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ENDOTOXIN PROTECTION OF RATS FROM OXYGEN TOXICITY: ROLE OF LUNG PHAGOCYTES

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ENDOTOXIN PROTECTION OF RATS FROM OXYGEN TOXICITY: ROLE OF LUNG PHAGOCYTES

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCES (PHYSIOLOGY)

AUGUST 1985

By

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I wish to express my sincere gratitude to Drs. Richard Smith, Martin Rayner and S. Ramanathan for their continued guidance, support and friendship during the course of this study. Appreciation is also due to my committee members, Drs. Nancy Lind, and David Lally for their numerous contributions and to Dr. Y. Hokama for answering a multitude of questions. Finally, I wish to thank my father, John W. Berg, for providing encouragement and support when it was most needed. Financial support from Leahi Trust (Hawaii Thoracic Society) is gratefully acknowledged.
Endotoxin (1 mg/kg body weight, i.p.) greatly reduces lung damage and pleural edema in rats exposed to > 99% oxygen. Endotoxin also activates and depletes complement in vitro. Both complement and polymorphonuclear leukocytes (PMN) have been shown to play a role in the development of lung damage in several models of lung inflammation. PMN may injure tissue by releasing free radicals or proteolytic enzymes. This dissertation was designed to evaluate the possibility that the mechanism of endotoxin protection involves changes in either 1) the number of PMN or their ability to release free radicals or 2) serum complement levels following exposure of rats to > 99% oxygen for up to 3 days at 1 ata (sea level). The potential of lavaged phagocytes to generate free radicals was determined using zymosan stimulated chemiluminescence (CL). Values were then expressed as peak CL/10⁶ PMN. PMN peak CL fell progressively with time of oxygen exposure. Peak CL by PMN from saline pretreated rats breathing oxygen for 3 days was 80% lower than peak CL by PMN from paired rats pretreated with endotoxin. In addition, complement hemolytic activity was not altered in serum from endotoxin pretreated rats following exposure to > 99% oxygen or air for 65 hours. Depletion of complement in rats prior to oxygen exposure also failed to provide protection from the pleural edema of oxygen toxicity. These results suggest that endotoxin does alter the ability of alveolar PMN from oxygen breathing rats to release free radicals but does not alter serum complement.
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GENERAL INTRODUCTION

History

In 1775, shortly after discovering oxygen, Priestley warned of the potential for pulmonary oxygen toxicity when he wrote:

"From the greater strength and vivacity of the flame of a candle, in this pure air, it may be conjectured, that it might be peculiarly salutary to the lungs in certain morbid cases...But, perhaps, we may also infer from these experiments, that though pure dephlogisticated air might be very useful as a medicine, it might not be so proper for us in the usual healthy state of the body: for, as a candle burns out much faster in dephlogisticated than in common air, so we might, as may be said, live out too fast, and the animal powers be too soon exhausted in this pure kind of air."

(Quoted from J. W. Bean, 1945, p. 1).

Priestley's warning that "the breathing of pure dephlogisticated air might...not be so proper for us" gained support from several experiments which were conducted at the time. In 1783 Lavoisier (see J.W. Bean, 1945) reported his observations following the autopsy of a guinea pig which had died in the 'air vital'. The lungs were very red and death was attributed to 'maladie inflammatoire'. In 1796 Beddoes and Watt (Bean, 1945) exposed a kitten to 80% oxygen for 17 hours prior to autopsy and noted the lungs as florid red with livid spots marking the edges. The pleura were inflamed while those of the kitten breathing air were pale.

Surprisingly, in view of these early experiments which are consistent with present observations, the medical community at that
time did not generally accept the idea that breathing purified oxygen could be toxic. In fact, it became quite fashionable during the 1800's to treat numerous maladies by exposure to oxygen. It remained for Paul Bert in the 1870's to establish firmly that oxygen could be toxic and because of his contribution he is regarded as the "Father of oxygen toxicity". Bert's account of the observations which led him to question the concept of increased oxidation, proposed a hundred years earlier by Priestley, is quite interesting:

"When for the first time I saw a sparrow struggling in violent convulsions under the influence of compressed oxygen, I imagined at first that the intra-organic oxidations had been so overstimulated in this bird that it was dying from burning itself out too quickly, producing thus a quantity of exaggerated heat, which perhaps became the direct cause of death. I thought, therefore, that the thermometer would show me a rise in the bird's temperature. Great was my surprise when I noted an absolutely opposite result.

(Quoted from Paul Bert, 1878, p. 743).

Several other observations reported by Bert in 1878 are especially relevant since they clearly illustrate that purified oxygen can influence life processes in a negative manner. Frog hearts, beating in oxygen, were found to 'die' more quickly than frog hearts beating in air. Bacterial action was found to be hindered by oxygen since meat could be stored for extended periods of time in purified oxygen without spoilage. Enzymes were also protected from the damage caused by bacteria since they retained their catalytic ability when stored in oxygen. Enzymes stored in air lost catalytic ability within a day or two.
Numerous reports, subsequent to the work of Paul Bert, have further established that breathing purified oxygen can produce detrimental effects on living organisms. In 1927 Binger et al. demonstrated that oxygen, and not an impurity in the gas, was responsible for the pathology observed under oxygen. They prepared oxygen by both air reduction and electrolysis and bubbled the generated gas through olive oil. Since this purified oxygen still caused oxygen toxicity they concluded that oxygen, and not a contaminant, was the responsible agent.

In 1932 Smith et al. discussed shortcomings of previous work:

"No previous work on the toxicity of increased oxygen tensions in the respired air has been conducted under conditions in which all the other factors were constantly and perfectly controlled, and most of the investigations on this subject have been based upon data obtained from observing a limited number of animals."

