously provided laboratory space for me at the University of Adelaide. I established a small laboratory and continued observations in human PAM function.

I was successful with NH&MRC funding, and laboratory space in the Hanson Center, within the Royal Adelaide Hospital, was then made available to me. With limited time available for research, I put effort into supporting my own Ph.D. students and Advanced Trainees in Thoracic Medicine, including providing external funding support for salaries and consumables where necessary, as well as daily supervision.

At about this time, I also realized that a larger funding base for lung research in Australia was required. To that end I established the Australian Lung Foundation, with support of colleagues within the Thoracic Society of Australia and New Zealand, the British Lung Foundation, and Bedford Industries in South Australia, as well as business friends and associates. This effort, which I had to initiate from the very beginning, in an Australian economy during a downturn in economic activity, was much greater than I had realized at the beginning, taking six very full years of constant hard work. The result is a solid well-

organized research funding body for the benefit of all in Australia. The Australian Lung Foundation is now a very active supporter of lung research in Australia. However, this effort did greatly restrict the time available for my own thesis preparation.

However, I did finally take accumulated vacation time, and over this 3 month period, finished most of the statistical calculations. At that point I was ready to complete my thesis, but unfortunately I developed a malignant soft tissue sarcoma. This required wide surgical excision, and subsequent extensive radiation therapy. This further reduced my efforts towards my own Ph.D. thesis presentation, although I continued very actively supporting my own Ph.D. students, and Advanced Trainees in Pulmonary Medicine. Indeed, it took several years to regain excellent health after this serious illness. Even at this time, the active discouragement of my research continued in the Department of Thoracic Medicine at the Royal Adelaide Hospital.

Throughout all of this period, I did continue with a strong time and effort commitment to community service through volunteer work with the St John Ambulance Brigade, as Corp Surgeon in South Australia.

I feel very fortunate to have been able to complete my thesis, by virtue of recently being in a very supportive environment in the Pulmonary Division at the University of Iowa. In this environment I am currently very well funded with external grants and am actively pursuing several of the areas arising from this thesis.

ACKNOWLEDGEMENTS

The work towards this thesis began in the same year the motion picture 'Star Wars' was first released, and is being submitted in the year that Star Wars is being re-released. Over this span of time there have been a number of people who have contributed to the work in this thesis, some of whom have gone from being colleagues to very close friends.

Throughout this time my family have been entirely supportive of all of my initiatives and in particular of the research and educational effort, some of which is included in this thesis. This particularly applies to my wife, Christine, who not only has assisted with the preparation and presentation of papers, grants, abstracts and the thesis, but has also been happy to participate in, and sometimes lead, the voyage of discovery embodied in this thesis, a voyage not just in science but in attitudes, ideas, cultures and technology.

Throughout the thesis there have been two primary supervisors, Professor Ann Autor, initially at the University of Iowa, and currently at the University of British Columbia, and Professor Barry Vernon-Roberts at the University of Adelaide. Professor Autor was instrumental in providing research space and support in Iowa in the early stages of this thesis, as well as providing guidance and an opportunity to study pulmonary toxicity and alveolar macrophage function. Professor Vernon-Roberts has been fundamentally important in the later stages of the research and thesis development. Without support from both of these mentors this work would not have been possible.

In the early stages in Iowa, Professor George Bedell (Director, Pulmonary Medicine) provided essential time for bench research, and my family and I remain always appreciative of his kindness and generosity (and that of Miriel) towards us. Dr Jeffrey Stevens Ph.D. (post-doc in the Autor lab) introduced me, on a daily basis, to bench research with a great degree of patience, practical advice and helpful criticism. The radiation experiments were supported with practical help and enthusiasm, by now Professor Larry Oberley, at the University of Iowa. The transmission electron microscopy

was practically helped by now Professor Kenneth Moore, and Dr Eugene Shih patiently supported the scanning electron microscopy, also at the University of Iowa. The initial BAL studies were supported by Dr Autor, and were performed by me in the University of Iowa. On my return to Adelaide, after a period of time, Mr. Grantley Gill, Reader, Department of Surgery, University of Adelaide, kindly provided laboratory space. This allowed for further BAL and alveolar macrophage work. During this time laboratory Mr. Neville DeYoung ably and enthusiastically provided supervision for research staff, and the work on phagocytosis and other alveolar macrophage functions continued using the technical expertise of Ms. Karen Martin. During this time, as well, I began much more work with protein chemistry in the lung, with very strong support by Robert Walsh, Clinical Chemistry, at the Institute of Medical and Veterinary Science. I am indebted to Bob for not only his enthusiastic support, but his enormous technical skill and knowledge, manifest by early protein chemistry, and also with subsequent work. Mark Stevens, Cytologist, also at the Institute of Medical and Veterinary Science, has been extremely helpful in corroborating BAL cell counts and differentials in the normal volunteers, and in providing reports on the cell counts for the patient studies.

I would also ackknowledge the patient help from the University of Adelaide, in allowing me to complete this thesis.

I have had the opportunity also to have strong and sincere personal support over the course of this project from Dr. Michael Drew at the Royal Adelaide Hospital and in the last few years from Mr. Bill Menzel AOM (and his wife Gerda). Finally, Professor Gary Hunninghake (and Margie) at the University of Iowa, continue to be significant in providing great personal and professional support.

Academic Staff Supported

Ph.D. students

Mr. A. Wozniak, Completed and Awarded 1994.

Ms. T.J. Dillon, Completed and Awarded 1995.

Mr. R.L. Walsh, In Progress.

Ms. R. Uppaluri, Completed and awarded 1997.

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Dr. A. Puddy, Research Fellow in Pulmonary Physiology, Winnipeg, Canada. Now in Adelaide, South Australia.

Dr. S. Miller, Pulmonary Fellow, Royal Adelaide Hospital, Adelaide, South Australia. Now in Hobart, Tasmania.

DEDICATION

This thesis is dedicated to several people.

To my father Alfred and my mother Audrey

Who, by example, have provided the spirit of discovery,

and of the need for extending oneself intellectually,

morally and socially, for the benefit of others.

To my children

Benjamin, Rebecca and Samuel

who may understand for the future.

- - - -

DECLARATION

This work contains no material, which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my Thesis, when deposited in the University Library, being available for loan and photocopy.

Signed : ____

Geoffrey McLennan, MBBS, FRACP, SBStJ

Dated this **sixteenth** day of March, 1997.

ABSTRACT

The work in this thesis encompasses oxygen free radical related inflammation in the peripheral lung and in lung cells. Animal and human studies have been used. Methods include cell culture with function studies, protein chemistry, animal and human physiology, and cell and lung structure through histopathology, and various forms of electron microscopy.

Chapter 1 provides a summary overview of pulmonary oxygen toxicity and radiation effects. This is not meant to be an exhaustive review, as there are already excellent texts on this subject. The intention is to establish the grounds for the work presented in this thesis, and to provide a focus around which the thesis is based. It concentrates mainly on oxygen free radical production, using pulmonary oxygen toxicity as an experimental tool, with reference also to acute radiation injury. The review also includes perspectives which I have gained from meeting and discussing the field with many of the authors quoted.

Chapter 2 reports the profound effects of temperature on pulmonary oxygen toxicity in the rat. It is clearly shown that the rat's temperature did not alter with these exposures. Perhaps the animal's respiratory rate increased in the warm conditions, and slowed in the cooler conditions, leading to a change in oxygen flux across the very fragile alveolar capillary membrane. There are a number of hypotheses that need to be tested with regard to this observation, including more work on the effect of inhaled gas temperature in the absence of an environmental temperature change, and questions regarding how temperature alters the oxygen free radical defense enzymes.

Chapter 3 reports the protective effect of exogenously administered superoxide dismutase (CuZnSOD) in modifying pulmonary oxygen toxicity in the rat. In these experiments the CuZnSOD was biologically purified from an animal source, and was very expensive. Nevertheless, the experiments did show that continuous administration of CuZnSOD to a whole animal, before and throughout >95% hyperoxic exposure, did reduce

the pulmonary oxygen toxicity manifestations. These experiments were very carefully performed, with attention to the animal environment, including monitoring of environmental temperatures and humidity, and the removal of animal waste products from affecting the animal. This was achieved through using a large exposure chamber, with a high flow of oxygen through the chamber, and absorption of waste products through regular cleaning of the cages, and using activated charcoal. Many other groups working in the field at the same or subsequent times, exposed animals to hyperoxia with small plastic "bag" chambers and very low oxygen flows, with no environmental controls or animal waste removal.

Chapter 4 outlines initial studies with pulmonary alveolar macrophage, especially with efforts to find major differences between alveolar macrophages from smokers and non-smokers. Major differences were not found in phagocytosis, or in hydrogen peroxide production. Thee studies were undertaken as a preliminary look, with the expectation that if major differences were found, these would be evaluated from the point of view of oxygen radical production, as one explanation as to why smokers are more like to develop lung diseases than non-smokers. Morphological differences, including more electron dense bodies in the PAM's from smokers, were noted, but otherwise not pursued. The morphology was performed to test whether this could be used as a qualitative or quantitative outcome measure in subsequent free radical injury models. This chapter also details BAL findings in normal human subjects, including standardization of methods that would be used later to harvest alveolar macrophages. It is clear that there is an expansion of the PAM population in smokers' lungs, suggesting that in the region sampled by BAL, that there is chronic inflammation present. The simple yeast phagocytic assay, which was developed, does, in my view, provide important insights into human macrophage phagocytosis. That only a small proportion of the human pulmonary alveolar macrophages actually engages in phagocytosis under these conditions is against traditional beliefs and knowledge that have been based upon more global measurements of phagocytosis. The primary role of the non-phagocytic PAM's is open to conjecture, as is the question as to whether these non-phagocytic cells can become phagocytic under some circumstances. The comparison between human and rat macrophages shows striking differences, not seen between smokers and non-smokers, and lends support to the hypothesis that there are species differences in PAM function, that may possibly be environmentally determined.

Chapter 5 examines the mechanism of radiation damage in a non- dividing cell, the alveolar macrophage, using a series of well-constructed oxygen free radical inhibitor studies. The conclusion that free iron is important, with hydroxyl radical being generated, in addition to superoxide anion and hydrogen peroxide, has helped to foster interest in the role for chelation therapy in free radical mediated diseases, in addition to radical inhibitors. This also suggests those natural proteins that function to regulate free iron or copper may have an important role in modifying oxygen free radical injury.

Chapter 6 provides a list of several conclusions. The thesis provides the basis for subsequent studies, some of which I am currently studying.

Chapter 7 provides the bibliography.

Chapter 1

Introduction

A system as complex as life requires organization for appropriate and timely regeneration or repair of damaged parts, for reproduction of like units, for protection against other potentially invading life forms, or for protection from the damage that might be effected by toxic materials in the surrounding environment. There are a large number of environmental hazards that continually put life at risk, and which also have the potential to inflict acute, subacute or very chronic non-lethal damage. After damage has occurred, or even whilst damage is still being inflicted, there is a substantial response to that damage in complex organisms. This response is known as the inflammatory response; and the inflammatory response may in many instances inflict further localized or generalized damage whilst the cause of the primary damage is contained and controlled. Often an entire organ system may be damaged and destroyed by the inflammatory response.

The human respiratory system is composed of a fragile gas exchange surface at the level of the alveolus and pulmonary capillary, together with the conducting airways, the neuromuscular system and rib cage that acts as the respiratory bellows, and the central and peripheral chemo-receptor control that drives respiration. There is a well-defined lymphatic system that helps drain the peripheral lung and that commences just above the level of the pulmonary alveolus. There is a complex innervation in the lung, with cholinergic, adrenergic and tachykinin mediated neurons. The lung receives all of the cardiac output from the right heart through the low pressure high flow pulmonary circulation as it functions to exchange oxygen and carbon dioxide, and receives nourishment from the left heart through the independent low flow high pressure bronchial circulation. This delicate gas-exchange surface is exposed to the environment intimately and constantly through the air that is breathed. It is therefore, more than any other organ system, at constant risk from exposure to environmental infectious particles, to non-infectious organic and inorganic particulates, to changes in environmental temperature, and to changes in gas composition. The gas composition may change by varying the oxygen, nitrogen, carbon dioxide or water vapour tensions of the normal atmosphere, or by adding pollutants such as the oxides of nitrogen, or ozone.

The lungs have only relatively recently been understood to be primarily involved with gas exchange. The very early anatomists, such as Galen (Claudius Galenus - 129-199), only portrayed the lungs of animals especially monkeys, and represented these as the human anatomy. This was not too surprising as undertaking anatomical dissection in the human was difficult, and when performed the lungs were one of the first organs to

undergo putrefaction, with destruction of the anatomical detail. Nevertheless, the teachings of Galen influenced European thought in Medicine for 1300 years. In Europe, in the 1500's, morbid anatomy was again studied. Alessandro Benedeti, from Verona, (1450-1512) in a treatise published in 1497, and entitled "The History of the Human Body" identified the lungs and lung lobes and wrote:

"The lungs form a cushion for the heart, and with their cool air temper the heat of the heart. The lung changes the breath, as the liver changes the chyle, into food for the vital spirit; the rest of the smoky or noxious air, especially the vapors of fevers, is expelled".

Niciola Massa, from Venice, (1485-1569), published an anatomical treatise in 1536. About the lungs he wrote:

"The function of the lung is to cool the heart with its intake of air drawn through the rough artery (trachea). This air is not a pure element, but an airy body that can nourish, although Galen, that great philosopher, thought he understood it in another sense, for air prepared in the lung itself nourishes or restores the spirit."

It is of interest that none of the anatomists in this era saw fit to publish illustrations of their works.

Leonardo da Vinci, (1452-1519), provided very pleasing anatomical drawings of the human. However, he did not draw the human lungs, and the illustrations that he shows of the human upper airways are most likely that of a pig. It is likely that he had problems in seeing the human lung because of early putrefaction, although he regularly observed cadaver dissections. One of his body cavity drawings is reproduced on the right. Note that the heart is displayed, but that the lungs are virtually absent.

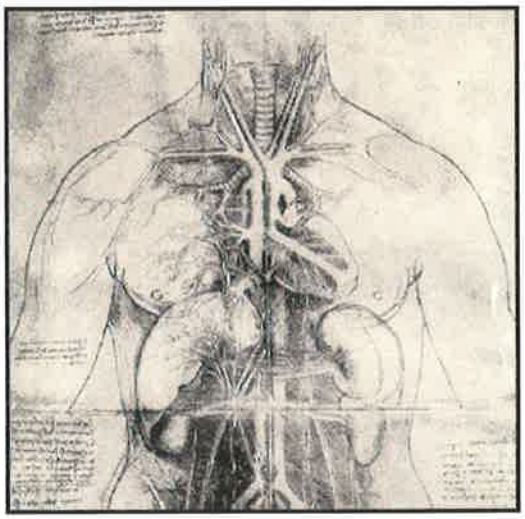
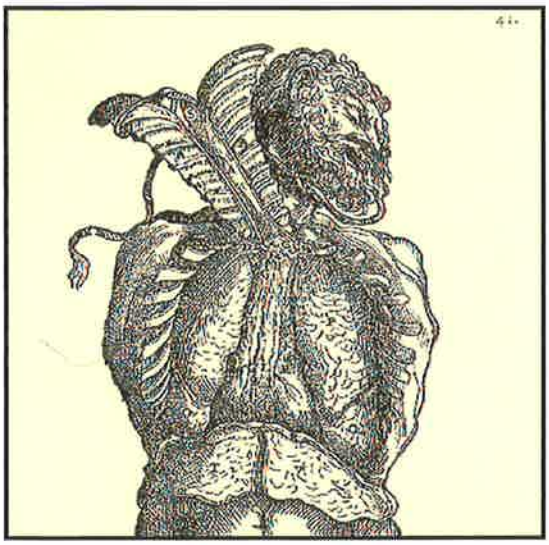


Illustration from Leonardo da Vinci. The drawing shows the trachea and main bronchi, with no lung detail.

The more correct structure of the human lung was produced by Andreas Vesalius, (1514-1564), who was appointed Professor of Surgery and Anatomy in Padua at the age of 23, in 1537, and left there in 1542 (1). He practiced public as well as closed dissection of human corpses. He is pictured on the right. Each "anatomie" or dissection would apparently last 3

days before there was too much putrefaction to continue. He showed that the teachings of Galen, still followed in Europe at that time were demonstrably wrong in many areas. He arranged for publication of De Humani Corporis Fabrica (On the Structure of the Human Body) in 1543, and again in 1555. A pupil of Titian, Jan Stephanus Calcar, probably did the illustrations for this (see illustration on the right). Vesalius moved to the court of Charles V in 1544, and the pro-Galen physicians there attacked his views, and gravely defamatory accusations were made against him. The reputation that his now published book had, seems to have been helpful in allowing him to maintain his position. However, he stopped doing his dissection work. It is also of interest that he did not name body parts that he discovered after himself as was the custom of the day (such as Fallopius and Eustachius). Vesalius' death remains a mystery.

Portrait of Vesalius from 1542. Shown with a dissection of the arm.



From the Fabrica, showing the dissected human lungs within the thoracic cavity.

Marcello Malpighi, (1628-1694), utilized the microscope that had been improved by van Leeuwenhoek, to describe histology, and he described the microscopic anatomy of the lung, especially the alveoli. He had major contributions to embryology, using chicken eggs as a means of noting when and from where the organs developed (2). Jean Lamarck recognized the concept of a cell in 1809, and the notion that cells cooperated to make plant or animal structures was proposed by Matthias Schleiden, and Theodur Schwann in 1838-1839. Rudolf Virchow proposed in 1858 that cells came from other cells,

Portrait of Malpighi, by Carlo Cignani.

and the field of cell biology was developed. Since then there has been the development of molecular biology, and increasingly advanced imaging techniques (electron microscopy and radiologic techniques), allowing for the mammalian body to be studied in unbelievable detail.

The human respiratory system has evolved, not surprisingly, a well-developed and complex system for protection from environmental hazards. With the recognition of adverse air quality, the initial host response is to cough or sneeze, in an effort to evict the offending stimulus. Removal from the scene may occur. If this fails, a proportion of the population experience chest tightness, breathlessness, increased mucous production or wheeze, consequent upon airway narrowing (in some instances classified as bronchial hyper responsiveness or asthma) (3). A further line of defense is the ciliated mucosa in the airways with it s associated thick mucous coating. Next, there are the cellular and biochemical defenses that exist in the proximal and, to an even greater extent within the distal lung (4). Finally, the lung has mechanisms by which effector cells and molecules can enter the lung from the circulating blood.

Inflammation is increasingly being characterized down to the molecular level. However, the classic description of inflammation into the component observational changes of redness, swelling, warmth and loss of function of the affected part, still provides the basis by which inflammation can be easily appreciated. The redness is the result of increased blood supply, the swelling the result of oedema fluid and effector cells extravasating from the blood vessels, and the loss of function from either pain from the swelling, or from loss of an effective capillary bed, as a result of the swelling.

Our bodies derive energy from the metabolism of molecular oxygen, which is delivered to all cells after it enters the blood system through the alveolar region of the lung. Most of the molecular oxygen is metabolized within cells in organelles that probably at one time were independent life forms, namely the mitochondria. Within the mitochondria, the molecular oxygen gives up four electrons through a series of tightly coupled single electron steps known as the electron transport chain. This ultimately results in the production of adenosine triphosphate (ATP), subsequently used by most energy requiring reactions within all cells in the body.

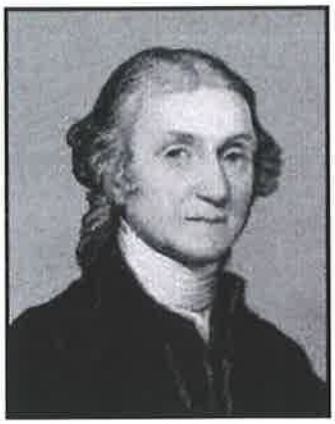
Whilst molecular oxygen, and the final product of the four electron reduction of oxygen, namely water, are not *per se* toxic, the metabolites that occur during the single electron reduction steps may be. The primary metabolite is the superoxide anion radical (O_2^-). Hydrogen peroxide (H_2O_2) can be produced from the O_2^- by the action of the enzyme superoxide dismutase, which converts the O_2^- into oxygen and H_2O_2. Other enzymes such as catalase and glutathione peroxidase usually remove the H_2O_2. The hydroxyl radical ($HO^\bullet$) can be produced by the Fenton reaction. In this reaction a metal, usually iron in the ferrous form (but also other metals such as copper), can react with H_2O_2 to form $HO^\bullet$ and ferric iron. Whether this occurs to a significant degree in biological systems remains controversial. In addition, there is continued debate as to whether O_2^- can also react, in biological systems with H_2O_2, in the presence of ferrous salts, to form $HO^\bullet$. This reaction is called the iron-catalyzed Haber-Weiss reaction, or sometimes the superoxide driven Fenton reaction. This has been recently very well summarized (5).

These metabolites of oxygen are commonly called oxygen-derived free radicals (ODFR), or reactive oxygen species, in view of their marked propensity to capture or donate the free unpaired electron to or from other molecules in close proximity. This often leads to loss of the target molecules functional or structural integrity, and to the development of more complex organic radicals in a chain reaction process. One common radical, for instance, is the peroxynitrite radical, the product of the reaction between superoxide anion and nitric oxide. Hydrogen peroxide is not strictly a free radical, but is usually included as a reactive oxygen species by virtue of established use of this term. It is clear that, to preserve health, that these ODFR must be inhibited or scavenged when they appear. Indeed this appears to be the case, and there are a series of enzymatic ODFR that have been discovered over the last 30 years. These are superoxide dismutase (for O_2^-), catalase or glutathione peroxidase (for H_2O_2), and the compounds which eliminate free iron (such as chelators), may reduce or eliminate the production of $HO^\bullet$.

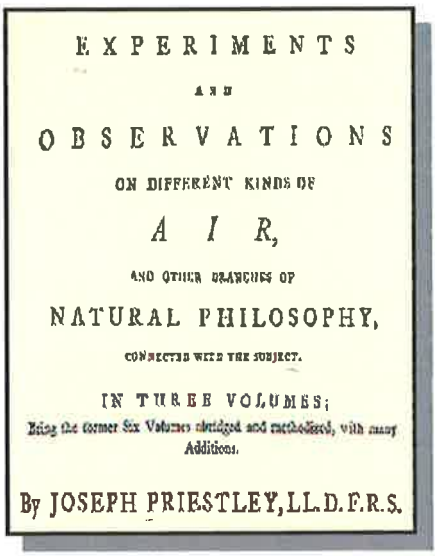
It is reasonable to note that iron metabolism is very regulated in the body to try and eliminate free iron. There are proteins which carry iron (transferrin), proteins that store iron (ferritin) and proteins that bind any iron that may be released by the destruction of haemoglobin, namely hemopexin (binds to free haem) and haptoglobin (binds to free haemoglobin). The same tight control also occurs in the case of copper (caeruloplasmin binds and transports copper) (5, 6).

Reactive oxygen species are also produced by the reaction of ionizing radiation with water. This has recently been summarized (7). It also must be noted that oxygen derived free radicals are produced by some cells as a means of defense or attack - the cell most commonly studied in this regard is the blood polymorphonuclear neutrophil (8-10).

Early observations on the effect of oxygen at greater than ambient concentrations and/or pressure began soon after oxygen was isolated by Joseph Priestley, 1733-1804 (11, 12). Priestley (see likeness on the left) was not a trained scientist, but primarily a theologist, but wrote an impressive large text of 3 volumes describing his experiments with various gases, one of which he discovered supported combustion, and which he called "dephlogisticated air". People who did not agree with his beliefs deliberately burned down the laboratories that Priestley worked in; and he moved because of that from England to Pennsylvania.



Joseph Priestley



EXPERIMENTS
AND
OBSERVATIONS
ON DIFFERENT KINDS OF
A I R,
AND OTHER BRANCHES OF
NATURAL PHILOSOPHY,
CONNECTED WITH THE SUBJECT.
IN THREE VOLUMES;
Being the former Six Volumes abridged and methodized, with many Additions.
By JOSEPH PRIESTLEY, LL.D. F.R.S.

Lavoisier, 1743-1794, named "dephlogisticated air" oxygen, and proposed the oxygen theory of combustion. A very innovative scientist, Lavoisier was executed by the guillotine in the French revolution.

Lavoisier

Paul Bert (13) [Bert, 1943 - translation #946] in 1878 demonstrated that it was likely that the increase in tensions or partial pressure of oxygen, rather than the concentration of oxygen in the inspired atmosphere, was responsible for adverse effects in exposed animals. The observations of Bert established better conditions for the use of oxygen under

pressure, and provided a firm basis for the ultimate development of relatively safe diving apparatus. The finding of Bert, noted above, was referred to as a fundamental law by Smith (14) in the report of his studies in 1899. Smith began these studies to examine whether high pressures of oxygen were toxic to bacteria. Although he began these studies with animals that had been infected, he quickly became engrossed with studying the toxic effects of normobaric oxygen on otherwise healthy mice and birds. In part, he performed the studies to refute the claims of Regnault and Reiset (15) that no pathological changes ensued on the exposure of animals to atmospheres rich in oxygen.[1]

Smith worked at Queens College, Belfast and it is of interest to review his experimental methods and results. He worked with small animals, mostly mice and larks, although did expose an occasional guinea pig or rat. His exposure chamber was "*constructed out of strong brass tubing of 6" in diameter and 15" in length. The capacity was almost exactly 6 litres. To permit observation of the animal during the experiment one end of the chamber was made of thick glass. This was fitted very carefully, and the fittings were covered with layers of modeller's wax. The opposite end of the chamber would be detached, and was formed out of a disc of gunmetal 3/8" in thickness. This disc rested on a strong collar, and between it and the collar was placed an inter-rubber washer. To close it down tightly, a number of bolts were used, arranged around the circumference, on a plan similar to that adopted in constructing the lid of an autoclave. The inner surface of the chamber was painted white. Two pieces of brass tubing were soldered into openings in the metallic disc, and by means of them a current of air or oxygen could be passed through the chamber after it was closed.*".

Once the animals were placed into the chamber and the lid screwed down, no further access to the animals was allowed until the experiment was complete. Oxygen or air was fed into the chamber until the required concentration of oxygen, and/or pressure, was obtained – and then the chamber completely sealed with the pressure being observed from a 10' high mercury manometer attached to the chamber. Whilst "*a supply of food sufficient for 2 or 3 days*" was provided, there was apparently no water for drinking.

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[1] The differing observations with regard to oxygen toxicity at this very early time have continued to be reflected in the reports dealing with oxygen effects up until today.

Sawdust and cotton wool was provided "*to keep the animal warm and dry*". A gauze basket containing potash lime "*to absorb the carbonic acid*" was also provided.

There was no measurement or control of humidity in the chamber. Temperature measurements were sometimes reported with the average temperature of the chamber being 19°C, with variation from 17°C to 22°C. Clearly Smith was concerned about the effects of temperature. He reports "*Special care was taken to avoid any fallacy from a fall in the body temperature of the mice*" and he indicates that at times "*the pressure chamber was kept warm by a gas flame*".

Smith made a number of casual observations, not otherwise obvious from his results. Firstly he comments that a "*young mouse gives way more quickly than one that is fully grown*". He found that there were two kinds of oxygen toxicity – one form at oxygen tensions close to 100% that resulted in death or disease from a severe pulmonary oedema, and the other form being severe convulsions which occurred at very much higher oxygen tensions (on one occasion exploding his chamber). He also showed that sub-lethal exposure to near 100% oxygen subsequently seemed to protect animals from higher oxygen tensions – the animals did not develop convulsions as early. He also concluded that whilst the effects on the lungs was similar in birds and mice, but that birds were much more sensitive to developing convulsions at high oxygen tensions than were mice.

In all he studied 33 mice (including one half grown one), 1 guinea pig, 1 rat and 15 larks. He was able to relate his conclusions to the possible toxicity of oxygen and pressure seen in men working underwater in caissons – this was very much an occupational hazard at that time. Indeed, Sir William Osler in the 1892 edition of the Principles and Practice of Medicine writes "*This remarkable affection, found in divers and in workers in caissons, is characterized by a paraplegia, more rarely a general palsy, which supervenes on returning from the compressed atmosphere to the surface....*" This observation, of course, deals with the problem of dissolved gases bubbling in the blood or tissues, rather than the toxic effects of oxygen *per se*.

An interesting study was performed on turtles and frogs in 1927 (16). This study showed that turtles could tolerate 90% oxygen exposure and independently, exposure at

37°C. However, when the 5 turtles were exposed to these conditions simultaneously, they died from pulmonary oedema. The study also reported that young turtles were susceptible to the same phenomenon, but were not as sensitive to the oxygen. The conditions were repeated with frogs. Frogs were found also to be oxygen tolerant, with no sign of discomfort, but the temperature effects on oxygen tolerance were not possible, as the frogs did not survive at 37.5°C in room air.

In 1981 a somewhat similar study was reported (17). This study examined only hyperbaric hyperoxia, but across species and temperature. The authors studied water-breathing animals (eels, trout and frogs) and concluded that under hyperbaric conditions oxygen toxicity, as measured by animal death, is directly related to the aerobic metabolic rate.

Animal exposure studies continued to be performed to elucidate evidence for oxygen toxic effects on the lung. In 1967 an excellent study of pulmonary oxygen toxicity in rat lungs was published (18). This was a comprehensive study of pathology including electron microscopy. The study defined the time course of the lung injury. They studied 90 Sprague-Dawley rats, divided into 5 groups. The first 4 groups had exposure to 98.5% oxygen at one atmosphere pressure for 6, 24, 48 and 72 hours respectively. The last group had exposure to room air only. The temperature of the exposures was 72-76F with the relative humidity of 45-47%. Results showed that the animals exposed to 6 and 24 hours of oxygen had normal pathology by light microscopy, with animals exposed to 48 hours starting to get peri-vasular oedema. At 72 hours the lungs were very abnormal with thickening of the interalveolar septa, and alveolar spaces filled with a haemorrhagic exudate containing fibrin, leukocytes and macrophages. Morphometry showed that 65% of all alveoli were obliterated – with about 30% being normal. Using transmission electron microscopy interstitial oedema was found at 48 hours of exposure. At 72 hours the pulmonary capillary endothelial cells showed *"drastic changes".* The endothelium became detached from the basal lamina, with variable levels of endothelial cell necrosis. The epithelium of the alveolus appeared to be normal even at 72 hours. The authors concluded, *"the primary cellular damage was located in endothelial cells"*

In 1969 a similar study was undertaken in monkeys. Sixteen monkeys (*Macaca mulatta*) were studied. They were exposed to 99-100% oxygen for 2,4,7 and 12 days.

Temperature and humidity of the exposures are not given. Two animals were exposed to 8 and 13 days of oxygen, and were then weaned back to room air for 56 and 84 days before final study. Four additional animals were used as controls. Of these 22 animals, 9 were not studied as they either died during the oxygen exposure, or their lungs were infested with mites. There were minor changes after 2 days of oxygen. At 4 days the alveolar walls appeared severely damaged. Most type 1 alveolar cells were swollen or detached from their basal lamina. Indeed, 90% of these cells were very damaged. Alveolar oedema was observed involving 13% of the air spaces. There was interstitial oedema and some damage to endothelial cells. At day 7, a regenerating layer of type 2 alveolar cells, involving 80% of the alveolar surface replaced the denuded epithelial type 1 cells. There appeared greater endothelial cell destruction and damage, with continuing interstitial oedema. At day 12, the proliferative process was even more advanced with increasing type 2 cells, and interstitial oedema. The study concluded that there were 2 phases in the oxygen toxicity response in the monkey lung – an exudative reaction lasting 4 days, and a proliferative reaction prominent on day 12, but reverting substantially to normal after the oxygen exposure stops; with some septal scarring, capillary dilatation, and minimal air-blood barrier thickening. These changes are different to the rat studies noted previously – this may be a species difference, or a matter of sampling different time points.

Another study using rats and electron microscopy was performed in 1972 (19). Pertinent to this discussion are the results of exposure of 3 rats to 90-96% for 6 hours, and 3 rats for 24 hours to 99-100% oxygen; both exposures at one atmosphere. Temperature and humidity of the exposures were not recorded. After 6 hours of exposure there were no changes at the light microscopy level, at 24 hours there was peri-vascular and alveolar fluid. With electron microscopy at 6 hours they reported interstitial oedema, that seemed to *"cause the endothelial cells to encroach on the capillary lumen".* They report that the capillary lumens appeared empty, with alveolar type 2 cells being a little more electron dense, but otherwise no real change in appearances of the alveolar type 1, 2 or 3 cells. [1] They also report proteinaceous material as being present in the alveolar lumen. Minimal quantification was performed, demonstrating small changes in alveolar size, and a slight reduction in alveolar macrophage numbers. The authors conclude that *"after 24 hours both*

[1] (N.B. this group is one of the few to identify a separate alveolar type 3 cell in the rat lung, but since that time alveolar type 3 cells have generally not been separated out in rat lung).

endothelial and epithelial damage was seen with the electron microscope and there was also leakage into the alveolar space". They contrast these findings to that of Kistler above (who observed no changes for 48 hours), but offer no explanation for the differences observed. However, Meyrick used 23cm of pressure for intra-tracheal fixation (as opposed to Kistler who used 20 cm of pressure), the species of rat used by Meyrick is not given, and Meyrick used specific pathogen free animals, in contrast to Kistler who used non-pathogen free animals – indeed of the 90 rats that Kistler studied five were rejected because of pneumonia. Kistler also studied "young" rats, which are probably different in age to the adult rats studied by Meyrick.

A further study looking at structural changes in rat lungs soon followed in 1980 (20). Specific pathogen free Charles River CD adult male rats were studied. Exposure to 100% oxygen was in small chambers, with gas changes 7-8 per hour, with food and water provided. Humidity and temperature were not recorded. Lung fixation was at 20 cm of water pressure through a tracheal cannula. Comprehensive morphometric studies were performed. Eight control animals exposed to air, and four animals each at 40 hours, 60 hours and "terminal" 100% oxygen exposures were analyzed. The air-exposed animals are not reported as being exposed in the small chambers. The terminal group had a mean survival time of 64 hours. A qualitative description of changes is given as follows. At 40 hours of exposure there was a slight but definite interstitial oedema, with more platelets and red cells in the capillary lumen. Small regions of disruption to capillary endothelial cells were observed. After 60 hours of exposure, packing of red cells within the capillary bed was observed. Interstitial oedema was present. The endothelial cells exhibited swelling, margination of nuclear chromatin, with, at times, endothelial cell necrosis. In other regions the alveolar region appeared almost normal. Alveolar *"epithelial changes were subtle"* and are not otherwise described. The morphometry supported the notion that there is a large reduction (>30%) in pulmonary capillary cells after 60 hours of 100% oxygen, as well as interstitial thickening, and an increase in interstitial polymorphonuclear leukocytes (from 0% to 11%). It should be noted that in the control group, 27% of cells were "indeterminate", but that in the 100% oxygen exposed group 49% of cells counted were "indeterminate" – described as small round cells with no distinguishing cytoplasmic organelles.

Coincident with this study was a light and electron microscopy report of the effects of breathing 100% oxygen in rabbit lung (21). Temperature was kept at 21-22°C for the

exposures, which occurred with a control group of 3 animals, and groups of 3 animals exposed to 100% oxygen for each of 24, 48, 66 or 72hours. Intra-tracheally administered cytochrome c (MW 12,523 Dalton, molecular radius approx. 17 Ang.) was used to examine the permeability of the alveolar membrane. Results showed no evidence of interstitial oedema, or changes in the epithelial or endothelial cells. Inflammatory cells were observed in the interstitial space by 48 hours, and increased with exposure time. At 72 hours focal alveoli were filled with a homogenous, occasionally flocculent electron-opaque material. The cytochrome c marker was visualized along the basal lamina of the alveoli, as well as within vesicles of alveolar type one cells, and of capillary endothelial cells. The authors postulate an increased vesicular transport as one cause of increased permeability seen.

In 1985, a further study examining oxygen toxicity in rat lung was reported (22). Two genetically different rat strains were examined – the Fischer and the Sprague-Dawley. This study examined only isolated perfused lungs that were exposed to 3 hours of hyperoxia, or an oxidant challenge in the form of perfused hydrogen peroxide. Basically, this study suggested that the Fischer strain is more susceptible to oxidant lung damage than is the Sprague-Dawley strain.

Crapo continued with his series of pathology studies in the rat lung associated with 100% oxygen toxicity with the publication of a study showing the pattern of inflammatory cell accumulation in the rat lung after 100%, and after the oxygen tolerance inducing 85% oxygen exposure (23). In this study specific pathogen-free adult Charles River CD rats were used. This study showed that under both exposure conditions, platelet aggregation occurs first in the capillary lumen followed by neutrophils. These changes are initially seen at 40 hours and are more obvious at 60 hours in the 100% oxygen exposed group, and with a slower time course, that is of much less intensity in the 85% oxygen exposed group.

In 1991, a further study on the effects of breathing 100% oxygen was undertaken in a primate, namely, the baboon (*Papio papio* and *Papio cynocephalus*) (24). This study examined a total of 35 male adult baboons, exposed for intervals of 40, 66, 80 and greater than 80 hours. After 80 hours exposure the animals were intubated and maintained on a ventilator, to reduce undue animal stress. Baseline pre-exposure pathology was taken from each animal by removing the right middle lobe at thoractomy one month before the exposure commenced. Exposures occurred in a large plexiglass container, with an

ambient temperature of 20-22°C, and relative humidity of 40-70%. Lung inflation for histology was at 30 cm H_2O. Leukocyte imaging studies were performed in the whole animal using ^{111}In-labeled oxine, lung permeability studies using radio-labeled intra-vascular tracers (^{51}Cr-labeled erythrocytes, ^{125}I-labeled albumin, $^{3}H_2O$, and ^{14}C-labelled urea). Morphometry of the lung tissue pathology was not performed. Broncho-alveolar lavage studies were also not performed. Results indicated no injury at 40 hours. At 66 hours there was endothelial cell swelling and increased intravascular neutrophils – the radio-labeled studies mentioned above were not performed at this time point. At 80 hours there was alveolar wall thickening, extensive endothelial thickening, with some endothelial cell destruction, and some disruption of the alveolar type 1 epithelium. Pulmonary accumulation of leukocytes had increased by 111%, and extravascular lung water increased by 254% (the paper in the text says 154%) as measured by the radio-labeled studies. Apparently chest radiographs and room air blood gas studies remained normal at 80 hours, but no information about these parameters are presented. As the exposure time exceeded 80 hours further pathology changes occurred, with now extensive destruction and swelling of endothelial cells, extensive interstitial oedema, obstruction of some capillaries by cells and cell debri, and destruction and swelling of alveolar type 1 cells, with hypertrophy of alveolar type 2 cells. Extravascular lung water increased by 391% over baseline. Cardiovascular and pulmonary lung volumes did not change appreciably until the 80 hour time point. The authors note that the response seen in the baboon is different to the rat, in that the alveolar type 1 cell is much more injured in the baboon. They also note similarity in this regard to the previous primate study that Kapanci had reported in 1969, and which is summarized above – although it must be noted that Kapanci found considerably more type 1 cell damage and much less endothelial damage. Whether these differences reflect the different primates used, the exposure conditions, or tissue-sampling problems is not able to be determined. The authors conclude *"The data indicate that histological changes by electron microscopy precede physiological responses to hyperoxic pulmonary injury in baboons by as much as 14 hours and that the physiological responses to early hyperoxic injury are relatively insensitive to the pathological injury".*

The effects of oxygen in the lung in humans remained largely anecdotal with limited information available from retrospective studies. Interest was, of necessity, aroused with the widespread use of mechanical ventilation of patients and the need for inspired oxygen

in normal subjects at environmental extremes such as high altitudes (in particular, military flights and space flight) or undersea exploration.

An initial study in 1945, reported by Comroe, was undertaken in normal men. Comroe used a 24-hour oxygen exposure period at sea level and a simulated altitude of 18,000 feet (25). These studies were performed to define if oxygen toxicity occurred in normal humans, as several previous studies, reported in the late 1930's reported either oxygen intolerance after seven hours, or absolutely no toxicity at all. Comroe questions the oxygen delivery systems employed by these earlier studies and notes that no control subjects were used. In this 1945 study, six subjects were exposed to 98-99% oxygen through an oxygen tent. Eighty-four subjects were exposed using a full-face mask. Some of the face mask subjects were exposed to bottled air in a blinded fashion. The temperature of the inspired gas in the hood was 21°C, and in the facemask experiments it was 31°C. The findings were that around 60-70% of subjects experienced substernal discomfort with oxygen breathing, and that this was present during breathing of >75% oxygen, but not seen with breathing bottled air, or oxygen concentration of <50%. Conjunctival irritation occurred in 23% of those breathing 100% oxygen, and in 10% of those breathing room air, an insignificant difference. The vital capacity was also noted to be reduced in 63 of 80 cases breathing 100% oxygen through the facemask, but no cause for this was found, although the authors speculate about the reduction as follows. "*In view of the alveolar damage that occurs in animals with longer exposure to 100% oxygen, we believe that these subjects had signs of early pulmonary irritation*". There was no change noted in chest x-rays or in clinical examination to support the notion of an alveolar injury.

In 1965, a retrospective autopsy human study from Sweden was reported (26). The study included 150 autopsies that were stratified according to the amount of oxygen that these patients had been exposed to preceding death, by retrospective chart analysis. Hyaline membranes in the alveolar region were found in 28 of the 150 cases, and the authors felt that oxygen therapy was a likely cause in at least 3 of these subjects.

In 1966 a further human study was reported from the 6570th Aerospace Medical Research Laboratories (add 111). Four normal human volunteers were studied in a controlled environmental chamber, breathing 98% oxygen for 30, 48, 60 and 74 hours respectively. The temperature was 72-74°F and the relative humidity 40-60%. Spirometry,

lung elastic recoil and diffusing capacity were all measured, as well as other lung function. Chest radiographs were also taken, and physical examinations were performed. Results indicated that all subjects developed ill-defined, migratory chest pains during the first 24 hours – these subsequently disappeared. No change in chest xray or physical examination findings was demonstrated. The vital capacity decreased in all subjects ($p<0.05$ as measured by the maximum fall). No table is presented with the actual values, although they are plotted on graphs. The subject exposed to 30 hours of oxygen had about a 2-liter fall in vital capacity. The subject exposed to 48 hours of oxygen had no change in vital capacity during the exposure. The subjects exposed to 60 and 74 hours of oxygen appear to have had minimal change in vital capacity of less than 500mls. Resting arterial blood gases showed no significant change. Lung elastic recoil was only measured in 2 subjects and showed no significant change. Diffusing capacity was measured manually, both breathing room air, and 73% oxygen. There were minor changes breathing room air, namely from values of 30 (ml CO/min per mm Hg) to 22, from 32 to 26, from 32 to 24, and from 20 to 19 before and after exposures in each subject respectively. Breathing 73% oxygen the changes were 21 (ml CO/min per mm Hg) to 21, from 18 to 17, from 24 to 19, and from 15 to 16 in each subject respectively. No statistical comparisons were performed. These diffusing capacity changes do not appear to be very marked, with no change at all in the last subject exposed to 74 hours of oxygen. Yet the abstract for this study reports *"There was a fall in vital capacity which was rapidly progressive after 60 hours of exposure and three subjects exposed longer than 30 hours had drops in pulmonary diffusing capacity."*

Nash (27), in 1967, in a study that is widely quoted in current literature, reported the development of pulmonary lesions associated with oxygen therapy and artificial ventilation. This study was a retrospective autopsy study of 70 patients who had prolonged artificial ventilation and either 21-90% oxygen, or 90-100% oxygen. The control group was 70 patients who died during the same period as the study group matched for age and gender, and somewhat matched for underlying disease, but who had no mechanical ventilation. The study found that "hyaline membranes" occurred in 17 of the 70 patients from the ventilator group and in 2 of the non-ventilated controls. They also found severe interstitial oedema and early fibrosis in 23 of the ventilated patients compared to 1 case in the control population. Statistical analysis of these numbers was not performed. The study observed that those subjects who died on a ventilator had heavier lungs, and that the group who had

been ventilated with 90-100% oxygen had heavier lungs than the group ventilated with 21-90% oxygen; this same group also had more interstitial oedema and fibrosis on microscopy. No morphometric analysis to quantitate the pathology was performed. Nevertheless, these results are somewhat self-evident in that it would be expected that patients on a ventilator and receiving a maximum amount of oxygen replacement would have greater lung disease than those receiving less oxygen replacement, or those not requiring ventilation. In sub-group analyses there was no significant difference in the presence of "hyaline membranes" in those subjects who had 90-100% oxygen compared to those with less oxygen. The authors conclude *"It should be emphasized that we have not established a definite cause-and-effect relation between the characteristic pathological appearance and <u>any</u> particular facet of therapy".* The authors further hypothesize that there are two phases in the pathology associated with oxygen exposure – an exudative and a proliferative phase, and they state *"It is our impression that these two categories are stages of a progressive deterioration"* but that *"a definite cause and effect relation has not been established by this study"*

These studies were followed by further studies using subjects with normal lungs. Two of these studies are discussed in more detail – both were reported in 1970. In the first (28), 9 normal male volunteers were exposed to 100% inhaled oxygen at ambient pressure, through a full-face mask, and monitored for cardio-vascular and lung problems during and for an hour after the exposure ceased. The first 5 men were exposed to 100% oxygen for 6 hours, and the remaining 4 men exposed to 9, 11, 12 and 12 hours respectively. The only abnormal effect was a burning sensation in the eyes of 4 of the subjects with conjunctival injection. There were no other effects noted. Environmental or body temperatures were not mentioned in this study. In the second study (29), 10 young patients with irreversible brain damage and on ventilators were studied. Five patients were studied breathing air, with 5 patients breathing pure oxygen. Periodic cardio-pulmonary physiological measures were made, and autopsies performed (in 2 subjects, one from the air group and one from the oxygen group the autopsy studies of the lungs were incomplete). The authors further comment that "*both groups of subjects were mildly hypothermic, but there was no significant difference in body temperature between the groups*". To standardize arterial blood gas measurements the air breathing group were ventilated intermittently for periods of 30 minutes with pure oxygen. The most striking

differences reported in the two groups was that the paO_2 measured after 100% oxygen breathing for 30 minutes fell significantly after 41 hours of 100% oxygen exposure. On closer inspection of the data however, it is not really such a striking observation. The oxygen and air breathing groups are slightly different at the 41-50 and 51-60 hour intervals ($p<0.05$), but more different at the 61-70 hour interval ($p<0.01$). However, there is no information given in the paper as to how many of the subjects survived until this time period, so the data at the 61-70 hour period may be based only on a very small number of observations. The other striking observation was that the two groups did not vary much in other measurements – lung compliance was not different; the chest radiography tended to have more bilateral disease in the oxygen exposed group; the lungs tended to be heavier in the oxygen exposed group; there tended to be greater intrapulmonary shunting in the oxygen exposed group; and the histology of the lungs in the two groups was not different, (and hyaline membranes suggested to be characteristic of oxygen toxicity were not seen). These observations were interpreted to indicate that 100% oxygen does adversely affect the normal human lung, certainly after 41 hours of continuous exposure at ambient pressures. However, the lack of the characteristic hyaline membranes in the pathology, and the control patients also having extensive and similar lung changes, is possibly indicative of no major oxygen effect in this group.