(Quoted from Smith et al., 1932, p. 63).

To overcome these shortcomings Smith built a chamber out of one half inch steel measuring 8' in diameter and 32' in length. In 1932 Smith reported the results of experiments using this chamber. 244 rats were exposed to 83.2% oxygen for 72 days. 40 days were then allowed for the survivors to recover in room air before being reexposed to oxygen for a final 10 days. Perhaps the most significant aspect of this study was the correlation drawn between age, survival and alveolar morphology. Rats younger than three months were found to be especially resistant to oxygen and showed a high degree of what Smith
called 'cellularity' in their alveolar walls. Surviving adults exhibited a similar degree of alveolar cellularity. Air breathing adult rats and rats dying of oxygen toxicity showed a lung morphology in which the alveolar membrane consisted of only one or two layers of cells. Smith concluded that oxygen inhibits cell division so that alveolar cells in adult rats succumbing to oxygen toxicity had lost the ability to revert to the cellularity seen in the lungs of young rats surviving oxygen. Though this interpretation is not directly in line with current concepts of the mechanism of oxygen toxicity, it is important since Smith drew attention to the fact that changes were occurring in lung morphology at the cellular level as a result of exposure to increased concentrations of oxygen at atmospheric pressure.

Other reports in the 1920's and 30's are particularly relevant to the theory that free radicals are involved in producing the lung pathology of oxygen toxicity. In 1927 Faulkner and Binger found that turtles were not affected by oxygen if exposure occurred at 23 °C. Interestingly, when exposure occurred at 37 °C the animals developed oxygen toxicity. These authors suggested that the increase in temperature allowed a chemical reaction to occur between oxygen and pulmonary tissue so that a chemical irritant was produced. At the lower temperature the hypothesized reaction did not occur for kinetic reasons. In 1932 Boycott and Oakley reported the results of experiments using rats. Pulmonary edema was seen in association with an increase in pulmonary monocytes. These workers proposed that the increase in oxygen concentration irritated the endothelium of
pulmonary capillaries so that plasma from the blood could leak through. This reasoning, so similar to current theory, leads to the concept of free radicals.

The chemistry of oxygen free radicals

Free radicals are short-lived, unstable chemical intermediates which owe their high reactivity to the presence of a single unpaired electron in the outer orbital. Ground state molecular oxygen contains two unpaired electrons so each oxygen molecule must gain two electrons to fill the outer valence orbital and satisfy the octet rule. The fact that oxygen's unpaired electrons have parallel spin while donor electron pairs have opposite spin necessitates the step-wise addition of single electrons as oxygen is reduced to water; i.e., only one electron can be added to each of molecular oxygen's unpaired electrons at a time because of the spin restriction. This addition of single electrons to ground state molecular oxygen leads first to the formation of the superoxide radical ($O_2^-$) and then to the formation of the hydroxide radical (OH') with $H_2O_2$ occurring as an intermediate. A simplified reaction sequence for the generation of superoxide and the hydroxide radical during the reduction of molecular oxygen to water can be summarized as follows:

$$O_2 + e^- \longrightarrow O_2^-; + e^- + 2H^+ \longrightarrow H_2O_2$$

$$H_2O_2 + e^- + H^+ \longrightarrow OH^- + H_2O; + e^- + H^+ \longrightarrow 2H_2O$$
Though superoxide and hydrogen peroxide are toxic to cells, the species of major concern is generally hypothesized to be the hydroxide radical. Susceptibility to this radical is due to the fact that the body does not possess protective enzymes to remove OH⁻; superoxide dismutases (SOD's) are present to quench O₂⁻ and catalase will detoxify H₂O₂ according to the following reaction sequences:

\[
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2^- + \text{O}_2 (\text{SOD's})
\]

\[
\text{H}_2\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 (\text{Catalases})
\]

In 1970 Beauchamp and Fridovich demonstrated that O₂⁻ and H₂O₂ can react to form the hydroxide radical via the Haber-Weiss reaction:

\[
\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{OH}^- + \text{OH}^+ + \text{O}_2
\]

They found that methional is oxidized to ethylene when exposed to both O₂⁻ and H₂O₂. If either reactive species were used alone, or if either superoxide dismutase (SOD) or catalase were added to the reaction mixture to scavenge O₂⁻ or H₂O₂ respectively, the formation of ethylene did not occur. Scavengers of OH⁻ (ethanol or benzoate) were also found to inhibit the conversion of methional to ethylene. The proposal that the Haber-Weiss reaction occurs in biological systems has attracted criticism since it occurs too slowly
in the test tube to be of significance in the generation of OH'.

(Fee, 1981; Sawyer and Valentine, 1981). Fridovich, however, has replied in defense of his theory that Fe$^{3+}$ may serve as a catalyst in living systems and speed up the formation of the hydroxide radical according to the reaction sequence:

$$\text{Fe}^{3+} + \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2$$

the Fe$^{2+}$ then reacts with H$_2$O$_2$ according to the Fenton's reaction:

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}'$$

to yield OH'. A quote from Fridovich expresses the current state of the debate concerning the role of the Haber-Weiss reaction in the generation of OH' in living organisms:

"Whatever the actual mechanism, it is clear that O$_2^-$ and H$_2$O$_2$ do conspire in the production of an oxidant more potent than themselves."

(Quoted from Fridovich, 1978, p. 876).