To add further to the human observations, and reported as a companion article to the above, Singer (30) found no difference in post-operative cardiac surgery patients ventilated for 15-48 hours with 100% oxygen, as compared to an otherwise similar "control" group ventilated with less than 42% oxygen for periods of time from 15-44 hours. Indeed they conclude that "*Artificial ventilation with pure oxygen in this series of patients with intrapulmonary shunting after cardiac surgery had no demonstrable effects during the period of this study.*"

The subject of human lung toxicity from normbaric oxygen exposure was re-visited in an excellent review article from Deneke and Fanburg in 1980 (31). They reference most of the studies quoted above and conclude that normal human beings "*can tolerate 100% oxygen at sea level for 24-48 hours with little if any serious tissue injury. Pulmonary damage results from longer periods of exposure,*". They go on to say "*Inspired oxygen delivered at concentrations between 50-100% carries a risk of lung damage over a longer period of time, and the duration required to produce damage seems to be inversely*

proportional to the concentration of inspired oxygen." The direct evidence for that in human subjects seemed somewhat conjectural based on the evidence from human studies, but their conclusions were likely to be true, taking into account as well the effects of oxygen on other mammals.

Further studies followed. These suggested that the development of retro-sternal discomfort, first noted by Comroe in 1945, was likely the result of involvement of the central airways with tracheitis (32). In this study ten normal human subjects were exposed to 90-100% inhaled oxygen through a full-face mask, with a relative humidity of 60-70%, for a period of 6 hours. The inspired gas temperature was not given. None had any retrosternal discomfort. One had conjunctivitis. None had changes in lung function tests. Fiberoptic bronchoscopy (this was performed 3x during the study in each subject) indicated *"focal areas of redness, edema, and injection of small vessels in the trachea of all of the subjects".* This was not otherwise quantitated. Tracheal mucus velocity was reduced in the oxygen exposed group, as studied with the movement of teflon discs in the trachea, observed at bronchoscopy. There was no control group studied, and no blinding performed. Since none of the subjects studied had retro-sternal discomfort it is an assumption made by the authors that retro-sternal discomfort observed by others was related to the tracheitis observed in this study.

The more distal alveolar injury, inferred from the animal and the human studies already quoted, was evaluated with bronchoalveolar lavage (BAL) [Davis, 1983 #957]. This study was performed on sixteen normal non-smoking volunteers. The subjects had an initial base-line BAL performed breathing room air (as well as lung function and physical examination) and then two weeks later this was repeated after breathing 95-100% oxygen through a face mask for 18 hours. Temperature and humidity of the inspired gases are not quoted. Appropriate controls (6 of the 16 subjects) were performed to check that results were not due to the facemask, or to the BAL procedure. Substernal discomfort occurred in 9 of the 14 subjects exposed to hyperoxia and mild erythema in the airways was noted after oxygen exposure in 6 of 14 subjects – the paper does not indicate whether these two observations were linked in the same subjects. The study found an increase in the lavageable albumin and transferrin in the oxygen exposed group, but no difference in cells recovered by the lavage procedure. Of interest was the additional observation that alveolar macrophages in the oxygen treated group did not spontaneously release

neutrophil chemotactic factor (but did release fibronectin and alveolar-macrophage-derived growth factor).

A further study examined BAL albumin as well as the clearance from the lung of inhaled 99m Tc-diethylenetriamine pentaacetate (Tc-DTPA) (33). Eighteen non-smoking normal human subjects were allocated to one of 4 groups. Four subjects had exposure to 21% oxygen, four subjects to 30% oxygen, four subjects to 40% oxygen and six subjects to 50% oxygen. The gas mixtures were delivered through a facemask for 44 hours. The gas mixture was humidified in two subjects only, and temperature not defined. Subjects underwent Tc-DTPA clearance and BAL studies x2 in an alternate manner 7 days apart. There were no symptoms reported by the subjects, no changes in pulmonary function, and no change in the appearance of the airway mucosa at bronchoscopy. There were abnormalities demonstrated in those subjects breathing 50% oxygen suggesting increased distal lung leakiness – these were increases in BAL albumin, and increased clearance of Tc-DTPA. There were also increases in the BAL albumin in subjects breathing 30% and 40% oxygen suggesting to the authors *"a dose-dependent relationship"*.

These last two studies, quoted above, suggested an early alveolar damage in human subjects exposed to normobaric hyperoxia.

A further study (34) examined six non-smoking, physically well, human volunteers who breathed an oxygen mixture through a mouth piece for three periods of 17 hours, randomly allocated to 21%, 40% and 100% oxygen, and at least one week apart. Here the inspired gases were maintained at 37°C, and humidified, with no report as to how that was achieved or monitored. The main end-point measured was Tc-DTPA uptake, with each subject as his or her own control, and symptoms. All subjects noted retrosternal chest pain after 6 or so hours of breathing 100% oxygen, but the study was not double-blinded. No conjunctivitis was reported. Further, no change in Tc-DTPA was noted indicating no peripheral alveolar leak as measured by this test. The authors concluded that the retrosternal chest pain is the result of tracheitis, although they had no measures of this in their study.

The sum of the literature on the effects of 100% oxygen breathing in the human subject remains therefore relatively incomplete, with conflicting reports as to the nature and time course of pulmonary oxygen toxicity.

Irwin Fridovich, at the Department of Biochemistry, Duke University Medical Center, reported in 1976 (35), on the function of a protein that had been isolated and purified some years before. This protein was called superoxide dismutase. From this observation a whole new field has arisen, namely that of oxygen free radicals in biology.

In the late 1970's and early 1980's energetic debate was occurring at scientific presentations whenever oxygen free radicals were being evaluated or ascribed a role in the generation of disease. On one side of the debate were scientists using such enzymes as superoxide dismutase to infer, by observation of the effects of these in excess or by their absence, that abnormal physiology was related to oxygen free radicals. On the other side of the debate were physical chemists who did not believe that oxygen free radicals existed in biological systems for long enough to have any relevance to a disease process.

Nevertheless, scientists now had at least one variable that they could examine in biological systems, namely superoxide dismutase, and more oxygen free radical scavengers were soon discovered in biology.

As the early planet earth evolved it is believed that free oxygen was not present in the early atmosphere. The 20% atmospheric oxygen that is now so critical to most life forms on earth was produced into the atmosphere from photosynthesis, with the free oxygen being produced in this process from water. As the level of oxygen rose in the atmosphere, life forms adapted to use the oxygen as a convenient and efficient way of deriving energy from food. However, the metabolism of oxygen in this way, as with so many convenient developments, was accompanied by the production of toxic compounds during the process. It is not surprising therefore that to accompany the adaptation to oxygen metabolism, there needed to be the adaptation to protect against these toxic metabolites.

Oxygen itself is a complex and unique molecule. Compounds at a molecular level have a nucleus and the surrounding electrons. The positive change of the nucleus is

balanced by the negative charge of the electrons. Although usually portrayed as a simple solar system type structure, the nuclear constituents are themselves complex, as are the electrons. The electrons behave according to the laws of quantum mechanics. The electrons have a "spin" direction, which generates a small magnetic field; in stable molecules the electrons are paired so that the spin of one electron in the pair is counter-clockwise to the spin of the other. This effectively generates two magnetic fields that neutralize each other. These molecules are called diamagnetic. The oxygen molecule is distinctly unusual because it has two unpaired electrons, and they have, of course, the same "spin" state. Thus oxygen is a para-magnetic molecule.

In most biological reactions between molecules, electrons are exchanged as a spin-opposed pair, maintaining the magnetic neutrality. Oxygen cannot accept a spin-opposed pair, as these would be joining the parallel-spinning unpaired electrons, and one of the added electrons would continue to have the same "spin" state as its partner to be, an energetically very unfavorable situation.

This problem is overcome in biology by having oxygen accept one electron at a time. Under these conditions electron "spin" states can be inverted by interaction with nuclear spins and the process becomes energetically favorable. Indeed in biological systems, oxygen has electrons added one at a time. With the addition of four electrons, oxygen is reduced to water. With this single electron reduction three intermediates are produced, namely superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$). These intermediates are believed to be responsible for the toxicity of oxygen, although precise details of this remain to be established. These and other toxic products of oxygen metabolism can also be produced through the following reactions, commencing with O_2^-, by itself a relatively weak oxidant, as follows:

$O_2^- + O_2^- + 2H^+ \Leftrightarrow H_2O_2$ – this reaction can occur spontaneously, or be catalysed by the dismutases;

$O_2^- + H_2O_2 \Leftrightarrow OH^- + HO^\bullet + O_2$ – this reaction is the Haber-Weiss reaction and is catalysed by free Fe^{+++};

O_2^- + $^{\bullet}NO \Leftrightarrow ONOO^-$ – in this reaction the superoxide anion reacts with nitric oxide to produce the peroxynitrite radical.

The last reaction with the production of the peroxynitrite radical has only recently been the recognized (36, 37). The peroxynitrite radical is a further key radical which can modulate tissue damage, and which can be produced from activated rat alveolar macrophages (38). However, under some conditions nitric oxide appears to be protective against the cellular damage by reactive oxygen species (39). In these studies, however, the nitric oxide was generated chemically, rather than from the enzyme nitric acid synthetase; and a subsequent study from the same group showed that nitric oxide greatly augmented the toxicity of hydrogen peroxide against E. coli (40). The chemistry of peroxynitrite has been recently reviewed (41). Nitric oxide is a powerful mediator of vascular tone. What is emerging is that nitric oxide, superoxide anion and superoxide dismutase may be intimately involved with the complex regulation of each other, and therefore of the biological effects that each may be primarily responsible for.

Oxygen derived free radicals are currently associated to a greater or lesser extent with almost all inflammatory disease states, including those that are thought to be mediated in part by the polymorphonuclear neutrophil (PMN) or other phagocytic cells. Activated macrophages kill micro-organisms and tumor cells by two separate oxidative pathways involving the synthesis of reactive oxygen species (42-44) and nitric oxide (45). Indeed, oxygen free radicals released by activated phagocytes and other effector cells were at one stage considered to be the most likely source of the toxicity of 100% normobaric hyperoxia (46). Polymorphonuclear cell depletion was shown to partially ameliorate the toxic effects of normbaric hyperoxia (47). However, the relative contribution of free radical damage compared to other agents released by the phagocytes such as proteases (48), and the precise site of the free radical damage (i.e. cell membrane, DNA or a specific protein) is poorly understood in most instances.

Indeed, most oxygen consumed by respiring cells is reduced by cytochrome c oxidase – this manages the four-electron reduction of O_2 to $2H_2O$ without the release of any of the toxic intermediates. However, in a bacteria (Escherichia coli or E. coli) it has been estimated that approximately 0.1% of the oxygen reduced by E. coli produces release

of the toxic intermediates – enough to cause cell death were defenses against their intermediates not present.

Superoxide anion (O_2^-) is relatively stable at the neutral pH that is usually found in mammalian systems. In water, O_2^- undergoes the following reaction –

$$O_2^- + O_2^- + 2H^+ \Leftrightarrow H_2O_2 + O_2$$

However, at neutral pH this is relatively slow and the O_2^- also has the opportunity to interact with other available targets such as, for instance, sulphates. It is evident that the interaction of a free radical will lead to chain propagation until the radical species is removed or quenched by an "anti-oxidant". The sulphite interaction can be represented by:

$$SO_3^{--} + O_2^- + 2H^+ \Leftrightarrow SO_3^{-\bullet} + H_2O_2$$

And this reaction can then continue to be propagated without any further O_2^- being needed. O_2^- reacts with many biological compounds including the polyunsaturated lipids that occur in the cell membrane. This lipid peroxidation is one feature that can be measured in oxygen free radical damage.

Biological systems need therefore to defend against O_2^-. Indeed the superoxide dismustases catalyze the reaction –

$$O_2^- + O_2^- + 2H^+ \Leftrightarrow H_2O_2 + O_2$$

- thereby removing O_2^- from interacting with biological important molecules. It is not surprising that there exist a family of superoxide dismutases that are abundant and widely distributed within the mammalian cells, and in the extra-cellular fluids.

One of the first of these discovered was the superoxide dismutase found in the cytosol of eukaryotic cells. This protein contains both copper and zinc – the copper undergoes a valence change during the enzymatic interaction with superoxide anion, whilst

the zinc is thought to be a structural component to the molecule. It is usually referred to as CuZnSOD (copper zinc superoxide dismutase).

Of interest is that mutations in CuZnSOD have been associated with familial amyotrophic lateral sclerosis – the paralysis appears to be the result of some toxic gain in function, rather than a loss of enzymatic activity (49).

The second group of superoxide dismutases associated with eukaryotic cells largely occurs within the mitochondria of the cell. This is a manganese containing protein that is present as a dimeric protein in aerobic prokaryotes such as E. coli, and as a closely related tetrameric protein in eukaryotes. The manganese superoxide dismutase (MnSOD) in eukaryotes is not structurally related to the CuZnSOD: no doubt the similarity to the prokaryotic MnSOD reflects the origin of mitochondria from prokaryotes. Nevertheless, MnSOD has similar activity to CuZnSOD in metabolizing superoxide anion.

Finally, an extracellular superoxide dismutase (ECSOD), a glycoprotein not structurally related to the previous dismutases has also been described (50, 51). This very exciting new finding has added a further complexity to the protection of tissues from oxidant stresses. The ECSOD has been further classified as types 1, 2 and 3, depending on the affinity to heparin. The ECSOD's are glycoproteins (52), and are found within the lung interstitial spaces along with collagen (53). They are also bound to the endothelial capillary luminal aspect (54). They appear to be secreted by tissue fibroblasts. The ECSOD's have been studied across species, and generally are much less present (often barely detectable) in tissues than are CuZnSOD and MnSOD (55).

Since the superoxide dismutase catalyzes the reaction –

$$O_2^- + O_2^- + 2H^+ \Leftrightarrow H_2O_2 + O_2$$

- it is clear that there will be a requirement to deal with the potentially toxic hydrogen peroxide. Mammals, with their complex arrangements of eukaryotic cells, manage this process in two ways. The first mechanism is through the action of catalases, which facilitate the following reaction –

$$2H_2O_2 \Leftrightarrow O_2 + 2H_2O$$

Or through the action of peroxidases which facilitate the reduction of hydrogen peroxide as follows –

$$H_2O_2 + H_2O_2 \Leftrightarrow 2H_2O$$

Mammalian catalases are ferriheme containing enzymes of about 60kDa that co-exist in the eukaryotic cell peroxisomes; therefore in the same physical location that much of the hydrogen peroxide may be produced within the cell. In mammals, catalases are not present in the extracellular environment.

The principal peroxidases in mammals are the glutathione peroxidase(s). These are selenium rich proteins. During the interaction with hydrogen peroxide, the glutathione is oxidized, but then is converted back to its original form by glutathione reductase. There are several closely related forms of glutathione peroxidase, one of which can be secreted from the cell into the intracellular fluids, but in mammals seem to be not present in the extracellular space.

The excess production of O_2^- and of H_2O_2 that might occur in hyperoxic environments clearly requires the superoxide dismutases and the catalase/peroxidases to be present at the cellular and extracellular sites that might be the target for these powerful oxidants.

Recently the oxidant defenses have been shown, in a prokaryotic system (again E. coli) to be under complex genetic regulation (56). In these bacteria, under oxidant stress, the SoxRS regulon is activated. This is a family of approximately 12 genes. The SoxRS regulon transcribes a protein, the SoxR protein, which acts as a sensor molecule. In conditions of oxidant stress SoxR is oxidized and transcriptionally activates the SoxS protein, which in turn activates all of the other genes in the regulon. Pertinent to this review is that one of these genes controls MnSOD.

It is likely that a similar family of genes occurs in eukaryotic cells; whether there is an equivalent SoxRS regulon in the mitochondrial DNA of these cells and an equivalent regulation associated with the nuclear DNA remains to be determined.

Indirect evidence to support the notion that anti-oxidants were important in the modulation of pulmonary oxygen toxicity came initially from observations regarding the relative tolerance of young as compared to adult animals, to pulmonary oxygen toxicity. The observation of this phenomenon was made in a systematic way in 1932 by Smith (57). As indicated above, the major components of the antioxidant enzyme system are the superoxide dismutases, catalase, and the glutathione-redox system enzymes, namely glutathione reductase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase. Hyperoxic exposure leads to increased production of damaging radicals, usually at sites near radical production, such as mitochondria and in cytoplasmic microsomes, and possibly in cell nuclei (58-61). An increase in oxidant production and failure of rapid removal leads to tissue damage.

There appear to be three important phases in the development of the antioxidant enzyme system, and these have been well reviewed (62). Firstly, there is a burst of production of the antioxidant enzyme system in the final 15-20% of fetal maturation that is superimposable on the maturation pattern for lung surfactant. This has been demonstrated to occur in rats, hamsters, guinea pigs, rabbits and in sheep (63-67), and may also occur in normal human development (68).

The second phase of antioxidant development occurs in neonatal animals, with the third phase the transition to the adult. In many species, neonatal animals are much more tolerant of normobaric hyperoxic exposure when compared to adult animals. This has been consistently shown to be associated with the induction of the antioxidant enzymes in neonatal, but not adult animals. This important finding seems to have been made initially by Stevens and Autor in 1977 in rats (69, 70), and later confirmed by other investigators. (71). Further studies have shown that this phenomena occurs in rabbits and mice, but not in the neonatal stage in guinea pig, hamsters and sheep. Indeed, these neonatal animals are as sensitive to the effects of normobaric hyperoxia as the corresponding adult animals of these species. Other explanations of the relative differences in neonatal and adult responses to hyperoxia have been postulated. These include the notion that adult cells

produce more free radicals for a given oxidant load (72), or that the inflammatory cell infiltrate noted in oxygen damaged lungs are more toxic to normal lung tissue in the adult than in the neonate. Further suggestions, are that the lipid composition of the neonatal lung is relatively protective compared to the adult lung (73-76), or that there is a greater and more effective replacement/repair of oxygen damaged cells in the neonatal compared to the adult lung, with, therefore, less evidence of overall tissue damage (77).

More direct evidence to support the notion that anti-oxidants were important in the modulation of pulmonary oxygen toxicity has come from attempts to replace or augment the antioxidant enzyme system in the lung by endogenous or exogenous administration.

Soulie in 1939 (78), and later Barach in 1944 (79) showed that pre-exposure to adult rats to 60% oxygen for 1-2 days, then 70% for 2 days, then 80% for 3-4 days, then 90% for 1-2 days, protected them from subsequent lethal damage from 100% oxygen exposure. Barach tried to reproduce this effect in mice, guinea pigs and dogs without success. He also showed that intermittent exposure to air and 100% oxygen also produced relative protection in adult rats from subsequent 100% oxygen inhalation. He also noted *"thickening of the cells in the alveolar wall"* in those rats that developed tolerance, and pulmonary oedema and pleural effusions in those rats that developed acute signs of oxygen damage. One of his conclusions was that *"Whether changes in the oxygen enzyme system take place in six to twelve days during acclimatization to increasing oxygen concentrations cannot be decided from these studies".* Barach tried to prevent his exposure chambers from heating up by running water down coils in the outside walls.

Later Rosenbaum in 1969 (80) studied the ultrastructure of the lungs of adult Sprague-Dawley non-pathogen free rats that had been either exposed to 100% normobaric hyperoxia, or to an "adaptive " dose of 85% oxygen for 7 days, and then subsequent 100% exposure. Appropriate controls were performed. Temperature of the exposures was 23°C, and lungs were fixed at 20cm water pressure for histology. The main finding of the study was the development of enlarged and abnormally shaped mitochondria within the alveolar type 2 cells, as well as an increase in mitochondrial numbers, in the lungs of rats that had been made oxygen tolerant. These changes were amplified when these animals were subsequently exposed to 100% oxygen. No formal morphometry was performed.

Crapo in 1974 (81) repeated the observations that pre-exposure of adult rats to sublethal 85% oxygen for a period of 5-7 days resulted in lung changes which allowed these animals to survive subsequent exposure to >95% normobaric oxygen exposure. He showed that this phenomenon was associated with elevated lung superoxide dismutase, as well as catalase and glutathione peroxidase levels. Pre-treatment with oleic acid (82) also produced the same effects. Since these experiments were published, there were other less damaging methods shown for augmenting the antioxidant defenses in the adult lung. These include pre-exposure of adult rats to 10-12% oxygen (hypoxic conditions) (83); treatment of adult rats with non-toxic doses of endotoxin (84, 85); and a protocol of intermittent short exposures to >95% hyperoxia (86). Of interest is that all of these therapies increase the antioxidant defenses, but only protect against pulmonary oxygen toxicity in the adult rat. The lack of effect in mice, guinea pigs, or hamsters continues to be a great mystery, and to date has not been satisfactorily explained.

The phenomenon of tolerance of adult rats to hyperoxia has also been studied with specific interest in the antioxidant enzyme activities and their genetic regulation (87). This study used specific pathogen free Sprague-Dawley male rats. Exposures were at >95% normobaric hyperoxia, with a relative humidity of 40-60% and temperatures of 22-25°C. Endotoxin when used was administered by intraperitoneal injection. Protein levels and mRNA levels for MnSOD, CuZnSOD (protein levels only measured), catalase and glutathione peroxidase were measured in the lungs. Results, in summary, showed that with oxygen exposure over 60 hours, CuZnSOD levels stayed the same, MnSOD levels fell (40%), catalase levels rose (60%) and glutathione peroxidase levels rose (40%). With the endotoxin therapy, and now oxygen tolerant animals, after 60 hours of oxygen exposure, CuZnSOD was still unchanged, MnSOD had risen (x3), catalase levels were now similar to baseline, and glutathione peroxidase levels largely unchanged also, although at 48 hours these last two were elevated. All of these results were normalized to lung DNA levels, which also introduces a changing variable, as with greater lung damage there are more cells and greater DNA amounts. This will have the effect of under-estimating any newly produced protein. Results might have been also expressed as total lung levels. In addition the authors note that they are measuring whole lung gross effects, and cannot therefore comment on the changes that might be occurring in the micro-environment of the alveolar region. The findings of this study contradict a previous study by this group which showed induction and increases in CuZnSOD in a similar model (88). The authors' final comment is

an interesting one and relates to oxidant enzyme mRNA stability *"There is thus the very interesting possibility that a protein, perhaps a metalloprotein, senses the redox state of the cell and, dependent upon the state, is released from, or remains bound to, the antioxidant enzymes mRNA thereby influencing the RNA's stability and secondarily the synthesis of the enzyme."*

The interaction between oxygen toxicity, the oxidant enzyme defenses and cytokines is complex and interesting. From a number of studies, pre-treatment with tumor necrosis factor (TNF) and interleukin 1 (IL-1) (89-91) have been shown to confer protection against pulmonary oxygen toxicity in the rat. These studies were natural extensions of the observation that endotoxin pre-treatment protects against oxygen toxicity in the rat, and the knowledge that endotoxin induces many cells to produce TNF and IL-1. It appears that these two agents work most effectively in this way when given together, either by intraperitoneal or intravenous injection. These agents selectively induce MnSOD. Further studies (92, 93) have shown protection from 100% oxygen toxicity in adult, pathogen free, Sprague-Dawley rats by the separate intratracheal injection of either TNF or IL-1. Exposures were in a large chamber, with temperature not recorded. Outcome was survival, BAL parameters, pleural effusion volume, and lung histology (not quantified). Either treatment is effective in protective against pulmonary oxygen toxicity, with induction of MnSOD (to two times normal values) initially, and later (at 7 days) increases in CuZnSOD, catalase and glutathione peroxidase. Red cell insufflation into the trachea, but not red cell lysate also is protective against oxygen, and the mechanism seems to be induction of IL-1 and TNF (94)

These observations have been extended by examining the MnSOD and CuZnSOD mRNA levels, and the corresponding protein levels, in a model that studied adult male Sprague-Dawley rats exposed to hyperoxia in small chambers at 23-25°C and given IL-1, TNF or endotoxin by concurrent intravenous and intraperitoneal injections (95). Outcome was by pleural fluid volume and survival data. The findings were that IL-1 alone, IL-1 with TNF, and endotoxin, but not TNF alone, given by these routes, protected against oxygen toxicity. This was associated with a rapid induction of MnSOD within 4 hours, which on immunohistochemistry localized to the alveolar type 2 cells, and to a lesser extent to the alveolar epithelium and to the blood vessel endothelium. The cytokine associated induction was bimodal, as by 24 hours it had markedly decreased, increasing again in

hyperoxia at 52 hours, at which time the saline control also showed induction. By 24 hours there was a measurable increase in MnSOD protein. CuZnSOD had mild increases in mRNA and protein only, not reaching statistical significance. In the oxygen exposed controls the levels of MnSOD and CuZnSOD mRNA fell acutely. Longer time points were not examined in this study.

In a further extension of these findings IL-6, also known to be produced by cells after exposure to endotoxin (along with TNF and IL-1), has been shown in the adult rat to markedly enhance the TNF and IL-1 increases in lung MnSOD, and to reduce oxygen toxicity in the lung (96). Each of these agents were delivered by tracheal insufflation to adult Sprague-Dawley rats, not specifically non-pathogen free, on one occasion, and then exposed to 100% oxygen. Outcome was death. IL-6 given by as a sole agent did not affect oxygen toxicity and did not affect the levels of MnSOD mRNA. MnSOD protein was not measured. In this study lung levels of MnSOD were not examined however, with the results being based on changes in MnSOD mRNA levels in cultured bovine (not rat) lung derived endothelial cells, after 24 hours exposure to the index cytokine(s). No quantitative results of the combination study are given. CuZnSOD mRNA was not changed by any of the treatments in these cells.

Of great interest is a recent study examining the effects of IL-11 as a protective agent in pulmonary oxygen toxicity (97). IL-11 shares many activities with IL-6. This study examined oxygen toxicity in a murine model. They used transgenic mice that over-expressed IL-11 in the lung and showed that these animals were markedly protected from the effects of normobaric hyperoxia, when compared to transgene negative controls. Outcomes were death, BAL parameters, lipid peroxidation (malonaldehyde), light and electron microscopy. The IL-11 transgene animals had remarkable resistance to the toxic effects of oxygen in all parameters measured. Protection against oxygen toxicity was nearly complete. There was a 1.5-2.0 times increase in MnSOD protein activity and even higher increases in the precursor mRNA at 48-72 hours of oxygen exposure in the IL-11 transgene animals. There were also small increases in glutathione peroxidase and reductase, but no changes in CuZnSOD or catalase levels. IL-1 and TNF were not induced in these animals (and appear to have been inhibited). DNA fragmentation, suggestive of apoptosis was present in the oxygen treated controls, but significantly diminished in the IL-11 transgenic animals. Lipid peroxidation was reduced in the IL-11 transgenic animals at

base-line and after oxygen exposure, suggesting that some of the protection relates to either oxygen free radical scavenging, or less production of these. These findings, in the mouse, establish a new direction for research in trying to understand the pathophysiology of oxygen toxicity, as the reasons for IL-11 to be protective against 100% oxygen are not immediately apparent.

IL-11 is mostly recognised as functioning as a stimulatory protein for early hematopoietic stem/progenitor cells, including B lymphocytes (98). It was cloned based upon IL-6 bioactivity. It appears to be similar in some functions to IL-6, and may stimulate IL-6 and IL-1 production. IL-11 induces acute phase reactants, including type 1 (alpha-l acid glycoprotein, C3, haptoglobin, and hemopexin) and type 2 (fibrinogen). IL-11 induces the synthesis of tissue inhibitor of metalloproteinase-1 (TIMP), and causes fat cells to atrophy. IL-11 can also be induced by IL-1. In small rodents IL-11 causes a large increase in platelet count, but has no effect on other cell counts; acute phase reactants are all increased in the blood. IL-11 is protective against sub-lethal radio-chemotherapy in mice in several models, and does not seem to cause a febrile response when administered.

CuZnSOD exerts a protective effect on bacteria exposed to hyperoxia (99). One of the first pieces of additional evidence that CuZnSOD is an important part of the pulmonary defense mechanism against oxygen toxicity was provided by the demonstration that exogenously administered CuZnSOD prevented the hyperoxic-induced depression of pulmonary serotonin clearance in the isolated perfused rat lung (100). In the same year, nebulized bovine CuZnSOD was administered to rats to see if it would ameliorate the toxic effects of 100% oxygen breathing (101). Specific pathogen free adult Charles River rats were used. Oxygen exposures were in small chambers, with room temperatures of 23-24°C. One group of rats was treated with intraperitoneal injections of 1mg of CuZnSOD at the beginning of the oxygen exposure, and again every 8 hours throughout the exposure. There were 10 oxygen exposed rats and 10 control animals that had intraperitoneal normal saline and oxygen exposure. Other groups of rats (20 in each group) were exposed to nebulized CuZnSOD, the timing of which is difficult to establish from the paper, but is likely to have been continuous throughout the exposure to oxygen. The animals exposed to the aerosol had significant increases in lung CuZnSOD, and it appeared to be distributed homogeneously throughout the lung. There was no detectable increase in the animals

receiving the intraperitoneal injections. There was no protection or augmentation of the effects of 100% oxygen, with animal death being the end-point.

Intratracheal injection of bovine CuZnSOD or catalase was also performed (102). In these experiments the free radical scavenger was encapsulated by liposomes, to create a longer tissue half-life, and injected into the trachea of animals just prior to the exposures. Adult Sprague-Dawley rats were used in these experiments. The oxygen exposures were in small chambers, with temperatures 23-25°C. Outcome was animal mortality. CuZnSOD and catalase levels in the appropriate groups nearly doubled and remained high throughout the exposure period. I will note at this time that the liposome was made from phosphatidylcholine and phosphatidylserine. There was a striking improvement in mortality in the animals receiving either the CuZnSOD or the catalase, when compared to the control 100% oxygen exposed animals. For instance, 26 of 28 CuZnSOD and 26 of 27 catalase treated animals survived 72 hours of hyperoxia compared to 8 of 68 of the oxygen exposed control animals (the controls included animals that had received a single injection of CuZnSOD or catalase or the liposome material). They also found that 6 animals that had been treated with the CuZnSOD, and 8 animals that had received catalase continued to survive without mortality for 12 days in 100% oxygen.

Liposome encapsulated bovine CuZnSOD or catalase was given by intratracheal injection to reduce the chronic pulmonary hypertension seen in young rats after recovery from 8 days of breathing 100% oxygen (103). In these studies young (27 days old) male Sprague-Dawley rats were studied. Endpoints were right ventricular pressure measurements at 58 days, and lung and heart morphometry at 60 days. The liposomes were made from lecithin, dipalmitoyl phosphatidyl glycerol and cholesterol. Intratracheal injection was performed daily during the exposures, and there were appropriate controls. The measured lung increase in CuZnSOD as a result of the liposomes was not changed until day 5 of exposure, when it was about 33% higher than baseline. The measured lung increase in catalase as a result of the liposome administration was also not changed until day 5, when it was about 12% above baseline. Both of these measures were normalized to lung DNA. The outcome results showed that the administration of the liposomal catalase, but not the CuZnSOD partially protected the animals from the chronic pulmonary hypertension that occurs after hyperoxia in the juvenile rat.

Also anti-oxidant defenses have been augmented in other experiments in animals. This has been undertaken with liposomal encapsulated antioxidant enzymes (CuZnSOD, and catalase) (104), where these were given by intravenous injection. The animal model here was adult Sprague-Dawley rats, exposed to hyperoxia. The animals had 3,000 U of SOD, and 90,000 U of catalase injected every 12 hours, through the rat tail vein (in liposomes). This injection increased lung CuZnSOD and catalase by 1.7X and 3.1X respectively. Outcome was survival time and pleural fluid volume, and both were improved (mean survival time 118 hours in the active liposome group vs. 69.5 hours in the control group). The liposomes themselves, injected with free SOD and catalase had a slight protective effect.

Lung treatment with polyethylene glycol-conjugated (PEG) superoxide dismutase and catalase (105) has also been tested in adult rats. In this study, Sprague-Dawley rats treated with a single intravenous injection of PEG-CAT / PEG-CuZnSOD survived longer (a mean of 79.1 hours vs. a mean of 60.7 hours), and had lower BAL albumin levels and smaller pleural effusions than control animals. The administration of these conjugates did not increase lung SOD levels, but did increase lung catalase levels by a factor of X2. The positive protective effect was not as pronounced as with liposomal delivery of the enzymes. Exposure temperatures were 24-26°C, with relative humidity of 60-70%. PEG with any protein, also had an apparent slight protective effect. A further study has reported intratracheal insufflation of red blood cells (106), relatively rich in CuZnSOD, to be shown to be protective in rats.

More recently piglets have been used to examine the effects of concurrent nitric oxide and hyperoxic exposure (107), and to assess whether administered CuZnSOD was protective. This model was developed in response to the theoretical synergism in pulmonary damage that might occur by creating conditions that favor the excess generation of the peroxynitrite radical from superoxide anion. In this model piglets (I-3 day old) are mechanically ventilated with 100 ppm NO and 90% hyperoxia for 48 hours, with pulmonary damage measured by BAL parameters including cell counts, surfactant quantity and function, neutrophil chemotactic factors, total protein and an index of lipid peroxidation (malondiadehyde). Inhaled gas temperature and humidity were not reported. Recombinant human CuZnSOD was tested in this model (107). The CuZnSOD was given by inhalation in doses of 5mg/kg at time zero (IT instillation), at time zero and again at 24

hours (IT instillation), and 10mg/kg at time zero (nebulized). Results showed a reduction in neutrophil chemotactic factor, reduction in total cell counts (that seemed to increase more with the higher dose of CuZnSOD), reduction in malondiadehyde, reduction in total protein, and no differences in functional surfactant, with the nebulized treated group doing best. This is a complex system with many variables, including the mechanical ventilation, and unfortunately no control groups, such as a group ventilated but with no nitric oxide or hyperoxia were reported.

In a further study examining nebulized delivery, recombinant human MnSOD was used (108). In this study baboons were the animal used; and the hyperoxic injury was from 100% oxygen for 96 hours delivered whilst the animals were anesthetized and ventilated. End points analyzed were lung microscopy with morphometry; distribution of the MnSOD was assessed by immunohistochemistry. Historical ventilated control animals were included in the analysis. The MnSOD was administered by nebulization at doses of 1 mg/kg, 3 mg/kg, and 10 mg/kg; with this being the total daily dose administered throughout the exposures, and divided into 12 hourly dosing. In all 24 animals were studied, 6 in each group (i.e., three treatment groups, and one group exposed to hyperoxia, but no drug). The 3 mg/kg dosing was performed first and analyzed. There was significantly less alveolar type 1 cell injury in the MnSOD treated animals ($p<0.05$), with no change in the neutrophil interstitial influx, in the thickness of the alveolar membrane, or in the volume of the endothelial cell. The other doses of MnSOD had similar qualitative effects, but were much less significant when compared to control animals, and on the data presented do not appear to reach statistical significance. The administered MnSOD was distributed along the epithelial surface, and in the airways, and in alveolar macrophages, but with no staining in epithelial cells or in the interstitium or interstitial cells.

In a companion paper (109) the authors detail changes in physiology and biochemistry attributed to the 1 mg/kg, 3 mg/kg, and 10 mg/kg dose of MnSOD. There were no differences in animal survival. There were minor differences in mean arterial pressures and systemic vascular resistances. There was significant improvement in the shunt fraction in the 3 mg/kg dosing group ($p=0.001$), but not in the other groups. There were improvements in all treated groups in oxygenation ($p<0.001$). The lung weights (increased by pulmonary oedema), were significantly reduced in the 3 mg/kg group ($p=0.01$), but not in the other groups. This was mirrored by BAL protein being reduced by

the MnSOD 3mg/kg dosing, although this was not statistically significant ($p=0.17$). BAL surfactant measures were increased above baseline in the 3 mg/kg treatment group, reaching statistical significance ($p<0.01$), suggesting that surfactant components were protected by the administered MnSOD. These studies together show a minimal effect on the course of oxygen toxicity in the baboon, and the bulk of the effect is protection of the type 1 epithelial cell in this model. It would be of great interest to know whether the improvement in the epithelial survival was absolute, or whether with additional exposure time to oxygen the type 1 epithelium would have ultimately continued to be damaged.

Using *in-situ* hybridization techniques the localization of the induced MnSOD in the oxygen tolerant rat (after 85% hyperoxia) has been defined as being within the alveolar type 2 cell, the septal tips of alveolar ducts (probably fibroblasts) and within arterioles and the visceral pleural mesenchymal cells (110). This is no different in distribution to that in the normal rat lung as assessed by the same technique. Whilst qualitatively there appeared more signal in these regions in the oxygen tolerant animals, the authors note the limitations of using *in-situ* hybridization techniques as a quantitative measure.

Interestingly, the relative resistance of rabbits to the toxic effects of hyperoxia have been suggested to be the result of higher intrinsic lung levels of CuZnSOD, with levels greater than double that of other mammals tested (55). However, other authors note that the rabbit is almost as sensitive to normobaric hyperoxia as is the rat, making it the second most sensitive mammal studied (111).

There has been increasing interest in using genetic models to more closely examine and define the various roles of the antioxidant protection systems in mammals.

Several observations have been made of the spontaneous or familial genetic problems in humans. There is a case report (112) that indicates that a human with partial monosomy of chromosome 21, and with diminished CuZnSOD activity consequent upon that, has marked sensitivity to pulmonary oxygen toxicity. Mutations in the CuZnSOD on chromosome 21 also occur in patients with familial amyotrophic lateral sclerosis (as we have indicated previously); there may be reduced CuZnSOD activity in some of these patients. At this stage there is no systematic report on whether these patients have greater susceptibility to oxygen toxicity, or indeed to the effects of ionizing radiation (113, 114).

A knockout mouse with complete absence of cytosolic CuZnSOD has been developed – the homozygous animals have no CuZnSOD, the heterozygotes have about 50% of normal levels. These animals seem to be phenotypically normal and as yet have not been systematically studied for the effects of hyperoxia.

Transgenic mice overexpressing cytosolic CuZnSOD have also been developed. In two studies so far reported it appears that overexpression of the cytosolic CuZnSOD (110-180% of control) may confer protection from hyperoxia (115), although a further study suggests that this may not be the case (116). In the study reported by White, the endpoints were survival, and lung pathology using a graded scale. No survival advantage was noted in mice at sea level, but in mice exposed to hyperoxia at altitude (in Denver), survival and pathology advantages were reported in the transgenic animals. The survival advantage was greater for younger animals than for older mice. Exposure temperatures were not recorded, but relative humidity was 50-70%. The transgenic animals, as well as having elevated levels of CuZnSOD, also had significant levels of glutathione peroxidase in their lungs, but not MnSOD, or catalase.

MnSOD gene knockout mice have also been produced. Homozygous mutant mice die within 10 days after birth with a dilated cardiomyopathy and lipid accumulation in the bone and skeletal muscle. The mechanism of these changes is not clear. The heterozygous mutant has approximately 50% MnSOD and normal levels of cytosolic CuZnSOD, and appears to be phenotypically normal (117). The response of these animals to a hyperoxic environment has recently been tested (118). The heterozygote animals also have normal levels of catalase and glutathione peroxidase. In this study oxygen exposure was in small chambers, with no monitoring of temperature or humidity. Lung fixation pressures are not stated. They found no differences in survival or pathology in hyperoxia, as a consequence of having a reduction of MnSOD to 50% of wild-type levels. The authors conclude *"only 50% of MnSOD activity may be sufficient for normal resistance to 100% oxygen toxicity"*. Another conclusion could be that MnSOD is unimportant for protection against the toxic effects of oxygen.

Transgenic mice overexpressing the MnSOD gene have also been developed. Conflicting results in potential protection against oxygen toxicity have been shown. In one

model [Wispe, 1992 #999] where overexpression was in type II alveolar and Clara cells within the small airway epithelium, protection against oxygen toxicity was reported. In another model (116), with MnSOD overexpression in type II alveolar cells, endothelium and fibroblasts, no protection was demonstrated. However, this group has a subsequent publication where they reach a different conclusion (119). In this study they used human MnSOD transgenes inserted into mice; they demonstrated the expressed product in the mitochondria of lung type 1 and type 2 alveolar cells, endothelial cells and fibroblasts. The study did measure levels of MnSOD and CuZnSOD and of glutathione and catalase, and indicate that there were no changes in these apart from the MnSOD. The transgenic mice had a 180% and 350% increase in immunolabelling density in mitochondria for MnSOD respectively for the heterozygote and the homozygote. The animal exposures were in small chambers, with no temperature or humidity monitoring stated. Also, the exposures to oxygen were undertaken in two separate geographic locations – in Durham, NC. and in Detroit, MI. The only end-points were animal deaths. Results show that there was no difference in survival between non-transgenic mice and heterozygous or homozygous transgenic mice exposed to 100% oxygen. Separate groups of animals were also exposed to 90% oxygen. There was a small difference in survival in one series of experiments (15 heterozygous transgenic animals cf. 13 non-transgenic animals; p=0.012) with a mean survival of 6.3 cf. 5.3 days, and 100% animal death at 6.8 cf. 8 days. The paper indicates that homozygous animals had a similar outcome, but the data is not shown. The authors conclude that *"....increase activity of MnSOD it provides modest protection to B6C3 mice against hyperoxic lung injury".*

EC-SOD gene knockout mice have also been produced. The homozygous knockout animals with no detectable EC-SOD activity are phenotypically normal. However, these mice have increased sensitivity to normobaric hyperoxia (120) with pulmonary toxicity.

One transgenic mice overexpressing EC-SOD has been developed, but these animals do not have measurable changes in their lung levels of EC-SOD. These animals do have greater cerebral oxygen toxicity under hyperbaric conditions (121), suggesting that they may be over-producing hydrogen peroxide from superoxide anion.

Another model with overexpression of the lung EC-SOD has recently been developed in the mouse (122). In this model, the third fraction of human EC-SOD is overproduced in the alveolar type 2 cells and in the non-ciliated airway cells by heterozygote animals up to 3X the levels of control animals. These mice were produced using the human ECSOD gene, which was inserted into the human surfactant protein-C transgene, and then transfected into fertilized eggs isolated from mice, the eggs being transplanted into pseudopregnant foster mothers. The mice had levels of ECSOD, CuZnSOD, catalase and glutathione peroxidase measured, as well as immunohistochemistry to determine the location of the human ECSOD. Oxygen exposures were undertaken in small chambers, with temperatures between 25-26°C and relative humidity of less than 40%. Histology was performed, as well as BAL to assess lung injury. The authors report that 12/30 wild-type (wt) mice died in hyperoxia at 84 hours compared to 5/43 transgenic (tg) mice. There were less inflammatory cells in the tg compared to the wt mice as shown by BAL counts at 72 and 84 hours, and less total protein and LDH in the BAL fluid at the same time points ($p<0.05$ for all). The tg mice, after exposure to hyperoxia, had significantly greater catalase and glutathione peroxidase levels. The tg mice had less lipid peroxidation at the 72 hour exposure time ($p<0.05$), and less damage to the lung by light microscopy. The study also showed in a subgroup of animals that prior polymorphonuclear depletion tended to reduce the damaging effects of the oxygen exposures on the lung.

The uses of transgenic and knockout mice for studies in oxygen toxicity are clearly powerful new tools. This has been reviewed recently (123). However, when these animals are used it needs to be firmly established that there have been no compensatory changes in the other anti-oxidants by enzyme induction during fetal and neonatal development. Further, the genetic control of the oxidant defense enzymes in mammals needs to be further investigated so that the various possible contributions of the nuclear DNA and of the mitochondrial DNA is understood.

Wilhelm Roentgen

Roentgen, 1845-1923, discovered the invisible rays that we now know as x-rays, and was awarded the Nobel prize in physics for this in 1901, the first to be awarded. His discovery, like so many great advances in science was made accidentally, while he

Pierre Curie

was investigating the nature of cathode rays. Roentgen only worked with x-rays for a very few years, and left the field partly because of colleague rivalry directed against him for the credit for this discovery. Shortly afterwards Marie Curie (1867-1934) together with Pierre Curie (1859-1906) discovered the radioactive nature of radium and polonium, for which they received the Nobel prize for physics in 1903 (shared with Bacquerel who had initially discovered naturally occurring radiation). This represented the first woman to win a Nobel prize. Marie Curie continued to work on the chemistry of radium, and received the Nobel prize in chemistry in 1911. Radiation poisoning and burns and other damage to living organisms became obvious shortly afterwards, and indeed Marie Curie died of acute leukaemia, likely a late effect of radiation exposure. It is of great interest to read about the Curie's, the tragic accidental death of Pierre, and the poor manner with which the French scientific community treated Marie. It is also of interest to note that their daughter (Irene Curie; 1897-1956) and son-in-law (Frederic Joliot; 1900-1958) were awarded the Nobel prize in Chemistry in 1935, for the development of new radioactive materials, notably radio-phosphorus.

Marie Curie

By 1954 it was starting to be recognized *"that oxygen poisoning and radiation injury have at least one common basis of action, possibly through the formation of oxidizing free radicals"* (124). The authors summarized previous observations and concluded *"that anoxia decreases the acute lethal effects of ionizing radiations on rats and mice, but also that increased oxygen tensions enhance the effects of radiation"*. They also report a series of experiments that demonstrated that ionizing radiation markedly enhances the toxic effects of hyperbaric hyperoxia in mice. This phenomenon is referred to as the oxygen effect.

Ionizing radiation is associated with the production of oxygen free radicals, and this has especially been studied in respect to the effects of ionizing radiation with water. This field is surprisingly a relatively complex area of chemistry and has been recently summarized (125). Much of the early work on examining radiation chemistry was driven by

a need to understand the effects of radiation in liquids, and in biological systems, initially because of the use of radiation in medicine. A greatly increased effort began in 1938 because of the development of the atomic bomb, and after that the development of the nuclear power industry. By 1962, the reaction with water was understood to be:

$$H_2O \Rightarrow H^+ + OH + OH^- + e_{aq}^-$$

The understanding that a hydrated electron might be a product was slowly accepted after pioneering work by Czapski (126) and Matheson (127).

There is relatively (compared to hyperoxia) less reported work on the protective effects of the antioxidant defense system in ionizing radiation damage of eukaryotic cells and whole organisms. However, interest has been recently rekindled by the finding that overexpression of MnSOD protects against radiation induced injury in the SK-N-SH cell line. This is only effective if the increased MnSOD is located within the mitochondria of the cells. Additionally, CuZnSOD also was protective in these experiments if this was artificially increased within the mitochondria (128). Indeed, it continues to be argued as to the identity of the damaging oxygen radical species in ionizing radiation effects. The work by Wong, cited above, suggests that superoxide anion is the damaging species, and that the cellular target is the mitochondria, at least in this cell culture model.