**A role for free radicals in membrane damage**

Our understanding of the biochemical basis for tissue and cell membrane damage during oxygen toxicity received a major boost during the 1940's when Stadie, Riggs and Haugaard (1945) and Dickens (1946)
reported that enzymes with sulfhydryl groups were especially sensitive to increased concentrations of oxygen. These workers proposed that the inactivation of enzymes containing sulfhydryl groups (S-H) can occur in two ways, the outcome being the formation of a disulfide bridge (S-S) in both cases. In the first instance, separate thiol molecules may react according to the reaction scheme:

\[
2 \text{RSH} + \text{H}^+ + \text{free radical}^- \rightarrow \text{R-S-S-R} + \text{H}_2\text{O}
\]

In the second case, disulfide bonds (S-S) may be formed internally within a molecule which contains two or more SH groups. Numerous essential enzymes and cofactors (e.g., coenzyme A, lipoic acid and reduced glutathione) possess S-H groups and exhibit conformational changes and a loss of catalytic function when these groups are reduced to form disulfide bonds. Structural alterations in these molecules may therefore be very detrimental to cell metabolism and the maintenance of membrane integrity.

A second mechanism was proposed in 1970 by W. A. Pryor to explain the role of oxygen free radicals in producing membrane damage. According to this scheme, free radicals react with carbon atoms in membrane lipids to produce secondary free radicals (symbolized C') by abstraction of a hydrogen. This initiates a chain reaction so that many C' are formed in an area of membrane. C' on neighboring lipid molecules then react to fill their outer orbital with electrons and satisfy the octet rule. Cross-linkages are formed between
membrane lipids and membrane function is impaired since such characteristics as permeability, membrane fluidity and receptor conformation will be altered. It is believed that polyunsaturated lipids are especially vulnerable to free radical attack since the conjugated double bond system renders the C-H bond weaker than usual. Vitamin E and reduced glutathione (GSH) may be of special significance in providing protection from free radical induced membrane damage since they are presumed to serve as free radical quenchers by donating protons. These free radical quenchers can therefore block the development of a chain reaction before it begins.

The role of free radicals in lipid peroxidation and cross-link formation has been demonstrated in several ways. First, Taylor (1958) found that vitamin E deficient animals are especially sensitive to the toxic effects of oxygen, an effect reversible by the administration of vitamin E. Matsumara et al., (1966) followed with the observation that solutions of synovial fluid show a decrease in viscosity following the generation of free radicals. This was presented as evidence for the involvement of free radicals in cross-link formation. A role for free radicals in this model was further supported by the finding that the addition of SOD to the synovial fluid prior to the generation of free radicals abolished the viscosity change; the addition of heat inactivated SOD did not.

The work of Kosower and Kosower (1978) is especially interesting and supportive of the concept that free radicals play a role in membrane damage following exposure to oxygen. They used azoester (a
compound which oxidizes GSH to GSSG while producing an end product which creates free radicals in the presence of oxygen) to demonstrate that tissue damage occurs only at the site of free radical generation. Red blood cells (rbc's) containing a high intracellular concentration of oxygen were incubated in a solution containing azoester. The result was denaturation of intracellular hemoglobin. When oxygen was removed and hemoglobin bound with carbon monoxide no damage was seen.

A recent report by Scott et al., (1985) provides an elegant example of the use of ingenuity to prove a point. These workers reasoned that the antibody to an intracellular molecule would not appear inside a cell unless damage to the cell's membrane occurred. They tagged an antibody to the intracellular molecule myosin with a fluorescent marker and found that the uptake of antibody by cultured myocytes occurred only in the presence of oxygen. When oxygen levels were reduced during incubation the uptake of antibody by myocytes decreased. Free radical quenchers were also found to block the oxygen dependant uptake of antibody. Interestingly, increases in oxygen concentration above atmospheric did not produce an increase in antibody uptake. These workers concluded that the rate of free radical production was maximal during ambient levels of oxygen and did not increase with hyperoxia. This observation may be significant when the role of phagocytes in pulmonary oxygen toxicity is considered (see below).
Changes in lung morphology during oxygen toxicity

The morphology of the lung reflects its function as an organ specialized for gas exchange. The alveolar membrane is composed of epithelium, interstitium and endothelium and separate the alveolar air space from the blood to form a network which resembles a fishing net in appearance. The interstitium is lined on the alveolar side with type 1 epithelial cells which are thin and provide minimal obstruction to gas diffusion. Endothelial cells line the capillary side of the alveolar wall. Normally, close contact is maintained between endothelial cells to prevent the leakage of plasma and blood proteins into the interstitium. Following hyperoxic exposure the gap between endothelial cells widens and the delicate morphology of the interstitium typical of the healthy lung becomes altered dramatically (see Balentine, 1982 for an excellent review of the pathology of oxygen toxicity). The interstitium widens as protein-rich plasma leaks through the vascular endothelium and an impairment to gas exchange develops (the processes involved in edema formation have been reviewed recently by Staub, 1984). Water may follow the leaked protein and contribute to interstitial thickening. PMN also begin to appear in the interstitium and air space as they migrate from the capillaries across the endothelial cell layer. This response to hyperoxia is in actuality an acute inflammatory response and is called the "exudative" phase (Kaplan et al., 1969; Clark and Lambertzøen, 1971). If hyperoxic exposure is removed at this time the lymphatic system will return the leaked fluid and protein to the circulation and
gas exchange across the interstitium will be restored to normal in most instances. Continued exposure of rats to hyperoxia at this time usually results in the death of the animal. If the animal survives the exudative phase the thin type 1 epithelial cells will be replaced by thicker type 2 cells and gas exchange is further impaired. This response is called the "proliferative phase" and represents a chronic response of the lung in animals which survive the exudative phase of oxygen toxicity. This study will focus on the contribution of phagocytes to the exudative phase of pulmonary oxygen toxicity and uses the oxygen toxic rat as a model of acute inflammation.

A role for free radicals in pulmonary oxygen toxicity

The theory that free radicals are responsible for the lung pathology associated with oxygen toxicity was first expressed by Gerschman et al. in 1954 when they noticed that radiation and oxygen produce similar effects on the lung. Though the free radical theory of lung injury is therefore over 30 years old it is significant that two of the classic review articles on oxygen toxicity (Haugaard, 1968; Clark and Lambertsen, 1971) view free radicals with passing interest.

Much of the impetus for the current popularity of the theory of free radical involvement in oxygen toxicity resulted from the discovery of the superoxide dismutases (SOD's) by McCord and Fridovich in 1969. The existence of these free radical quenching enzymes provided a useful tool for assessing possible free radical involvement in experimental protocols. Also of major importance in establishing a
role for free radicals and SOD in oxygen toxicity were reports from Fridovich's laboratory at Duke University following an extensive survey of a wide variety of microorganisms (McCord et al., 1971). These workers found that only aerobes contained significant amounts of SOD activity. Obligate anaerobes, which do not use oxygen as an electron sink and are killed by an oxygen atmosphere, were found to totally lack SOD. Intermediate levels of SOD were found in aerotolerant anaerobes (microorganisms which can tolerate oxygen but lack a cytochrome chain and generate energy by anaerobic glycolysis).

In 1973 McCord et al. reported a temperature sensitive mutant of *E. coli* whose ability to survive in an oxygen atmosphere varied as a function of temperature. The parental strain can survive in oxygen at both 30 °C and 42 °C and can synthesize SOD at both temperatures. Aerobic survival of the mutant strain, on the other hand, was limited to the 30 °C environment as was the ability to synthesize SOD. The ability to survive anaerobic conditions was the same for both groups of bacteria. Further evidence emerged from Fridovich's laboratory in 1973 (Gregory and Fridovich, 1973a). SOD levels were found to increase in *E. coli* and yeast when the organisms were grown in an atmosphere of increased oxygen concentration. Control organisms, grown in nitrogen, showed no increase in SOD. When exposed to 20 atmospheres of pure oxygen the preexposed group of microorganisms, with their high levels of SOD, were much more resistant to oxygen toxicity than the control group which had been grown in nitrogen and lacked enhanced levels of SOD. Also in 1973 Gregory and Fridovich
reported the results of an experiment based on the ability of the antibiotic streptonigrin to produce $O_2^-$ only in the presence of oxygen. Because of this characteristic bactericidal activity in streptonigrin is only seen in the presence of oxygen. Reasoning that increased levels of SOD should provide protection against the lethality of streptonigrin Gregory compared the viability of *E. coli* which possessed high levels of SOD via previous induction to the viability of normal *E. coli* with lower levels of SOD. *E. coli* with high SOD levels were found to be much more resistant to streptonigrin than control *E. coli* with unaltered levels of SOD.

Crapo and Tierney (1974) found that enzyme induction also occurred in Mammalia; SOD levels in whole rat lung increased following one week exposure to 85% oxygen. Survival also increased following exposure to 100% oxygen in a manner related, over time, to the increase in SOD. Likewise, SOD activity was found to decline during the period following removal of the animals from 85% oxygen. Again, the decline in SOD was correlated, over time, with the loss in ability to survive exposure to 100% oxygen.

More evidence supporting a free radical involvement in oxygen toxicity comes from work reported by Stevens and Autor in 1980. By basing their experiments on the observation that many species show an age dependant change in tolerance to increased concentrations of oxygen, these workers demonstrated a positive correlation between the induction of protective enzymes (SOD, catalase and glutathione peroxidase) and resistance to increased concentrations of oxygen. Of
special significance is their finding that ten day old rats exhibited increased levels of antioxidant enzymes in association with a complete absence of lung pathology following a four day exposure to 100% oxygen. Rats 15-30 days old failed to show an increase in lung SOD activity following a four day exposure to 100% oxygen though catalase and glutathione peroxidase were maximally induced. Lungs examined in this group had minor signs of tissue damage (focal edema and hemorrhage). The majority of adult rats exhibited no change in antioxidant enzyme levels and died of massive lung injury within 72 hours exposure to 100% oxygen. A second experiment found that pretreatment of rats with the protein synthesis inhibitor, cycloheximide, before exposure to 95% oxygen lead to an increase in mortality with the dead animals showing all the symptoms of oxygen toxicity. The control group, which received cycloheximide and exposure to air, showed 100% survival and an absence of lung pathology.

Work reported by Del Maestro et al., 1980 provides a direct demonstration of the role free radicals play in producing vascular injury and leakage. These workers perfused the microvasculature in a hamster cheek pouch preparation with fluorescein-labeled dextran (molecular weight, 150,000) and demonstrated that vascular leakage developed only after free radicals were introduced. In control cheek pouch preparations free radicals were not present and the fluorescent dextran was retained within blood vessels. The addition of free radical scavengers (SOD, catalase, L-methionine, or dimethylsulfoxide) to the vasculature before free radical generation significantly
lessened the severity of vascular leakage. Severe vascular leakage still developed when SOD was inactivated by boiling prior to its introduction to the hamster cheek pouch preparation.

Phagocyte activation and chemiluminescence

Phagocytes provide a first line of defense in protecting the body from invasion by bacteria and other pathogens. In the absence of pathogens, phagocytes are quiescent and display only minimal levels of metabolic activity. When they encounter bacteria or other substances which possess a surface foreign to the body the response of phagocytes is quite different. This response is called the 'respiratory burst' and is characteristic of activated phagocytes. The respiratory burst develops rapidly after contact with pathogens and can occur without phagocytosis (Morton et al., 1969; Henson and Oades, 1973). It is associated with dramatic changes in phagocyte metabolism and biochemistry (i.e., rapid increases in the rates of glucose catabolism, oxygen consumption, NAD(P)H generation and free radical release) (see Babior, 1980; Rossi et al., 1980; Nathan, 1982). Alterations in phagocyte shape and function also occur during the respiratory burst. Cells increase in size, become more able to attach to surfaces, show an increase in surface receptor density and develop greater phagocytic ability.