The lung is also particularly sensitive to radiation injury. This has been excellently reviewed by Gross in 1977 (129). Initial studies on the effects of radiation on the whole lung suggested a pattern of injury and repair that was very similar to that seen with toxicity from inhalation of 100% oxygen (130, 131). Most of this published work has examined the effects of radiation in the murine lung. The pattern of lung injury after sublethal bilateral thoracic radiation in the rat has been recently studied (132). In these experiments specific pathogen free adult Wistar rats were used. The investigators examined histology after fixation of the right lung at 25 cm H_2O pressure, and for EM studies they fixed the lungs at 21-23 cm pressure for ultra-structure. An endothelial cell leak, without inflammation or gross oedema was first noted at two weeks. The lungs exhibited an inflammatory alveolitis with protein leak at 4 weeks, and this was associated ultrastructurally with the transformation of type 2 alveolar cells to type 1 alveolar cells, with some oedema of the type 1 cell walls, and detachment of endothelial cells with sub-endothelial oedema. They also noted enlargement of alveolar macrophages, with multinucleated forms. Following

these changes pulmonary fibrosis occurred. This study did not count pulmonary capillary numbers, but it is likely that they were diminished as a consequence of the radiation, again similar to the situation in oxygen toxicity. The endothelial cell appears to be preferentially damaged by radiation (133), with endothelial cell dysfunction, death and regeneration all occurring. The initial increase in permeability is thought to be both a de-polymerization of the intercellular muco-polysaccharide cement that constitutes the tight junctions between these cells, as well as liberation of vaso-active amines such as histamine and serotonin (134).

The genetic models that have had changes in expression of the oxidant enzyme defenses, noted above, do not seem to have been used to examine radiation sensitivity or protection to date. Some observations have been made to suggest there may be some relationship, as in a high level of Mn-SOD expression that seems to be associated with radiation insensitivity in a mesothelioma cell line (135). More recently, it has been found that increased induction of MnSOD in a glioma derived cell line, increases radiation sensitivity, possible by the over production of H_2O_2 (136). Finally, it has also been shown, that in some instances, radiation can induce the oxidant enzyme defenses (137), again suggesting that a similar relationship exists.

The work presented in this thesis has been performed over several years, and extends knowledge with regard to the occurrence, and the nature, of toxic effects of oxygen derived free radicals on complex biological systems, especially with regards to the lung and to the alveolar macrophage. There are a number of unique observations, including the first observation of the relative insensitivity of the alveolar macrophage to radiation damage, the first observation that native CuZnSOD modifies, but does not ameliorate pulmonary oxygen toxicity in a living animal, and confirmatory evidence for the role of hydroxyl radical in acute radiation damage in biological systems. The work has provided a very good understanding for me of the macroscopic, microscopic, cellular and biochemical processes that occur in the lung under a variety of inflammatory conditions. Finally, this work has generated a number of interesting hypotheses.

Each chapter has been written as a stand-alone section, containing relevant background, relevant methods, results and discussion. The figures and tables are presented as printed word documents rather than as glossy photographs, largely as the current technology allows this to be done with an acceptable degree of accuracy, and

because the photographs were creating an unacceptable thickness in the manuscript. The concluding chapter summarizes the new knowledge and provides a basis for further experiments in the area.

Chapter 2

Pulmonary Oxygen Toxicity - Effect of Environmental and Inhaled Gas Temperature.[1]

[1] Communications arising from this work are as follows: (138)

INTRODUCTION

Mammals breathing 100% oxygen at one atmosphere develop pulmonary oedema, which usually leads to the death of the animal after several days. The LT_{50} is dependent upon a variety of factors, including age and species (139). Cold-blooded animals, which are relatively resistant to oxygen toxicity, are also poisoned by oxygen if their body temperature is warmed to 37°C (139). Hibernating mammals are relatively protected from oxygen toxicity during the hibernating phase, but develop typical oxygen-induced pulmonary damage when exposed to hyperoxia during the non-hibernating phase of their annual life cycle (140).

Decreased body and environmental temperatures concomitant with exposure to hyperbaric oxygen result in increased animal survival. Several investigators have related this increased survival to decreased oxygen consumption (141, 142). In a study of mice maintained at environmental temperatures of 4°C to 40°C in normobaric hyperoxia, a substantially reduced LT_{50} in both extremes of temperature was found (143).

In reported studies investigating the effect of temperature upon pulmonary oxygen toxicity remain very limited, especially in mammals, and the influence of altered temperature of the inhaled gas, in addition to the effects of altered environmental temperature, are unknown.

METHODS

Adult male Sprague-Dawley rats (Biolab) weighing 200 - 250g (unless otherwise indicated) bred and maintained in a pathogen free environment were used in all experiments. Two types of exposure chambers were used; one in which animals were completely contained in the controlled environment in a large exposure chamber, and

another in which only the head of the animal was exposed to the controlled environment, so that the temperature of the inhaled gas could be modified. For experiments in which the temperature of the total environment was altered, a large chamber with a capacity of 430 liters was used. Animals were exposed in groups of ten and housed, unrestrained, 2-3 per cage within the chamber. Animals had free access to food and water, which was contained within their cages. Air or oxygen flowed into the chamber at a rate of 30-40 liters per minute. This produced 4-6 changes per hour of air or oxygen within the chamber, thus maintaining oxygen partial pressure at the desired level. Animal waste trays were filled with activated charcoal, which absorbed methane and ammonia. In addition, the chamber atmosphere was recirculated through a reflux condenser cooled by water at 5°C to maintain a reduced relative humidity (approximately 65% of saturation unless otherwise stated), and then through a series of chambers containing sodium hydroxide granules and activated charcoal. The chamber was opened briefly twice per day in order to remove selected animals, to replace food and water and to change the animal waste trays.

The oxygen and carbon dioxide levels were monitored using Beckman OM12 and OM11 Monitors, respectively. Relative humidity was measured within the chamber using a hygrometer. Temperature within the chamber was measured with an inserted dry mercury-filled thermometer and was regulated by a thermostatically-controlled electrical heater. To achieve temperatures below room temperature, the chamber was housed in a cold room at 5°C, and heated against this temperature.

The second type of chamber used for exposing animals provided only higher inhaled gas temperatures, with maintenance of the temperature around the body of the animal at 23°C (room temperature). With this chamber, only the head of the animal was placed within the temperature-controlled environment (Figure 2.1). Thus, the rats breathed only the desired concentration of oxygen at the elevated temperature. The inhaled gas temperature was regulated by using thermostatically-controlled, heated water which circulated through an outer jacket surrounding the chamber. The animals in these experiments were lightly anaesthetized with ether and then placed in individual wire restraining jackets, with heads placed within the chamber and the neck and outside of the chamber (Figure 2.1). The animals, although restrained, had free access to food and water, and recovered rapidly from the ether anaesthesia.

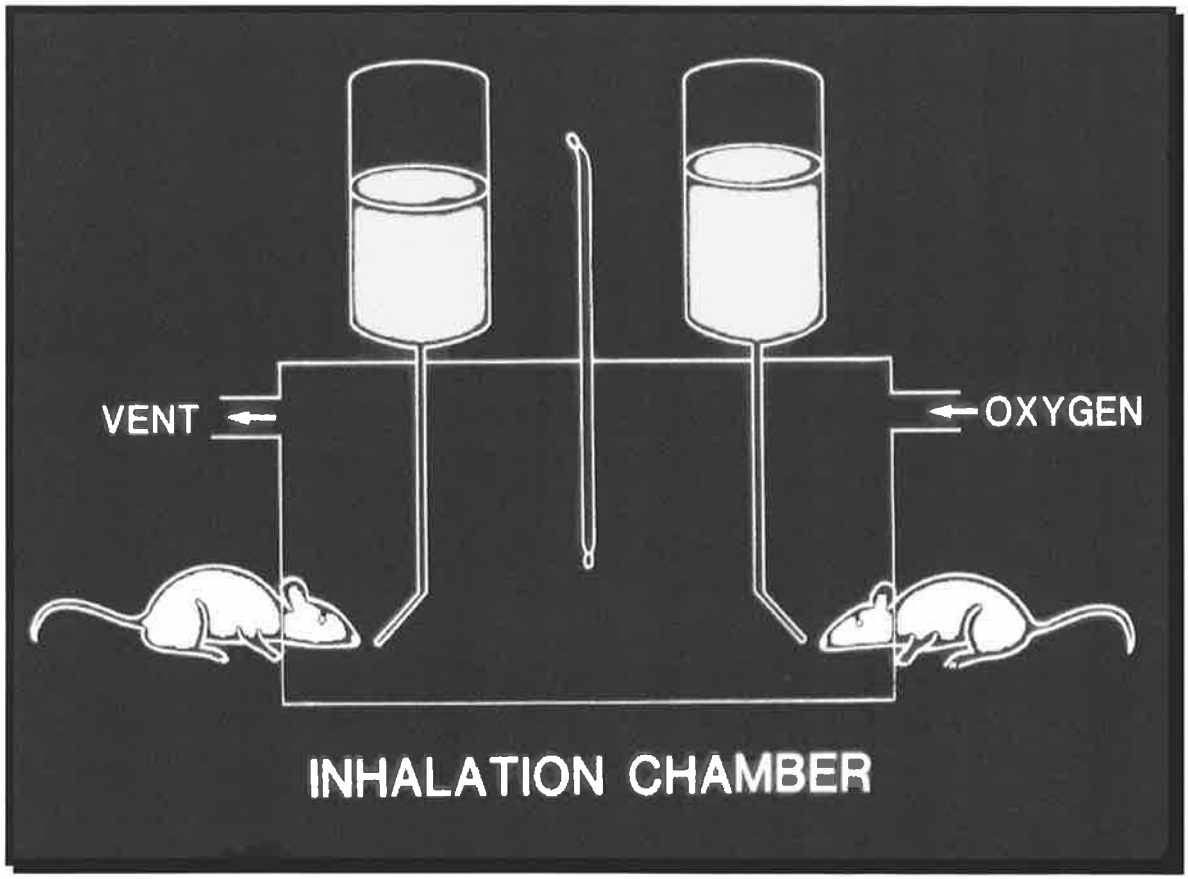


Figure 2.1 : Inhalation chamber, for exposure to rats to an increased inhaled gas temperature.

Using a thermistor probe inserted 2 cm into the rectum, body temperatures were measured in representative animals after 6 hours of exposure to the specified experimental conditions, with the only changes being in the temperature of the inhaled gas.

Toxicity was assessed by animal survival at the completion of the prescribed exposure period. In addition, histopathology of the lung parenchyma of surviving animals was evaluated after formalin fixation of the excised lungs at 30 cms water, and staining of the paraffin sectioned lung samples with hematoxylin and eosin. In other experiments, animals were removed from the exposure chamber at specified intervals for histopathologic evaluation of the lungs using the light microscopic. Lung sections were also examined by transmission electron microscopy (TEM) after fixation of the tissue with glutaraldehyde followed by staining with osmium tetroxide. Small (about 1mm) blocks of lung tissue were also examined under a scanning electron microscope using two methods of fixation - in the

first method glutaradehyde was instilled through the trachea as above for the TEM, but in the second method fixation was as follows. The normal healthy adult rat was anaesthetized with ether anaesthesia and the lungs prepared in a novel way to preserve the alveolar contents, together with the capillary contents. Several experiments were performed to develop this method, as it was found that intratracheal fixation with glutaraldehyde removed most alveolar macrophages and distorted the vascular pattern, and fixations via the pulmonary artery removed the blood cells from the alveolar capillaries. The trachea of these animals were cannulated via a tracheal catheter whilst the animals were anaesthetized but still breathing. The tracheal cannula was then tied off coincident in time with the animals end inspiratory maneuver thereby holding the lungs in inspiration, yet air-filled. The dorsal aorta was then transected, and the lungs gently removed from the animal by dissection. Still inflated, the lungs were totally immersed in the glutaraldehyde fixative for 24 hours. A small piece of subpleural lung was then removed for subsequent scanning electron microscopy (SEM) as well as transmission electron microscopy. Using this method it was found that about 2 mm of subpleural lung was excellently fixed. In all instances the lungs were inflated at 30 cm water pressure for fixation via intratracheal instillation of the appropriate fixative.

Statistical evaluation of survival curves was performed by Professor Peter A. Lackenbruch, Department of Preventative Medicine and Environmental Health, University of Iowa. The methods involved a product-limit life table analysis, with Mantel-Cox statistics.

RESULTS

Figure 2.2 : The survival of adult rats at 25°C

Initially, the survival times of rats at an ambient temperature of 25°C was established within this large exposure chamber, (the exposure chamber also that included methods for extracting contaminating CO_2 and other gases that might accumulate in these conditions such as ammonia or methane). A typical rat survival curve under these conditions is shown in figure 2.2.

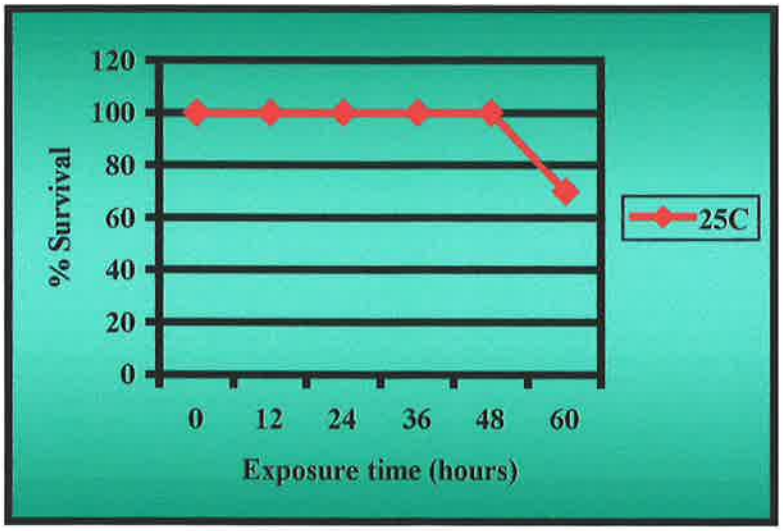
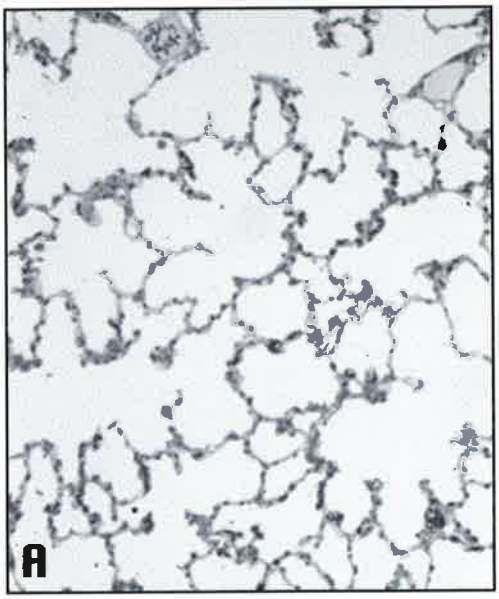


Figure 2.2 : The percent survival of rats exposed to >95% hyperoxia at 25°C. There were 10 rats in the group. Each rat was enclosed completely in an environment maintained at the indicated temperatures and a relative humidity of 65 ± 10%.

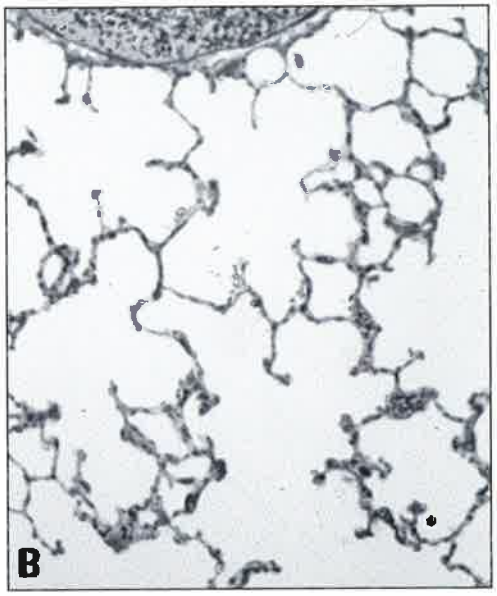
The pathological evolution of hyperoxic (>95%) lung damage at 25°C

Initial experiments were conducted to assess the evolution of the lung pathology changes from hyperoxic exposure at the light , TEM and SEM levels. The light level histology of the evolution of the oxygen toxicity at ambient temperature of 25°C is shown in Figure 2.3.

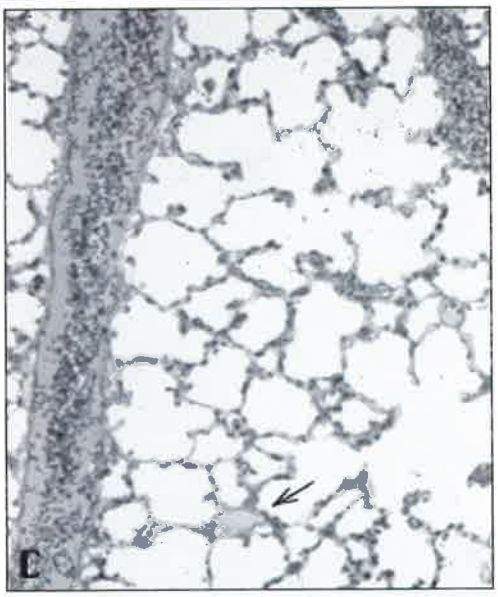
Figure 2.3 : The evolution of pulmonary oxygen toxicity in the rat at 25°C as shown by light microscopy (All panels photographed at X 100 magnification).



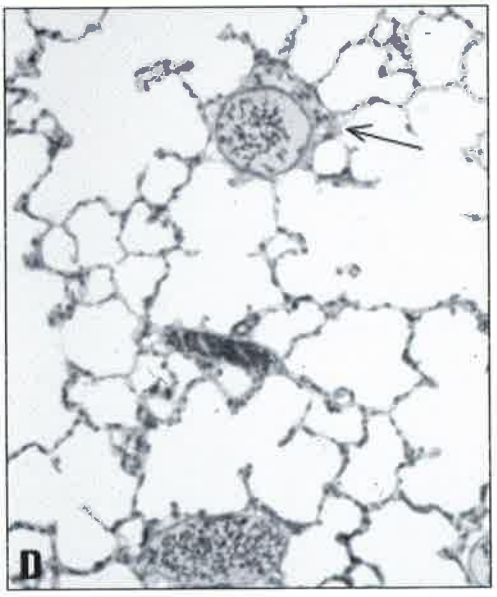
Panel A shows normal histology at 12 hours.



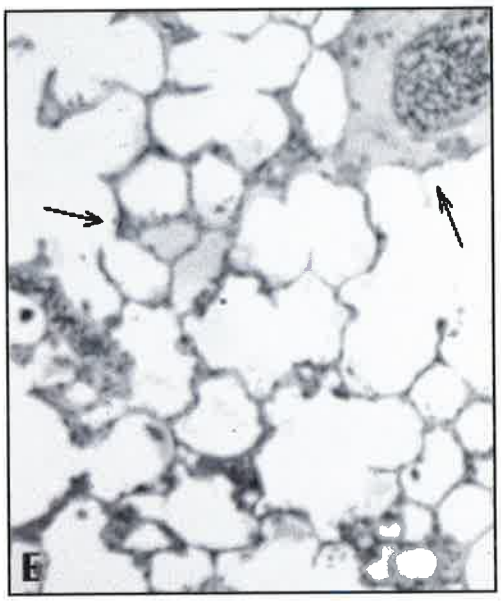
Panel B representative normal histology at 24 hours.



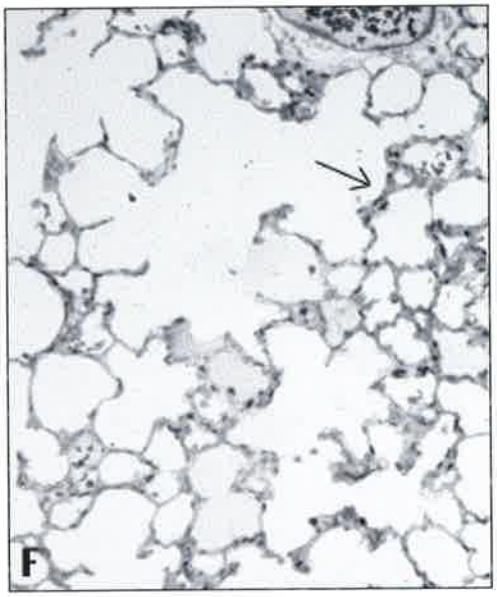
Panel C shows histology at 36 hours. The arrow indicates an alveolar region filled with oedema fluid.



Panel D shows changes at 48 hours. The arrow shows the beginning of perivascular oedema.



Panel E shows changes at 60 hours. Arrows indicate severe perivascular oedema.



Panel F shows representative histology at 72 hours in a surviving animal. In addition to perivascular and alveolar oedema, there is alveolar wall thickening with some increased cellularity of the alveolar wall, alveolar lumen, and alveolar fluid filling.

The evolution of pulmonary oxygen toxicity as demonstrated by transmission electron microscope is shown in the following figures (figure 2.4), under the same ambient conditions, at 25°C. The normal rat lung is shown first, followed by the changes in the alveolar region under hyperoxic (>95%) conditions.

Figure 2.4a : Transmission electron microscopy of normal rat lung

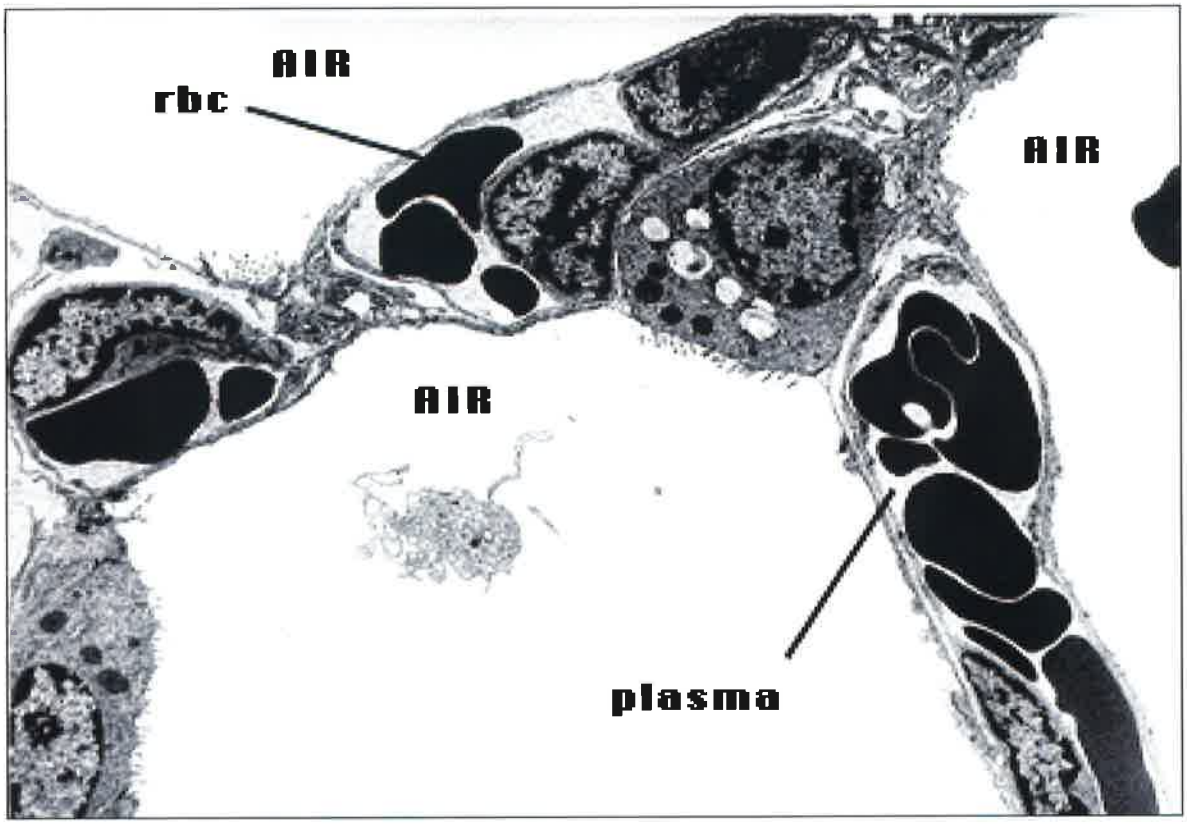


Figure 2.4a : This TEM shows the peripheral alveolar region o the normal rat lung (X10,000) The components are illustratec in Figure 2.4b

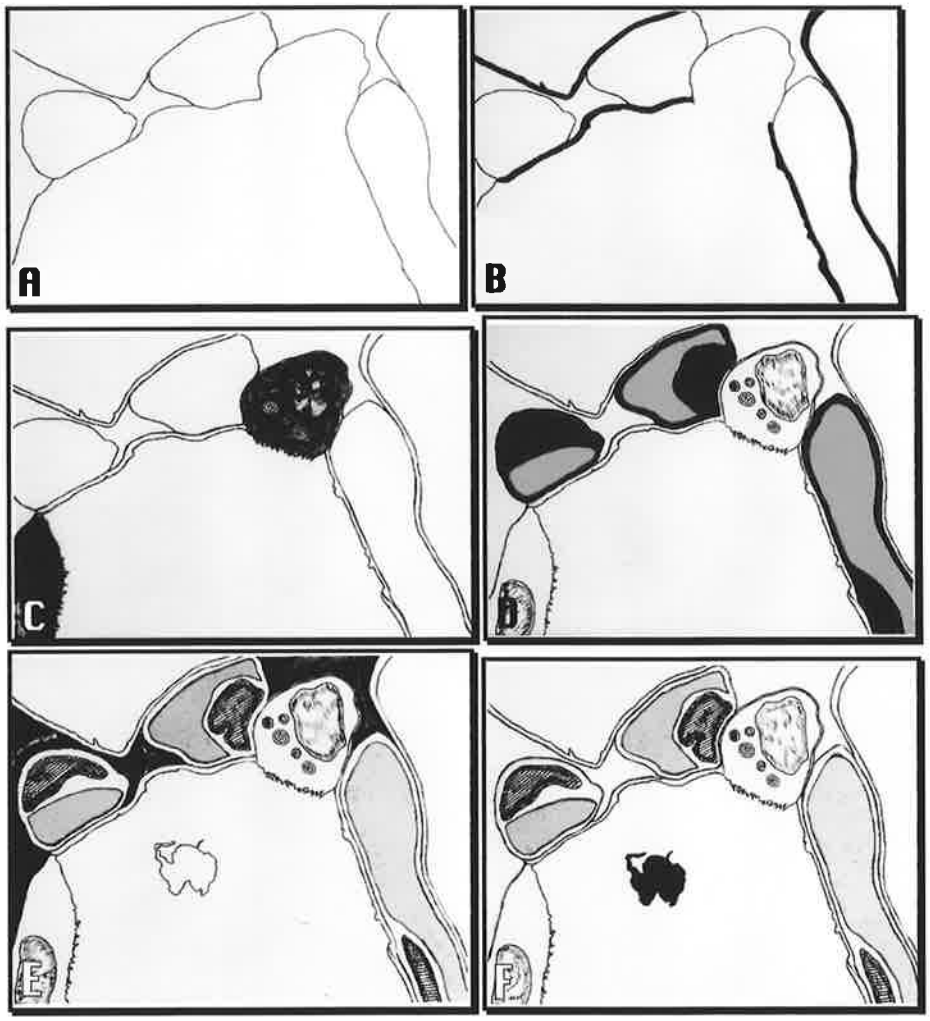


Figure 2.4b : In this Figure the Panel A indicates the basal laminae in the peripheral lung. This separates the endothelial cell from the type I and type II epithelial cells, and is therefore a conjoint basement membrane. The regulation and function (in terms of sieving) of this membrane remains unclear. Panel B indicates the Type I epithelial cell that is built onto the basal laminae. The Type I cell has a complex transport system for solutes and for larger molecules that currently is still being investigated, and the intercellular junctions are "tight". Panel C highlights the Type II cell, nestled usually at the alveolar corners. This cell has the surfactant containing lamellar bodies, and by replication can replace the much flatter Type I cell. The Panel D indicates the pulmonary endothelial cell, that continuously lines the capillaries. This cell has a transport system that is transmembranous, as well as allowing transport of fluid and cells through the intercellular "gap" junctions. Panel E highlights the interstitial area. This area contains collagen and elastin, as well as interstitial fibroblasts, myo-fibroblasts and interstitial macrophages. There are no lymphatics, but fluid entering this space "flows" adjacent the air spaces into the bronchial lymphatics. Panel F highlights the resident pulmonary macrophage (at least the edge of one). The blood components, plasma proteins and cells, are not specifically illustrated, but constitute the final component of this complex structure.

Figure 2.5 : The evolution of pulmonary oxygen toxicity in the rat at 25°C as shown by transmission electron microscopy. (all panels ^{X}10,000)

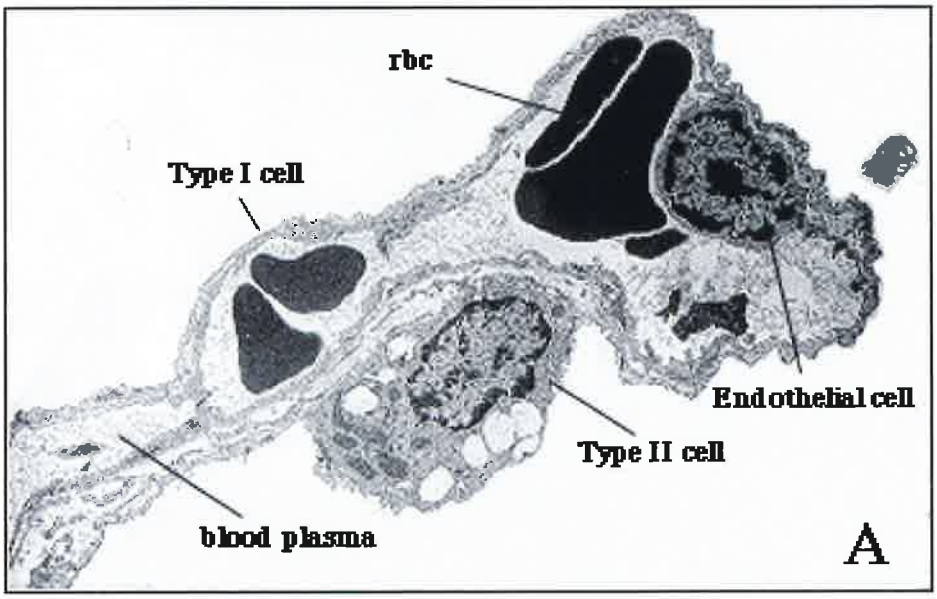


Figure 2.5a : Shows a normal appearing alveolar region, with capillary endothelium, a type II cell inferiorly (with lamellar bodies), and plasma proteins appearing granular in the capillary lumen together with red blood cells (dark bodies). This was from an animal exposed to hyperoxia for 12 hours.

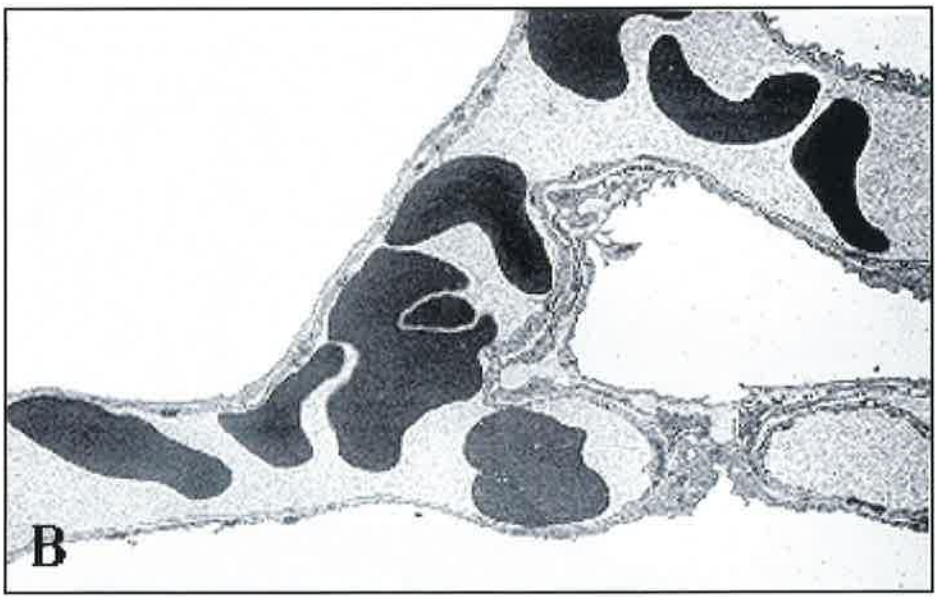


Figure 2.5b : This demonstrates the appearances of the alveolar-capillary region of the lung at 24 hours of >95%oxygen exposure. There is no abnormality apparent

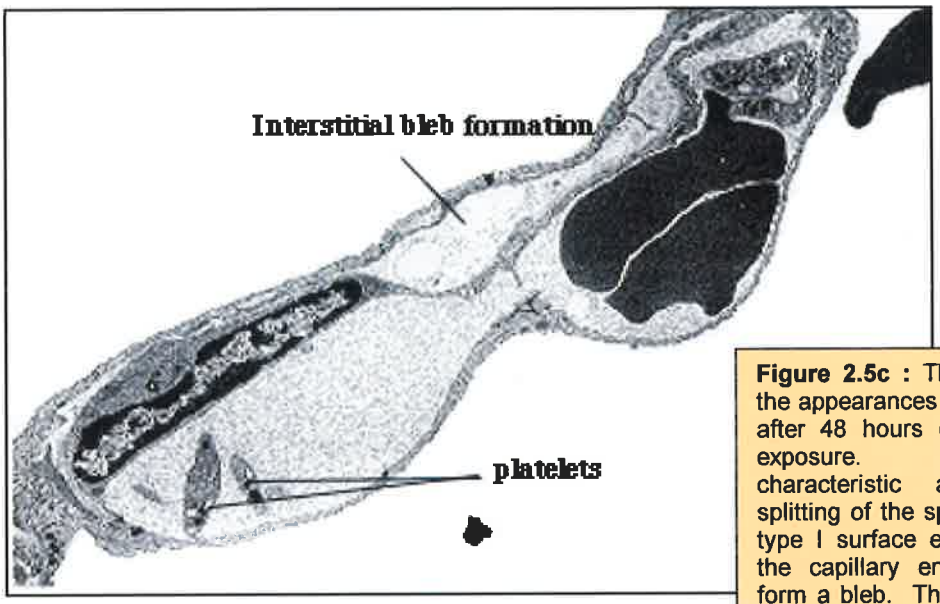
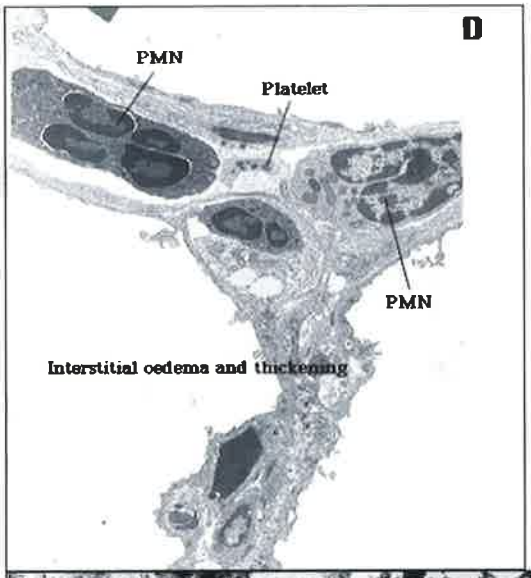


Figure 2.5c : This demonstrates the appearances of the distal lung after 48 hours of >95% oxygen exposure. There are characteristic appearances of splitting of the space between the type I surface epithelial cell and the capillary endothelial cell to form a bleb. This space appears to contain proteinaceous fluid. The second characteristic event, from many observations, is the appearance in the capillary lumen of many platelets, in addition to the red blood cells. There is no alveolar oedema.



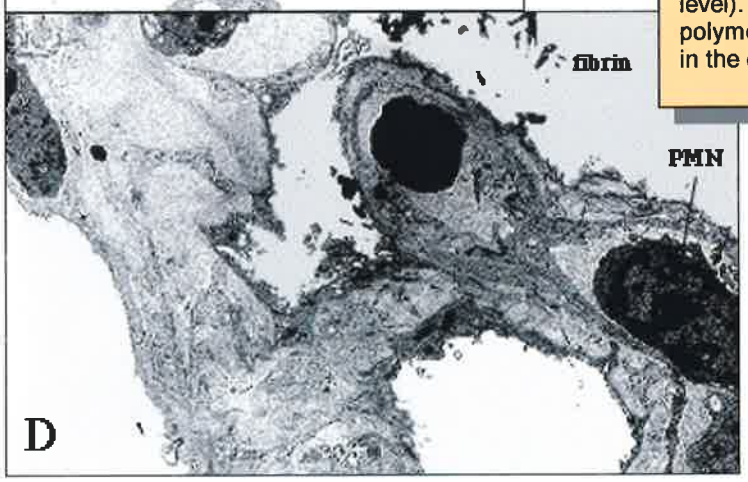


Figure 2.5d : These 2 panels, both marked D, illustrate changes typical of severe oxygen toxicity at 72 hours of >95% exposure. The alveolar wall is thickened, with proteinaceous material, and severe thickening of the alveolar capillary conjoint membrane region. There is also alveolar luminal debris, having the appearances of fibrin (hyaline membranes at the light microscope level). In addition there are now a polymorphonuclear neutrophils present in the capillary lumen.

Figure 2.6 : Representative views using Scanning Electron Microscopy are shown for normal rat lung, fixed by intratracheal fixation, and by the novel transpleural method

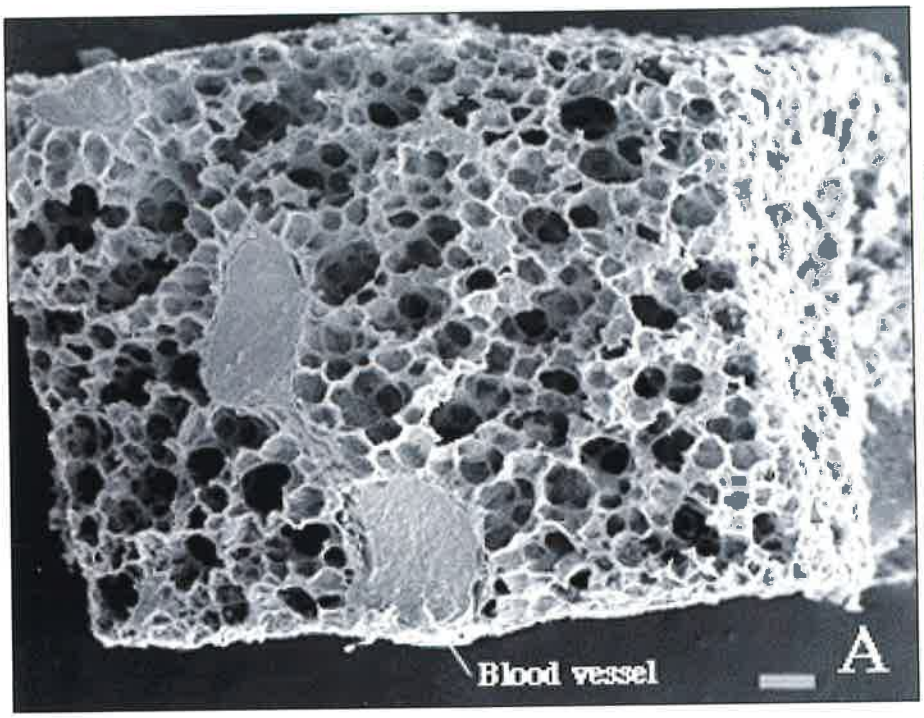


Figure 2.6a : This shows a block of normal rat lung (about 1.5 mm cubed), fixed by tracheal fixation (as per text). Note the blood vessel filled with fixed blood cells and protein, and the fine network of alveoli. This view was taken X48 magnification. The bar at the bottom right is 100 microns

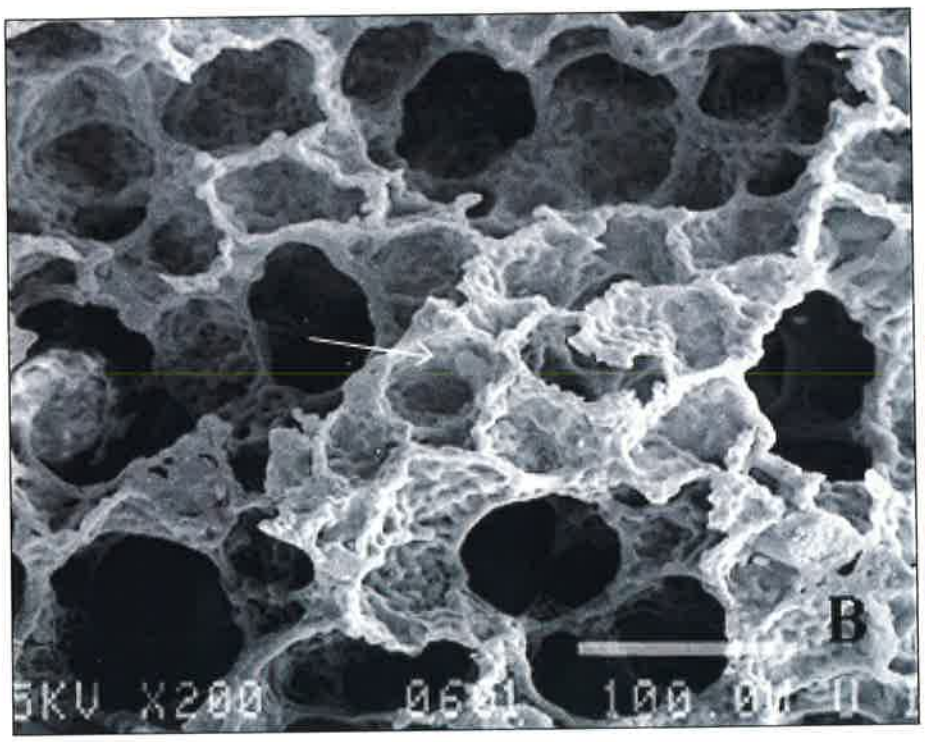


Figure 2.6b : This shows the same block of lung at higher magnification. Note the capillary loops in the alveolar wall, the extensive alveolar lumen connections, and the alveolar macrophage just appearing in the alveolar lumen in the center of the picture (arrow). Magnification X 200. The bar at the bottom right is 100 microns long.

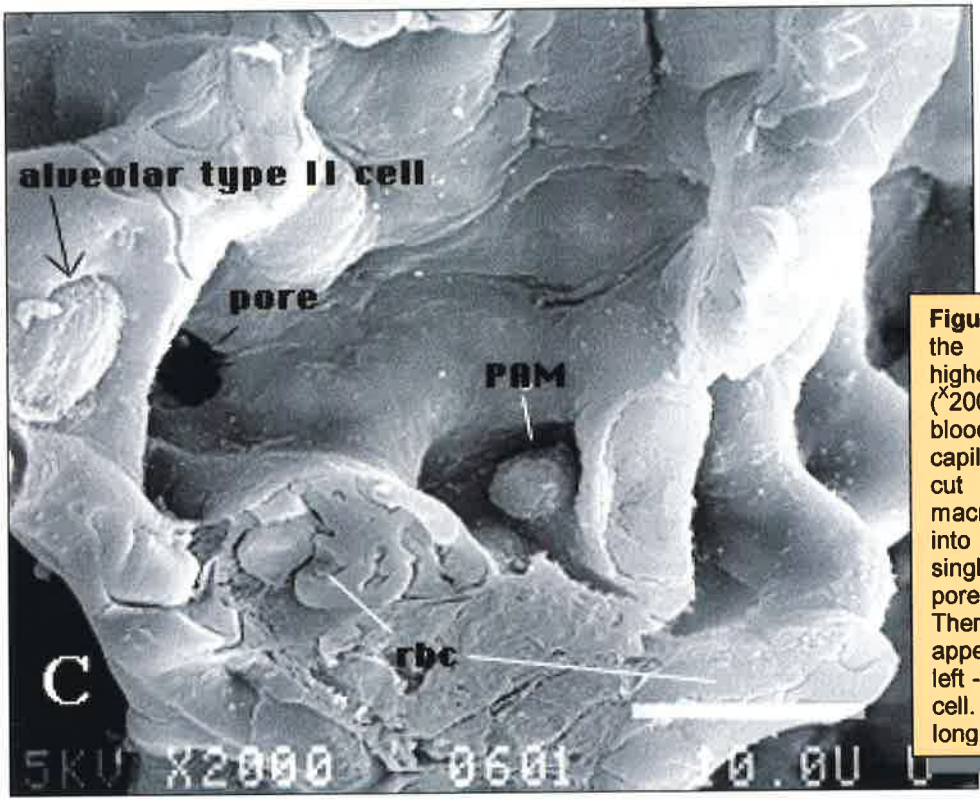


Figure 2.6c : This shows the normal rat lung at higher magnification (^{X}2000). Note the fixed red blood cells in the several capillaries that have been cut through, the alveolar macrophage protruding into the lower part of the single alveolus, and the pore of Kohn on the left. There is a cell which appears as a button on the left - this is a typical type II cell. The bar is 10 microns long.

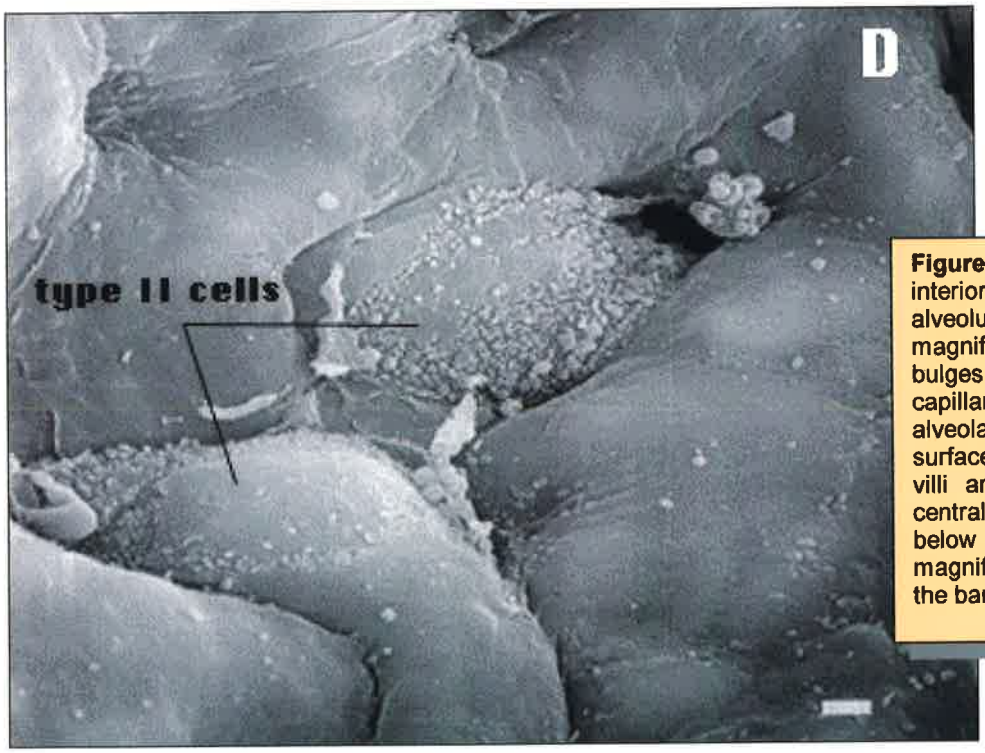


Figure 2.6d : This shows the interior of a normal rat alveolus at even greater magnification. The folds or bulges are alveolar capillaries covering the entire alveolar surface. The two surface cells with the short villi are type II cells (one central and the other just below and slightly left). The magnification is ^{X}4000, and the bar is one micron.

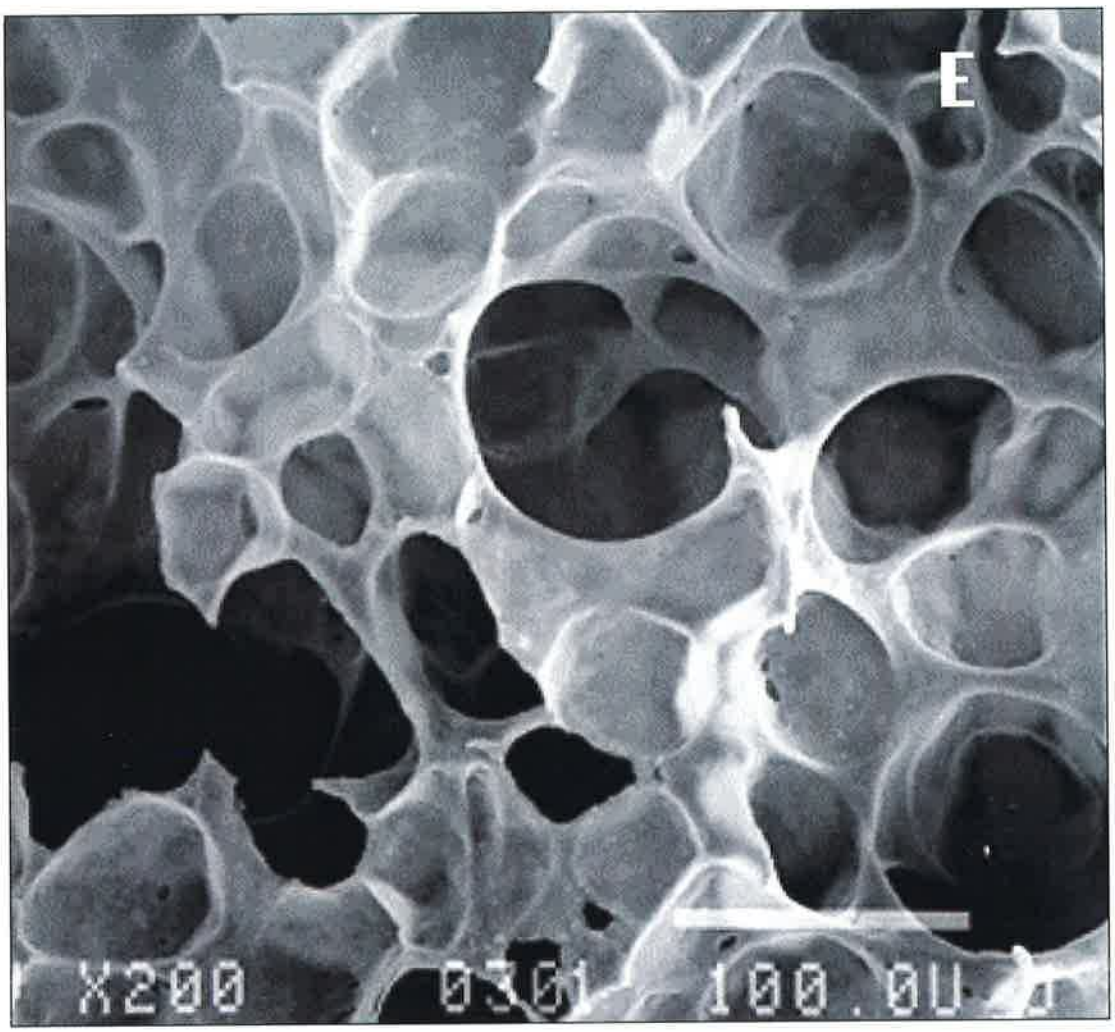


Figure 2.6e : This shows the normal rat lung fixed with the transpleural method (see text). This can be directly compared with 4b, taken at the same magnification, but fixed via the trachea. Note that the surfactant layer coating the alveolus has been preserved, and the alveoli now take on a smooth rounded appearance. Capillary loops are obscured by the surfactant coating. Magnification X 200. The bar at the lower right is 100 micron.

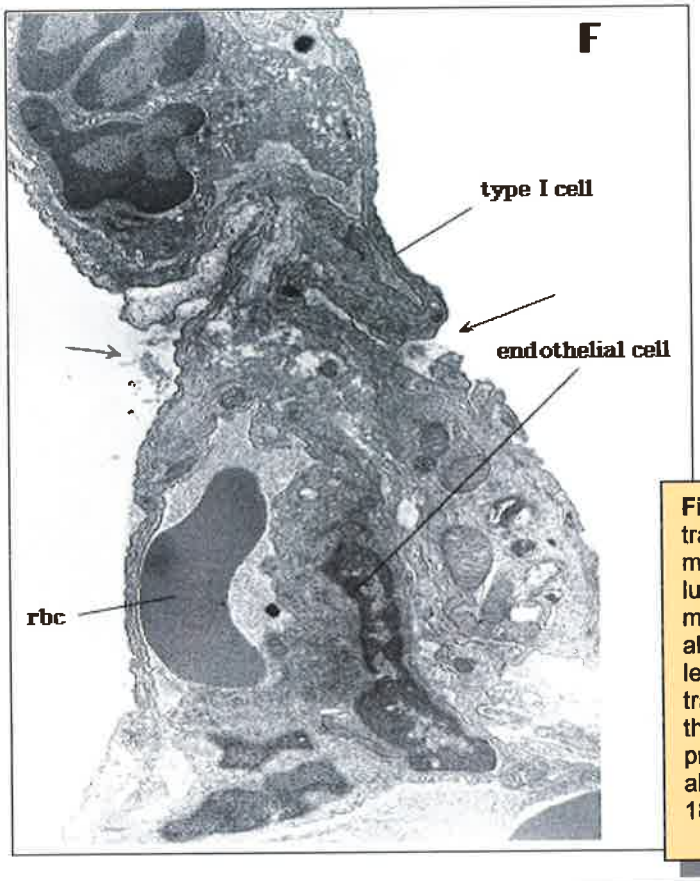


Figure 2.6f : This shows a transmission electron micrograph of the normal rat lung fixed with the transpleural method (see text). The alveolar walls appear to be less stretched than with tracheal fixation. Note that there is very little surfactant or protein present along the alveolar lumen. TEM X 18.000.

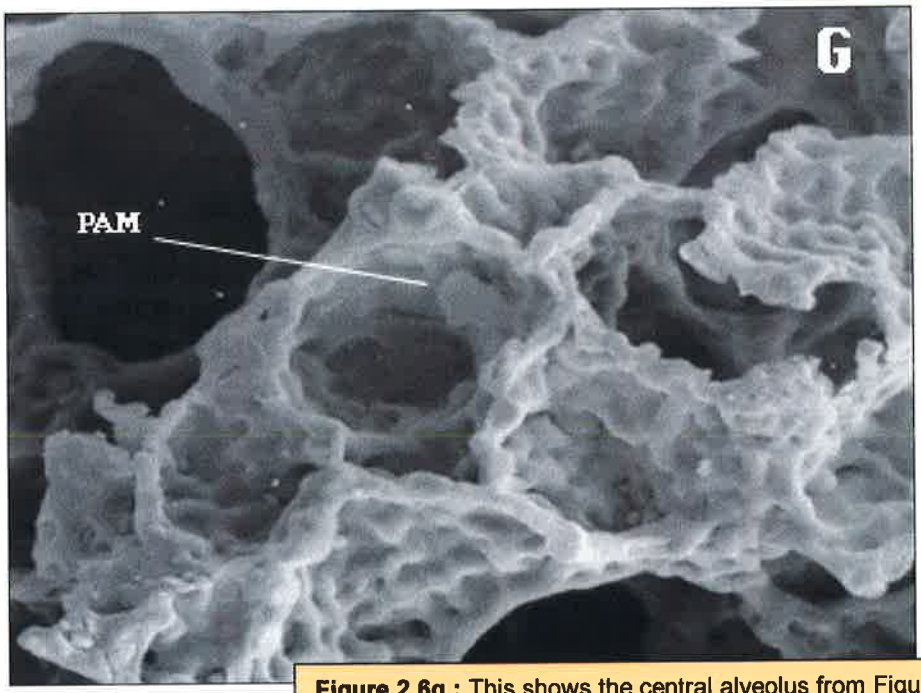


Figure 2.6g : This shows the central alveolus from Figure 2.6b above with the PAM within the lumen SEM.

Figure 2.7 : The evolution of pulmonary oxygen toxicity in the rat at 25°C as shown by scanning electron microscopy, with transtracheal fixation

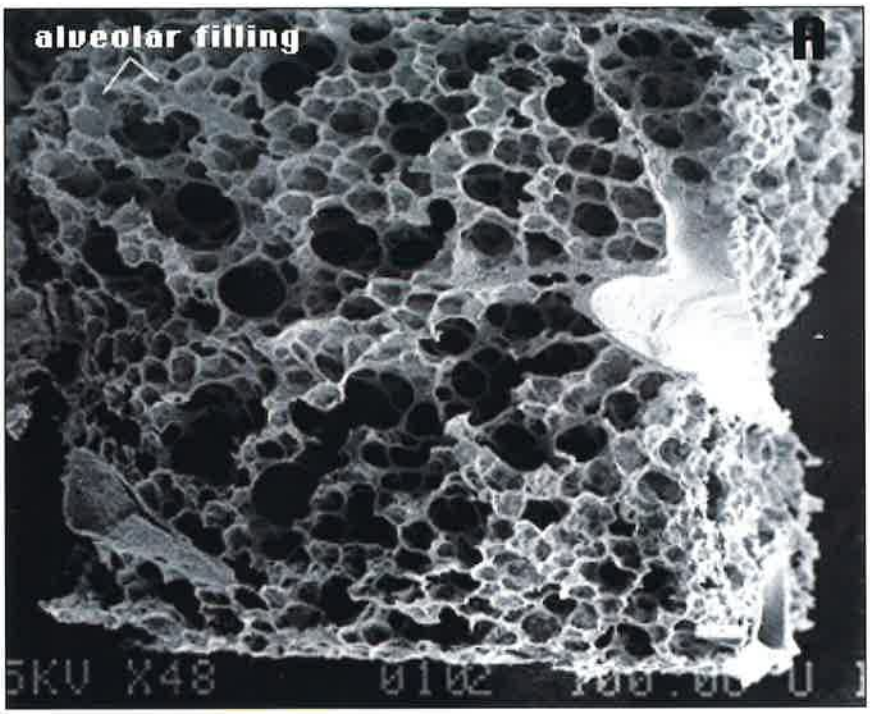


Figure 2.6a : This shows a cube of lung with the airway on the right. This rat has had 48 hours of >95% hyperoxia exposure. The alveoli show some fluid filling especially in the top left. Magnification X 48.

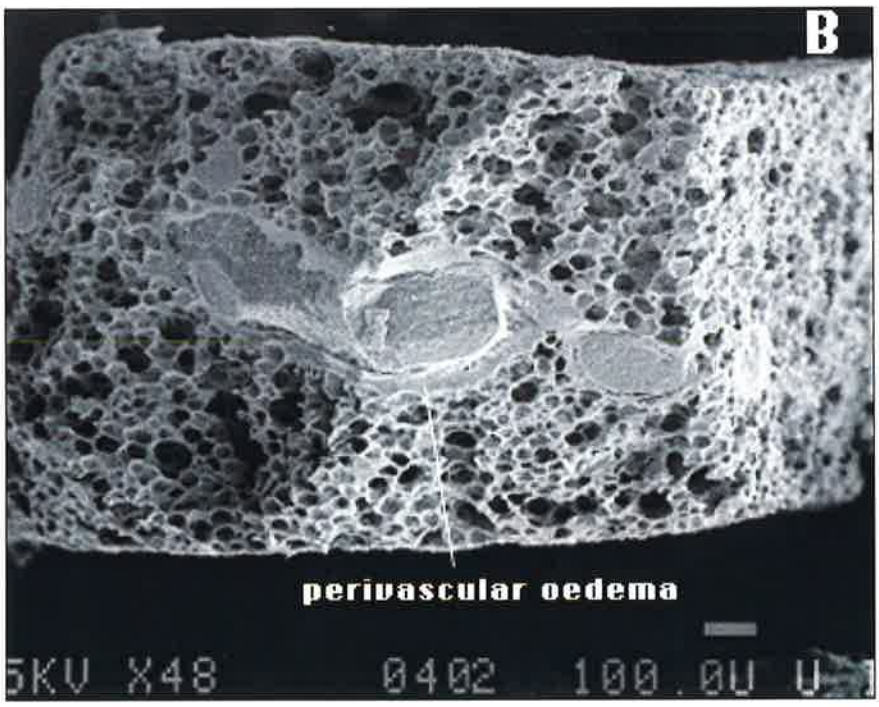


Figure 2.6b : This shows the effects of >95% oxygen exposure on the lung after 72 hours. The lung looks contracted (even though fixed at a standard inflation pressure), with smaller alveoli. A lot of the alveoli are fluid filled. There is very marked perivascular oedema all around the large central blood vessel. Magnification X 48, and the bar on the lower right is 100 micron.

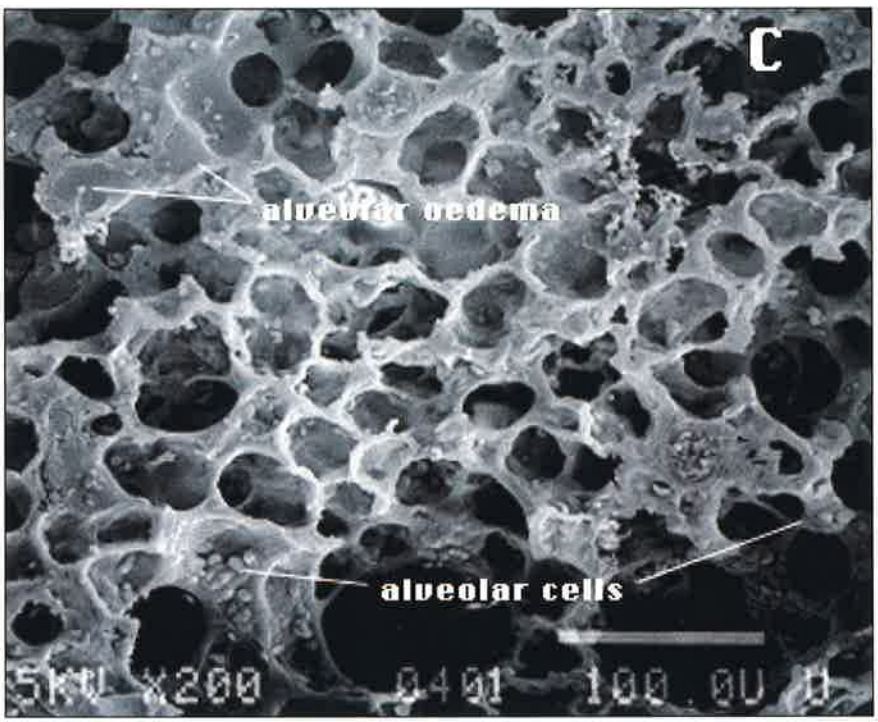


Figure 2.6c : This shows the effects on the lung of >95% hyperoxia at 72 hours, magnification X 200. The alveoli are small, and many are fluid filled. This is by tracheal fixation. There is an increase in the number of cells in the alveolar lumen, especially inferiorly. These may be red blood cells, or polymorphonuclear neutrophils or alveolar macrophages. The bar on the lower right is 100 microns long.

Figure 2.7 : The evolution of pulmonary oxygen toxicity in the rat at 25°C as shown by scanning electron microscopy, with transpleural fixation

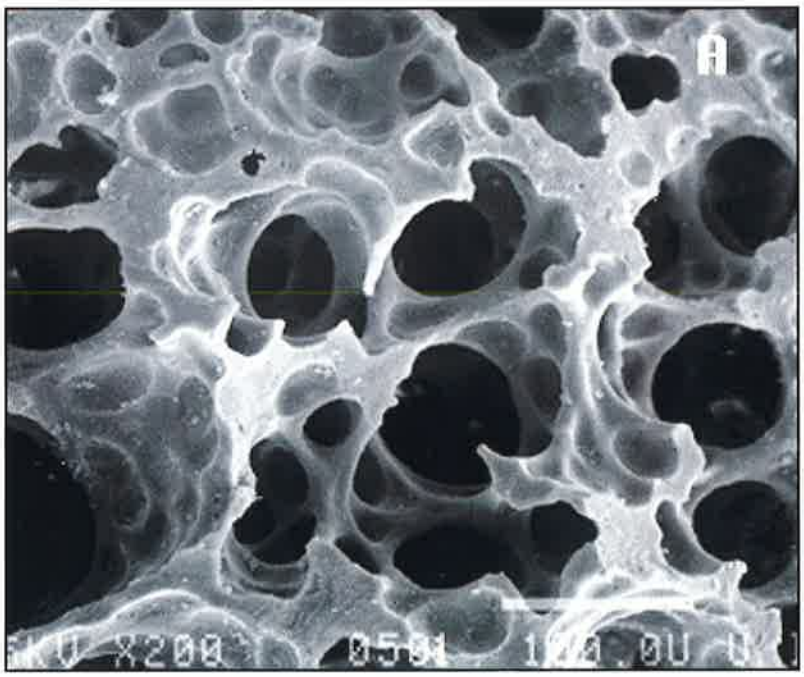


Figure 2.7a : This shows the rat lung after 72 hours of >95% hyperoxia, with marked thickening of the alveolar walls, and retention of the surfactant layer. The alveoli are much smaller than in the normal rat lung. Magnification X 200. The bar at the lower right is 100 microns long.

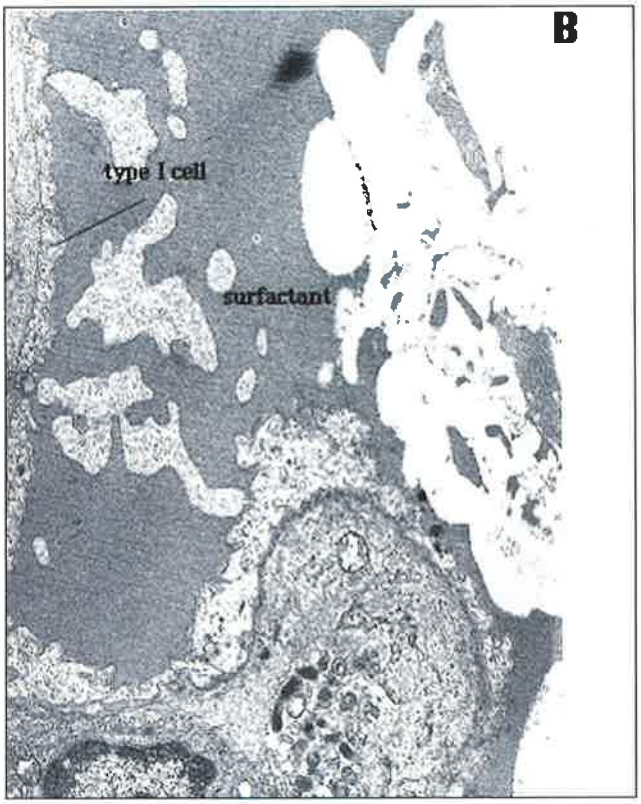


Figure 2.7b : This shows the rat lung after 72 hours >95% hyperoxia under transmission electron microscopy, with the pleural fixation method. Note the large amount of surfactant and oedema fluid covering the convoluted alveolar epithelium. There is some tubular myelin off to the right. TEM X 18,000.

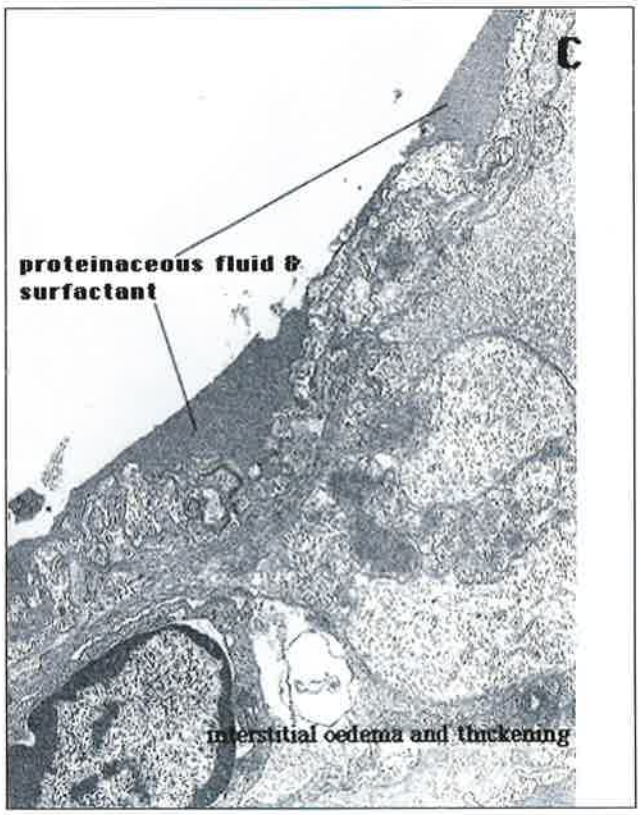


Figure 2.7c : This shows the rat lung after 72 hours >95% hyperoxia under transmission electron microscopy, with the pleural fixation method. Note the large amount of surfactant and oedema fluid covering the convoluted alveolar epithelium. Compare to 3.4f. TEM X 18,000.

Figure 2.8 : Effect on rat survival from pulmonary oxygen toxicity (>95%)of increasing or decreasing the environmental temperature

Survival curves for groups of rats completely exposed to >95% oxygen at several different environmental temperatures showed that the median survival at 8°C is 96 hours, at 30°C is 60 hours, at 35.5°C is 48 hours and at 37.5°C is 24 hours (Figures 2.8a and 2.8b).

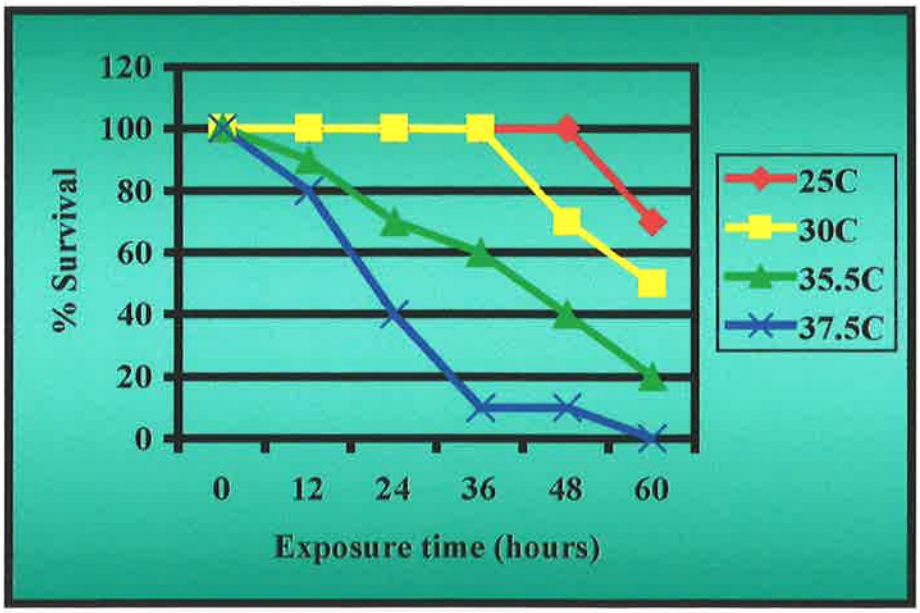


Figure 2.8a : The percent survival of rats exposed to >95% hyperoxia at varying environmental temperatures. There were 10 rats in each group. Each rat was enclosed completely in an environment maintained at the indicated temperatures and a relative humidity of 65 ± 10%. Statistical comparisons were calculated as described in Methods. All groups differ from each other at a significance of $p < 0.001$.

The survival curves indicate that there is an inverse relationship between environmental temperature and survival time during hyperoxia. Clearly, the animals maintained at a higher environmental temperature had a markedly shortened survival, and those at an environmental temperature that is low, have an improved survival time. Gross pathologic examination of all of the animals that died after hyperoxic exposure revealed

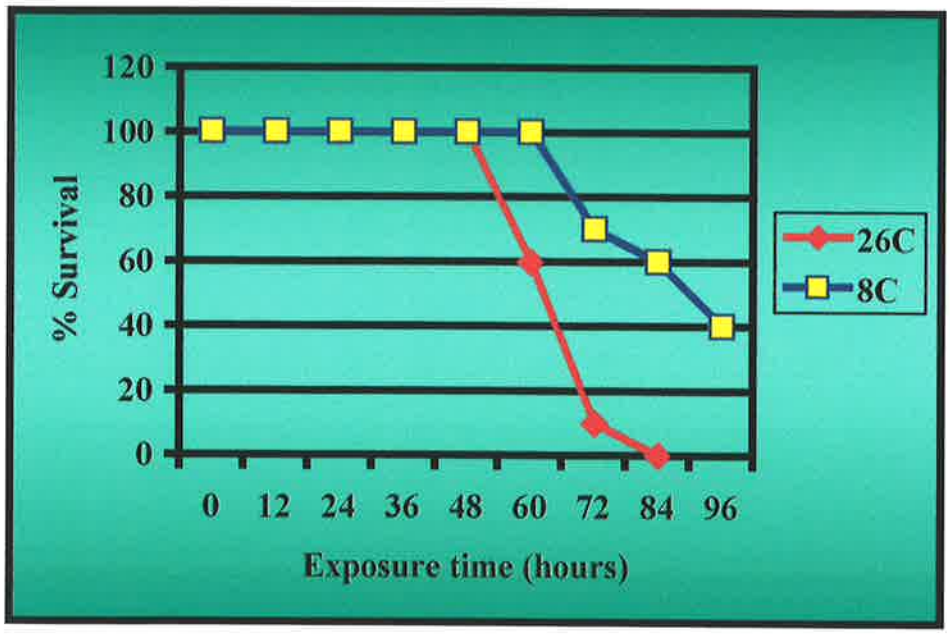


Figure 2.8b : The percent survival of rats exposed whole body to >95% hyperoxia at varying temperatures. Rats were enclosed completely in an environment maintained at the temperatures indicated and with a relative humidity of 65 ± 10%. There were 10 rats in each group. The two groups differed from each other at 72 hours at a significance of $p < 0.001$.

macroscopic evidence of severe pulmonary oedema with bilateral pleural effusions and haemorrhagic lungs. The animals, which survived hyperoxia to the end of the exposure period, showed detectable pulmonary edema, evidenced by perivascular edema, alveolar edema and hyaline membrane formation observed by light microscopy.

Effect on rat survival and lung pathology of increasing or decreasing the environmental temperature in an ambient oxygen environment

In a further series of experiments, animals were exposed to air in environmental temperatures ranging from 8°C to 37.5°C. Animals appeared to be at all times normal during normobaric normoxic exposures to 8°C. However, in two control exposures with the animals breathing ambient oxygen tensions (i.e. room air), using groups of ten rats at 37.5°C for a period of 5 days, one rat died and the others showed signs of severe stress with refusal to eat, relative immobility and decline in general animal appearance. These animals were clearly under stress. However, pathologic analysis of the lungs of all of these animals showed no evidence of pulmonary damage at either the electron microscopic or light levels (Figure 2.9), and there were no pleural effusions.

Figure 2.9 : Transmission electron microscopy of rat lung after 72 hours of exposure to 37.5°C, exposed to ambient levels of oxygen

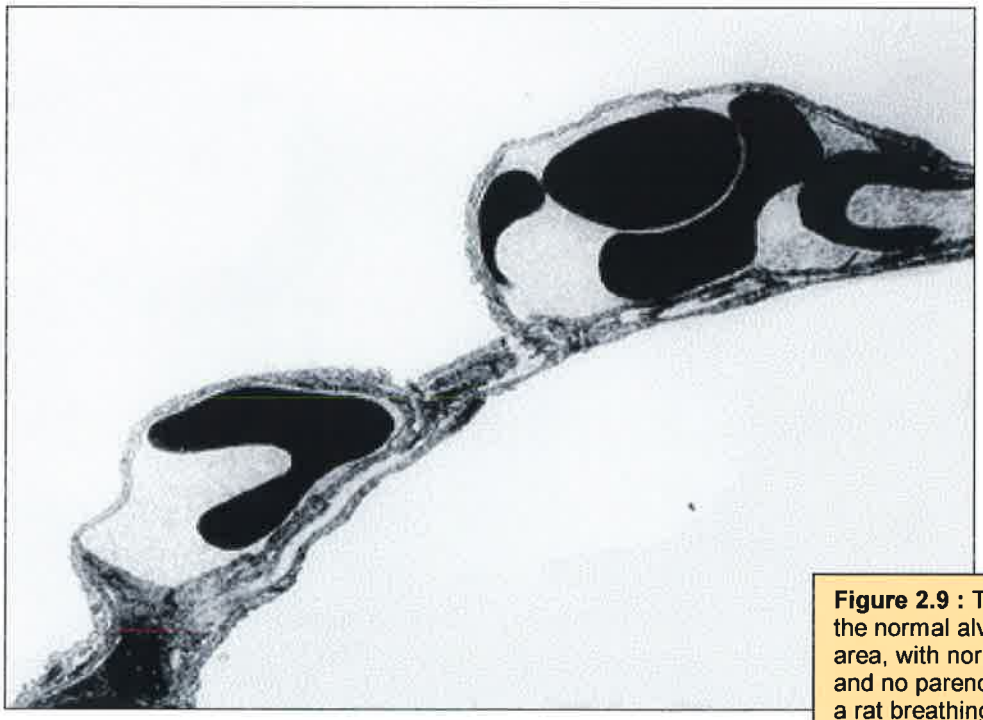


Figure 2.9 : This demonstrates the normal alveolar/capillary area, with normal blood vessels, and no parenchymal oedema, in a rat breathing ambient levels of oxygen at 37.5°C. TEM X 10,000.

Rat body temperature under conditions of higher and lower environmental temperatures[1]

An initial attempt was made to assess core body temperature of rats exposed to various environmental temperatures by recording the mean rectal temperatures during exposure to >95% oxygen, although it has been reported that the rectal temperature of rats provides only an approximation of core body temperature (144). The mean rectal temperature of rats breathing and housed in room air (ambient temperature 23°C) was 37.2°C. At environmental temperatures of 37.5°C, 35.0°C, 30°C and 8°C the mean rectal temperatures of the exposed rats were 37.7°C, 37.8°C, 37.2°C and 37.2°C and 37.2°C respectively. Rats are known to be incompletely homeothermic; that is, their core body temperature responds to some degree to changes in environmental temperatures which do not affect complete homeotherms (145). Because the rectal temperatures of rats exposed to a range of environmental temperatures from 8°C to 30°C did not differ statistically from rats maintained at room temperature, additional measurement of core body temperature was undertaken. This was to substantiate the conclusion that core body temperature was indeed not depressed at an environmental temperature of 8°C. Folk has shown that liver temperature obtained immediately on decapitation of the test animal accurately reflects core body temperature (144). Therefore, the temperature of the liver and lungs of rats maintained at 8°C and 25°C in air for 24 hours was determined by this method. No difference in liver temperature was observed between the two groups, thus confirming that core body temperature was not depressed during maintenance at 8°C (Table 2.1). In addition, it is worth noting that, in all of these animals, the temperature of the lungs was 1.5°C lower than liver temperature. Core temperature by this method for animals exposed to 8°C and 25°C at the same time as 100% oxygen was not performed because of technical difficulties.

[1] These experiments were performed in Dr Folk's laboratory, with his assistance and advice.

	Lung Temperature	Liver Temperature
Control RT/22.7°C [n=10]	36.9	38.2
	37.1	38.0
	37.0	38.1
	36.3	37.5
	35.6	38.0
	37.2	38.2
	36.3	37.8
	36.6	37.7
	37.1	38.7
	-	37.5
	36.7 ± 0.18	**38.0 ± 0.12**
Cold 8°C [n=10]	37.1	37.9
	37.0	38.0
	36.4	38.1
	37.3	37.8
	36.5	37.8
	36.4	37.8
	37.3	38.2
	-	37.8
	36.3	37.6
	37.0	37.9
	36.8 ± 0.14	**37.9 ± 0.17**

Table 2.1 : Rats weighed between 180-200 gm. Temperature measurements were made as described in the text. Temperatures of the liver and lungs of rats [180-200 g] obtained immediate after decapitation. Animals were maintained at environmental temperatures of 23°C [O] and 8°C [O]. Each point represents an individual animal. Within the lung and liver groups the same temperatures are seen irrespective of the environmental temperature, but the lungs are different to the liver temperatures ($p<0.0001$ at both temperatures studied. The **bold** numbers in each group are the means, ± SEM.

Effect on lung pathology of increasing the environmental temperature to 37.5°C in an oxygen environment of >95%

Pathological assessment using light and electron microscopy was performed on lung tissue recovered from the animals killed after exposure to >95% oxygen at 37.5°C for 12, 24 and 36 hours. Alterations in lung architecture consistent with pulmonary oxygen toxicity (139) were observed (Figure 2.10), as already previously demonstrated at 25°C in hyperoxia in figure 2.5. However, the changes were accelerated at this higher environmental temperature.

Figure 2.10 : The electron microscopic changes of the lung in >95% hyperoxia at a 37.5°C environmental temperature (all panels ^{X}10,000)

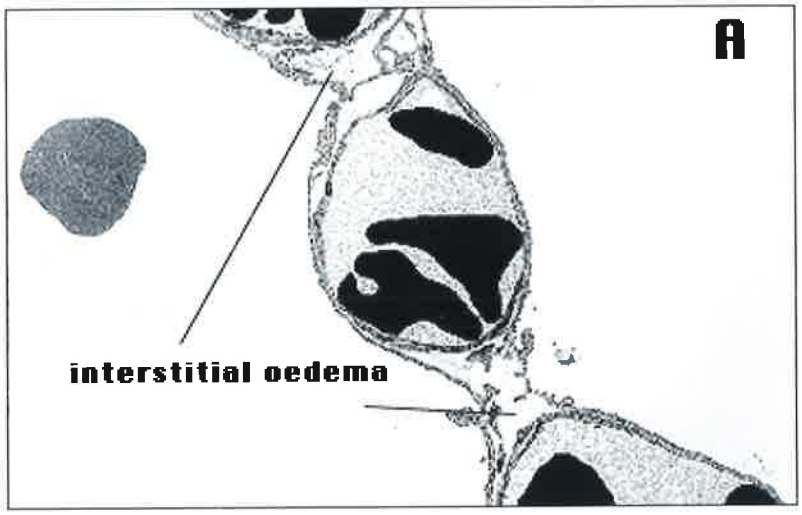


Figure 2.10a : This shows the TEM picture of rat lung after 12 hours of >95% hyperoxic exposure at 37.5°C. Note the early interstitial oedema. At this time point at ambient temperatures there was no evidence of any interstitial oedema.

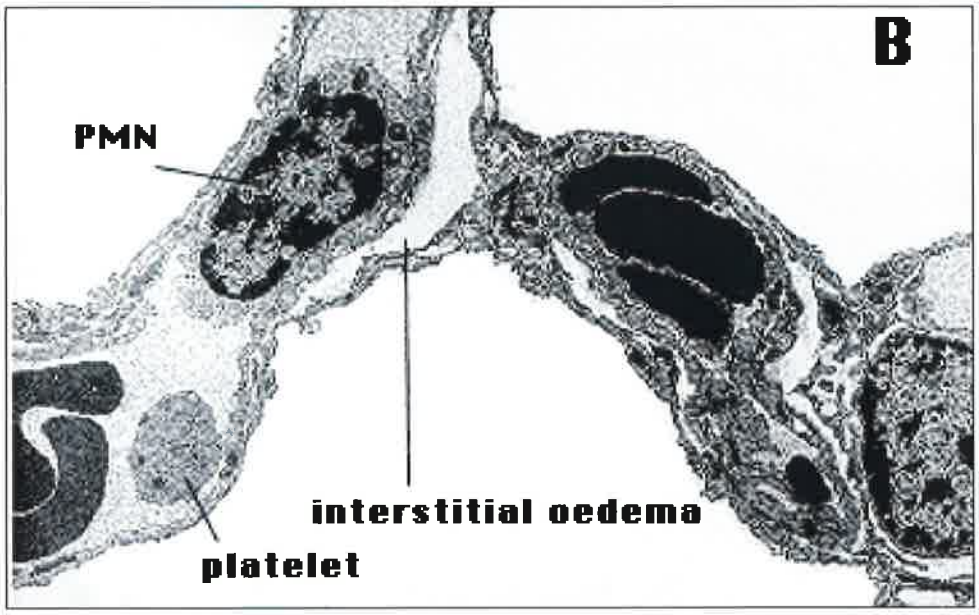


Figure 2.10b : This shows the TEM picture of rat lung after 24 hours >95% hyperoxic exposure at 37.5°C. Note the developing interstitial oedema with blebbing, and neutrophils and platelets already in the alveolar capillary.

For purposes of comparison, the pathological assessment performed in the same manner for animals exposed at 25°C has been previously shown in Figure 2.5. At both temperatures, interstitial pulmonary oedema was the first microscopically observable pathologic change. The rate of onset of pulmonary oedema was much more rapid, however, in animals exposed to oxygen at the higher temperature.

Effects of increasing the inhaled gas temperature on hyperoxic (>95%) lung damage, with an environmental temperature of 23°C

In these experiments, rats inhaled air or >95% oxygen at temperatures above or at ambient temperature, but the general environmental temperature was maintained at 23°C. As determined by rectal temperature, no change in core body temperature occurred as a result of temperature changes in inhaled gas. Although restrained, the animals tolerated the exposure chamber well. The survival curves indicate that at high-inspired gas temperatures there is an increased mortality (Figure 2.11).

Figure 2.11 : Effect on rat survival of increasing the inhaled gas temperature (>95% inhaled oxygen), with an ambient external environmental temperature

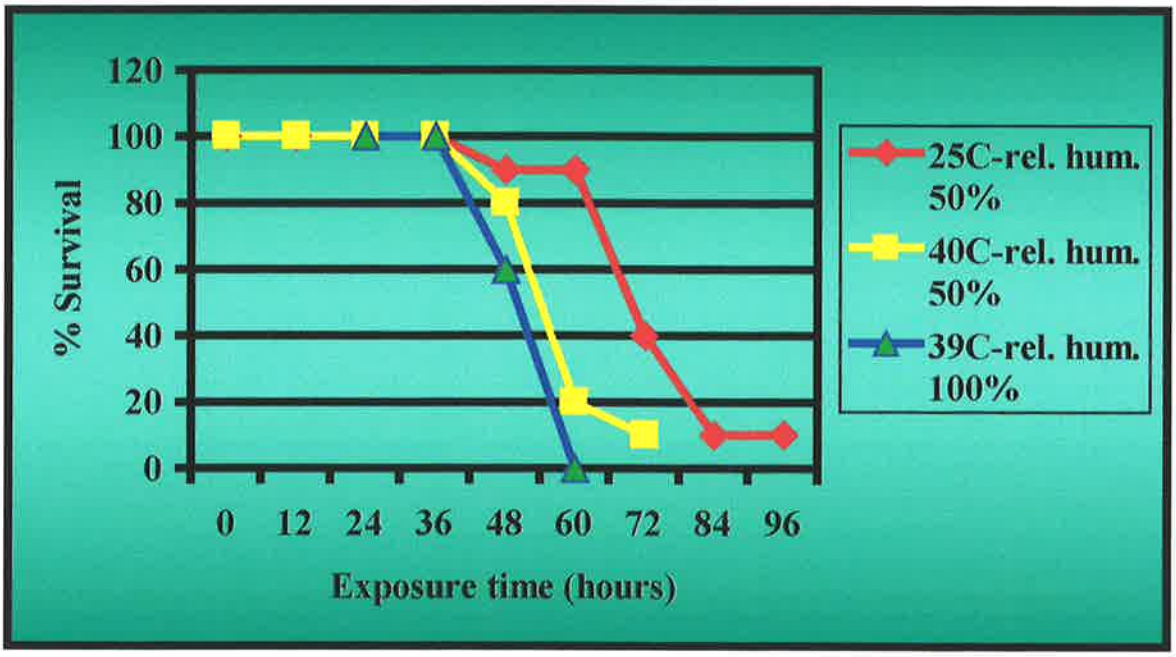


Figure 2.11 : This shows the effect of breathing >95% hyperoxia at room temperature, but with the inhaled gas heated to the temperature shown. The median survival of animals breathing oxygen at 25°C (ambient temperature) was 72 hours and at 40°C was 60 hours. The difference in mortality at the two temperatures was significant ($x^2 = 11.6$, $p < 0.01$). Histopathological examination revealed the presence of severe pulmonary oedema consistent with hyperoxic exposure. There were 10 rats in each group. Electron microscopic studies were not performed on this group of animals.

Effects of breathing 40% oxygen at an elevated environmental temperature (37.5°C)

In order to determine whether increased environmental temperature caused greater oxygen sensitivity, a group of ten rats was exposed to an environmental temperature of 37.5°C but only 40% inspired concentration oxygen. All of these rats survived for 72 hours, the LT_{50} for 95% oxygen at room temperature.. Histopathological assessment of the lungs taken from two rats after 24 and 48 hours of exposure showed no abnormalities, but at 72 hours there were minor abnormalities as shown in Figure 2.12.

Figure 2.12 : Assessing the effects of 40% hyperoxic exposure at high environmental temperatures

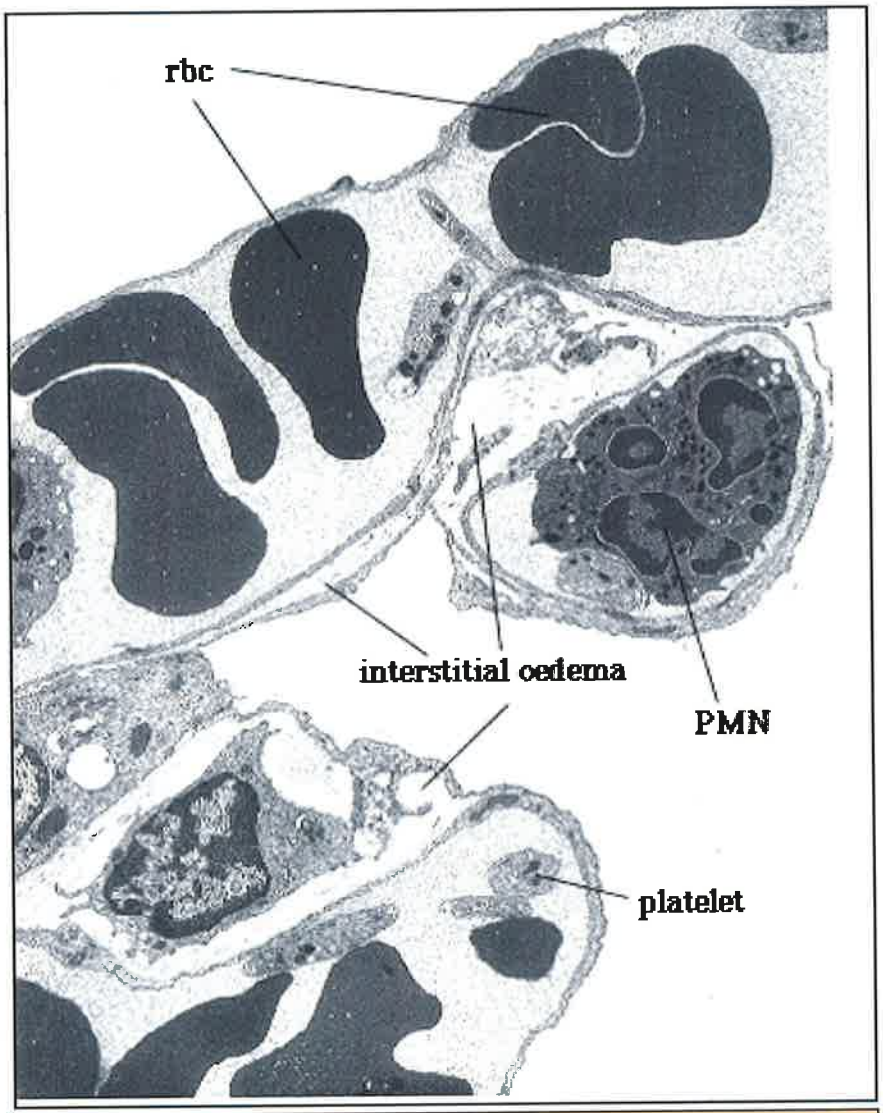


Figure 2.12 : The TEM of rat lung after 72 hours of 40% hyperoxia at elevated environmental temperature. There are mild changes similar to early pulmonary oxygen toxicity at >95% hyperoxia. TEM ^{X}12,000.

Macroscopic evaluation of the lungs of the animals which survived to 72 hours showed no pathology. In three of the surviving animals at 72 hours, only mild perivascular edema and mild alveolar congestion were observed on histopathology. The remaining five animals had normal lungs on histopathological examination.

Assessing the effects of relative humidity on normobaric oxygen toxicity.

The relative humidity in the exposure chamber used above for the experiments described was 65 ± 10%. The effect of relative humidity alone on oxygen toxicity was examined by subjecting rats to >95% oxygen at 40°C in either 50% relative humidity or 100% relative humidity. No difference in mortality was seen in >95% oxygen when the temperature remained constant and the relative humidity was increased from 50 to 100%.

Figure 2.13 : Assessing the effects of temperature on pulmonary oxygen toxicity, combining all results.