NAD(P)H oxidase and myeloperoxidase (MPO) are membrane bound enzymes which play a major role in initiating the respiratory burst following stimulation of the phagocyte membrane (Klebanoff, 1968;
Rossi et al., 1980). In addition, these enzymes produce $O_2^-$ and $H_2O_2$ and have been assigned major roles in the production of chemiluminescence (CL) (see Allen, 1980 for a review of this subject). Robert Allen first used CL to quantitate free radical release by activated phagocytes in 1972. He proposed that the potential for CL develops when ground state molecular oxygen uses the energy released during oxidation-reduction reactions to promote an electron to a higher orbital and form singlet oxygen ($^1O_2$). $^1O_2$, in turn, generates CL when the high energy electron reverts to a lower orbital with photon emission. The identity of the free radical species responsible for the generation of CL has not been established with certainty. Allen further proposed that CL represents increases in the activity of both NAD(P)H oxidase and MPO and results from their contribution to the generation of $^1O_2$. The historical development of this concept is quite interesting and worthy of a brief digression at this point. In 1927 Mallet discovered that light is emitted during the reaction of hypochlorite ($OCl^-$) with $H_2O_2$. In 1970 Kasha and Kahn demonstrated that $^1O_2$ is generated during this reaction. Allen was aware of Klebanoff's previous demonstration in 1967 that MPO exhibited bactericidal activity when combined with $H_2O_2$ and various halides. He also knew of Zgliezyński's suggestion in 1971 that $OCl^-$ played a role in the bactericidal activity of MPO. This knowledge formed the basis of Robert Allen's doctoral thesis work in 1973 which supported the hypothesis that phagocytic cells would display CL during phagocytosis. The MPO
reaction on which Allen's reasoning was based is:

\[ \text{H}_2\text{O}_2 + \text{OCl}^- \rightarrow \text{H}_2\text{O} + \text{O}_2 + \text{Cl}^- . \]

\( \text{O}_2^- \) will also generate \( \text{O}_2 \) during the dismutation reaction:

\[ 2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O} + \text{O}_2 . \]

It is this reaction sequence that supports Allen's proposal that \( \text{O}_2^- \) plays a role in the generation of CL in addition to the MPO reaction.

Whatever the chemical basis of CL, the association between CL, the respiratory burst and free radical release by activated phagocytes has been firmly established. Jowa et al., (1981), for example, have shown that peak CL increases significantly when glucose is added to cells during zymosan activation. The addition of pyruvate or 2-deoxyglucose to similar cells did not effect peak CL indicating that the glucose contribution occurred via the hexose-monophosphate shunt and the generation of additional NAD(P)H. Stevens and Young (1976) added support to the validity of CL as an indicator of free radical release when they correlated \textit{in vitro} killing, oxygen consumption, visual phagocytosis and CL in human granulocytes. Trush et al., (1978) discuss this topic in detail and include the observation that PMN from patients with chronic granulomatous disease (CGD) show severely depressed CL (PMN from CGD patients are unable to generate
superoxide and therefore cannot kill bacteria although phagocytosis is unimpaired. PMN from patients with myeloperoxidase deficiency cannot produce H$_2$O$_2$ and also show a significantly reduced ability to generate CL (these PMN can produce superoxide but are unable to generate significant amounts of $^{1}$O$_2$). Perhaps the clearest evidence indicating that CL provides an accurate reflection of phagocyte free radical release is provided by two observations: 1) CL is not produced unless oxygen is present (Allen, 1973) and 2) free radical quenchers eliminate CL. These observations support the use of CL as a useful method for assessing free radical activity in phagocytes.

A role for phagocytes and complement in lung injury

The presence of phagocytes in lung tissue following exposure to increased concentrations of oxygen has been reported frequently in the literature (Boycott and Oakley, 1932; Fox et al., 1980; Smith et al., 1981). The possibility that phagocytes play a role in oxygen toxicity was suspected since they were known to produce proteolytic enzymes and other mediators of inflammation. In 1967 Klebanoff discovered that granulocytes produce H$_2$O$_2$. Six years later Babior made a major contribution with the discovery that phagocytes also produce O$_2^-$ and release it to the surrounding medium. These discoveries are especially significant since O$_2^-$ and H$_2$O$_2$ may react to generate OH$^-$ via the Haber-Weiss reaction. Phagocytes therefore possess ample potential to produce tissue damage.
Del Maestro et al., (1980) monitored granulocyte behavior within the microvasculature and found that the velocity of cell flow was significantly reduced following the introduction of a free radical generating system. Cells also spent a greater portion of their time near the capillary wall and a 20 fold increase in the number of cells adhering to the vessel wall was seen. If one postulates that granulocytes play a role in edema formation during pulmonary oxygen toxicity, these free radical induced changes in granulocyte behavior are highly significant.