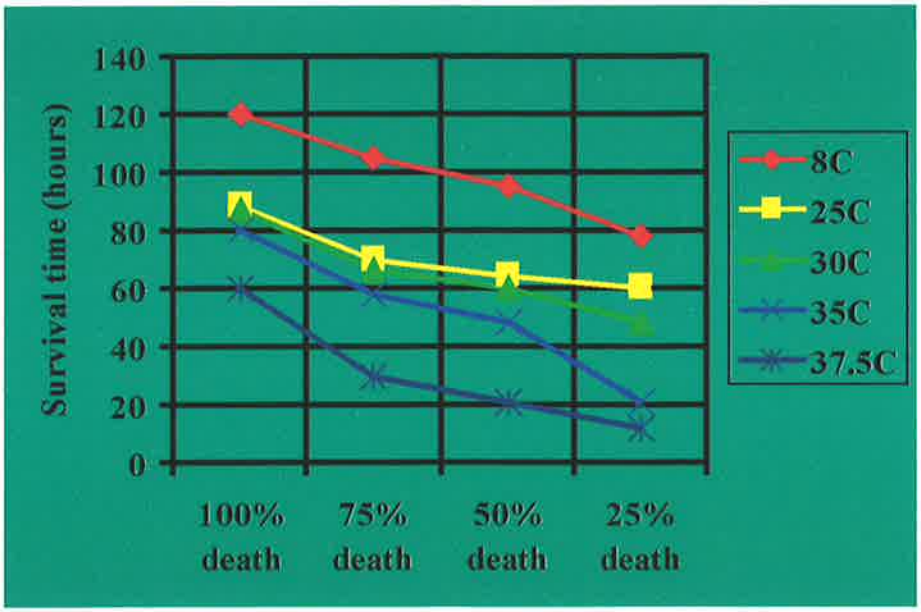


Figure 2.13 : The results of all of the rat mortality studies are combined, at a fixed relative humidity. This demonstrates the rat survival at different environmental temperatures over time. For instance, at an environmental temperature of 37.5°C, 25% of the study animals were dead at 12 hours, with 100% dead at 60 hours. The plots are generally parallel.

DISCUSSION

Two important factors regarding temperature and pulmonary oxygen toxicity emerge from the studies reported here. The results indicate significant enhancement of pulmonary oxygen toxicity at elevated ambient temperatures, and diminished oxygen toxicity at low environmental temperatures (e.g 8°C). Secondly, elevation of the environmental temperature is not the only factor for augmenting pulmonary oxygen damage in the rat model. Even when the environmental temperature is not elevated, inhalation of oxygen at a higher temperature is more damaging to the lungs than breathing oxygen at ambient temperatures. Relative humidity did not effect pulmonary oxygen toxicity, however, as increased or decreased, humidity had no measurable effect.

Higher environmental temperatures (up to 37.5°C) produced increases in rectal temperature and clearly worsened pulmonary oxygen toxicity. However, exposure of rats to a low environmental temperature (8°C), which diminished the rate of development of pulmonary oxygen toxicity, was not accompanied by a significantly lowered rectal temperature or core body temperature as measured by liver and lung temperatures immediately after death. It is possible, however, that after 24 hours the body temperature of the rats maintained at 8°C in high oxygen was depressed, a phenomenon observed previously (146) but which was not determined in this study since only air-exposed animals were examined for temperature changes. It appears from the data presented here, therefore, that a simple inverse relationship between body temperature and pulmonary oxygen toxicity does not exist.

The environmental temperature changes are capable of producing a wide range of physiological alterations within the rat that may alter the response to normobaric hyperoxia. These include changes in thyroid function, in adrenal pituitary axis function, and in basal metabolism (147), oxygen consumption (145), suppression of food intake and drug metabolism (148), reduced lung cilial function (149), and cardiac output (150). Other temperature-dependent lung functions are effects of surfactant (151, 152) and in the

hypoxic pulmonary vasoconstrictive response (153). Respiratory rate does not change as a function of environmental temperature, however (150). However, at extremes of temperature, the respiratory rate may rise due to heat induced hyperpyrexia.

Because of the wide variety of physiological responses to altered body temperature, it is difficult to attribute the augmentation of oxygen toxicity by elevated environmental temperature to any one specific factor. Furthermore, since body temperature is not depressed at low environmental temperatures where the severity of oxygen poisoning is reduced, factors other than a generalized physiological response may be involved. Perhaps local alveolar wall cooling or heating alters the response of the alveolar cells to inspired hyperoxic damage. One other possible effect is the likely increase in local production of interleukin 1 in response to the heat stress (154, 155), even though it is generally understood that interleukin 1 is produced during inflammation and elevates body temperature. Interleukin 1 may be produced by alveolar macrophages locally in the alveolar lumen as well as by monocytes (156). Chicken derived macrophage secretion of interleukin 1 is temperature dependant (157). Interleukin 1 has been shown to protect against the effects of subsequent hyperoxic exposure, and this protective effect is associated with an increase in Mn SOD, but not CuZnSOD (158, 159). This possible involvement of interleukin 1 is not a good explanation for the striking effects of environmental temperature on oxygen toxicity, but does raise the intriguing possibility that fever, or raised environmental temperature may protect against subsequent hyperoxia.

Mice, unlike rats, are affected adversely by cold stress with respect to the toxic response to hyperoxia (143). Indeed, as shown in these studies, cold-maintained rats are protected to a significant extent. The reason for this difference between the two rodent species is unclear.

The augmentation of pulmonary oxygen toxicity by breathing oxygen inspired at high temperatures without increasing environmental temperatures or core body temperature is not as dramatic as the cold-dependent protection via manipulation of the environmental temperature, but nevertheless, the effect is significant. I have no data on the effect of breathing cooled oxygen at normal ambient temperatures, however, as we could not develop a system of cooling the oxygen. In addition, the mild changes of pulmonary oxygen toxicity seen after inhaling 40% but at a high environmental temperature

supports the possibility that temperature is an important modifier of pulmonary oxidant stress.

These observations are potentially important, particularly with respect to the heat enhancement of oxygen toxicity for the management of patients requiring high concentrations of oxygen. Such persons are those who are febrile or persons who are, or could be, exposed to the inspiration of oxygen at high temperatures, such as patients on ventilators (160-162) aircraft pilots (163) or divers (164). Indeed, there have been clinical observations that might support these observations as being relevant in people as well as in experimental animals (165). There still remains very little observation on the effects of environmental or inhaled gas temperature on pulmonary oxygen toxicity, apart from these studies reported in this chapter. An isolated rabbit lung preparation has confirmed the effects of temperature on lung oedema formation (166). Recent studies in the fly have suggested very strongly that the higher mortality of these insects under warmer environmental temperatures is the result of an increased oxidant stress (154).

Conversely, these observations suggest that ventilation with cooled oxygen may be an effective means to protect both patients and healthy individuals exposed to high concentrations of oxygen from developing the lung lesions typical of pulmonary oxygen toxicity. In some medical centers, patient cooling in the setting of acute lung injury is being evaluated (167).

Further observations regarding the effects of the temperature of inspired gases in oxygen induced pulmonary damage would seem to be warranted.

Chapter 3

Effect of Intraperitoneally Administered Superoxide Dismutase on Pulmonary Damage resulting from Hyperoxia[1]

[1] The results were presented at an International Symposium on Superoxide Dismutase, and publications resulting from this work are as listed, (168-171).

INTRODUCTION

As indicated in Chapter 1, prolonged exposure to 95-100% oxygen at a pressure of 1 atmosphere, causes progressive loss of pulmonary function accompanied by structural damage to the lungs in all mammalian species studied (139, 172). The pulmonary damage resulting from hyperoxic exposure is probably accompanied by the production of oxygen-derived free radicals. Superoxide anion (O_2^-) hydrogen peroxide (H_2O_2) are produced in biological systems by the single-electron reduction of oxygen (35). By a mechanism that is yet to be clarified, but is apparently non-enzymatically catalyzed by iron, O_2^- and H_2O_2 together can produce a highly reactive species resembling a hydroxyl radical ($HO^\bullet$) (173). This is one free-radical species that has been proposed to be the damaging agent in oxygen-induced cell lysis, membrane damage, and lipid peroxidation (174). However, as indicated in Chapter 1 there are a variety of other potentially damaging radical species, including peroxynitrite radical.

The superoxide dismutases, the enzyme(s) that catalytically convert the substrate, O_2^-, to H_2O_2 and O_2 , are present in mammalian cells in three forms. The manganese-containing enzyme (MnSOD) is found in the mitochondrial matrix, the copper/zinc (CuZnSOD) containing form is in the cytosol, and there is a form, ECSOD, with strong affinity for heparin, which is found within the interstitial tissues, between cells. All of these are unique and are not structurally related. There is now a great deal of evidence that the function of the superoxide dismutases is to prevent the accumulation of cellular O_2^- [initially Fridovich, (175)]. Hydrogen peroxide, which is produced by a number of biochemical reactions, including the action of the superoxide dismutases, is a substrate for catalase and glutathione peroxidase, enzymes that are both present in normal mammalian cells. The catalytic destruction of either the superoxide anion or hydrogen peroxide is therefore potentially an important factor in preventing the production of damaging amounts of $HO^\bullet$.

Under normoxic conditions the endogenous cellular levels of the superoxide dismutases, the enzymes of the glutathione peroxidase system, and of catalase, appear to

be adequate for the control of the cellular flux of reactive oxygen metabolites. It has been established that elevation of the levels of pulmonary catalase, glutathione peroxidase, and the superoxide dismutases through oxygen-mediated enzyme induction, is an important factor in the neonatal resistance to pulmonary oxygen toxicity [initially Stevens, (69)] although this phenomenon is largely confined only to the rat. This has been reviewed in more detail in Chapter 1.

Intermittent systemic administration of the superoxide dismutase(s) has been tried in an attempt to reproduce oxygen tolerance in the adult rat. However, this is not satisfactory as a means of testing the possible protective effects of the enzyme because of a very short half-life after IV administration (several minutes); the result of rapid renal excretion (176, 177). As also indicated in Chapter 1, other measures to increase the biological half-life of these enzymes, such as administration in liposomes or polyethylene glycol, have had variable results. Continuous systemic administration could provide a more adequate means of testing the efficacy of the exogenous superoxide dismutases in possibly protecting against pulmonary oxygen toxicity. The purpose of the study described here was to assess the effect of continuously administered CuZnSOD in preventing or modifying pulmonary damage provoked by continuous exposure to normobaric hyperoxia, in the rat.

METHODS

Adult male Sprague-Dawley rats (Biol-Lab, 175-200g) were used for these studies. Animals were specific pathogen free. Animals to be exposed to hyperoxia were lightly anaesthetized with ether, and a catheter was inserted through a small incision at the nape of the neck. The distal tip of the catheter was then passed subcutaneously to an intraperitoneal position and held in position with a silk suture. The proximal end of the catheter was left exiting the animal in a subcutaneous position at the nape of the animal's neck. The animals tolerated this catheter extremely well, and when housed in individual cages, could be free to move about in the cage without any restraint. This novel system was then used for the continuous delivery of an intraperitoneal infusion of either the test substance or placebo, without the need for further manipulation (Figure 3.1).

Figure 3.1 : Rat with intraperitoneal catheter inserted into the nape of the neck. The catheter then passes subcutaneously around the body into the peritoneal cavity. The rat can move freely in the cage, and if housed as one animal per cage the catheter is very well tolerated.

After waking, these animals were given either sterile normal saline or sterile normal saline with added superoxide dismutase (1500 units/ml; copper/zinc superoxide dismutase prepared from bovine liver, Truett Laboratories) as a slow intraperitoneal infusion (12-15 ml per 24 hr) using an infusion set (Venoset). The animals were exposed to a 95% oxygen atmosphere for 72 hours in a controlled atmosphere chamber with continuous monitoring of oxygen, carbon dioxide, and water vapor pressure according to a previously described procedure (69).

Animals were exposed in the same large oxygen or air chamber(s) in individual cages. Four animals were used in each 72 hour experiment, two treated with N-saline only, and two treated with superoxide dismutase carried in normal saline. The saline and superoxide dismutase was administered for 12 hours prior to beginning hyperoxia exposure. The amount of superoxide dismutase was determined in prior experiments to be that producing a serum level of approximately 10 units/ml.

Oxygen toxicity was assessed at 72 hours using animal mortality at 72 hours; in addition, arterial blood gas tension analysis, measurement of static pulmonary compliance and histological assessment of the lung were used as objective indices of pulmonary damage.

Animal deaths were recorded every 6 hours and totalled at the end of the 72 hour period. Surviving animals were lightly anaethetized with ether, and an arterial blood gas sample was quickly drawn with a heparinized syringe from the abdominal aorta under direct vision. The arterial blood gas samples were placed immediately in ice water, and blood gas tension analysis was performed within 20 minutes (Instrumentation Laboratory Blood Gas Analyzer 713). The lungs were then removed *en bloc* and inflated to 25 cm of water pressure with 10% buffered formalin and fixed at this pressure. Lungs were embedded in paraffin and then sectioned, and the slices stained with hematoxylin and eosin. These tissue sections were evaluated histologically by a very experienced pulmonary pathologist with no prior knowledge of the experimental protocol. A grading system to describe these changes was devised by the pathologist to allow comparisons or contrasts to be made. The pathologist who participated in these studies was Dr Earl Rose MD, Pathologist at the University of Iowa Hospitals and Clinics.

A further group of animals were studied for other changes in lung function. This involved the measurement of the lung elastic recoil properties after 72 hours into oxygen exposure. To measure this, animals were anaesthetized with ether, the chest wall split open by cutting anteriorly along both sides of the sternum and removing carefully the anterior ribs together with the sternum. The trachea was then cannulated and the lungs inflated to 35cm H_2O pressure and then allowed to spontaneously deflate. The pressure volume curve was then constructed by inflating the lungs again to 35 cm H_2O, and measuring the volume of the lungs in mls. The lungs were then passively deflated by 5-cm H_2O pressure decrements and the lung volume change measured at each pressure. To calculate % predicted total lung capacity (TLC) a series of preliminary experiments were performed establishing the total lung capacity in relation to the body weight of the rats using inflation at 35 cm H_2O pressure as the TLC equivalent - the resulting relationship enabled each study animal to have TLC calculated from the pre-treatment body weight. It should be noted that in all cases the lungs were left in the thorax with the anterior chest wall removed - this was found to be necessary as the oxygen treated animals sometimes had significant pleural adhesions, as well as pleural effusions, making complete lung removal very difficult without producing a visceral pleural leak.

Another group of animals similarly fitted with intraperitoneal catheters received sterile normal saline with added superoxide dismutase, as well as a control group receiving equivalent volumes of normal saline, as previously described. These animals were not exposed to hyperoxia. The lungs of these animals were prepared for enzymatic analysis. After 24 hours, rats were anaesthetized with ether, and a blood sample was collected by venepuncture of the tail vein. The blood sample was allowed to clot and the serum collected. The lungs of these animals were perfused *in situ* with 30 ml of ice-cold phosphate-buffered saline, pH 7.4, by injection into the right atrium. The lungs were removed *en bloc* from the animals, blotted dry, and excess tracheal and non-lung tissue was removed. The lungs were weighed and homogenized in sterile normal saline using a Sorvall Omnimixer. The homogenate was subjected to sonic disruption for 3 minutes. Superoxide dismutase activity was measured in this preparation. The group of control animals, which received an intraperitoneal infusion of normal saline only, were treated similarly.

Superoxide dismutase activity was measured by the method of McCord and Fridovich, modified by the addition of 5 x $10^{-5}m$ M NaCN to the assay mixture (178). This

assay was performed in 3ml of 0.05M potassium phosphate buffer at pH 7.8, containing 10^{-4}M EDTA in a 1.0-cm cuvette thermostated at 25°C. The reaction mixture contained $1X10^{-5}$M ferricytochrome *c*, $5X10^{-5}$M xanthine, and sufficient xanthine oxidase to produce a rate of reduction of ferricytochrome *c* at 550mμ of 0.025 absorbance unit per minute. Under these conditions the amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome *c* by 50% (i.e. to a rate of 0.0125 absorbance unit per minute) is defined as 1 unit of activity. Total lung DNA was measured according to the method of Richards (179), using diphenylamine reagent (4% diphenylamine and 0.01% paraldehyde), with absorbance read at 600 nm. Haem concentration was determined from the carbon monoxide difference spectra of blood, serum and tissue homogenate samples (180). Superoxide dismutase activity attributable to contaminating blood in tissue samples were calculated from the known enzyme concentration in blood for all samples analyzed, and was subtracted from the measured values.

RESULTS

Body fluids such as serum normally contain little superoxide dismutase (181). A very low level of activity was present in the serum of control rats receiving saline in these experiments. This was possibly attributable to haemolysis during the preparation of the serum sample, but more likely (now in retrospect) represents small levels of circulating EC-SOD. However, the superoxide dismutase level in the serum of animals receiving the intraperitoneal infusion of superoxide dismutase was significantly greater than that of the control animals (Table 3.I).

Table 3.1 : Superoxide Dismutase in Rat Serum

Saline Treated Rats (Units of SOD/ml)	**Superoxide Dismutase Treated Rats** (Units of SOD/ml)
3.8	11.0
2.5	10.7
4.0	12.3
4.5	
MEAN 3.7 ± 0.43 SEM	MEAN 11.33 ± 0.85 SEM

Table 3.1 : Rats were given either saline or a total of 18-22.5 x 10^3 units of superoxide dismutase (in saline) in a 24 hour period. Blood samples were taken and allowed to clot. Serum was removed and analyzed for enzyme activity as described in Methods. Values are reported for each animal tested. The difference between the values for saline treated and for enzyme-treated animals is significant to $p < 0.05$. Statistical method Mann-Whitney U Test.

Enzyme analysis of the homogenates of perfused lungs from the superoxide dismutase-treated animals demonstrated a significant increase (32%) in the enzyme level compared with the level in saline-treated rats (Table 3.2).

Table 3.2 : Superoxide Dismutase in Rat Lung Homogenates

Saline Treated Rats Units of SOD/µgm DNA)	**Superoxide Dismutase Treated Rats** (Units of SOD/µgm DNA)
2.4	3.1
2.0	3.3
2.6	3.1
2.6	
MEAN 2.4 ± 0.14 SEM	MEAN 3.17 ± 0.067 SEM

Table 3.2 : Rats were treated as described in Table 2.1. Lung homogenate samples were obtained as described in Methods. Values are reported for each animal tested. The difference between the values for saline and for enyzme-treated animals is significant to $p < 0.05$.

The assessment of animal mortality showed that, whereas 42% of saline-treated animals died after 72 hours of continuous exposure to 95-100% oxygen at 1 atmosphere, only 20% of the superoxide dismutase-treated oxygen-exposed rats died (Table 3.3). Postmortem examination of the animals that died during oxygen exposure showed dark hemorrhagic lungs and bilateral pleural effusions in both the saline treated and superoxide dismutase-treated animals without any obvious difference (Table 3.3).

Table 3.3 : Mortality after 72 hours in a Controlled Atmosphere

	Mortality	Gross Pathology
Saline-treated Air exposed	0/4 (0%)	Normal
Saline-treated O2 exposed (95-100%)	5/12 (42%)	Haemorrhagic lungs* Bilateral pleural effusions
Superoxide Dismutase Treated O2 exposed (95-100%)	2/10 (20%)	Haemorrhagic lungs* Bilateral pleural effusions

* Conducted only on those animals which died during the exposure period.

In the surviving animals, arterial blood gas tension analysis demonstrated a significant difference between the saline treated, oxygen exposed animals and the superoxide dismutase-treated, oxygen-exposed rats in both arterial oxygen tension (paO_2), and blood pH ($p < 0.05$, Student t test). The paO_2 of saline-treated, oxygen-exposed animals was a mean of 34.3 mm Hg, compared to 55.3 in the CuZnSOD treated, oxygen exposed animals. The blood pH of the saline-treated group was 7.05, and that of the CuZnSOD-treated group was 7.26. The arterial carbon dioxide tensions were not significantly different between these groups(Table 3.4a and 3.4b) . These results indicate a major difference in gas exchange parameters between the two groups of animals. The CuZnSOD treated animals, as a group, had a reasonable state of oxygenation and maintained a satisfactory blood pH. The saline treated animals, as a group, had severe tissue hypoxia associated with a significant acidemia.

Table 3.4 : Rat arterial blood gas analysis

Normal Control			Saline Treated oxygen exposed			Superoxide treated oxygen exposed		
paO_2	$paCO_2$	pH	paO_2	$paCO_2$	pH	paO_2	$paCO_2$	pH
107.1	30	7.28	41.7	70.1	7.12	56.6	64.5	7.17
108.2	41.8	7.34	22.5	116.7	6.89	50	155	6.87
104.2	36.3	7.32	25.3	110.0	7.02	62	96.4	7.11
112.6	38.2	7.25	37.4	86.2	6.97	48.9	78.8	7.23
			52.4	91.7	7.12	39.1	64.0	7.34
			19.6	95.1	7.16	79.6	65.3	7.33
			41.0	106	7.06	51.2	68.7	7.34
MEAN	108.02	36.6 7.30	34.3	96.5	7.05	55.3	84.6	7.26
SE	1.74	2.47 0.02	4.56	5.99	0.36	4.84	12.53	0.06

paO_2	Normal cf	Saline treated $p < 0.01$
	Normal cf	Superoxide dismutase treated $p < 0.01$
	Saline cf	Superoxide dismutase treated $p < 0.02$
$paCO_2$	Normal cf	Saline treated $p < 0.01$
	Normal cf	Superoxide dismutase treated $p < 0.01$
	Saline cf	Superoxide dismutase treated $p < 0.1$
pH	Normal cf	Saline treated $p < 0.01$
	Normal cf	Superoxide dismutase treated $p < 0.5$
	Saline cf	Superoxide dismutase treated $p = 0.06$.

Table 3.4 : Showing the actual values of the arterial blood gas measurements in these animals.

The pressure volume lung deflation curves are shown in Figure 3.2. Whilst the elastic properties of the lungs in both oxygen exposed groups shows marked loss of elastic recoil compared to the normal animals, there was no significant difference between animals treated with saline or superoxide dismutase.

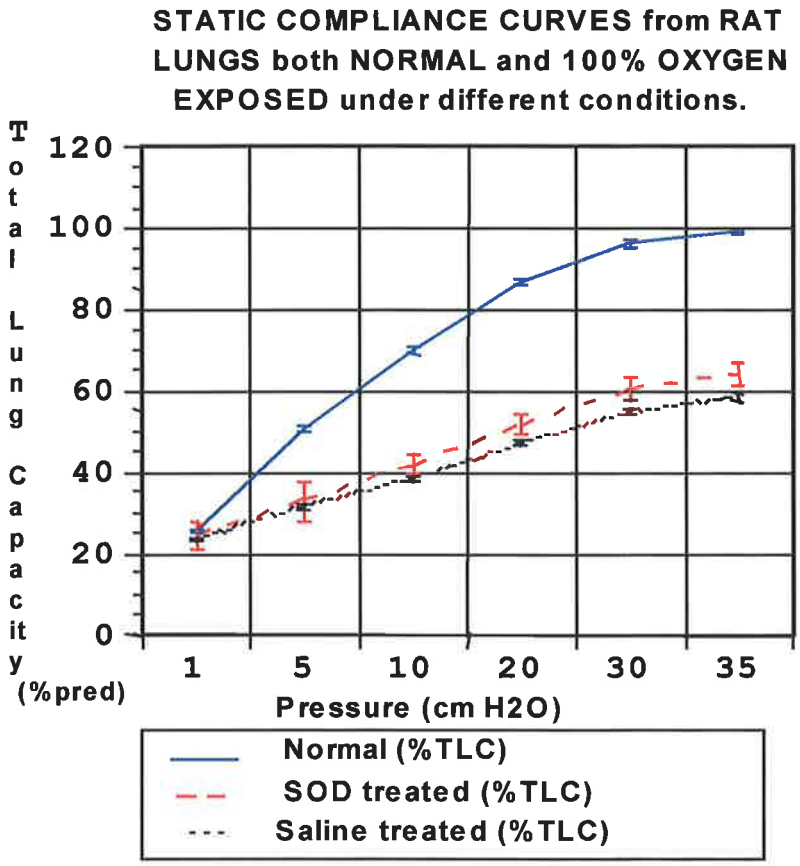


Figure 3.2 : This plot of the deflation pressure volume curves shows no difference in static compliance between the saline and SOD treated animals that survived 72 hours of 100% oxygen. These animals were the same animals that were assessed by blood gas analysis.

For the purpose of histological comparison, three major categories of pathology were developed by the pathologist. The pathologist examined the whole lung. (Figure 3.3).

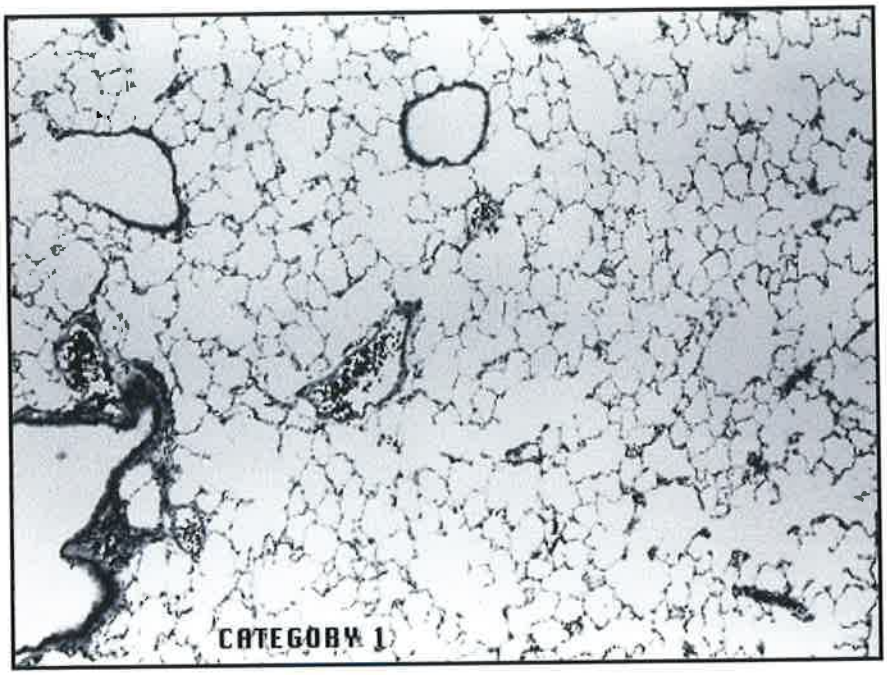


Figure 3.3a : Category I was assessed as minimal change (normal or nearly normal lungs). Note the normal airways at top/center and at the left of the picture. The pleural surface can be seen at the bottom right. In the center are blood vessels containing blood cells.

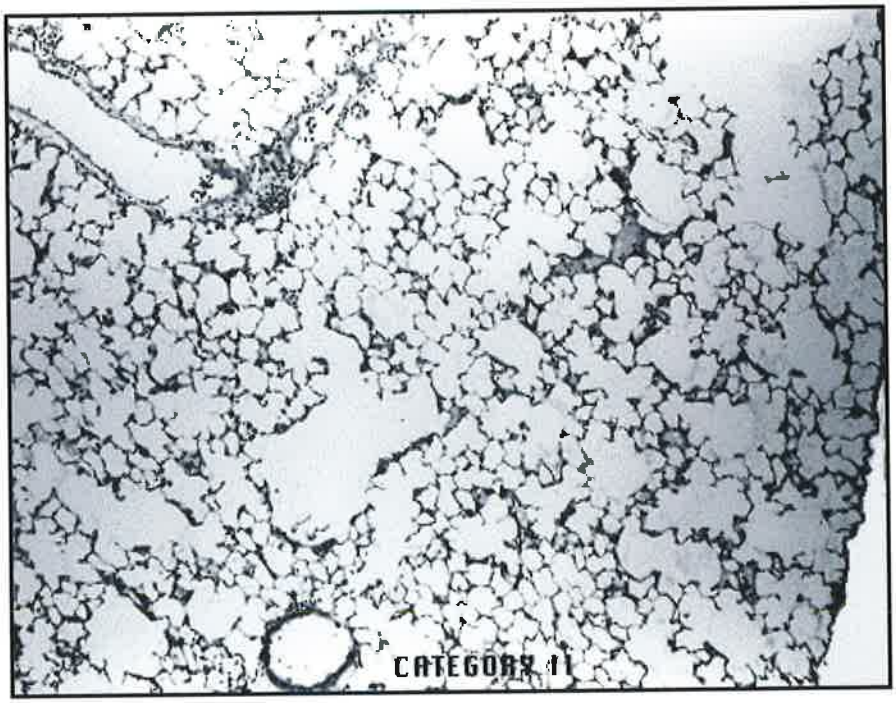


Figure 3.3b : Category II included mild perivascular edema, focal alveolar wall thickening, and focal areas of increased cellularity. Note the airway at the top left, with the beginning of peribronchial oedema, and the alveoli that are filling with oedema fluid at the mid region towards the right. The pleural surface is again shown on the lower right.

Figure 3.3c : Category III was defined by severe perivascular oedema, generalized alveolar wall thickening with increased alveolar infiltrate, haemorrhage, and well developed hyaline membrane formation. Note the haemorrhagic infiltrate in the alveolar regions, associated with alveolar oedema especially seen in the sub-pleural alveoli. The pleural surface is shown at the right hand edge.

Histological evaluation demonstrated a difference between the lungs of oxygen-exposed saline-treated rats and oxygen-exposed superoxide dismutase-treated rats. Whereas, four of six saline-treated, oxygen-exposed animals showed severe lung damage (Category III), only one of seven superoxide dismutase-treated rats showed similar damage (Table 3.5).

Table 3.5 : Histological Comparison of Saline and Superoxide Dismutase treated rats

Animals	Category 1 (a)	Cateogory II (b)	Category III (c)	Total number of animals
Normal	+ + +			3
Exposed with continuous infusion of N saline		+ +	+ + + +	6
Exposed with continuous infusion of superoxide dismutase	+	+ + + + +	+	7

(a) Normal or near normal lungs

(b) Mild perivascular oedema, focal alveolar wall thickening focal areas of hypercellularity.

(c) Severe perivascular oedema, generalized alveolar wall thickening with alveolar infiltrate, alveolar hemorrhage, well developed hyaline membrane formation.

A further statistical analysis of these data was conducted by combining the results obtained from mortality assessment with the histopathological evaluation. For this purpose, data from the two groups, Category I and Category II, were evaluated together. The extent of lung damage and the mortality rate were significantly higher in the rats subjects to 72 hours of continuous hyperoxic exposure and treated only with saline than in the oxygen-exposed animals treated with continuous superoxide dismutase (Fischer's Exact test, $p < 0.04$).

DISCUSSION

This study demonstrated, for the first time, that continuous intraperitoneal administration of CuZnSOD provides protection against the pulmonary damage, and subsequent mortality, associated with normobaric, hyperoxic exposure in this rat model. Although significant, the protection was by no means complete. This study did not measure pleural fluid accumulation as a further index of pulmonary damage.

The results demonstrated that the continuous infusion of CuZnSOD reduced the mortality from pulmonary oxygen toxicity. In the animals still surviving 100% oxygen exposure at 72 hours, there was also an improvement in the pulmonary oedema of the whole lung as assessed histologically. Further, there was significant improvement in oxygenation and improvement in carbon dioxide clearance and acidaemia in the CuZnSOD treated animals. There was, however, no significant improvement in the elastic recoil properties of the lungs in these surviving animals, although there was a definite trend indicating more normal static compliance in the CuZnSOD treated group. The pleural fluid accumulation is a feature also of oxygen toxicity in the rat, but was not objectively measured in this study - differences in pleural fluid may be another factor to explain the improved oxygenation not associated with improved elastic recoil properties.

These experiments required supervision of the infusion requiring frequent observations during the day, and 4 hourly checking overnight. The continuous infusion provided a demonstrable increase in both serum and lung levels of CuZnSOD, without causing any obvious animal distress. The outcome measures of mortality, of gas exchange, of pulmonary compliance, and of pulmonary pathology in the surviving rats serves to provide a variety of pulmonary assessments to objectively assess outcome. Larger numbers of animals were not used, partly because of cost, with the biologically isolated CuZnSOD being very expensive. In addition, to maintain the oxygen chambers with good quality oxygen and air, high flow rates were used into the chambers, this also increased the cost.

Nevertheless, the evidence from these experiments indicated a significant, moderation of the pulmonary oxygen toxicity by the infusion of CuZnSOD during, and 12 hours prior to the oxygen exposure. This effect in whole animal studies has not previously been demonstrated.

Optimal protection against the toxic effects of oxygen may only be possible if adequate levels of CuZnSOD are continuously present at the site of superoxide anion generation. By the maintenance of a relatively constant serum level through continuous infusion, an equilibrium between blood and lung interstitial space can be established in a manner similar to that known to occur with plasma albumin. Albumin equilibration occurs in 3-5 hours in adult rodents (182, 183). It is not known whether CuZnSOD penetrates into the cytoplasm of the pulmonary endothelial or epithelial cells under these conditions. Some cytoplasmic uptake of exogenous superoxide dismutase has been demonstrated in bone marrow cells, however. In addition, indirect evidence indicates some cellular accumulation of CuZnSOD on *in vitro* incubation of pulmonary macrophages in cell culture, after the enzyme has been added to the medium. It is worth noting that cells may develop a compromised permeability barrier in their cell membrane when exposed to prolonged hyperoxia *in situ*.

The site of lung damage after normobaric hyperoxic exposure in the adult rat, as well as the origin of the subsequent oedema formation, is the pulmonary capillary endothelial cell. This has been demonstrated by sequential electron microscopy (184), (and confirmed by studies in this thesis) and also by endothelial cell regeneration studies after oxygen exposure (185). However, oedema formation occurs before any recognizable morphological change in the capillary endothelial cell (184). The precise mechanism by which oxygen exposure causes the initial pulmonary oedema is unknown but may be related to damage to the fluid transport mechanism of the endothelial cells, or damage to the cell permeability, either through altering the gap junctions, or the plasma membrane permeability.

The moderate effectiveness of CuZnSOD in diminishing the pulmonary damage occurring under normobaric, hyperoxic conditions is strong evidence that the superoxide anion is involved at least in part in causing this damage. The superoxide anion could be generated under at a variety of intra- or extra-cellular sites in close association with endothelial cells. A potential source of oxygen radicals later in the process may be the

polymorphonuclear leukocytes recruited to sites of chemically-induced injury. These cells constitute a significant source of biologically generated oxygen free radicals (42, 43). Because CuZnSOD probably does not cross the normal cell membrane to a great extent the protective effect noted here is most likely related to removal of the superoxide radical at the membrane of the endothelial cell. The superoxide radicals may also be generated from recruited polymorphonuclear leukocytes. The time course of the superoxide anion production can not be answered by these experiments. However, It is possible that the administered CuZnSOD reduced the O_2^- insult from near the endothelial cell membrane early in the hyperoxic exposure, and from polymorphonuclear cells late in the course of the injury. Further, one of the products of the catalytic activity of CuZnSOD is hydrogen peroxide. The extracellular level of CuZnSOD available from continuous infusion may remove some, but not all of the superoxide anion, and thus contribute to the pool of H_2O_2. Under these conditions, therefore, in the presence of both reactants, which appear to generate $HO^{\bullet}$, tissue damage will occur. This may also be an explanation as to why the protection afforded by the exogenous enzyme may be incomplete. Also, the O_2^- may also react with nitric oxide to form the peroxynitryl radical. By removing superoxide anion, CuZnSOD does reduce the potential for hydroxyl radical production, and also peroxynitryl radical, and subsequent radical-initiated tissue damage via these mechanisms.

CuZnSOD is available in reasonable quantities through recombinant techniques and may be conjugated to polyethelene glycol to extend the half-life; in addition, animals expressing increased levels of the SOD's have been made available through molecular genetics. Studies using a variety of SOD enhancing systems (186-189) have all shown various protection from hyperoxia, as reviewed along with other studies in Chapter 1.

Chapter 4

Alveolar Macrophage Function

Differences between non-smokers and smokers, and observations on phagocytosis of rat compared to human cells.[1]

[1] Communications arising from this work are as follows: (190-194).

INTRODUCTION

The pulmonary alveolar macrophage (PAM) is a component cell of the mononuclear phagocytic system - a system which comprises other cell types such as the blood monocyte, the peritoneal macrophage, the Kupffer cell in the liver, and the microglial cell in the central nervous system. In inflammatory conditions, cells such as epithelioid cells and multinucleated giant cells are also considered to be part of the mononuclear phagocytic system. The mononuclear phagocytic system was classified as a specific cell system by Langevoort (195) in 1970, and this has been modified subsequently by van Furth (196).

There have been many reviews of the PAM (192, 197-203). In this following section, the literature regarding alveolar macrophage structure and function is very briefly summarized, and some controversies regarding this cell are reviewed.

The PAM is resident within the alveolus, where it lies within the alveolar lumen on alveolar epithelium and coated with alveolar fluid containing predominantly pulmonary surfactant and soluble plasma type proteins, including the immunoglobulins (194). The cell therefore occupies a relatively unique position within the body, being exposed directly to a relatively hyperoxic environment, and in intimate contact with air borne and blood borne materials.

Therefore the PAM is likely to be abnormal in smokers and to non-smokers because of the direct and indirect effects of cigarette smoke. However, it remains unclear as to the nature and magnitude of differences. Also, the PAM frequently is subject to radiation challenges, either low level through inhalation of radon gas, or at a higher dose rate through medical radiation treatment for such diseases as lung or breast cancer.

PAM's may be obtained for study by bronchoalveolar lavage, first described in 1961 in animals (204), and adapted to human studies by Reynolds in 1974 (205). Lung mincing and differential centrifugation is another way of obtaining these cells, either from lung biopsy specimens or from small laboratory animals (70).

Fiberoptic bronchoscopy has enabled human PAM's to be recovered by bronchoalveolar lavage with relative ease. When recovered from normal human lung they are a fairly uniform population of cells seen with light microscopy with greater than 90% of the cells being PAM's. The PAM's from smokers are pigmented and are therefore easily distinguished from PAM's obtained from non-smokers. PAM's have a characteristic appearance on transmission electron microscopy, with the macrophages from non-smokers and smokers often being distinguishable.

There are 2 to 20 times the numbers of PAM's in the lungs of smokers compared to non-smokers. PAM's may be studied in culture as suspended cells, as they were in most of the initial studies. This may not have been ideal because, as adhered cells, in culture, they certainly have different magnitudes of function compared to suspended cells; and it is with adhered cells that the majority of the studies are now performed. Culture conditions generally employed are 37°C in a liquid medium, a 95% air, 5% CO_2 atmosphere, and pH 7.4; this may not be entirely appropriate as the temperature and oxygenation of the micro-environment in which the PAM exists may be different.

In tissue culture, approximately 45% of glass adherent PAM's from a normal non-smoker will appear as rounded cells, with the rest spreading out. PAM's from smokers tend to spread out more in tissue culture with only 25% of the cells having the rounded appearance seen in cells from non-smokers. These appearances have been described by scanning electron microscopy (206) and found to be significant. However, other workers have found no significant differences in such comparisons (207). With time, in culture, PAM's from non-smokers spread more and appear indistinguishable to those from smokers. Typical macrophages are shown in Figure 4.1.

Figure 4.1 : Examples of normal alveolar macrophages in tissue culture

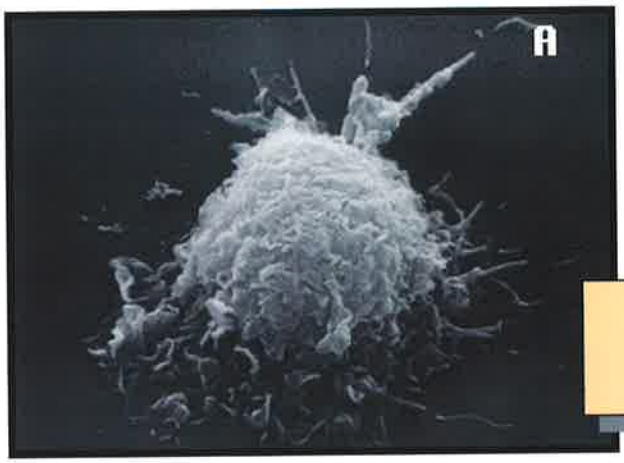


Figure 4.1a : Normal alveolar macrophage. SEM X 4000.

Figure 4.1b : Side view of a normal alveolar macrophage (SEM X 3800).

Figure 4.1c : A group of normal PAM's. SEM X 3600. The bar at the lower right is 10 micron.

PAM's originate from bone marrow precursors and migrate into the lung via the blood monocyte (208, 209). There has been much interest in a pulmonary interstitial "precursor" macrophage, and the intravascular macrophage, and these continue to be studied (210).

Mitosis of the PAM is rare. Mitosis can be observed in occasional PAM from normal bronchoalveolar lavage preparations, but this remains a potential means of generating larger numbers of macrophages in the lung if they are required (211).

The adaptation of PAM's with animal age has been of continuing interest. In particular, relevant to this thesis, is the PAM synthesis of superoxide dismutase and catalase as a mechanism for protection against the toxic effects of 100% oxygen in neonatal animals (212, 213).

The broad functions of the pulmonary alveolar macrophage may be considered under three headings -

1. Clearance;
2. Modulation of the immune system;
3. Modulation of surrounding tissue other than through the immune system, including direct cytotoxicity.

1. Clearance

The clearance function has been the predominant function that has been studied in the early investigation of this cell (214). Clearance may involve both macrophage migration and phagocytosis. Phagocytosis is generally of non-living organic or inorganic particulate material, or of infectious organisms. There are four components of phagocytosis; an initial recognition of something that requires phagocytosis, followed by attachment of the cell membrane to whatever is to be phagocytosed, ingestion, and then digestion (215). Phagocytosis may be studied using a variety of living and dead micro-organisms or manufactured particles.

There have been many reports on the effects of cigarette smoking on PAM function. These have been summarized (203, 216). In some reports there was no influence of cigarette smoke, and in others there seemed to be an inhibitory effect. Some of these differences may be explained by the different methods used, including using suspended cells rather than adhered cells and using different culture times prior to the various assays.

Particulates that are phagocytosed can be cleared either by destruction within the PAM, or removed with the PAM from the lung via the mucociliary elevator, or into pleural or airway lymphatics and then into draining lymph nodes. The interstitial PAM, which is sometimes seen in pneumoconiosis laden with particulate material, is not readily found in normal subjects but, in these diseases at least, it seems that PAM's can ingest particles and move into the pulmonary interstitium. There may also be an airway alveolar macrophage population, although this remains conjectural.

Alveolar macrophage migration is slow when compared to other monocytic cells and polymorphonuclear neutrophils. Migration is enhanced by C5a twenty fold (217), and by a very small polypeptide (formylmethionylphenylalanine) (218). Migration appears to be decreased after smoke inhalation in humans (219), thereby possibly reducing clearance of particles.

2. Modulation of Immune System

The second broad function of the alveolar macrophage is modulation of the immune system. The cell has a complex interaction with lymphocytes. The alveolar macrophage produces an increasing number of recognized lymphokines and cytokines. This area of macrophage immune modulation is rapidly developing, and further review is beyond the scope of this thesis.

3. Modulation of Surrounding Tissue

Apart from its effect on the immune system, the third broad function of the alveolar macrophage is the ability to modify surrounding pulmonary tissue. The PAM has many

surface receptors. These receptors enable the PAM to respond to a wide variety of chemical messages in the immediate environment. The function and importance of many of these receptors in normal and diseased lung remains largely unclear. The PAM also has a secretory function and, in human studies, lysozyme and elastase are secreted. There is some debate as to whether the elastase is actually produced and secreted by the PAM, or whether this reflects neutrophil elastase which has been incorporated into the PAM (220); however, there is now compelling evidence that there is a specific alveolar macrophage elastase (221). Nevertheless, all of these proteins may amplify pulmonary damage, as well as providing a possible protective role. Secretion of chemotactic factors for polymorphonuclear neutrophils (PMN) is also an important function that has changed the perceived role of the PAM's in inflammation. This factor was first described in monkeys in 1977 (222) and shortly afterwards in humans (223). There appear to be at least two chemotactic factors produced, and both are distinguishable from complement. The two factors are of small molecular weight and are released by PAM's within the lung, and in tissue culture. The role of this chemotactic factor in pulmonary inflammation and fibrosis continues to emerge.

The PAM's produce superoxide anion radical, hydrogen peroxide and hydroxyl radicals, all potentially damaging oxygen-derived species. This is particularly important because of the relatively hyperoxic environment in which the PAM resides. Hydrogen peroxide release by PAM's is a potential means of directly damaging surrounding lung, including the soluble proteins along the alveolar surface, such as $\alpha 1$ antitrypsin. One report (224) indicates that tobacco smoke exposure in rats results in increased hydrogen peroxide release from PAM's with phagocytic stimulation.

Another function of the PAM is antibody dependent cellular cytotoxicity (ADCC) (225). ADCC is a function of cells of the mononuclear phagocytic system which is believed to play a role in eradicating infectious agents or tumour cells. The contribution of direct PAM mediated cytotoxicity to lung damage is unknown, but it may be important in contributing to tissue damage in many pulmonary inflammatory conditions.

METHODS

Alveolar macrophages were obtained by the technique of bronchoalveolar lavage (BAL) in both animals and humans. Normal human volunteers were recruited, and the BAL performed at both the University of Iowa Hospitals and Clinics, and at the Royal Adelaide Hospital.

Smokers of tobacco and non-smokers were included. All subjects were otherwise healthy in all respects on clinical history, clinical examination of the chest was normal, and no subjects were on any medication. The age range was restricted to 20 - 50 years. A standard lavage volume was used for all these studies: 50 ml N saline at room temperature was instilled on 3 separate occasions followed, after each instillation by gentle aspiration at a pressure adjusted so as not to cause airway collapse. Lavage return was kept separate for this study, with differential cell counts being performed on each aliquot and, finally, on the combined sample. For cell counts, 0.5 ml of each aliquot was placed into EDTA for subsequent analysis, although when only one cell count for a subject is required in subsequently described studies, it has been taken from the second aliquot returned.

In normal subjects the fiberoptic bronchoscope was always wedged gentled into either a subsegment of the lingular, or a subsegment of right middle lobe, to allow gravity to assist the lavage fluid. Local anaesthesia using 0.5% lignocaine down the channel of the bronchoscope was always instilled, but only the minimal amount to ensure patient comfort.

In each instance the appropriate Institutional Ethics Approval was obtained, and signed consent provided by the subjects after verbal and written information was provided to them.

Cell counts of the lavage samples were performed by examining Jenner-Giemsa stained cytocentrifuge preparations to determine the proportion of cells present. A total cell count was obtained using a manual cell counting chamber.

Scanning electron microscopy was performed using a standard procedure. Briefly, the specimen was fixed in 2.5 - 3% glutaraldehyde in 0.1M cocodylate, then rinsed in buffer for 30 mins, then progressively put through increasing concentrations (30%, 50%, 75%, 95%, and 100%) of ethanol for 30 mins at each concentration. The 100% ethanol was changed three times for 20 minutes each time. At this critical point drying was undertaken, and the specimen then coated with an appropriate electron dense material.

Alveolar macrophages were concentrated in the lavage fluid by gentle centrifugation, and the pellet formed re-suspended to produce the appropriate cell concentrations. Generally the BAL fluid was centrifuged at 1900 RPM for 10 minutes to produce the macrophage pellet.

Scanning electron microscopy pictures of alveolar macrophages were taken from cells cultured on plastic (Aclar) for the required time and then fixed in 1% glutaraldehyde prior to the usual scanning microscopy preparation.