Work by Shasby, Shasby and Peach (1983) provided perhaps some of the most conclusive direct evidence linking phagocytes and free radicals with edema and tissue injury. These experiments used layers of cultured endothelial cells with the flux of tritiated albumin as a measure of fluid leakage. It was found that PMN must be activated and in direct contact with the endothelial cell layer before significant albumin leakage occurred. Direct contact of the endothelial cell layer with quiescent PMN did not alter the rate of albumen flux across the layer. In addition, simply placing a filter paper between PMN which had been activated by phorbol myristate acetate (PMA) and the endothelial cell layer prevented the increase in albumin leakage seen when direct contact was allowed between these cells. Apparently the thickness of the filter paper provided a sufficient barrier so that free radicals were rendered harmless by the time they diffused across the gap separating PMN and the endothelial cell layer (the half-life of free radicals at physiological pH and temperature is on the order
of $10^{-10}$ seconds) (Pryor, 1970). A free radical involvement was also suggested when SOD prevented albumin leakage across the endothelium following direct contact of this layer with activated PMN. Most importantly, the introduction of PMA-activated PMN from patients with chronic granulomatous disease (PMN cannot generate $O_2^-$) failed to produce an increase in albumin flux across the cell layer even though direct contact between cell types was allowed.

Reports from John Repine's laboratory at the University of Colorado in Denver (Shasby et al., 1982) have provided evidence supportive of a direct role for phagocytic cells in oxygen toxicity. These workers used nitrogen mustard to partially deplete PMN in rabbits prior to oxygen exposure and demonstrated protection from oxygen toxicity. Furthermore, they showed a correlation between the number of PMN obtained by bronchoalveolar lavage and the severity of lung edema.

Complement comprises a series of plasma proteins which act in concert with PMN, mast cells, blood coagulation proteins and the immune system to regulate the inflammatory response to injury (Muller-Eberhard et al., 1972). The interaction between complement and PMN suggests that complement may also be involved in oxygen toxicity. Numerous reports have indeed demonstrated a need for both complement and PMN in several models of lung edema (Shaw et al., 1980; Till et al., 1982 and 1983). G. O. Till has shown that the depletion of complement with cobra venom factor (CVF) will prevent the formation of pulmonary edema in rats which had been exposed to 75 °C water for
30 seconds. PMN depletion will also prevent the development of lung injury in this model. Peter Henson used a different model of lung inflammation and found that the administration of immune complexes as well as complement fragments into the air space of rabbits produced an inflammatory response with alveolar flooding and an influx of PMN (1982). Prior neutrophil depletion also prevented the development of lung injury in this model.

McCord and Wong (1979) at the University of South Alabama have studied antibody-induced edema formation in rat skin in a model which employed the reverse passive Arthus reaction. In this model human serum albumin is injected i.v., followed by the intradermal introduction of rabbit anti-human serum albumin. By coupling SOD to a polymer which delays in vivo degradation of the enzyme, these workers have demonstrated that $O_2^-$ plays a role in edema formation. Their conclusion was further supported by the demonstration that heat inactivation of SOD by boiling coupled to polymer prevented the reduction in edema formation seen with the active enzyme. Interestingly, McCord et al., (1980) reported the generation of a chemotactic factor for PMN in serum following exposure of human plasma to $O_2^-$. This factor is not produced if plasma is incubated with SOD prior to the generation of $O_2^-$. If phagocytic cells play a role in producing tissue injury during the inflammatory response then the absence of chemotactic factors may be significant, i.e. SOD may prevent the accumulation of phagocytes during acute inflammation by removing $O_2^-$ before chemotactic factors can be generated.
Endotoxin protection of rats from pulmonary oxygen toxicity: Potential mechanisms of action.

In 1978 Frank, Yam and Roberts discovered that pretreatment of adult rats with a single low dose of endotoxin (1 mg/kg body weight, i.p.) significantly reduced the lung injury and edema of oxygen toxicity. They measured the levels of several antioxidant enzymes (SOD, catalase and glutathione peroxidase) in lung tissue from these rats and found that they were significantly elevated. To explain the observed protection of rats from oxygen toxicity they proposed that endotoxin induced the synthesis of antioxidant enzymes and thereby lessened exposure of the lungs to free radicals.

Endotoxin is known to interact with living tissues in numerous ways (see reviews by Nowotney, 1983; Gans, 1984). For example, endotoxin will activate complement to generate anaphylatoxins (Fantone and Ward, 1983) and it will inhibit both in vitro free radical release by phagocytic cells (Proctor, 1979; Davis et al., 1980) and the chemotactic and degranulation responses of PMN to complement derived fragments (Rosenbaum et al, 1983). Because of the diversity of effects of endotoxin on the body it may be instructive to reevaluate the role of endotoxin in protecting rats from oxygen toxicity.

The need for reevaluation is further suggested since alternative hypotheses may be formulated which account for the increase in antioxidant enzymes found in the lungs of endotoxin protected rats. For example, Smith (personal communication) has shown that the total number of phagocytes increases dramatically in the lungs of endotoxin
protected rats during oxygen exposure (this observation is in direct opposition to the report of Fox et al., 1981a, that endotoxin causes neutropenia and a disappearance of PMN from the lung following hyperoxic exposure for 65 hours). Phagocytes are also known to contain high levels of antioxidant enzymes (Salin and McCord, 1974; Simon et al., 1977). It follows from these observations that the increase in antioxidant enzymes in the lungs of endotoxin protected rats may have resulted from phagocyte accumulation during oxygen exposure and not from enzyme induction.
Of numerous possibilities the following 3 hypotheses may be forwarded as alternatives for the hypothesis that endotoxin protects rats from pulmonary oxygen toxicity by the induction of antioxidant enzymes:

1) Endotoxin may cause a disappearance of PMN from the lungs of oxygen exposed rats and thereby lessen the possibility of phagocyte-induced lung injury.

2) Endotoxin may inhibit free radical release by alveolar phagocytes and thereby lessen exposure of lung tissue to potential damage.

3) Endotoxin may deplete complement during oxygen exposure and thereby lessen lung injury by PMN.

This study will evaluate these hypotheses as alternatives to the concept that the mechanism of endotoxin protection of rats from oxygen toxicity results from enzyme induction.
Breathing pure oxygen at 1 atmosphere absolute (1 ata) causes extensive lung injury in most mammals within a few days. Rats develop massive bilateral pleural effusions during the third day of exposure and die of suffocation as the pleural space fills with fluid and the lungs are unable to expand (Smith et al., 1981). Polymorphonuclear leukocytes (PMN) have the potential to cause tissue damage by the release of both proteolytic enzymes and free radicals and may therefore play a role in producing this vascular leakage. In addition, PMN accumulate in the lungs of rats at the same time that edema occurs. The concept that PMN contribute to the severity of permeability edema has gained support from work using monolayers of cultured endothelial cells or neutropenic animals. In these studies significant cell layer leakage or edema do not occur unless activated PMN are present and in direct contact with target tissues. The involvement of free radicals as mediators of tissue injury in these models has been suggested by the demonstration that the addition of free radical quenching enzymes will prevent injury.

Frank, Yam and Roberts (1978) have reported that a single i.p. injection of endotoxin protected rats from oxygen toxicity and greatly reduced intrathoracic edema. They found that the levels of antioxidant enzymes were elevated approximately 50% in the lungs of these rats and proposed that endotoxin provided protection from pulmonary oxygen toxicity by inducing the synthesis of free radical
quenching enzymes. This dissertation will extend their work and assess the possible involvement of phagocytic cells in endotoxin protection.

STUDY 1: Chemiluminescence and lung phagocytes

This study will evaluate the hypothesis that endotoxin protects rats by reducing \textit{in vivo} free radical release by lung phagocytes. Rats will be pretreated with saline or endotoxin and exposed to air or oxygen for periods of 1, 2 or 3 days. Following the exposure period phagocytes will be harvested by bronchoalveolar lavage and the potential of lavaged phagocytes to release free radicals quantitated by zymosan-stimulated, luminol-dependent chemiluminescence (CL). Additional data will be collected from rats to assess the magnitude of endotoxin protection and reconfirm earlier observations of Frank, Yam and Roberts. Microscopic examination of lavage fluid will also be conducted to determine if changes occur in the phagocytic cell populations between the different groups of rats.

STUDY 2: Endotoxin and complement

Complement fragments are known to activate PMN and have been shown to play a role in the development of lung damage in several models of inflammation. Endotoxin is also known to activate complement \textit{in vitro} and thereby generate complement fragments. The second study is designed to determine if endotoxin protects rats from the lung injury of oxygen toxicity by depleting complement. Two separate experiments will be conducted to evaluate this possibility.
Experiment 1). Rats will be pretreated with endotoxin or saline (1 ml, i.p.) immediately prior to oxygen exposure for a period of 60 hours. Complement levels will then be measured by a standard hemolytic assay on serum samples obtained following the exposure period in oxygen or after 60 hours in air.

Experiment 2). Cobra venom factor will be used to deplete complement in rats prior to oxygen exposure to determine if complement depletion provides protection from oxygen toxicity.
STUDY 1
CHEMILUMINESCENCE (CL) AND LUNG PHAGOCYTES

Introduction

In 1972 Robert Allen developed the technique which allowed a standard laboratory scintillation counter to be used to measure light emission (CL) by phagocytes following cell activation with particulate or soluble activators. In 1979 Proctor used this technique to measure free radical release in human PMN which had been incubated with endotoxin. He found that killing ability, oxygen consumption, superoxide production and CL were significantly reduced in these cells undergoing bacterial challenge. Davis et al., (1980) reported similar decreases in the functional capabilities of alveolar macrophages (AM) following incubation with endotoxin. These reports raise the possibility that endotoxin may protect rats from the lung damage of oxygen toxicity by reducing the ability of phagocytes to release free radicals \textit{in vivo} as well as \textit{in vitro} as observed by Proctor and Davis.

To test this hypothesis, phagocytes were harvested by bronchoalveolar lavage from the lungs of rats which had been pretreated with endotoxin or saline just prior to oxygen exposure for a period of 1-3 days. The ability of these cells to release free radicals was then quantitated \textit{in vitro} by measuring the generation of CL following maximal challenge with unopsonized zymosan.
Phagocytes from endotoxin pretreated rats breathing >99% oxygen for 3 days were found to generate almost 5 times as much CL as cells from paired rats pretreated with saline alone. The possible significance of this finding is discussed.

**Materials and methods**

**Animals:** Sprague-Dawley albino rats of either sex (200-400 gm) were obtained from the University of Hawaii Laboratory Animal Service. Animals were maintained on standard laboratory chow and water ad libitum.

**Gas exposure:** Rats for oxygen exposure were paired by weight, and colonic temperatures were recorded using a rapidly responding glass thermometer accurate to 0.1 °C. One rat of each pair then received endotoxin (E. coli lipopolysaccharide B, 055:85, Difco; 1 mg/kg body weight, i.p.) suspended in 1 ml of 0.9% NaCl while the other rat received 1 ml of 0.9% NaCl (saline) alone (i.p.). Three pairs of rats were housed individually in a plexiglass chamber during the exposure period. Oxygen (obtained from GasPro; medical oxygen, 99.5% pure) was maintained at a concentration >99% at 1 ata (sea level) and CO₂ levels were below 0.25% as measured by mass spectrometry (Perkin-Elmer, model 1100). Rats were protected from soda lime and wood shavings lining the floor of the exposure chamber by a wire mesh. Animals were fasted during the exposure period but
each had separate access to water. Pretreatment and exposure of fasted, air control rats was the same as for the oxygen exposed group except that filtered, compressed air replaced oxygen. Rats were removed from the chamber for lung lavage at 18 or 24 hours (designated Day 1 rats) or at 42 or 48 hours exposure (designated Day 2 rats). The time of removal for Day 3 rats varied and reflected the health of the animal. Saline and endotoxin pretreated rats had mean oxygen exposure periods (±SEM) of 61.4 ± 0.9 hours and 67.4 ± 0.7 hours, respectively.