Phagocytosis was assessed using heat killed yeast. The centrifuged alveolar macrophages were re-suspended from their pellet by gentle agitation with a glass pipette into Hams F12 medium with 10% Fetal Calf Serum added. The cell count was reassessed, and adjusted to 1 x 10^5 cells per ml, and 1 ml of the suspension added into sterile Leighton tubes containing glass coverslips. They were then cultured for 24 hours at 37°C in 5% CO_2 and 95% air without agitation. At that stage 0.1 ml of heat killed yeast, opsonized with 0.1 ml of guinea pig complement was added to produce a concentration of 150 yeast cells per macrophage. The Leighton tubes were then replaced into the incubator, and gently agitated mechanically. Tubes were removed every ten minutes, and the coverslips removed and washed with sterile saline at 4°C to remove excess yeast. The coverslip was then stained with 0.4% erythrocin B for 2 minutes, inverted on a glass slide and 100 macrophages with their adherent and/or ingested yeast counted. This technique was developed for this purpose after another phagocytic assay using staphlococci and lystostaphin was shown in studies conducted over six months, to be an unsatisfactory assay (data not shown). The procedure described and used here, enables the yeast that are adhered to the macrophages to be counted (as they stain with erythrocin B), and also the ingested yeast to be counted (they are visible, but dye is excluded by the macrophage membrane). The technique also allows for macrophage viability to be assessed at the same time.

RESULTS

Figure 4.2 : The typical survival of human alveolar macrophages in culture.

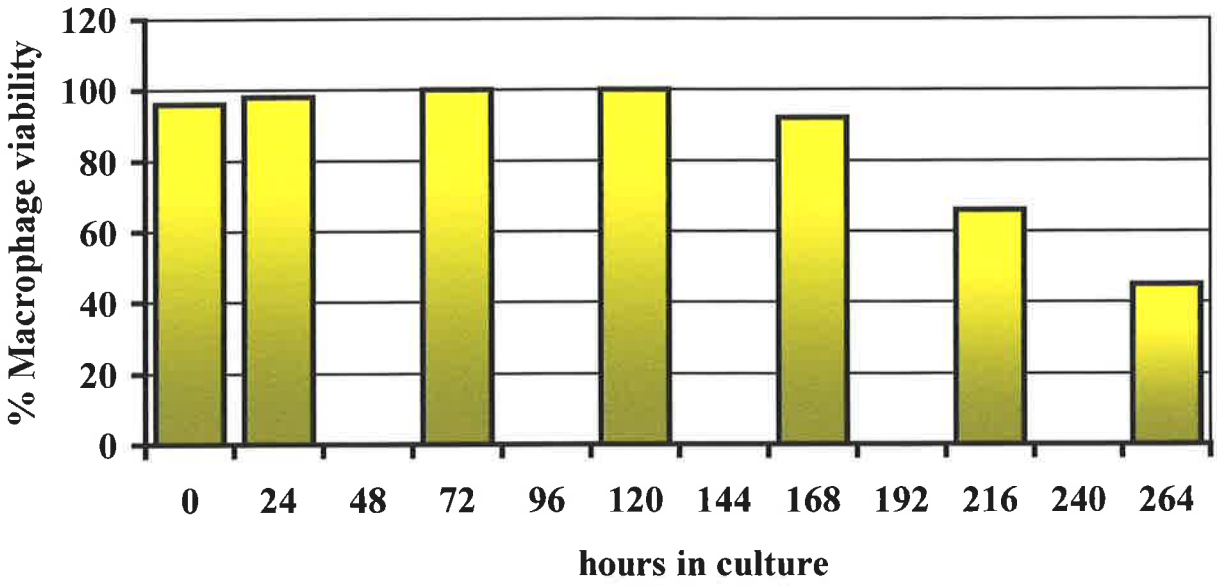


Figure 4.2 : Survival of PAM's in culture with time as detailed on the x-axis, with % viability on the y-axis.

The normal human volunteer BAL results are shown with age, fluid volume and cell return in Figures 4.3, 4.4, 4.5 and 4.6 with the volume returned for each aliquot of fluid instilled and the cell differential and numbers for each aliquot given. The mean ages for the normal human volunteers was 25.7±2 years for the non-smokers, and 26.1±1.7 years for the smokers. There was no significant difference between these two groups.

Figure 4.3 : The volume of the fluid returned for the first, second, and third aliquot returns, and the final total volume.

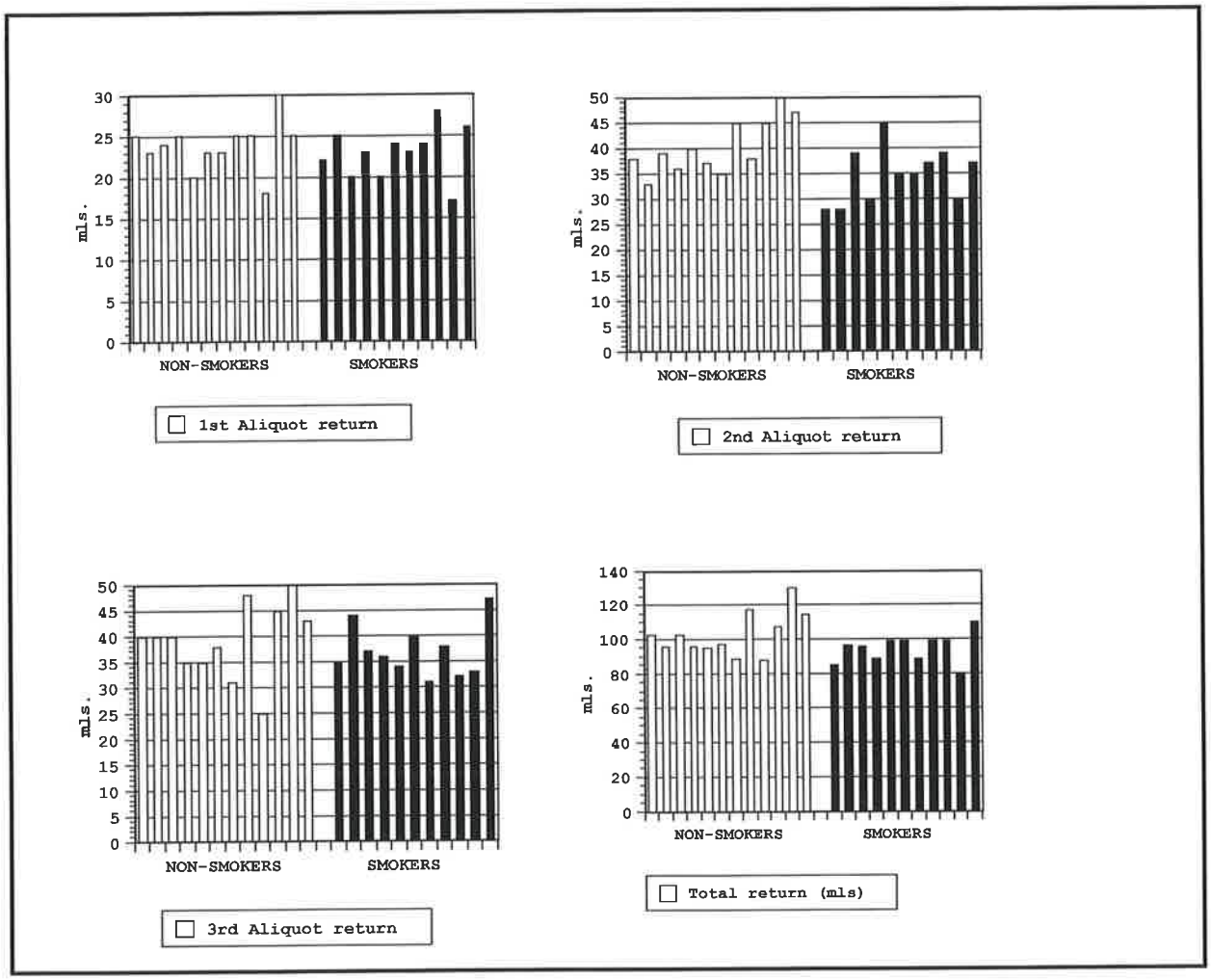
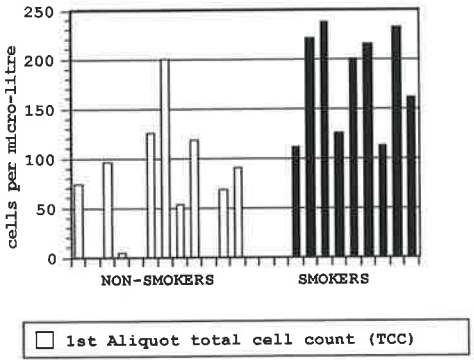
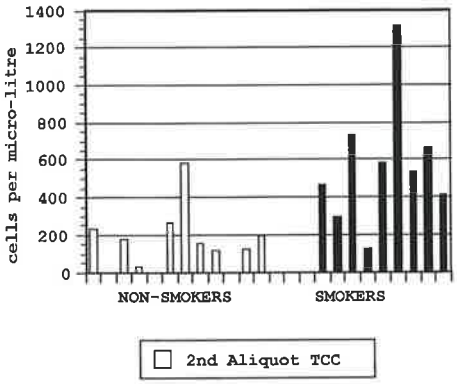
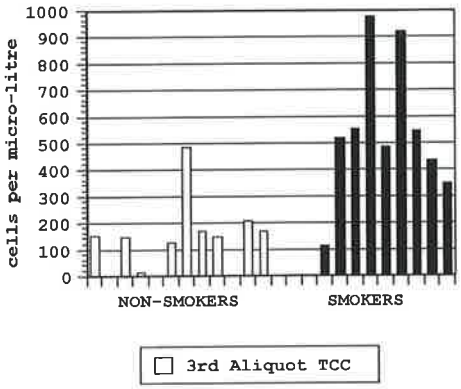


Figure 4.3a : The volumes of the fluid aliquots returned is shown for the first lavage. The mean for the non-smokers is 23.8±0.8, with that of the smokers being 22.9±0.9. There was no significant difference between the two groups.
Figure 4.3b : The volumes of the fluid aliquots returned is shown for the second lavage. The mean for the non-smokers is 40.2±1.5, with that of the smokers being 34.82±1.6. There was no significant difference between the two groups.
Figure 4.3c : The volumes of the fluid aliquots returned is shown for the third lavage. The mean for the non-smokers is 39.1±2, with that of the smokers being 37.0±1.5. There was no significant difference between the two groups.
Figure 4.3d : The volumes of the total fluid returned is shown for the total lavage. The mean for the non-smokers is 103.2±3.6, with that of the smokers being 94.7±2.5. There was no significant difference between the two groups with a p = 0.07.

Figure 4.4 : Total cell counts for the first, second, and third aliquot returns, and the final total cell count.







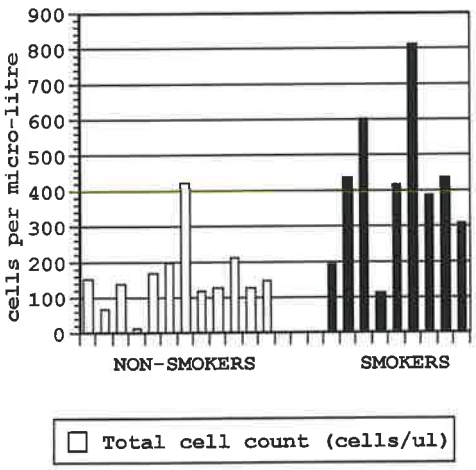


Figure 4.4a : The total cell count of the fluid aliquots returned is shown for the first lavage. The mean for the non-smokers is 91.6±18.2, with that of the smokers being 179±17.5. There was a significant difference between the two groups ($p < 0.02$). Absent values were not recorded.

Figure 4.4b : The total cell count of the fluid aliquots returned is shown for the second lavage. The mean for the non-smokers is 206±52.1, with that of the smokers being 565.8±111.7. There was a significant difference between the two groups ($p < 0.02$). Absent values were not recorded.

Figure 4.4c : The total cell count of the fluid aliquots returned is shown for the third lavage. The mean for the non-smokers is 180.4±42.1, with that of the smokers being 545.1±88.6. There was a significant difference between the two groups ($p < 0.02$). Absent values were not recorded.

Figure 4.4d : The total cell count in the combined fluid returned is shown. The mean for the non-smokers is 159.3±28.4, with that of the smokers being 414±69.7. These groups are statistically different, with $p=0.018$.

Figure 4.5 : Percentage of macrophages for the first, second, and third aliquot returns, and the final macrophage percentage.

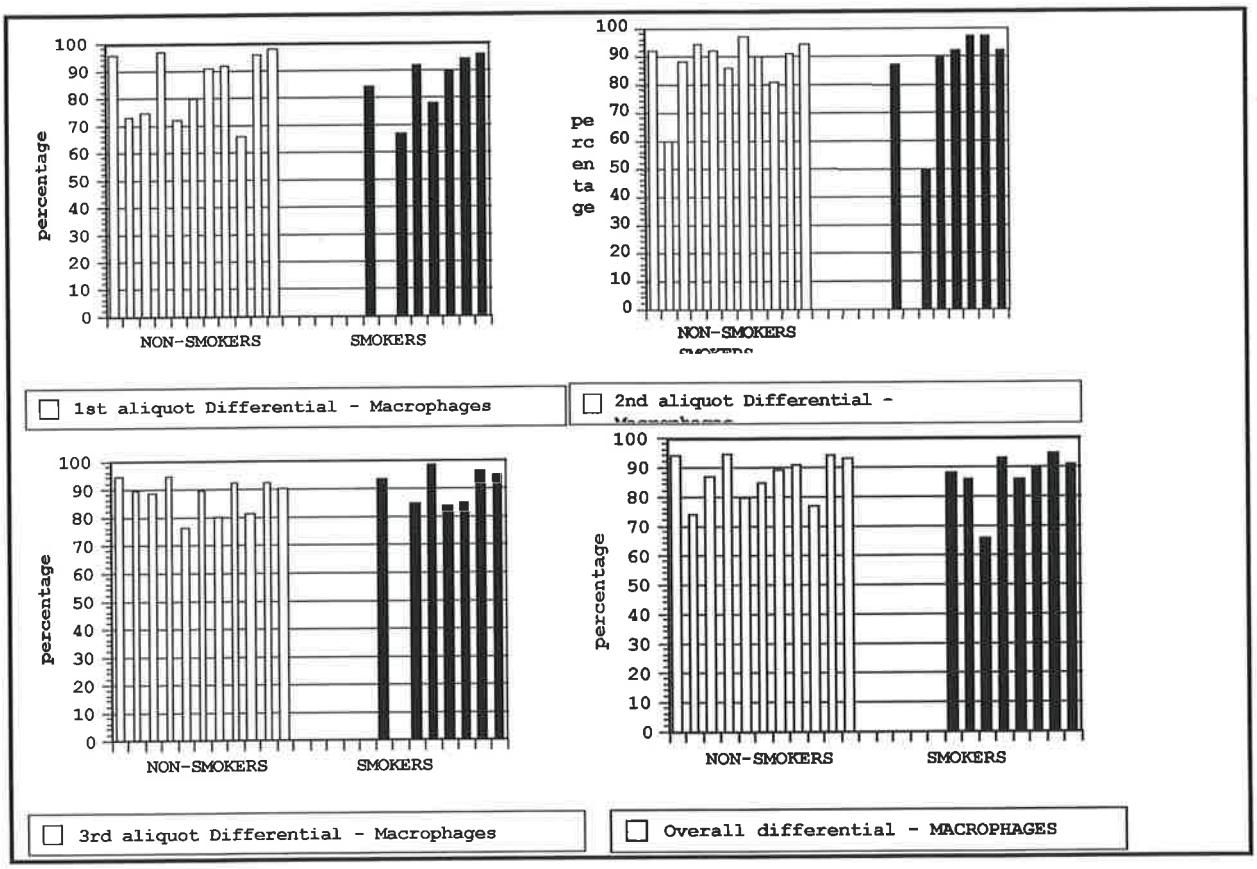


Figure 4.5a : The differential proportion of PAM's in the first lavage return. The mean proportion for the non-smokers is 85.1±3.6, with that for the smokers being 85.9±3.9. There is no significant difference between the two groups.
Figure 4.5b : The differential proportion of PAM's in the second lavage return. The mean proportion for the non-smokers is 87.7±3.1, with that for the smokers being 86.2±6.4. There is no significant difference between the two groups.
Figure 4.5c : The differential proportion of PAM's in the third lavage return. The mean proportion for the non-smokers is 87.7±1.8, with that for the smokers being 90.3±2.4. There is no significant difference between the two groups.
Figure 4.5d : The overall differential proportion of PAM's in the combined lavage return. The mean proportion for the non-smokers is 87.2±2.2, with that for the smokers being 86.9+3.2.

Figure 4.6 : Percentage of polymorhonuclear neutrophils for the first, second, and third aliquot returns, and the final neutrophil percentage.

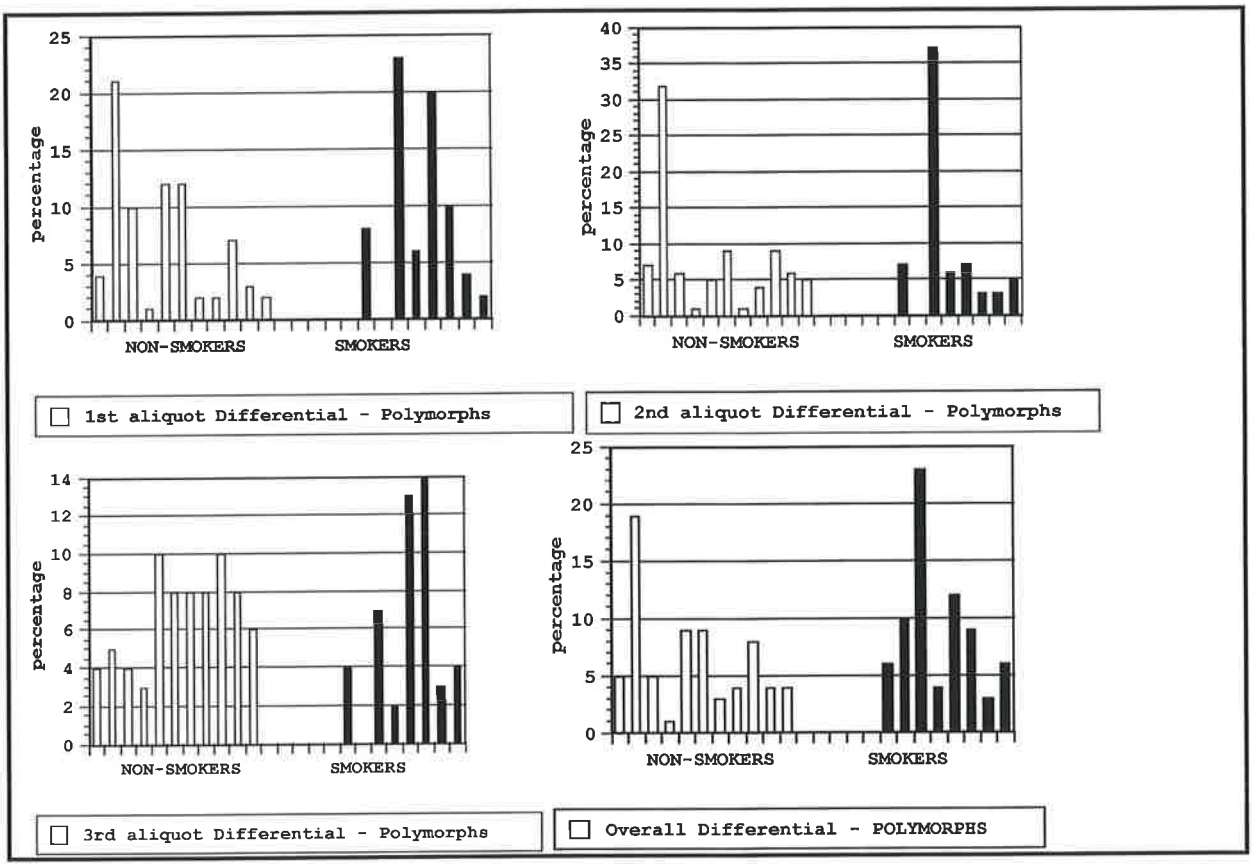


Figure 4.6a : The differential proportion of PMN's in the first lavage return. The mean proportion for the non-smokers is 6.9±1.9, with that for the smokers being 10.4±3.0. There is no significant difference between the two groups.

Figure 4.6b : The differential proportion of PMN's in the second lavage return. The mean proportion for the non-smokers is 7.7±2.6, with that for the smokers being 9.7±4.6. There is no significant difference between the two groups.

Figure 4.6c : The differential proportion of PMN's in the third lavage return. The mean proportion for the non-smokers is 6.7±0.7, with that for the smokers being 6.7±1.8. There is no significant difference between the two groups.

Figure 4.6d : The differential proportion of PMN's in the combined lavage return. The mean proportion for the non-smokers is 6.5±1.5, with that for the smokers being 9.1±2.2. There is no significant difference between the two groups, with p=0.063.

Figure 4.7 : Percentage of lymphocytes for the first, second, and third aliquot returns, and the final lymphocyte

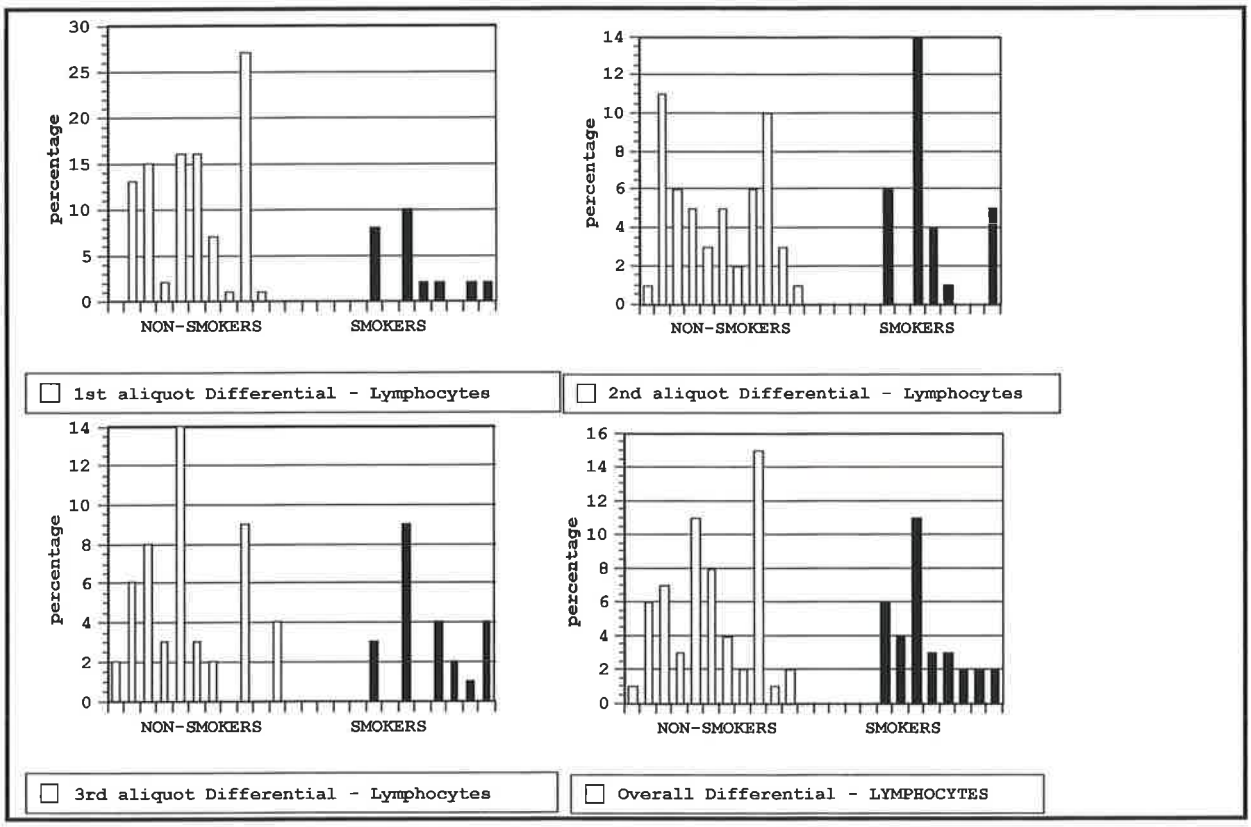


Figure 4.7a : The differential proportion of lymphocytes in the first lavage return. The mean proportion for the non-smokers is 8.9±2.7, with that for the smokers being 3.7±1.4. There is no significant difference between the two groups.
Figure 4.7b : The differential proportion of lymphocytes in the second lavage return. The mean proportion for the non-smokers is 4.8±1.0, with that for the smokers being 4.3±1.9. There is no significant difference between the two groups.
Figure 4.7c : The differential proportion of lymphocytes in the third lavage return. The mean proportion for the non-smokers is 4.6±1.3, with that for the smokers being 3.3±1.1. There is no significant difference between the two groups.
Figure 4.7d : The differential proportion of lymphocytes in the combined lavage return. The mean proportion for the non-smokers is 5.45±1.4, with that for the smokers being 4.1±1.1. There is no significant difference between the two groups, with p=0.44.

These results are of interest and demonstrate no difference in volume of fluid returned between non-smokers and smokers, nor the cell differential counts, although the total numbers of cells are greater in the smoking population. The mean number of cells obtained from the non-smoking group was 16,186,100 ± 2,536,300, and from the smoking group 39,470,220 ± 6,918,400. These groups are significantly different with p = 0.024. There was no correlation between the amount of fluid returned and the cell concentration, with the highest total cell count being obtained in a smoking male, aged 40 years, with a fluid return of 99 mls. The second highest cell concentration was found in a smoking female, aged 22, with a fluid return of 99 mls. The lowest cell count was from a non-smoking female, aged 26, with a fluid return of 96 mls.

Transmission electron micrographs of human alveolar macrophages are shown in Figure 4.8. The TEM photomicrographs show that the alveolar macrophages from smokers contain numerous electron dense bodies; these are usually ascribed to particulates from cigarette smoke, but could also represent cellular debris, perhaps arising from apoptosis, that the alveolar macrophages have scavenged.

Figure 4.8 : Transmission electron micrographs of human alveolar macrophages obtained from the lungs of normal non-smokers and from normal smokers
(All of the following images are taken from different subjects).

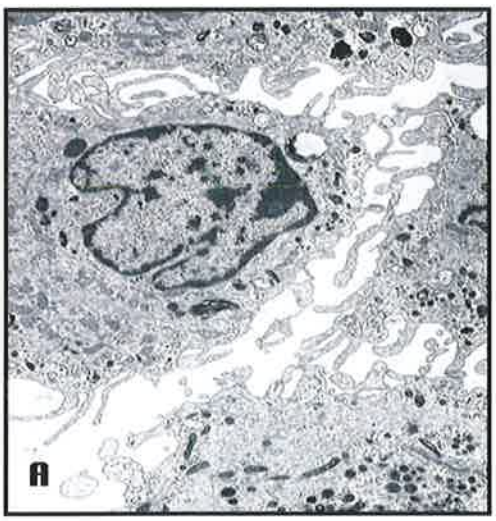


Figure 4.8a : Typical alveolar macrophages from a non-smoker.
TEM X 12,000

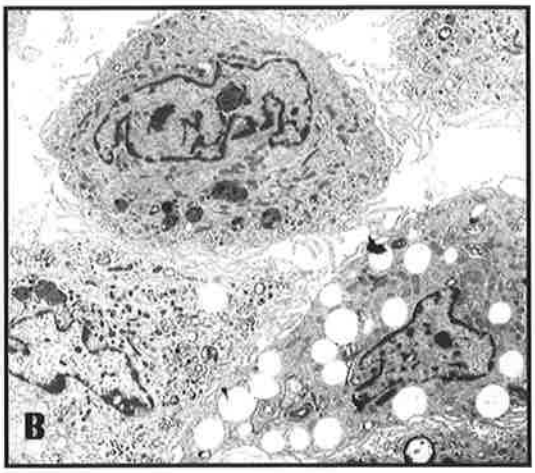


Figure 4.8b : Typical alveolar macrophages from a non-smoker. TEM **X** 7,200

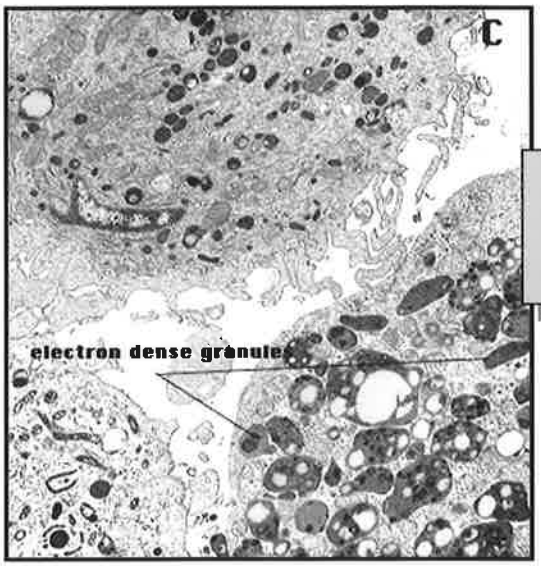


Figure 4.8c : Typical alveolar macrophages from a smoker. Note the electron dense granules. TEM ^{X} 12,000

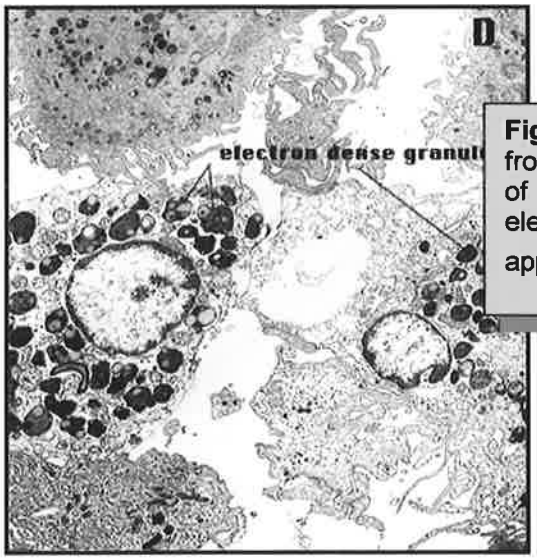


Figure 4.8d Typical alveolar macrophages from a smoker. As can be appreciated, not all of the alveolar macrophages contain the electron dense deposits, although those that do appear to be packed with them. TEM **X** 7,200

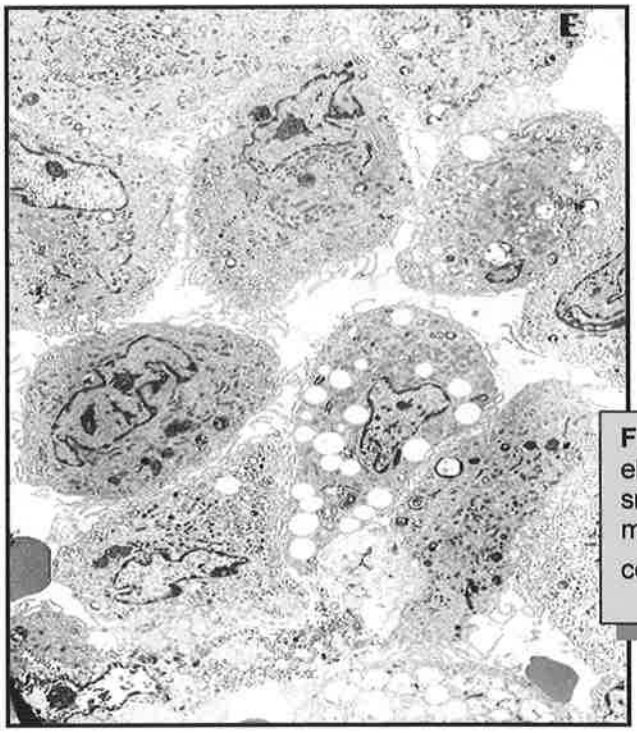


Figure 4.8e : Typical transmission electron microscopy from a non-smoker, showing the alveolar macrophages as a relatively uniform cell population. TEM X 4,800.

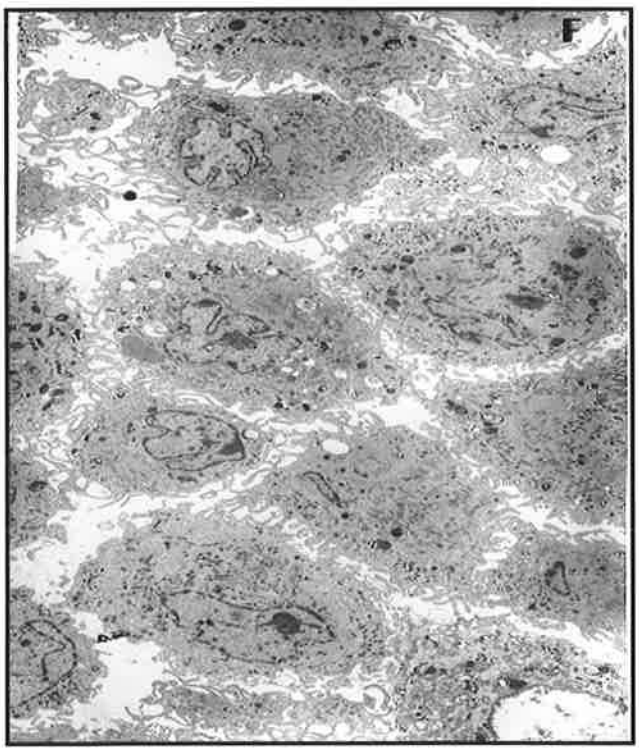


Figure 4.8f : Typical transmission electron microscopy from a non-smoker, showing the alveolar macrophages as a relatively uniform cell population. TEM X 4,800.

Scanning electron microscope pictures of cultured alveolar macrophages are shown in Figure 4.9. It was the clear impression that the PAM'S from non-smokers were much rounder than the PAM'S from a smokers lung, at the corresponding conditions in culture; however, this was not systematically measured.

Figure 4.9 : Scanning electron micrographs of alveolar macrophages from human non-smokers and smokers

Figure 4.9a : Normal alveolar macrophages from a non-smoker, after 7 hours in tissue culture. SEM X 600.

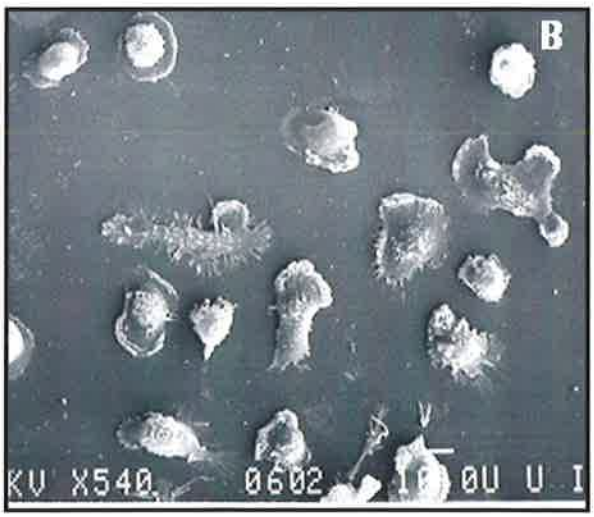


Figure 4.9b : Normal alveolar macrophages obtained from a smoker, after 7 hours in tissue culture. Note the spreading compared to Figure 4.10a above. SEM X 540.

The apparent differences between smokers and non-smokers in alveolar macrophage morphology demonstrated here is of interest, but it was not the primary purpose of this study to quantitate these changes.

The yeast phagocytosis results are shown in the next series of figures and tables. A typical appearance of the yeast adjacent a PAM is shown in Figures 4.10a and 4.10b, with a phase contrast photomicrograph shown in Figure 4.10c, and SEM shown in Figures 4.10d and 4.10e.

Figure 4.10 : Yeast phagocytosis appearances

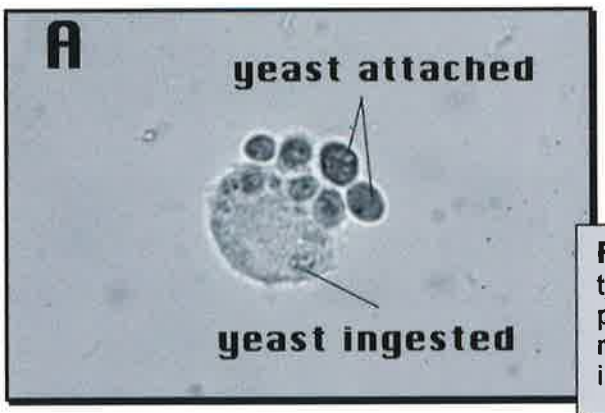


Figure 4.10a : The typical light microscopy of a pulmonary alveolar macrophage attaching and ingesting yeast.

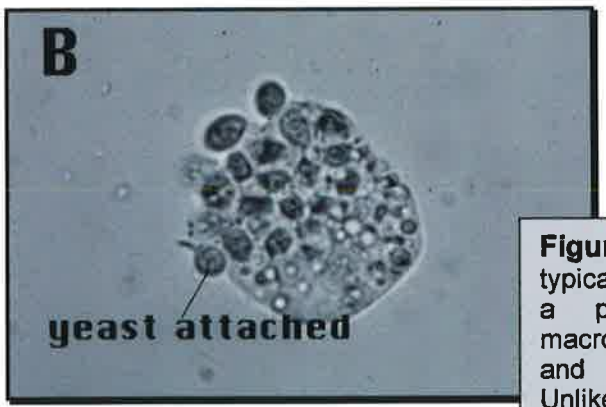


Figure 4.10b : The typical light microscopy of a pulmonary alveolar macrophage attaching and ingesting yeast. Unlike Figure 4.11a, here there are a many yeast making counting difficult.

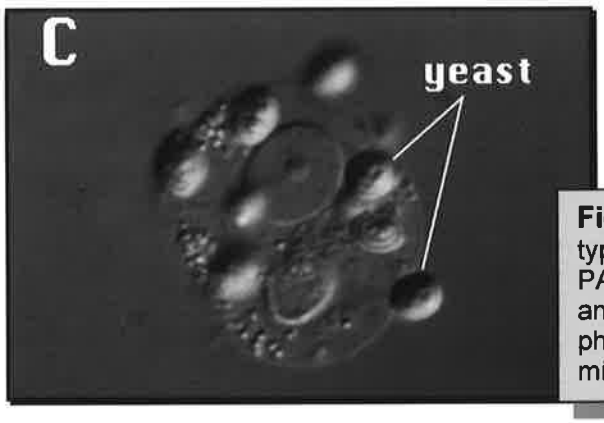


Figure 4.10c : The typical appearances of a PAM with yeast attached and ingested, using phase-contrast microscopy.

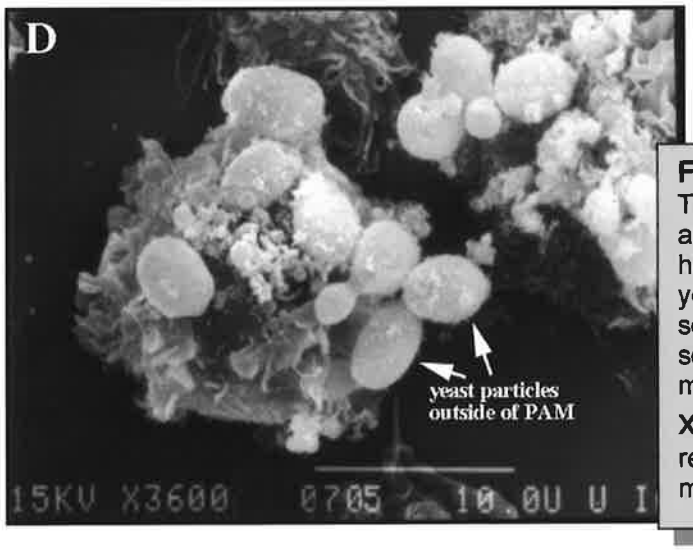


Figure 4.10d : The typical appearances of human PAM's with yeast particles as seen with the scanning electron microscope. SEM X 3600. The bar represents 10.0 microns.

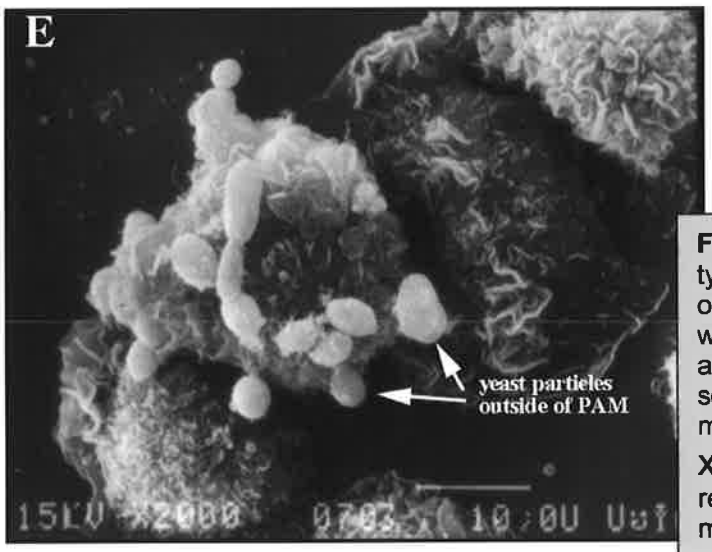
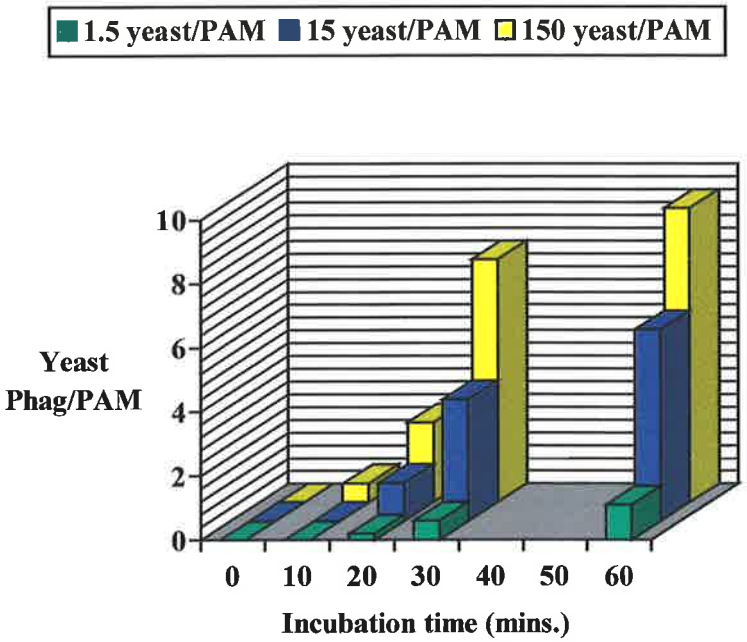


Figure 4.10e : The typical appearances of human PAM's with yeast particles as seen with the scanning electron microscope. SEM X 2000. The bar represents 10.0 microns.

Initially the optimum concentration of yeast per macrophage, and a time course was determined. These experiments were performed with rat PAM's and the results are summarized in Figures 4.11 and 4.12.

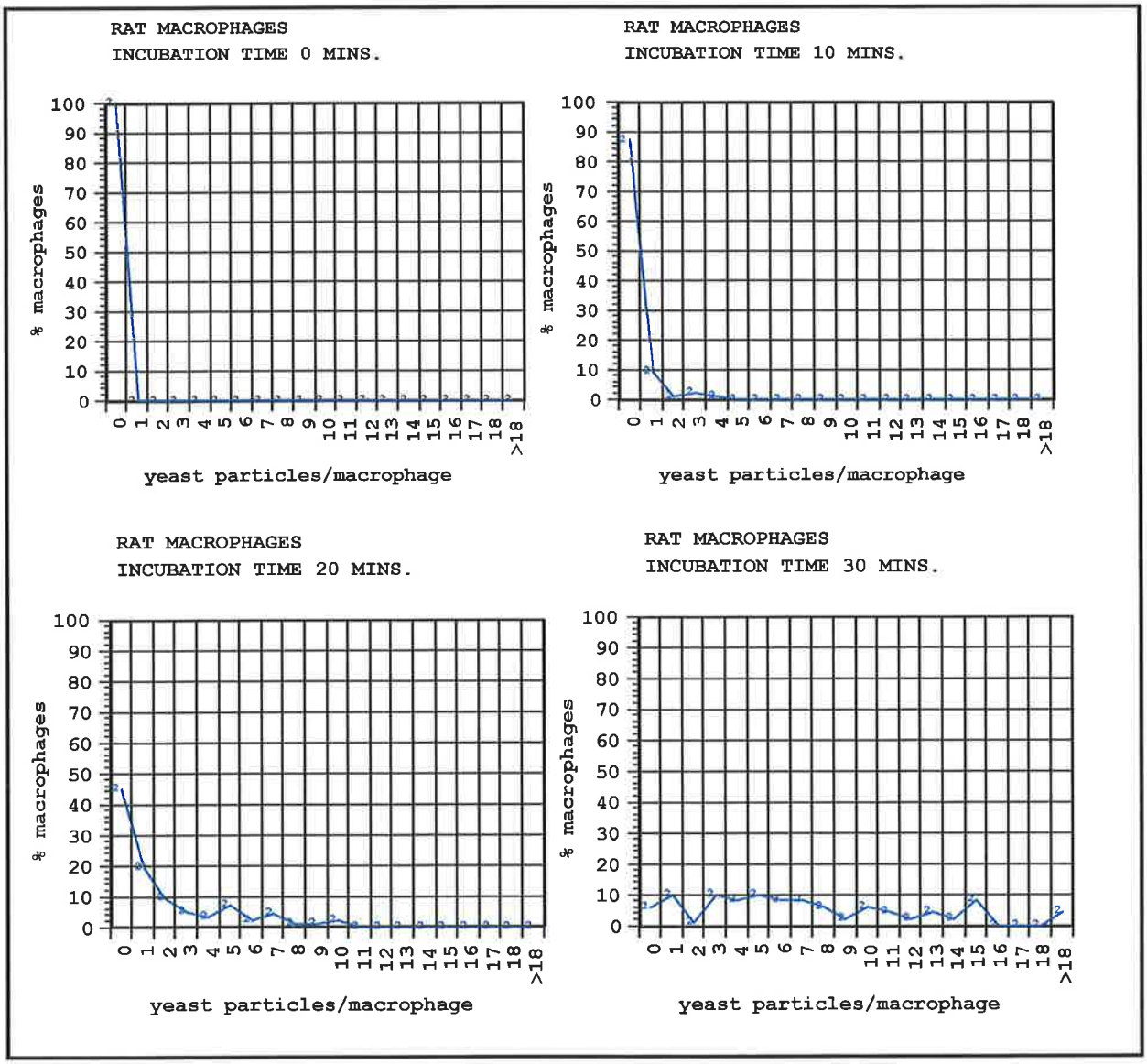
Figure 4.11 : Assessing the optimal yeast/macrophage proportions

F hown.



The X axis shows the incubation times in 10 minute intervals from 0 to 60 , with the Y axis showing the yeast phagocytosed/macrophage. The Z axis shows the various concentrations of yeast per macrophage, varying from 1.5, 15 and 150. It was concluded that 150 yeast per macrophage would be a satisfactory concentration for these assays. A saturation point for macrophage phagocytosis was not established by this technique as at high concentrations the yeast particles became impossible to count in a reliable manner.

Figure 4.12 : Assessing the optimal time course for phagocytosis



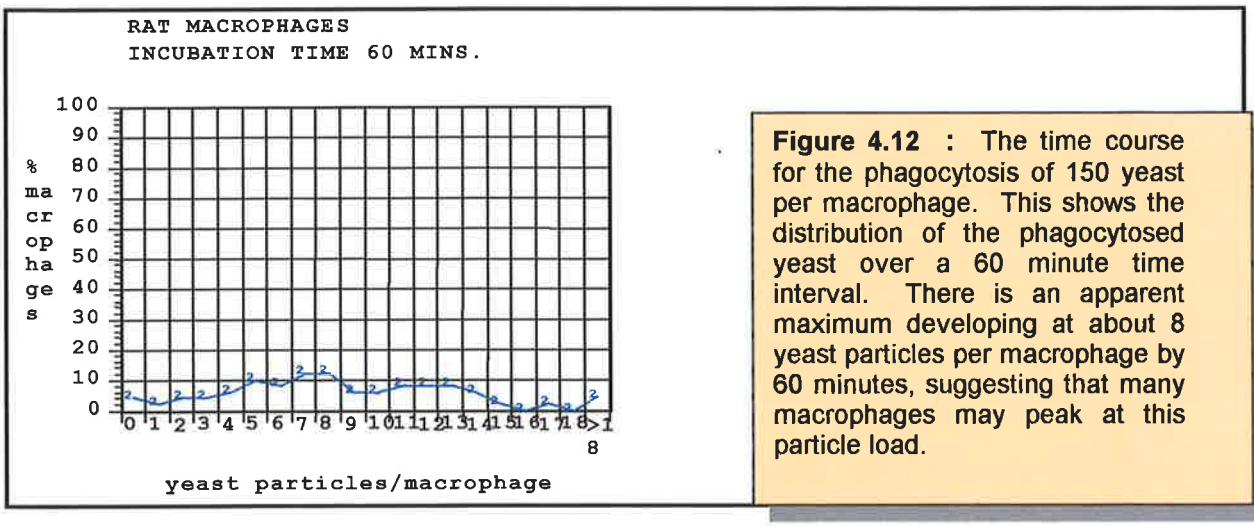


Figure 4.12 : The time course for the phagocytosis of 150 yeast per macrophage. This shows the distribution of the phagocytosed yeast over a 60 minute time interval. There is an apparent maximum developing at about 8 yeast particles per macrophage by 60 minutes, suggesting that many macrophages may peak at this particle load.

Subsequent experiments were performed with incubation times of 30 minutes, with 150 yeast particles per macrophage. This enabled a reasonable number of cell associated yeast to be counted with each experiment, as too many yeast around each macrophage led to problems with visual counting. Subsequent experiments used alveolar macrophages obtained from normal smokers and non-smokers detailed earlier in this chapter, as well as alveolar macrophages from adult rats. The results of the comparison between smokers and non-smokers is shown in Figures 4.13a, 4.13b and 4.13c.

Figure 4.13 : Human PAM phagocytosis with comparison between PAM's from non-smokers and smokers

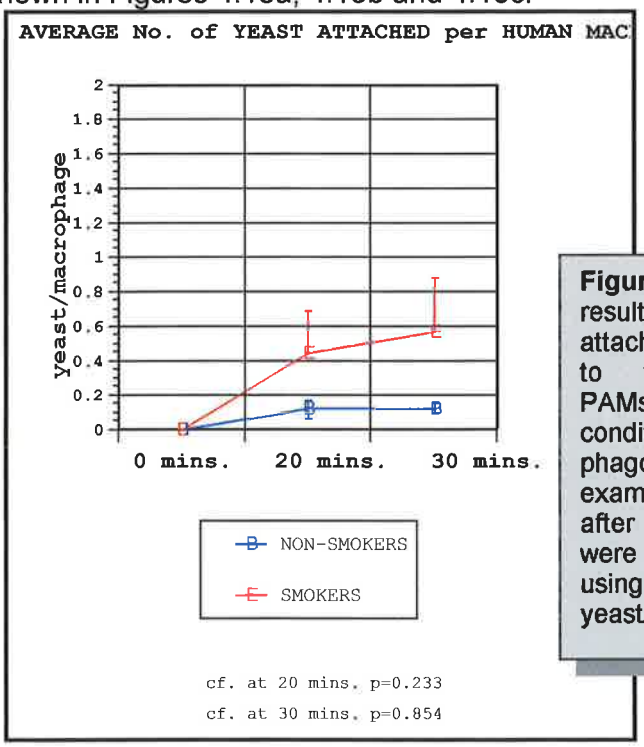


Figure 4.13a The results of the attachment of yeast to the cultured PAMs. The conditions were that phagocytosis was examined 24 hours after the PAM's were in culture, using 150 yeast/macrophage.

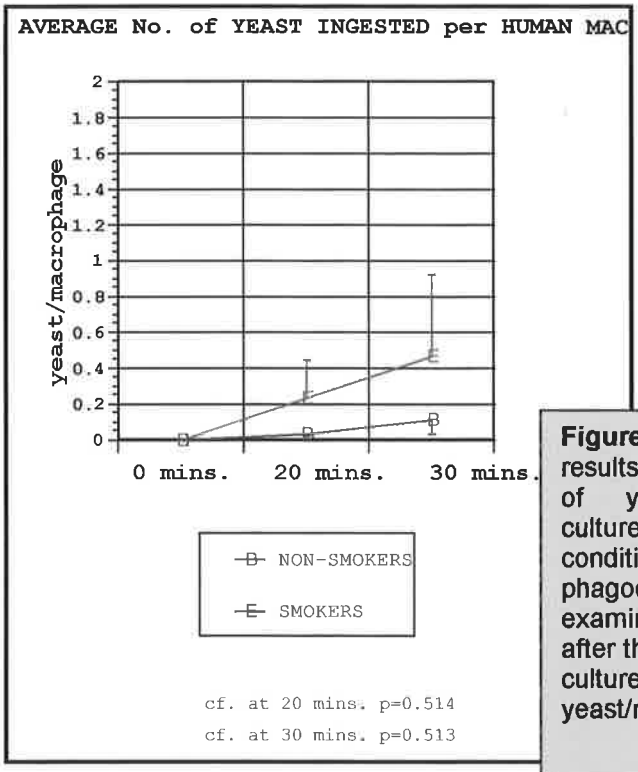


Figure 4.13b The results of the ingestion of yeast by the cultured PAMs. The conditions were that phagocytosis was examined 24 hours after the PAM's were in culture, using 150 yeast/macrophage.

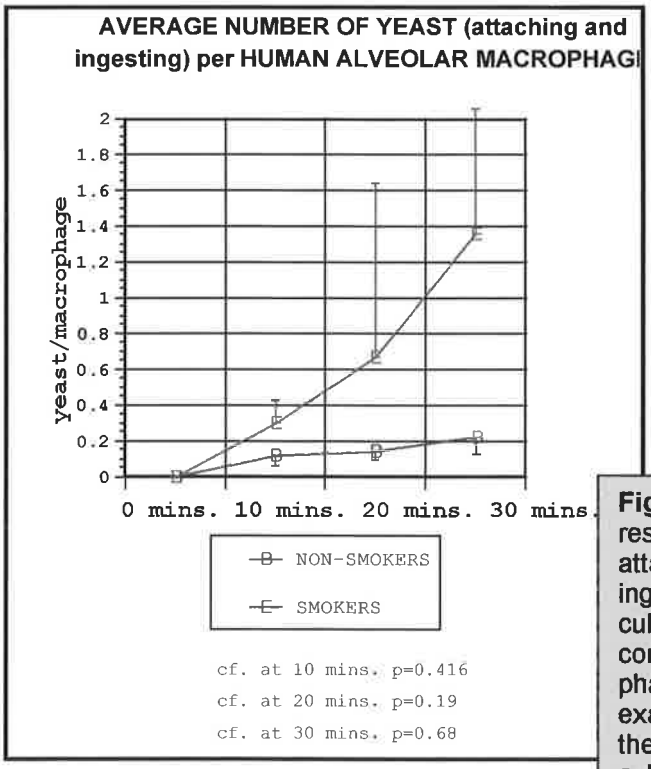


Figure 4.13c The results of the sum of attachment and ingestion of yeast by the cultured PAMs. The conditions were that phagocytosis was examined 24 hours after the PAM's were in culture, using 150 yeast/macrophage.

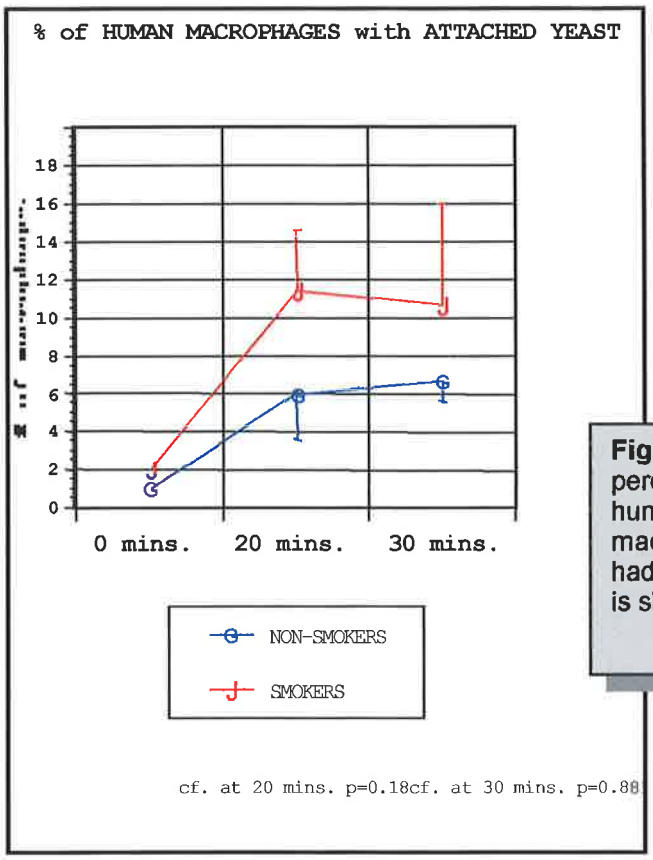


Figure 4.13d The percentage of human alveolar macrophages that had attached yeast is shown.

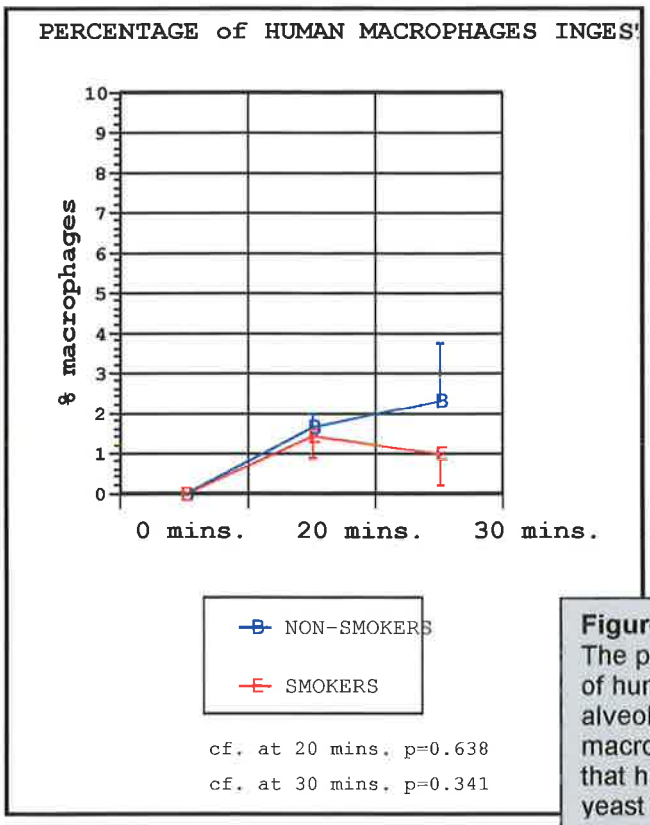


Figure 4.13e The percentage of human alveolar macrophages that had ingested yeast is shown.

In all instances, alveolar macrophages from smokers were trending to be "more active" than those from non-smokers, but with the numbers in these experiments, this did not reach statistical significance. Also, as shown in Figures 4.13d and 4.13e, the percentage of alveolar macrophages actively involved in the phagocytic act (either attaching or ingesting yeast) were higher in the cells derived from smokers, but not statistically so. However, it should be noted that only a small proportion of attached human alveolar macrophages are phagocytic under these conditions. Further, the overall phagocytic activity of rat and human derived alveolar macrophages are compared - showing that rat alveolar macrophages are significantly more active than those from human lungs (Figure 4.14).

Figure 4.14 : Comparing human and rat PAM's

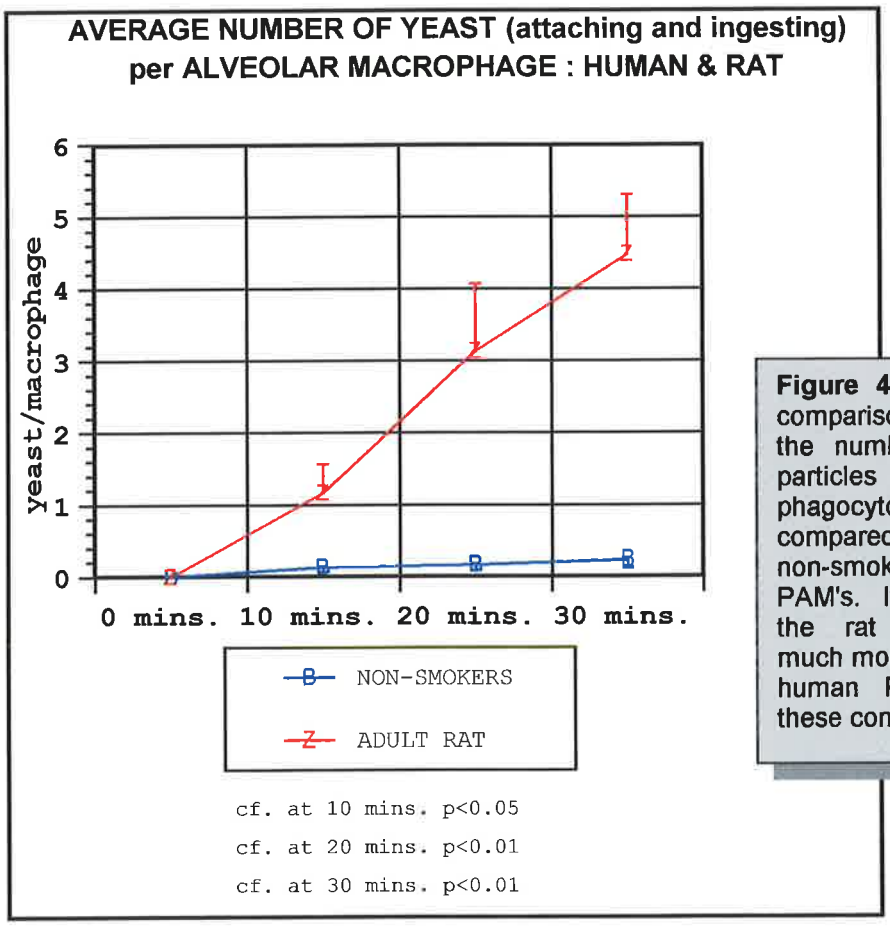


Figure 4.14 : The comparison between the number of yeast particles actively phagocytosed by rat compared with human non-smoker derived PAM's. It is clear that the rat PAM's are much more active than human PAM's under these conditions.

Finally, the proportion of alveolar macrophages actively phagocytic at 30 minutes is markedly different between rat and human PAM's. 94%of the rat alveolar macrophages are actively phagocytic, compared to 24% of human smokers and only 12% of human non-smokers.

DISCUSSION

The work in this chapter is important as it documents a number of observations that are important for further results in this thesis as well as for on-going research. Firstly, the volume of returned BAL lavage fluid on each lavage, together with the cell counts and cell proportions indicates there is a diminishing return of cells with each lavage, however, the proportions of cells do not change much. Filtration of BAL fluid through gauze was not used in this study, as this may also affect the cell proportions (226). The BAL return depends upon the region of the lung lavaged (227), the methods for cell preparation such as elutriation (228), and changes in lavage volume. The right middle lobe or lingula, with gravity assisted return is best to lavage in the normal subject, as was the standard employed here(229). Lymphocyte counts may vary in normal subjects (230). Also cell counts vary according to the methods used (231, 232), so standardization is extremely important. There is loss of protein with the filtration method for BAL fluids (233). The results presented in this chapter are in keeping with other studies examining a similar protocol (234-236) and served to establish the procedure for future studies examining for cell counts. Recently, similar studies have been reported in children (237).

The phagocytic method developed as part of this thesis, and described is simple. It allows for examination of populations of alveolar macrophages with a measurement of attachment as well as particle ingestion. At the same time the viability of the macrophages can be assessed. Most other methods that examine macrophage phagocytosis use some form of global measurement such as flow cytometer counting of a fluorescent particle (238), or scintilllation counting of a radiolabelled particulate. These global measures do not allow for the evaluation of participating macrophages, but instead express an average phagocytic load per macrophage. Newer techniques for assessing phagocytosis, however, are relying on measuring particle uptake and attachment by individual macrophages, as in more recent studies using fluorescent liposomes (239).

There has been increasing evidence over the last 20 years to show there are slight differences in alveolar macrophages derived from smokers and non-smokers; as indicated, the work in this chapter tends to support the notion that alveolar macrophages

from smokers contain more electron dense particles, that they tend to flatten out more in culture, and that they are more actively phagocytic - however, specific morphological methods were not carried out, and the differences for phagocytosis did not reach statistical significance. The smokers, however, in the studies reported in this chapter, were relatively young, with a mean smoking history of 10 pack years. Greater differences between smokers and non-smokers might be observed if the smokers have greater than 40 pack years of smoking. The differences observed between alveolar macrophages from smokers and non-smokers are minor, and in all of the published studies there are still no clear consistent functional differences noted between smokers and non-smokers derived alveolar macrophages.

One intriguing hypothesis, I would propose, but has not been tested, is that the particles observed in the macrophages from smokers represent not only smoke related debris, but also cell debris released from inflammatory cells (other macrophages or polymorphonuclear neutrophils) that have undergone apoptosis, or programmed cell death (240).

There has been information that acute exposure to cigarette smoke (in a mouse model) causes a decrease in the numbers of particles phagocytosed, as well as an increase in the percentage of alveolar macrophages engaged in this function (241). Subsequent studies by the same author using Candida as the particle, confirmed suppression of phagocytosis (242). Acute exposure to ozone seems to stimulate rat macrophage phagocytosis, with carbon black exposure having an inhibitory effect (243). There is inhibition after ozone exposure noted in alveolar macrophages derived from mice (244), with carbon black exposure having no effect in this study. There has also been increasing evidence that the macrophage population in the lung is heterogeneous with regard to position (245, 246) (i.e. airway macrophages, pleural macrophages, interstitial macrophages and alveolar macrophages). There is also heterogeneity in regard to function, such as adherence (247) density (248-251) and monoclonal antibody binding (252). Rat macrophage heterogeneity has also been shown in regards to complement receptors (253).

The demonstration in this thesis that not all alveolar macrophages are actively phagocytic has been questioned. However, this notion has recently been supported in a study in hamsters where 20-30% of alveolar macrophages were not actively phagocytic

(254). There are very few studies comparing phagocytic function across species, and only recently have there been other reports of differences between human and animal derived alveolar macrophage phagocytosis (255). There is a further report comparing rat, mouse, hamster and guinea pig; where it was shown that the rat is the most efficient rodent for alveolar macrophage phagocytosis (256).

With regard to the studies reported in this chapter, there could have been a much greater population of phagocytic alveolar macrophages if the experimental time course was longer. This is possible, given the greater proportion of human alveolar macrophages that attached yeast compared with those that had ingested yeast at 30 minutes. However, the proportion of cells attaching yeast appears to have reached a plateau, suggesting that no more alveolar macrophages were going to participate. Several experiments were carried through to longer time points, and in these the proportion of macrophages involved in phagocytosis did not increase, but with this assay, the yeast involved with the macrophages became increasingly difficult to count and to discriminate as being inside or outside of the cell. The human alveolar macrophages were certainly much less actively phagocytic than rat cells; with only a very small proportion of human cells being actively phagocytic (12% non-smokers and 24% of smokers), compared to 95% of the rat alveolar macrophages. This is not likely to be related to the isolation procedure which was similar, and is unlikely to be related to the effects of the local anaesthetic lignocaine used in the collection of the human cells (257), as the macrophages were washed, and then cultured for 24 hours to allow for recovery. This inter-species difference has not usually been taken into account by most studies that examine alveolar macrophage function. In addition, the observations in this thesis indicates two distinct functional populations of alveolar macrophages in the human, and raises the question as to the primary function of the macrophages that are not primed for phagocytosis. It is interesting to speculate as to the primary function of the non-phagocytosing human macrophages. It is possible that part of this non-phagocytic population reflects aged, non-functional macrophages that are being excreted through the airway muco-ciliary transport system. The differences between human PAM's and those from the rat may reflect the environment under which the two species exist, with the rat possibly being exposed to a greater infectious or particulate pulmonary burden.

Chapter 5

The role of oxygen derived free radicals in radiation induced damage and death of non-dividing eukaryotic cells.[1]

[1] Communications arising from this work are as follows: (212, 258-260).

INTRODUCTION

The toxic mechanism of ionizing radiation in non-dividing eukaryotic cells remains to be clarified, having been a controversial problem for many years (261). Although ionizing radiation is independently toxic, this toxicity is enhanced by oxygen. Several early studies have implicated the superoxide radical in the oxygen enhancement of radiation lethality (262-265). However, this view has met with considerable controversy, and it has been claimed that the superoxide radical plays no part in the oxygen effect (266, 267). Radiolytic cleavage of water produces e_{aq}, $H^{\bullet}$ and $HO^{\bullet}$ and secondary products of these radicals, O_2^- and H_2O_2, form in the presence of oxygen. The most toxic of these agents resulting from ionizing radiation is the $HO^{\bullet}$. This radical may arise by a secondary process as well as from the primary process via a modified form of the Fenton reaction according to the following equation -

$$H_2O_2 + O_2^- \xrightarrow{Fe^{+++}} HO^{\bullet} + OH + O_2$$

The superoxide radical, therefore, may play a crucial role in radiation-induced cell damage and death as one of the three required reactants, including iron and H_2O_2, required for the generation of $HO^{\bullet}$.

Isolated rat pulmonary macrophages were selected for this study of radiation toxicity because they constitute a good model of a non-dividing eukaryotic cell from higher animals, and are relatively easy to obtain as homogenous preparations. It is worth noting here that the non-dividing cell provides the best model in which to study the toxicity of ionizing radiation independent of its effect on the process of cell division. A method, which depended upon the effectiveness of specific radical scavengers to confer protection against radiation damage, was chosen to identify oxygen radicals.

MATERIALS AND METHODS

1. Materials and enzymatic assays

Superoxide dismutase free of contaminating catalase was obtained from Truett Laboratories (Dallas, Texas) or Biotics Research Corporation (Houston, Texas). The activity was determined by assessing the extent of the inhibition of ferricytochrome c reduction by superoxide anion (178). Catalase was obtained from Sigma, assayed for activity (268), and determined to be free of superoxide dismutase activity. Diethylenetrieminepentaacetic acid (DETAPAC) and disodium ethylenediaminetetra acetate (EDTA) were obtained from the Fisher Scientific Company.

Heat-inactivated superoxide dismutase and equimolar concentrations of bovine serum albumin were used as controls for native superoxide dismutase. Heat-inactivated catalase and equimolar concentrations of myoglobin were used as controls for native catalase. Heat inactivation of both enzymes was achieved by boiling for at least 110 minutes. The denatured enzymes were assayed after boiling to ascertain that no residual activity remained.

2. Cell preparation

Pulmonary alveolar macrophages were obtained by bronchial lavage from adult male Sprague-Dawley rats weighing from 250 to 350 g using 1mM phosphate-buffered saline (PBS), pH 7.4. The recovered cells were then centrifuged at 1900 rpm for 10 minutes and the cell pellet was re-suspended in Ham's F12 medium containing 10% fetal calf serum and gentamycin (10 mg/litre). The cell suspension was added to sterile Leighton tubes containing glass coverslips. The tubes containing the macrophages were incubated at 37°C in a mixture of 95% air and 5% carbon dioxide at 1 atm. The pulmonary macrophages in the cell suspension adhered to the glass coverslips were washed three times with sterile physiologic saline (0.85% NaCl). The non-adhered cells removed by the washing procedure were discarded. The coverslips with the adhered macrophages were bathed in sterile PBS alone or PBS containing the appropriate concentration of test

substance. The pH of the buffered saline was adjusted to 7.4. Microscopic examination indicated that the attached cells were >95% pulmonary macrophages.

3. Irradiation of Cells

Selected tubes were irradiated at room temperature with a GE Maxitron (250 kVp) for varying periods of time up to 2 hours (24 Krad). The dose rate was approximately 250 rad/min. The beam filters used were 1/4 mm Cu and 1 mm Al. Dosimetry was performed with Victoreen R meters and LiF dosimeters. At the end of the radiation period the PBS was decanted from the Leighton tubes and replaced with Ham's F12 media containing 10% fetal calf serum. The Leighton tubes containing the glass-adhered cells were incubated for 24 hours at 37°C in a 95% air, 5% carbon dioxide atmosphere. Cell viability was assessed 24 hours after irradiation using the exclusion of erythrosin B dye (0.4%; pH 7.4) as an index. During the course of the post-irradiation incubation period some cells in both irradiated and non-irradiated Leighton tubes became non-adherent. No difference in the extent of non-adherence was detected between the control and treated cells. The viability of these suspended or floating cells was found to parallel the viability of adhered cells for all levels of radiation.

4. Scanning electron microscopy of cells

Pulmonary alveolar macrophages prepared for viewing by scanning electron microscopy were obtained and treated as described above, except that they were adhered to plastic (Aclar) rather than glass. The coverslips with the adherent macrophages were placed in cold 3% glutaraldehyde in iso-osmotic cacodylate buffer (pH 7.4) and fixed overnight. They were then progressively dehydrated in alcohol, followed by critical point drying. Specimens were coated with a Hummer V pulsed-planar magnetron triode (Sputterer) using gold-palladium. Examination was performed with a JEOL scanning microscope. Each specimen was examined under low magnification. Representative samples showing cell membranes in surface detail were selected from groups of at least 10 cells subjected to the specified treatments.

5. Phagocytosis

Measuring the phagocytosis of heat-killed yeast particles (as developed in the previous Chapter) assessed pulmonary alveolar macrophage function. Although only a small proportion of alveolar macrophages are actively phagocytic, it is such a fundamental function of these cells, that it was considered reasonable to use this assay as a method of assessing functional changes. The macrophages were prepared and irradiated in an identical manner to that used for viability assessment. An exposure of 3000 R (2850 rad) of irradiation was chosen after an initial study of the cellular response from 1000 to 12000 R. The 3000 R exposure was the highest level of radiation that consistently showed inhibition of phagocytosis with no accompanying loss of cell viability. To each Leighton tube was added 10×10^4 macrophages. Twenty four hours after irradiation the killed yeast, opsonized with guinea pig complement (Pel-Freez Biol.; 0.1 ml added to 1 ml of yeast suspension), was added to each Leighton tube to produce a concentration of 15×10^6 yeast/ml. The cells were incubated at 37°C in a 95% air, 5% carbon dioxide atmosphere. At intervals of 15 minutes a single Leighton tube was taken from the incubator, and the glass coverslip with the adherent cells was removed and washed gently five times with 1mM PBS. Phagocytosis was inhibited by using washing solutions pre-cooled to 4°C, thus lowering the temperature of the cells. The coverslip was then placed into a 0.4% solution of erythrosin B at 4°C for 4 minutes. Excess erythrosin B dye was gently washed off with 1mM PBS. The cells were examined using a light microscope under oil immersion (x1000). Extracellular and intracellular yeast particles were easily distinguished from one another by exploiting the relative staining of each. Yeast particles which remained in the medium either freely suspended or attached extracellularly to the pulmonary macrophages were stained with the erythrosin B dye. Yeast particles within viable cells were unstained. This difference in staining response allowed the quantitation of both yeast particles attached to the cells and those actually within the macrophages, as well as an assessment of the viability of the pulmonary macrophages.

RESULTS

The marked radioresistance of non-dividing pulmonary macrophages compared to most non-dividing, nucleated eukaryotic cells is indicated in Figures 5.1a and 5.1b. In these experiments the viability of rat alveolar macrophages was compared to the viability of human derived alveolar macrophages. Radioresistance was independent of temperature up to 37°C under these conditions.

Figure 5.1 : Radiation effects on alveolar macrophages

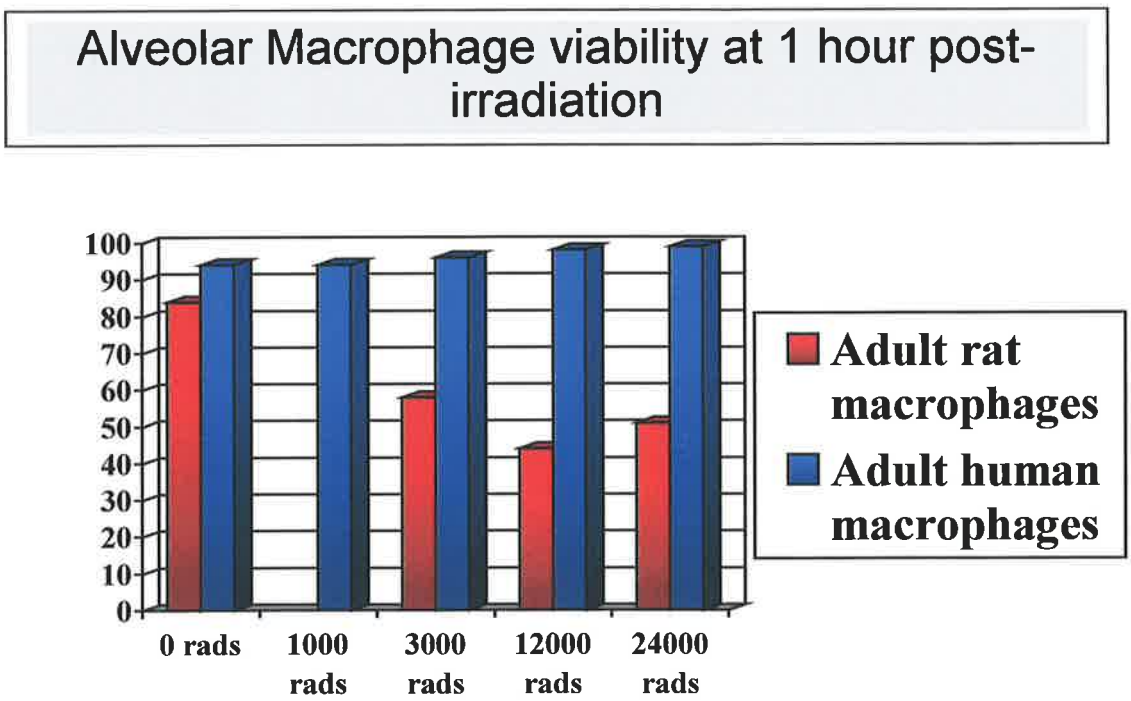


Figure 5.1a : Comparison of human alveolar macrophage radiation resistance with that of alveolar macrophages from adult rats. The human cells are much more radioresistant. A representative experiment, from 6 separate experiments, is shown. Alveolar macrophage viability is shown on the Y axis (%).

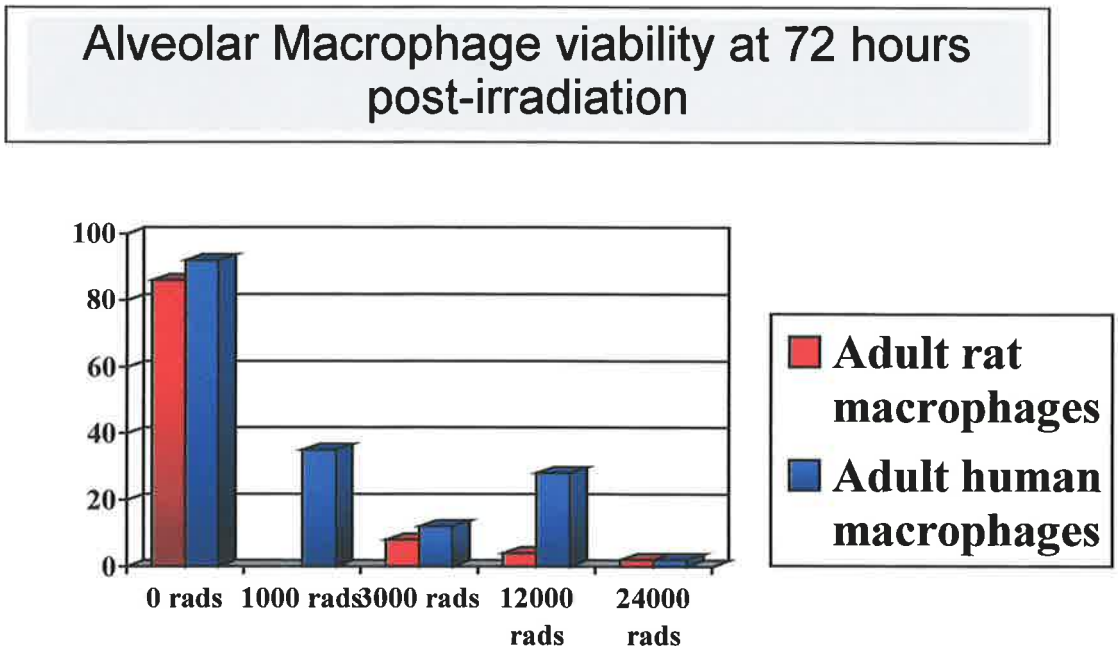


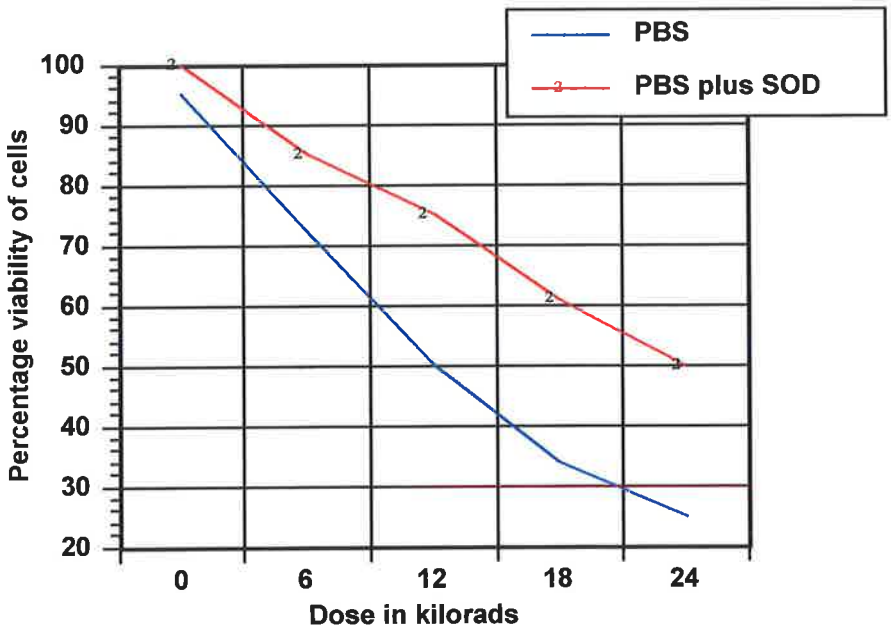
Figure 5.1b : The relative radioresistance of human compared to rat derived alveolar macrophages is also seen at 72 hours after radiation injury. A representative experiment, from 6 separate experiments, is shown. Alveolar macrophage viability is shown on the Y axis (%).

Assessment of cell viability conducted 24 hours post-irradiation revealed that a dose of 11.5 krad was required to kill 50% of the exposed cell population. Most other dividing eukaryotic cells have been shown to be nonviable at an order of magnitude lower dose. No radiation-induced loss of viability was observed at <3 krad. This is consistent with the characteristic shoulder region of survival curves seen with other mammalian cells in which no loss of viability is observed from zero to very low doses of radiation but begins at a critical radiation dosage. In preliminary experiments, noted above, there was an even greater radioresistance of human alveolar macrophages when compared to rat alveolar macrophages, under the same conditions.

The effectiveness of superoxide dismutase as a radioprotective agent at a single enzyme concentration, during increasing radiation dosage is summarized in Figure 5.2.

Considerable protection against radiation-induced lethality was provided when superoxide dismutase was present during irradiation and alveolar macrophage viability was assessed 24 hours later. Whereas 50% of the untreated cells remained viable following 11.5 krad of irradiation, the cell population bathed in media containing superoxide dismutase was 75% viable following the same treatment.

Figure 5.2 : Effect of superoxide dismutase on the radiation damage of PAM's



The PBS line refers to viability of cells radiated in phosphate-buffered saline (pH 7.4). The PBS plus SOD line refers to the same population of cells radiated in PBS containing 1000 units/ml superoxide dismutase. Log-linear regression analysis of variance demonstrates a significant difference ($p<0.02$) between the two groups. Combined data from five paired experiments.

Figure 5.2 : Viability of normal adult rat macrophages at increasing levels of radiation in the presence or absence of superoxide dismutase.

Radioprotection conferred by superoxide dismutase was dose dependent up to 100 units per ml of suspending medium, at which concentration the maximum protection was achieved (Figure 5.3).

Figure 5.3 : Dose response curve for the effect of superoxide dismutase on radiation protection of cultured PAM's

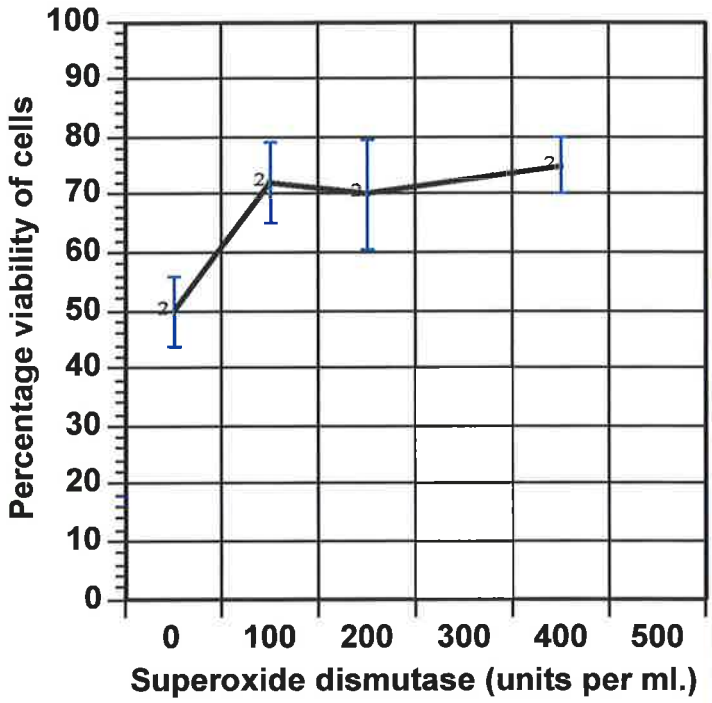


Figure 5.3 : The viability of normal adult rat pulmonary macrophages exposed to 11.5 krad of radiation as a function of superoxide dismutase in the medium. All cells were irradiated in 1 m M phosphate-buffered saline (pH 7.4) in the presence of the indicated concentration of superoxide dismutase. The viability of all cells was corrected to control cell viability (non-irradiated) of 100% for each individual experiment. Data are shown as mean with SEM.

To test whether the $HO^{\bullet}$ radical was directly responsible for radiation-induced lethality in these cells, the radioprotective effects of catalase and the iron-chelating agent DETAPAC were assessed and compared with superoxide dismutase. Following 11.5 krad of irradiation, 39% of the untreated cells were viable, whereas in superoxide dismutase 58% were viable (Figure 5.4). Heat-inactivated superoxide dismutase and catalase, native bovine serum albumin, and native myoglobin conferred no protection against radiation-induced lethality. EDTA when present during irradiation provided no significant protective effect (Figure 5.4). Similarly, 100 mM mannitol provided no protection against radiation-induced lethality (data not shown).

Figure 5.4 : Effect of various oxygen free radical scavengers on the viability of PAM's after radiation exposure

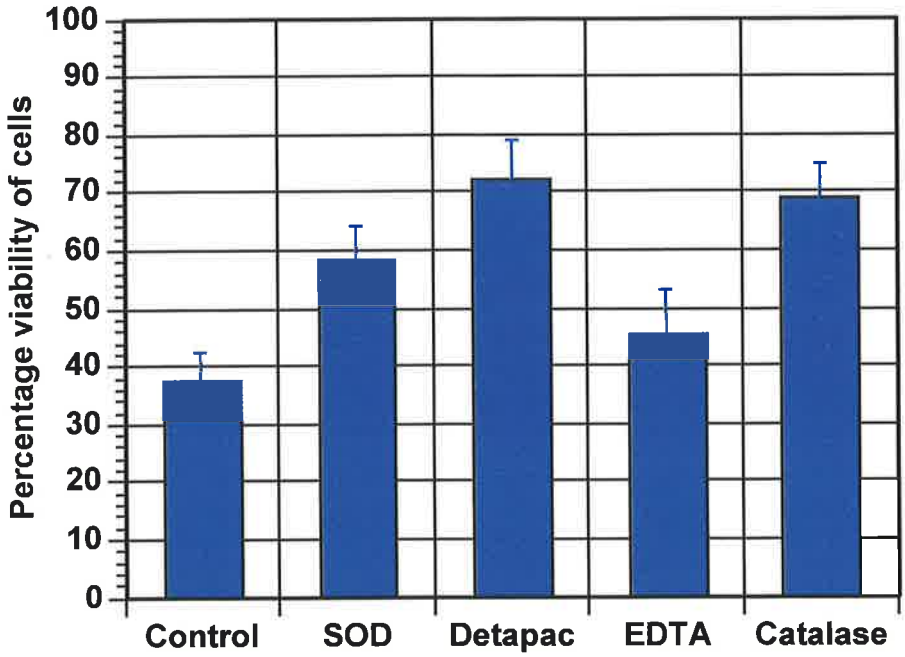


Figure 5.4 : Percentage viability of pulmonary macrophages irradiated in the absence or presence of the indicated agents. The cells were irradiated at 11.5 krad in 1 m*M* phosphate buffered saline, pH 7,4. The viability of cells in all instances has been corrected to a control (nonirradiated) viability of 100%. Student's *t* test was used for the statistical analysis of the results. Data is shown as mean plus SEM. The SOD was 200 units per ml., the DETAPAC was 1 x 10^{-4}M, the EDTA was 1 x 10^{-3}M, the catalase was 25 µg/ml. The SOD result was significantly different to control at the $p<0.05$ level; with the DETAPAC and catalase being significantly different to control at the $p<0.01$ level. The EDTA experiments were not different to the control group.

Although the primary lesion following exposure to low levels of radiation (<3000 rad) is not likely to be the cell membrane, at higher levels this may not be true. Cell viability studies at radiation doses of >3000 rad may in fact be dependent upon the integrity of the cell membrane. The appearance of this membrane following radiation in the absence and in the presence of radioprotective agents was examined by scanning electron microscopy. The general appearance of a normal pulmonary macrophage is shown in Figure 5.5a. The plasma membrane of this cell is shown at a higher magnification in Figure 5.5b in which the

complex structure of the membrane is clearly seen. Twenty-four hours after exposure to 11.5 krad of radiation, extensive destruction of the normal membrane architecture is seen. Typically, the projections of the membrane are greatly reduced and small holes or pits appear in the membrane surface following irradiation (Figure 5.5c). When superoxide dismutase (Figure 5.5d), catalase (Figure 5.5e), or DETAPAC (Figure 5.5f) is present during irradiation, it is clear that the normal architecture of the membrane surface is preserved to a great extent. The pits, which appear to be typical of radiation induced membrane damage, are not seen when any of the three radioprotective agents are present during irradiation. These results confirm the protective action of these agents as assessed by cell viability, and are presented in Figure 5.5.

Figure 5.5 : Scanning electron microscopy assessment of the radiation effects on PAM's in the presence or absence of various agents

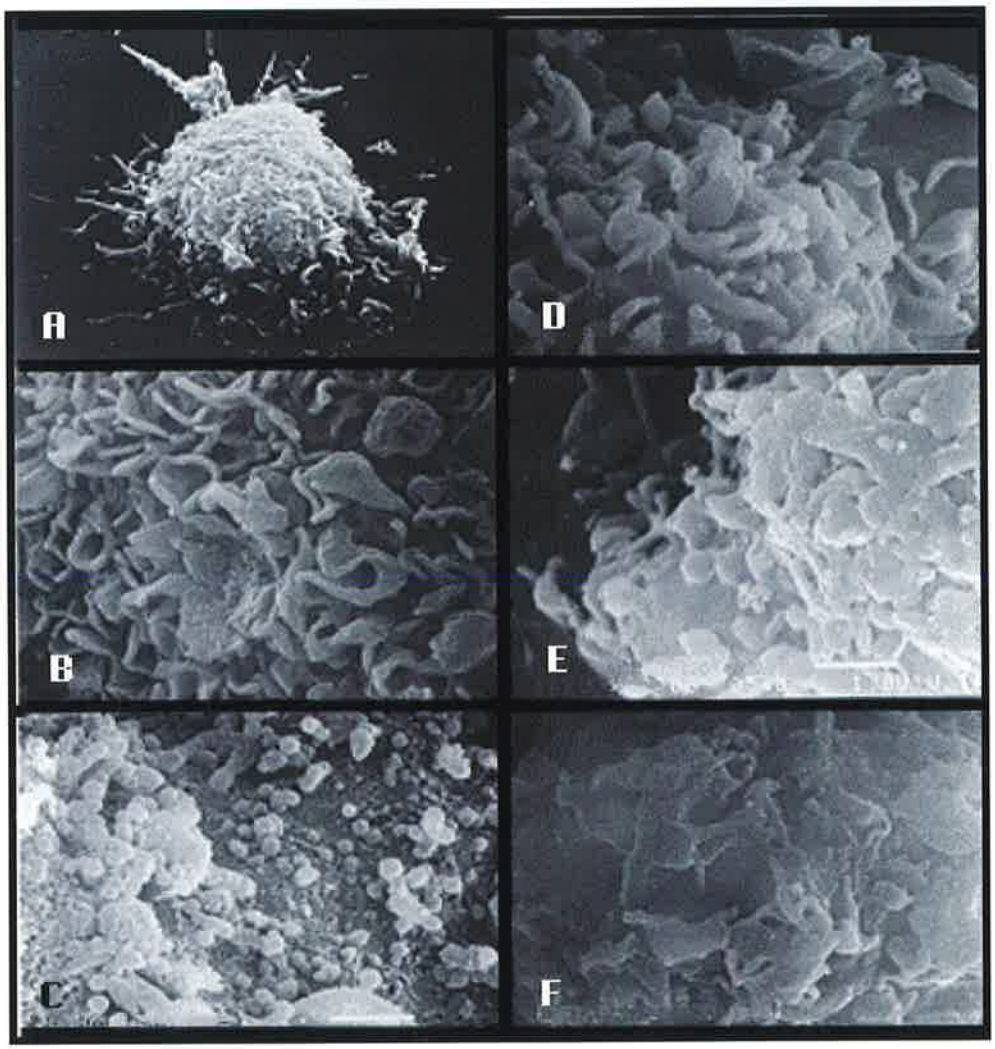


Figure 5.5a : PAM surface changes in the normal PAM in A and B (buffer, not irradiated), and after 12000 rads in buffer in C. There are minor surface changes seen in D (in the presence of SOD), in E (in the presence of catalase) and F (in the presence of DETAPAC).

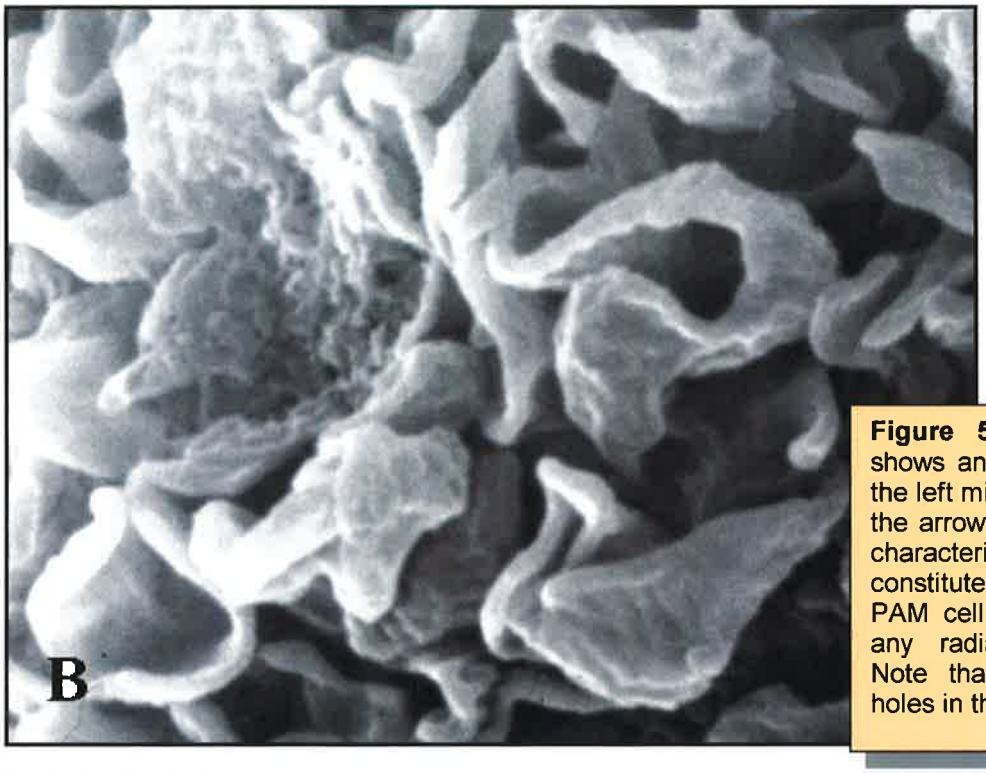


Figure 5.5b : This shows an enlargement of the left mid panel (B), with the arrows pointing at the characteristic ruffles that constitute the normal PAM cell surface without any radiation exposure. Note that there are no holes in the membrane.

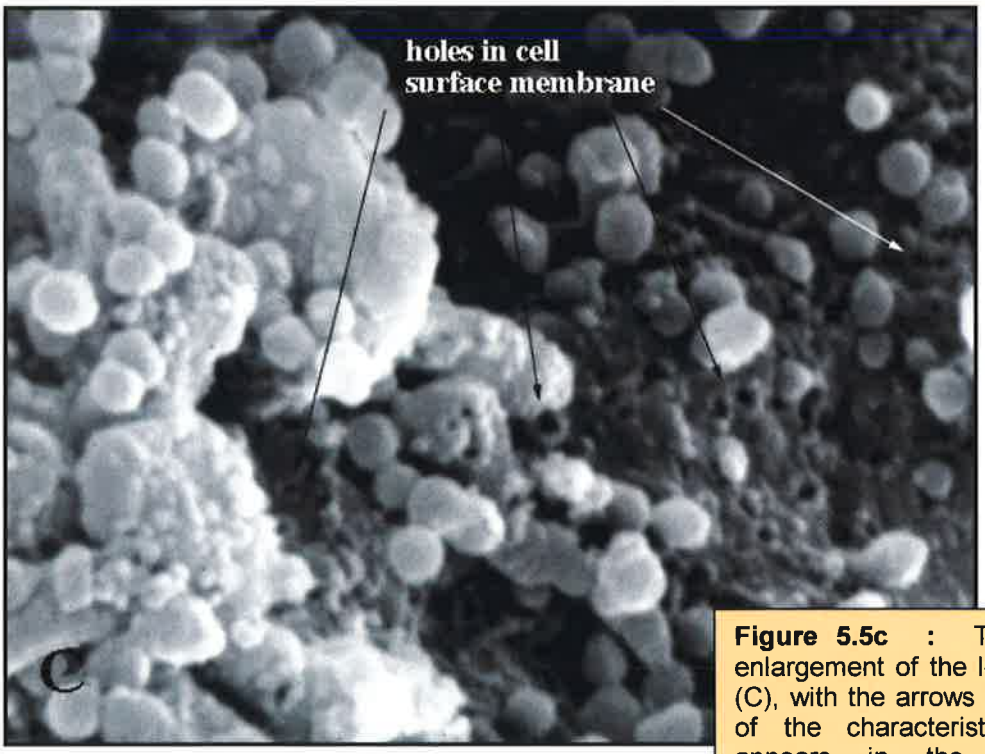


Figure 5.5c : This shows an enlargement of the lower left panel (C), with the arrows pointing at one of the characteristic holes that appears in the cell surface membrane after 12,000 rads of irradiation in the absence of any protective chemical. Note also the loss of surface ruffles and the small spherical surface blebs.

A further observation was also made from the examination of the SEM pictures under the various conditions. The PAM's that were irradiated in the control solution underwent characteristic surface changes with frequently a very characteristic blebbing (Figure 5.6a). or a complete absence of surface ruffling (Figure 5.6b), This was rarely seen in the PAM's that were not irradiated, or in the PAM's that were incubated and irradiated in the presence of DETAPAC or active SOD (Figure 5.6c). The differences in cell surface morphology following irradiation in the presence or absence of SOD is further shown in Figures 5.6d and 5.6e. For all of the experiments shown in Figure 5.6, uniform irradiation dose was used (20,000 rads).

Figure 5.6 : Further changes in the cell surface of irradiated pulmonary macrophages

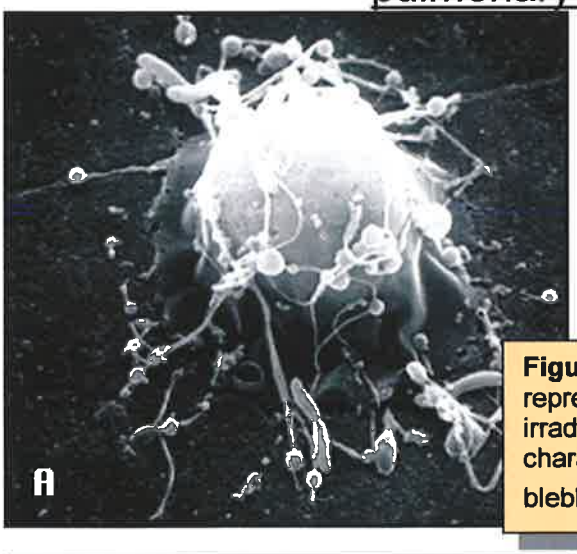


Figure 5.6a : A representative PAM following irradiation, showing characteristic surface blebbing. SEM X 3630

Figure 5.6b : A representative PAM following a large dose of radiation (20,000 rads) irradiation, showing loss of surface features. SEM X 5100

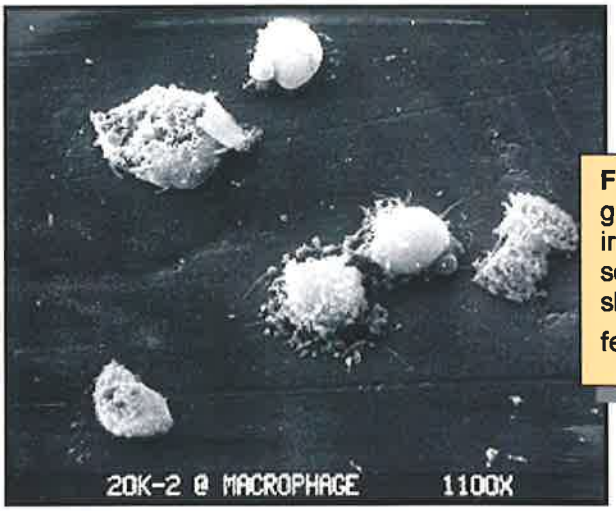


Figure 5.6c : A representative group of PAM's following irradiation (20,000 rads) in a solution containing DETAPAC, showing some retention of surface features. SEM X 1100

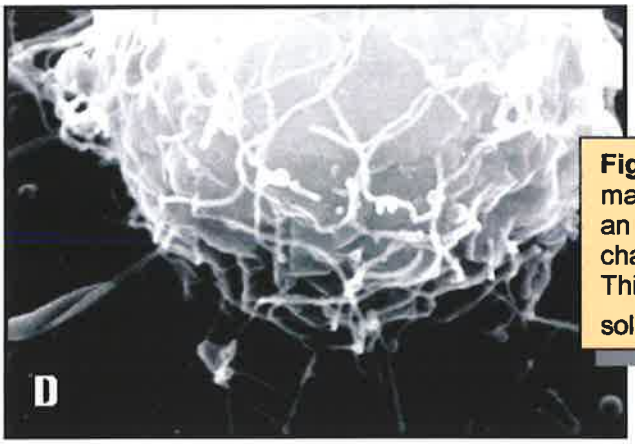


Figure 5.6d : A more highly magnified view of the surface of an irradiated PAM, showing the changes to the cell surface. This cell was irradiated in control solution. SEM X 5040

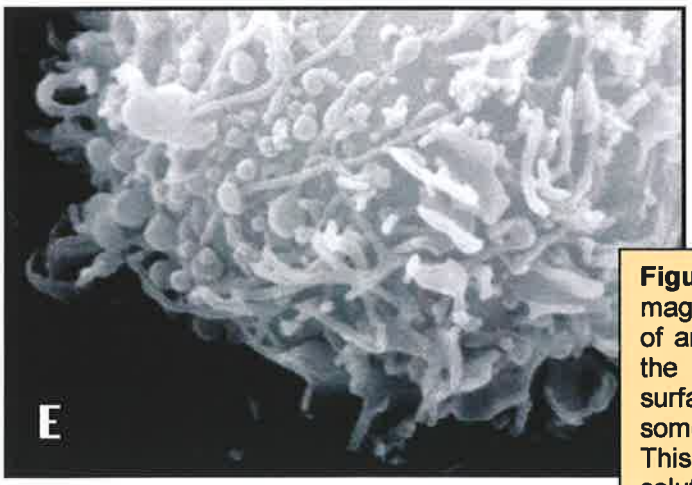


Figure 5.6e : A more highly magnified view of the surface of an irradiated PAM, showing the relatively normal cell surface, although there is some minor bleb formation. This cell was irradiated in a solution containing active SOD. SEM X 5040

Although the studies described above were based on observations of the morphological changes of the plasma membrane, and consequent loss of cell viability resulting from high dosage of radiation, it was necessary to probe the consequences to cells of radiation at dosages at which no lethality occurred, but which caused loss of some specifically defined function. This was performed in an attempt to define more clearly the possible intracellular sites that might be sensitive to radiation. Assessment of phagocytosis was selected as a representative cellular function that could be evaluated for radiation sensitivity at dosages of radiation where no lethality occurred. The extent of phagocytosis was determined by assessing the average number of killed yeast particles ingested by the pulmonary macrophages. Control values were established with non-irradiated and irradiated macrophages. Values obtained from each group were compared by statistical evaluation. Significant differences were those with p values of <0.01. Only 20% of the phagocytic function of control or untreated cells remained after exposure of the cells to 3000 R (2850 rad) (Figure 5.7a). Fifty percent of the phagocytic function remained, however, when superoxide dismutase was present during radiation. DETAPAC provided almost complete protection against functional loss. Catalase provided little protection against radiation-induced loss of function under these experimental conditions. However, when the glass-adhered cells were pre-incubated in catalase for a longer time, protection was observed. Following a pre-incubation period of approximately 180 minutes, compared with the usual 30 minutes of pre-incubation with both enzymes before irradiation, catalase provided virtually the same extent of protection as seen in Figure 5.7a for superoxide dismutase. Pre-incubation with superoxide dismutase for longer periods also increased the extent of radio-protection. These results are summarized in figure 5.7b. Heat-inactivated superoxide dismutase and catalase provided no protection from functional loss caused by radiation.

Figure 5.7 : PAM phagocytosis and the effect of radiation and various chemical agents

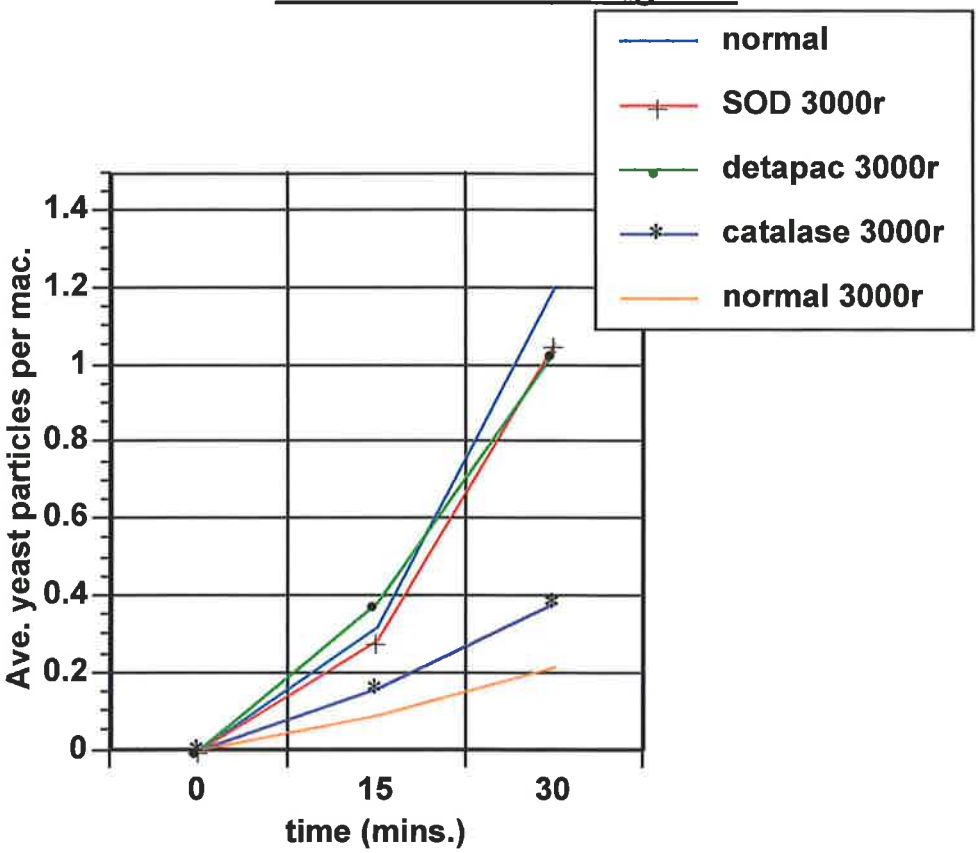


Figure 5.7a : Average number of yeast ingested by nonirradiated and irradiated alveolar macrophages in the absence and the presence of indicated agents. The alveolar macrophages were irradiated at 2850 rad (3000 R). Cells were pretreated for 30 mins. with the various added agents as indicated i.e. superoxide dismutase (SOD), catalase and DETAPAC. Heat inactivated SOD and catalase were also added in similar concentrations, with the phagocytic curve being almost identical to the normal cells irradiated at 3000 rad. This result is typical of 9 different experiments.

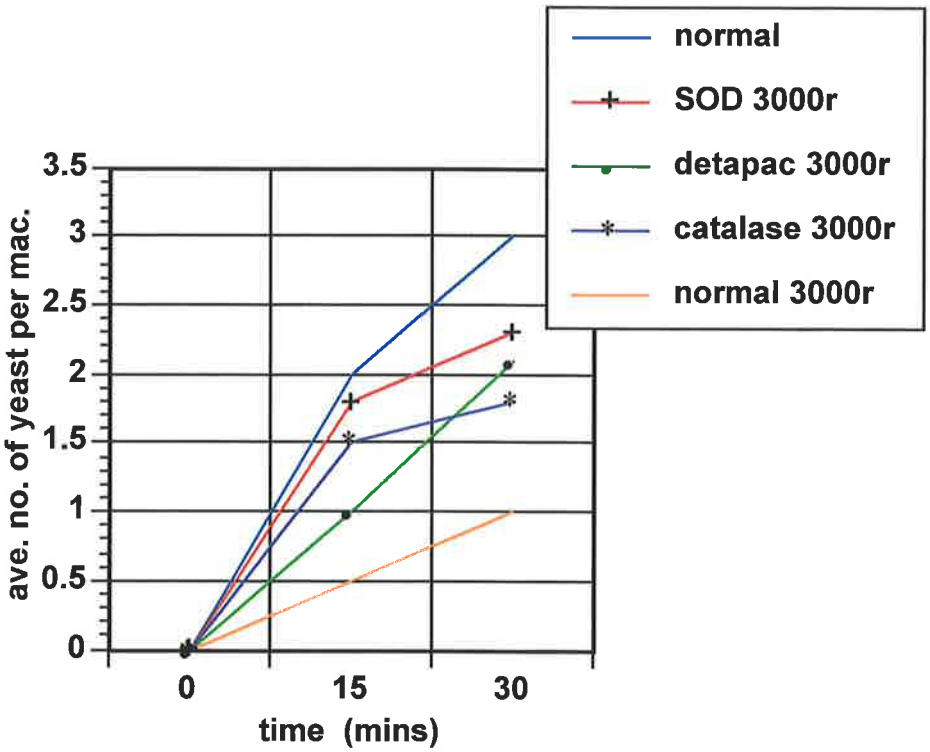


Figure 5.7b : Average number of yeast ingested by nonirradiated and irradiated alveolar macrophages in the absence and the presence of indicated agents. The alveolar macrophages were irradiated at 2850 rad (3000 R). Cells were pretreated for 180 mins. with the various added agents as indicated i.e. superoxide dismutase (SOD), catalase and DETAPAC. Heat inactivated SOD and catalase were also added in similar concentrations, with the phagocytic curve being almost identical to the normal cells irradiated at 3000 rad. This result is typical of 5 different experiments.

DISCUSSION

It has been previously shown (269, 270) that the hydroxyl radical, as measured by both chemical and physical means, can be generated from H_2O_2 and Fe^{++} in accord with the original observations of Haber and Weiss (271). Indirect evidence has accumulated that O_2^- participates in this reaction by converting Fe^{+++} to Fe^{++} (272). Catalase and superoxide dismutase block the formation of $HO^\bullet$ in systems containing H_2O_2 and O_2^-/Fe^{+++}. The iron-chelating agent, DETAPAC, also prevents the formation of $HO^\bullet$ from systems containing O_2^-/Fe^{+++} and H_2O_2. In contrast, EDTA, which does not prevent electron transfer by the chelated iron, has no effect on $HO^\bullet$ production (269). Because the results reported here demonstrated protection against radiation-induced toxicity by all three agents which remove the reactants for $HO^\bullet$ production, strong support is gained for occurrence of the iron-catalyzed reaction in irradiated biological systems. Although mannitol, a $HO^\bullet$ scavenger, did not protect against radiation-induced damage, this can be explained by competitive effects between mannitol and $HO^\bullet$ sensitive cell components. Such cell components are potent $HO^\bullet$ radical scavengers and are at a much higher concentration in the cell than is mannitol. Concentrations of mannitol higher than 100 mM could not be tested because the increased osmolarity of the suspending medium resulted in loss of cell viability of the non-irradiated samples.

In agreement with others it is concluded that ionizing radiation manifests its toxicity via the $HO^\bullet$ radical. However, the basis of the oxygen enhancement of radiation lethality is the generation of $HO^\bullet$ via O_2^-, which in turn is produced through the reduction of oxygen by radiation-induced radicals. The hydroxyl radical can be produced upon irradiation of oxygenated aqueous solutions through the action of a redox cycle with ferric iron, as described above. The best explanation of the data is that O_2^- may be nontoxic *per se* but is a precursor or reactant in the formation of $HO^\bullet$. The contrary opinion is that superoxide radicals have no role in the radiation-induced damage (267). This conclusion was reached from data showing that superoxide dismutase apparently did not provide protection against radiation lethality in a system containing formate in which all radicals were converted to O_2^-. In this system, however, it may not have been possible to generate $HO^\bullet$ for the following reasons -

1. If all radicals were converted to O_2^-, the other reactants necessary to produce $HO^\bullet$ may not have been present.

2. Formate may scavenge $HO^\bullet$ radicals once formed.

Other supporting data were cited in which cells were radiated in the presence of H_2O, an agent used to convert all radicals to $HO^\bullet$. Again the toxicity was said to exclude O_2^- since no oxygen enhancement was observed. The generation of $HO^\bullet$ from O_2^- may not have been possible in this system because of its intrinsic design. Therefore, our conclusions are not inconsistent with these data.

Protection by catalase, superoxide dismutase, and DETAPAC is also seen in other cytotoxic systems known to produce $HO^\bullet$. Pulmonary macrophages are protected by these three agents against the toxicity of auto oxidizing dihydroxyfumarate, which has been shown to generate $HO^\bullet$ (273). Similarly, pancreatic islet cells are protected against the cytotoxicity of the oxygen radical-generating diabetogenic agent alloxan by the same three agents (274). Superoxide dismutase has also shown protective efficacy against the side effects of radiation therapy. Preliminary reports from clinical trials designed as double-blinded and placebo-controlled have shown that superoxide dismutase when administered concomitant to radiation treatment greatly alleviated typical adverse effects of pelvic irradiation (275, 276), although these studies have not been confirmed.

At a high radiation dosage above approximately 3000 rad, the sensitive cellular sites appears to include the plasma membrane as well as intra-cellular targets. Extra-cellularly administered radical scavengers provided significant protection under these circumstances. When the dosage of radiation is lower, and function rather than cytotoxicity is monitored, the target is intra-cellular since there is no loss of membrane integrity as assessed by dye exclusions. These observations suggest that in this case intracellular protective agents such as endogenous superoxide dismutase and catalase provide significant protection against oxygen radical-induced intracellular damage. Furthermore, the data illustrating the effectiveness of oxygen radical scavengers in providing protection against radiation damage in the shoulder region (i.e. the protective effect on phagocytosis in the dosage range of 3000 R and below) can only be interpreted as oxygen enhancement of radiation damage in this region, contrary to many other reports.

Cellular targets of radiation-generated radicals therefore appear to vary considerably in their sensitivity. At low dosages of radiation the toxicity was assessed by loss of function. In this instance, intracellular targets appear to be most vulnerable and loss of function ensues. Apparently, only when the protective agents are available intracellularly does protection become manifest. DETAPAC must enter the cell freely, and as a function of incubation time both superoxide dismutase and catalase appear to enter the cell as well. At higher doses of radiation, the plasma membrane becomes vulnerable; the holes observed in the plasma membrane may reflect secondary lipid peroxidation in the cell membrane. In this instance, extra-cellularly administered protective agents are effective in preventing cell death even if they do not appear to enter the cell to any great extent. An alternative, and probably complementary, explanation for the morphological cell surface events observed with radiation exposure is that radiation has induced program cell death or apoptosis (277, 278). Cells undergoing apoptosis have characteristic surface blebbing similar to that observed here. In human alveolar macrophages, apoptosis has been induced by exposure of the cells to endotoxin (279). Lymphocytes have apoptosis induced by adjacent activated monocytes, by a process inhibitable by catalase, but not other oxygen radical scavengers including superoxide dismutase or iron chelators. The authors of this study concluded that this effect was mediated through H_2O_2 (280). If apoptosis is occurring as a result of the radiation exposure, then in this cell type, it is mediated in part by a combination of O_2^-, H_2O_2 and $HO^\bullet$. There seems to be at least two cellular pathways leading to apoptosis in normal cells (281), and it is possible that radiation provides a third pathway by directly affecting the cellular DNA.

These data strongly support the secondary generation of $HO^\bullet$ via the modified Fenton reaction following irradiation of oxygenated aqueous systems. The strong protection afforded the cells by the agents that remove the precursors of $HO^\bullet$ can at this time only be interpreted to demonstrate the indirect but unequivocal toxicity of both O_2^- and H_2O_2.

Chapter 6

Conclusion

When these studies began, oxygen derived free radicals were not considered likely to have any meaningful place in biological systems, largely because theoretically they were considered so reactive that they would not be present in a biological system. In addition, they could only be directly detected in physical systems that could not support biological substances. Their presence in biological systems could only be inferred by indirect measures such as using a specific inhibitor like superoxide dismutase. Superoxide dismutase had been discovered many years before, but it's potential function as a specific "inhibitor" of superoxide anion radical was only described in the late 1960's. This discovery provided the impetus to begin the search for oxygen free radicals and their effects in biological systems.

Now the oxygen radical field is an enormous one, with radical damage being potentially incriminated in normal biology such as aging, and abnormal biology such as cancer, drug action, nitric oxide metabolism, arthritis and inflammation generally as recently reviewed (282). However, therapies directed solely at removing damaging free radicals have been disappointing, and there are no recognized approved therapies for any disease process.

Pulmonary oxygen toxicity continues to be examined, with only small gains in knowledge. As noted in the text, the use of recombinant pure proteins such as superoxide dismutase, allows for better control of the experimental situation in using oxygen free radical scavengers, and the use of various genetic "knockout" animals has allowed for a clearer view of the importance of various free radical scavenging proteins.

The evidence available in the literature, and summarized in Chapter 1, is strongly suggestive that oxygen free radicals are important in the pathogenesis of pulmonary oxygen toxicity. The evidence comes from four main experimental areas. The first area is the expanding theoretical and chemistry aspects of oxygen free radical generation. This has continued to mature, with now a general consensus that oxygen free radicals exist in biological systems, and can cause disease. This has also recently, within the last few years, been strengthened by the discovery of a strong biological role for nitric oxide, together with the realization that nitric oxide and oxygen interact to form the peroxyradical.

The second experimental area is in the knowledge gained from understanding the neonatal resistance to pulmonary oxygen toxicity observed in the rat. The link of this to the induction of the oxygen free radical defense enzymes, has demonstrated that these may be biologically important. Linked with this is the finding of the same enzyme induction with various agents that induce oxygen tolerance in the adult rat. The initial observation here was made with endotoxin. Since endotoxin induces IL-1, TNF and IL-6 it is reinforcing to know that all of these induce the oxidant defense enzymes, and confer partial pulmonary oxygen resistance.

The third experimental area is in the administration of the various oxidant defense enzymes systemically to animals. It is worth tabulating these experiments.

Administered agent	Route of administration	Animal model	Effect on lung oxygen toxicity
CuZnSOD	Aerosolized	Rat	No effect
CuZnSOD	Intravenous	Isolated rat lung	Good effect
Liposomal CuZnSOD & catalase	Intratracheal injection	Rat	Very good effect
Liposomal CuZnSOD & catalase	Intravenous injection	Rat	Good effect
PEG CuZnSOD & catalase	Intravenous injection	Rat	Poor effect
Recombinant MnSOD	Nebulized	Baboon	Poor effect
Recombinant CuZnSOD	Intratracheal injection	Pig	Mild effect
Transgenic with CuZnSOD overexpression		Mice	Moderate effect
Transgenic with MnSOD overexpression		Mice	Poor effect
Knockout, MnSOD		Mice	No effect
Knockout, EC-SOD		Mice	Increased oxygen sensitivity
Transgenic with EC-SOD overexpression		Mice	Good protection in hyperbaric oxygen

Initially, rats were the experimental animal model of choice, but with the development of transgenic models in the mouse, this animal has been utilized more recently. It is unfortunate that the pathology of oxygen toxicity has been less well studied

in the mouse, and the mouse also does not exhibit neonatal resistance to oxygen toxicity, as is seen in the rat.

Nevertheless, putting these studies together it would seem that MnSOD administration, or manipulation of endogenous MnSOD levels, does not influence pulmonary oxygen toxicity appreciably in any animal model apart from the baboon, where the alveolar epithelium is relatively protected. Initial expectations were the opposite, as it was felt, with some experimental evidence, that excess oxygen radicals would most likely be produced within the mitochondrial under hyperoxic conditions.

It is also apparent that CuZnSOD has a partial protective effect. This is only seen when one condition is satisfied. This is that the CuZnSOD is administered with methods that prolong the half-life. Finally, increasing or decreasing the extracellular levels of ECSOD also moderates or exacerbates pulmonary oxygen toxicity respectively.

The studies reported in Chapter 2 clearly show improvement in lung histology, gas exchange and survival following the continuous intraperitoneal infusion of CuZnSOD in the rat, exposed to hyperoxia. This finding is further evidence that CuZnSOD has a protective role in against pulmonary oxygen toxicity. It is of interest that when this study was first reported by me, there was harsh criticism because of the finding of measurable levels of CuZnSOD circulating in the plasma of these animals. This was considered to not be biological possible, as CuZnSOD was felt to be only present within the cytoplasm. In retrospect, this finding likely represented not CuZnSOD, but circulating ECSOD, at that time not yet otherwise discovered. Various authors have felt that scavenging superoxide anion radical might generate even more damage by the over-production of hydrogen peroxide. However, removal of superoxide anion radical might on balance be protective as it is then unable to participate in the Haber-Weiss reaction (with production of the even more damaging hydroxyl radical), or the in the reaction with nitric oxide,(with the production of the peroxynitrite radical).

See footnote below. [1]

The now somewhat compelling evidence that sustained increased levels of CuZnSOD (or ECSOD), but not MnSOD, can partially protect against pulmonary oxygen toxicity in the rodent lung is of interest. There are several conclusions that relate to oxygen toxicity that come from these observations.

1) *That pulmonary oxygen toxicity is in part mediated through damaging oxygen radicals, at least one of which is super-oxide anion radical.*
2) *That damaging oxygen radicals are not generated in the mitochondria, and that mitochondria are not the primary site of lung cell injury.*
3) *That damaging oxygen free radicals, in particular, superoxide anion radical, is produced extracellularly in the lung.*

Whilst it has been noted in Chapter 1 that interstitial oedema is the first change in the rat lung during hyperoxic exposure, studies in other animal models suggest that the alveolar epithelium is the major site of damage. In this thesis, using light microscopy, transmission electron microscopy, and scanning electron microscopy, the sequential changes of occurring in the rat lung are shown clearly. These are: interstitial oedema, followed by endothelial cell blebbing and lifting away from the basal lamina, followed by alveolar luminal proteinaceous material. In concert with these changes are, initial platelet adherence to the endothelial cells, followed by polymorphonuclear neutrophil accumulation within the capillary lumen. These are the first scanning electron microscopy studies of the lung showing progression of this disease, and the first scanning electron microscopy images of the rat lung with preservation of the surfactant lining, that have not been prepared by intravascular fixation. The interstitial oedema clearly occurs before evidence of platelet or other cellular accumulation. These findings suggest the following conclusions in relation to pulmonary oxygen toxicity.

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[1] $O_2^- + O_2^- + 2H^+ \Leftrightarrow H_2O_2$ – this reaction can occur spontaneously, or be catalysed by the dismutases;

$O_2^- + H_2O_2 \Leftrightarrow OH^- + HO^\bullet + O_2$ – this reaction is the Haber-Weiss reaction and is catalysed by free Fe^{+++};

$O_2^- + {}^\bullet NO \Leftrightarrow ONOO^-$ – in this reaction the superoxide anion reacts with nitric oxide to produce the peroxynitrite radical.

4) That the initial site of injury in the rat lung is the endothelial cell.

5) That the endothelial cell is not damaged by the action of circulating platelets or polymorphonuclear neutrophils, at least not initially.

Combining these conclusions (1,2,3 4& 5) leads to the following summary conclusion:

6) Pulmonary oxygen toxicity in the rat lung is mediated in part through the production of superoxide anion radical, and this is likely to come from an extracellular source adjacent to the pulmonary endothelial cell.

This hypothesis is a reasonable explanation of the observations made in Chapters 2 & 3 of this thesis, and is supported also by the other experimental work summarized in Chapter 1. Hypotheses generated by others in past and recent literature have focused on the endothelial cell mitochondria as being the likely target of oxidant damage, or on the alveolar epithelium. Also, several years ago inflammatory cells, especially the polymorphonuclear neutrophil were postulated to be the source of the excess oxygen free radicals. However, this notion does not explain the early lung damage, although inflammatory cells may contribute to damage in the latter phases of injury. There are a number of questions that arise from the summary conclusion (6 above). These include how free radicals might be generated at this site, and in what way they cause cell damage? These questions will be addressed a little later.

In addition to the work reported here in this thesis, there were a number of studies that were performed which did not have a promising outcome. One of these was to study if penicillamine, by binding free metals, might moderate pulmonary oxygen toxicity. These studies examined if exogenously administered penicillamine could modify pulmonary oxygen toxicity in the rat (it did not).

[1]The focus was still the understanding of pulmonary oxygen toxicity. Of the various attempts to modify pulmonary oxygen toxicity, none were as dramatic as the effects of temperature. Indeed, even compared against more recent literature, the effects of temperature are more dramatic than most other manipulations.

As detailed in Chapter 4 of this thesis, higher environmental temperatures greatly augment pulmonary oxygen toxicity, and environmental temperatures lower than a comfortable ambient room temperature greatly reduce the effects on the lung. The environmental temperatures *per se* do not damage the lung, as measured by histology and lung ultrastructure. The increased death of animals at higher environmental temperatures is associated with an evolution of pulmonary disease that is histologically and ultrastructurally identical to that of oxygen effects at ambient temperatures. However, the damage is accelerated, and the animals die from pulmonary disease. These studies as outlined in Chapter 4 also indicate that the inhaled gas temperature is a critical determinate of pulmonary oxygen toxicity. Whilst these studies build upon previous literature, especially those dealing with hibernating, or cold blooded animals, they are still the most complete observations in a mammalian species. It is important to note that the animal core body temperature did not change as a result of these exposures (unlike hibernating animals). I have covered a wide range of possible explanations for the effects of environmental temperature on pulmonary oxygen toxicity as part of the discussion in Chapter 4, and there are several that invite further thought. The effect of temperature seems to be associated with the temperature of the inhaled gas, suggesting that there is modification of a local lung event. It is possible that the direct production of oxygen free radicals within the lung under hyperoxic conditions is temperature dependent. This could occur because of a change in the rate of oxygen metabolism, or in the balance between the various pathways for scavenging excess superoxide anion. An alternative explanation is that inhaled gas temperature modifies the anti-oxidant defense enzymes, although this is less likely because pre-exposure is not required. Finally, it is possible that the inhaled gas

[1] I also performed a number of other preliminary, but not rewarding studies such as examining chopped up lung explants in tissue culture to see if they would be a good model for studying lung toxicity (they were not as under TEM most of the cells had died within 4 hours, and what was left was a basal lamina preparation), attempting to isolate and grow pulmonary type I cells in tissue culture, using lung digestion and various fractionation techniques (this failed), using horseradish peroxidase (HRP) as an electron microscopy marker to establish where the leak was in the lung in hyperoxic injury (this failed as the HRP was not detectable in the concentrations used), and finally a series of experiments to track endogenously administered CuZnSOD by radiolabelling this (successfully), and then using photographic emulsion over tissue slices to find the CuZnSOD (the conditions for these autoradiographs was not established within the time frame for doing the experiments).

temperature modifies oxygen toxicity through rapid induction of TNF, IL1, IL6 or IL11. It is reasonable to broadly conclude :

7) That inhaled gas temperature is a critical determinate of pulmonary oxygen toxicity, and that this is likely to be a result of altered metabolism of oxygen free radicals.

Ionizing radiation has been thought to generate oxygen free radicals that damage tissue, and that this is the cause of the "oxygen effect" commonly observed in radio-biological interactions. The experiments detailed and discussed in Chapter 5 clearly demonstrate that non-dividing eukaryotic cells are injured by ionizing radiation through the Haber-Weiss reaction. There are several observations worth re-commenting on. Firstly, the relative resistance of alveolar macrophages to ionizing radiation. Secondly, the remarkable holes noted in the alveolar macrophage plasma membrane by scanning electron microscopy as a result of the radiation exposure. The observation that extracellular CuZnSOD is relatively protective under these circumstances also is of note. Overall the conclusion that is most reasonable to draw is

8) That radiation damage, under aerobic conditions, in a non-dividing eukaryotic cell is mediated in part via oxygen free radicals directly damaging the plasma membrane

In any event conclusions I - 8 can be combined to give a final unifying conclusion as follows :

Both oxygen and radiation damage to eukaryotes is mediated, in part, by the relative overproduction of superoxide anion, and the subsequent metabolism of this into other damaging oxygen radicals. Temperature is a critical modulator of this damage, probably by altering the relative production of superoxide anion, and of other radicals. A critical site of cellular damage is, in part, the plasma membrane, and in pulmonary oxygen toxicity it is the plasma membrane of the capillary endothelial cell, adjacent the alveolar conjoint basal lamina.

This conclusion is one that best explains the observations made in this thesis. The conclusion is also supported by positive and negative data from other published works. It can be summarized for pulmonary oxygen toxicity by the following figure:

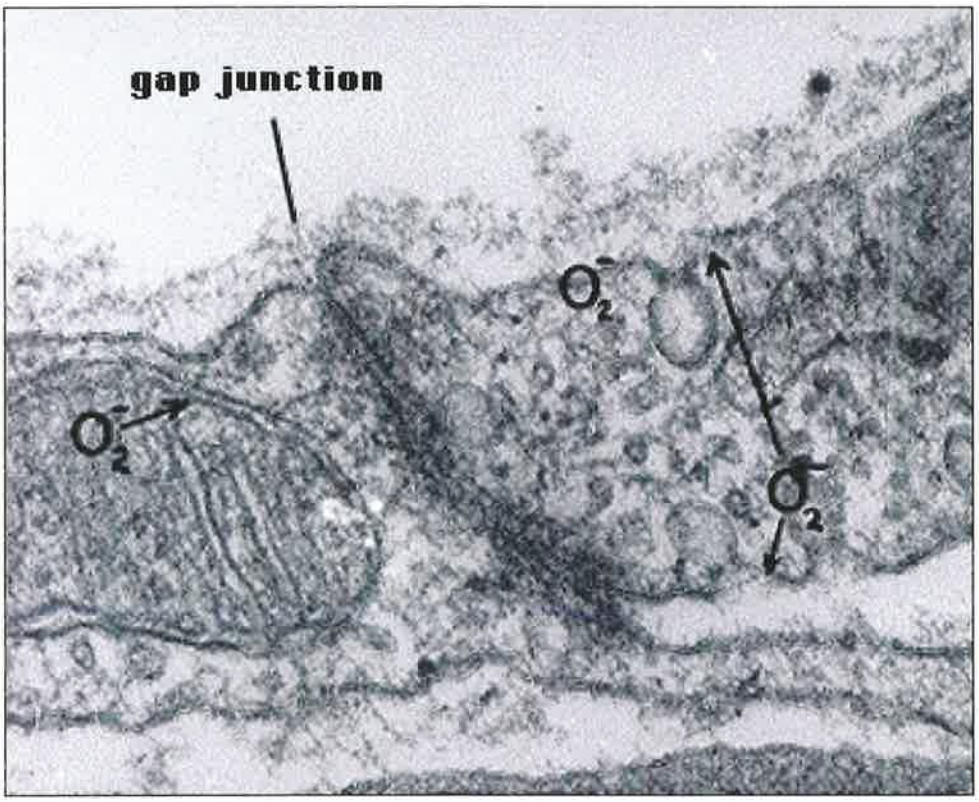

Figure 6.1 : This shows a section of capillary endothelial cell, with the intercellular gap junction. This cell is the likely target of oxygen toxicity in most animal models studied, particularly small rodents such as rats and mice. Although oxygen free radicals are generated from mitochondrial metabolism (a portion of an endothelial cell mitochondria is shown on the left in this image), it is likely from the results of studies using oxygen radical scavengers that, the endothelial cell membrane adjacent the interstitial space is the initial critical target. The rounded structures within the cytoplasm are thought to be transport vesicles, and the darker structure seen at the bottom left of the image is a portion of a circulating red blood cell. TEM ^{x}150,000.

This final conclusion raises a number of pertinent questions for future studies. Firstly, how does temperature increase or decrease resistance to pulmonary oxygen toxicity? It is possible that this is just the result of changes in metabolic rate. This is unlikely as altering metabolic rate, such as by manipulation of thyroid hormones has some effect on pulmonary oxygen toxicity, but not of the magnitude seen from manipulating the environmental temperature. It is most likely that there is either an increase in free radical

production, or that the oxygen free radical defenses are altered. The first premise is possible, but has not been tested to my knowledge at this time. The second premise, that is there is an alteration in the oxygen free radical defenses is also a possibility – and this could be either through alteration in the quantity of these enzymes or in their position(s) within the cell. These possibilities could be easily tested.

The second important question that arises from this thesis, is the relative importance of injury to the plasma membrane, compared to the mitochondria, compared to the nuclear DNA – especially in non-dividing eukaryotic cells. There will clearly be different cellular sensitivities in this regard. The effects of oxygen toxicity and of radiation may be quite different. Finally, the dose response and time course of the relative injuries clearly needs to be studied.

A third important question that arises from this thesis is how the plasma membrane of the endothelial cell is damaged? How could oxygen free radicals be generated, in excess, at or near this site? There are several potential explanations. There may be a free radical generating system that is more active at this site, and this could be related to an interaction between nitric oxide production and superoxide anion production (through plasma membrane bound NADPH oxidase), with excess local production of the peroxynitryl radical. Alternatively, the damage could still come from endothelial cell mitochondrial free radical production, especially as the mitochondria is in close proximity to the plasma membrane (see image above). This is not likely, however, as this would mean either that the endothelial cell plasma membrane adjacent the interstitium was more susceptible to radical damage, or that the mitochondria were extremely close to the plasma membrane (eccentrically arranged within the endothelial cell), or that the mitochondria exhibits polarity with regards to the release of free radicals – favouring the plasma membrane on one side only. Again, the likely excessive production of free radicals adjacent to the endothelial cell plasma membrane is a testable hypothesis.

I would note also that most animal studies involving pulmonary oxygen toxicity do not monitor or maintain the environmental temperature during exposures. The temperature of small exposure chambers can vary greatly depending on the number of animals within each chamber. Failure to address temperatures during the exposures makes it very difficult to interpret results, and in my view makes the results invalid. Of the 12 studies

tabulated above (and summarized in Chapter 1), only 4 made reference to the exposure conditions, with most indicating the ambient temperature of the exposures, but no actual temperature control. Five made **no** reference to the temperature of the exposures, and in three it is unclear.

The findings in this thesis have important translational research implications also. Within intensive or critical care areas, patients are routinely artificially ventilated. These ventilation systems deliver humidified gases, frequently rich in oxygen, directly into the patients' lungs. Importantly, the humidification is associated with direct heating of the inspired gases. There is no monitoring of the inspired gas temperature. Further, many of these critically ill patients already have substantial increases in body temperature. Many of these patients develop an acute lung injury, known collectively as the adult respiratory distress syndrome (ARDS); this has 50% mortality. The findings from this thesis would suggest that the heated inspired gases contribute to a substantial amount of lung injury and patient mortality. Further, a substantial reduction in lung injury would be expected by the maintenance of cooler temperatures. This should be studied, and addressed, as a matter of urgency.

There are a number of other observations that are worth mentioning. Firstly the novel way to prepare the lung for electron microscopy with preservation of the surfactant layer, has not to my knowledge been reported elsewhere. This method may have some advantage in understanding the surface action in the alveolus of endogenous surfactant and of administered artificial surfactants.

The observation that rat lung is 1.5 degrees lower than core body temperature is surprising, and raises the question of the correct temperature to study alveolar macrophage, surfactant and protein function in culture conditions. The major impact of temperature on pulmonary oxygen toxicity has direct relevance to ventilatory management of patients, and should be examined in different models (it is possible that low inspired oxygen, say 40%, is toxic under conditions of high temperature), and the mechanisms for this temperature effect established. It remains unclear what the safe level of oxygen exposure is as 60% can induce some disease in the rat (283) and 50% is enough to induce oxidant stress in the human lung (284). It may well be that oxygen, like radiation, has no safe level of exposure, and perhaps the use of oxygen therapeutically, needs to be more

carefully monitored and/or regulated in the ICU setting. The home long-term use of oxygen replacement especially in obstructive pulmonary disease should be continued only in the knowledge that there may be toxic effects, especially if there are significant environmental temperature increases or patient fevers. It would be of interest, for instance, to examine for mortality in patients on oxygen replacement during heat wave conditions with groups stratified for air-conditioning.

Whilst there is no major difference between the PAM's in non-smokers and smokers, the macrophage population in smokers is so expanded, that even a small change in individual cell function will impact on the alveolar region. The striking observation that only a minority of alveolar macrophages participate in phagocytosis (cf to rat macrophages), raises questions as to whether the human alveolar macrophage requires priming for this function, or whether there is true functional differentiation of these cells into different populations. Comparison with the blood monocytes (the precursor cell) would be of interest, and separating out the actively phagocytic cells, by density, and then examining the function of the remainder would also be of interest.

The work resulting from this thesis has formed an important basis for understanding acute and chronic lung injury. I have been particularly interested in establishing methods for the objective assessment and longitudinal followup of acute and chronic lung injury models. After spending a large effort on evaluating bronchoalveolar lavage for this purpose (and finding it not as reproducible as initially thought), I have moved into computer generated methods for evaluating the lung using ultrafast CT scanning. I intend to apply those methods, now well evaluated and funded, into continuing the studies on lung injury in animal and human subjects.

Chapter 7

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