**Bronchoalveolar lavage:** Following removal from the exposure chamber rats were reweighed and colonic temperature recorded. Rats were then anesthetized with sodium pentabarbital (60 mg/kg, i.p.). A mid-ventral incision was made and heparanized blood was collected from the abdominal aorta. Following exsanguination the diaphragm was pierced and the chest widely opened. The trachea was exposed and a cannula (P.E. 240) inserted and secured. The lungs were gently massaged in situ following each fill with saline at room temperature (5-10 ml volume) and the lavage terminated when 100 ml had been collected. Harvested cells were stored in plastic tubes, on ice, during and after the lavage.

**Preparation of phagocytes for CL measurement:** Lavaged cells were centrifuged at 350 g's for 10 minutes at 4 °C. They were then resuspended in saline for determination of total cell count using a
hemacytometer. If necessary, red blood cells were lysed osmotically by suspension for 90 seconds in 0.2% NaCl before restoration to isotonicity by addition of an equal volume of 1.6% NaCl. Cell viability was determined by dye exclusion following the addition of 5 microliters of 0.4% trypan blue to 0.5 ml cell suspension. Slides of the same cell suspension were air dried for later determination of the differential count using 0.01% toluidine blue. Cells were washed a second time (350 g's for 10 minutes at 4 °C) and resuspended in the dark at a final concentration of 1 x 10^6 cells/ml in dark-adapted Dulbecco's phosphate-buffered saline (PBS) (Merchant et al., 1964). PBS contains CaCl_2 = 0.9 mM; MgCl_2·6H_2O = 0.49 mM; NaCl = 137 mM; KCl = 2.7 mM; Na_2HPO_4 = 8.1 mM; KH_2PO_4 = 1.1 mM, but is glucose free. Blood phagocytes (monocytes and granulocytes) were isolated from fresh heparanized blood (collected from the abdominal aorta prior to lavage) by density gradient sedimentation with histopaque (Sigma). Blood cells were then prepared for CL measurement using the method described for lavage cells.

Chemiluminescence (CL): A Packard tri-carb liquid scintillation spectrometer (model 3375), operating in the out-of-coincidence mode with window settings at 50-1000, was used to measure CL (Trush et al., 1978). All steps were conducted in the dark. To initiate CL, 1.0 x 10^6 cells were added to a dark adapted vial which contained unopsonized zymosan and luminol dissolved in 3 ml of PBS. Final vial concentrations, following the addition of cells, were: Cells = 0.25 x 10^6/ml; zymosan = 0.75 mg/ml and luminol = 1.13 micromolar.
Analysis of CL data: Repeat counts per minute (CPM) were recorded from each vial at 0.5 minute intervals after the addition of cells. Counting was terminated when a maximal peak had been recorded, this time typically being within one hour following the addition of cells to the vial. Duplicate vials of each cell sample were counted and mean values used for data comparison. Peak CL represents the difference between the minimal and maximal CPM obtained following the addition of cells to the vial. Its magnitude serves as an accurate reflection of cell function since differences in minimal CPM between groups were small. The value for peak CL/10^6 mixed lung phagocytes was determined by dividing the peak CL by the product of % cell viability and cell concentration in the vial. Peak CL/10^6 PMN was then determined by dividing the value for peak CL/10^6 mixed phagocytes by the % PMN in that vial. This step is allowable since PMN produce more than 10 fold greater CL than AM (Allen and Loose, 1976; Williams and Cole, 1981). Up to 1500 cells were examined to determine the % PMN on air dried slides where PMN were fewer than 1%. Peak CL/10^6 blood phagocytes was derived in the same way as Peak CL/10^6 mixed lung phagocytes but was not adjusted for viability since blood phagocytes always showed 100% viability. The CL data were also analyzed to further quantitate cell function. For example, the temporal nature of the cell response in generating CL was expressed in two ways: 1) time to CL (i.e., the elapsed time following the addition of cells to the vial before the generation of CL began) and 2) time to peak (i.e., the elapsed time between the addition of cells
to the vial and attainment of peak CPM). Total free radical production to peak CL was determined by addition of individual 0.5 minute CPM values (adjusted for variations in baseline by subtraction) for the period of time between the rise and peak of the CL response.

Superoxide and CL: The specificity of the assay for superoxide was established by exposing cells to SOD during the measurement of CL and noting a dose dependant loss of signal. Prior boiling of SOD blocked this inhibition.

Other measurements: Hematocrits were determined on heparinized blood by spinning a capillary tube sample for 10 minutes in a microhematocrit centrifuge (Clay-Adams, Inc.). The plasma portion of the centrifuged blood sample was taken from the capillary tube for protein determination using a hand-held refractometer (Atago Co. Ltd., Japan) calibrated with distilled water.

Pleural effusion fluid was collected by glass pipette from the open chest cavity and the volume measured using a heparanized graduated cylinder. Effusion protein concentration was determined with the hand-held refractometer using a fluid sample taken directly from the chest cavity and not heparanized.

All values are expressed as mean ± SEM (n). Statistical analyses were done using the unpaired, two-tailed, Student's t-test with a significance level of p < 0.05; the same level of significance was preselected for routine least squares linear regression analysis.
Results

Lack of response in air exposed rats to fasting and endotoxin: When exposed to >99% oxygen rats tend to stop eating. Food was therefore withheld from all animals during the exposure period. Table 1 shows that significant differences did not occur in measured responses in air breathing rats as a result of fasting or pretreatment with endotoxin. The responses of alveolar phagocytes from these air exposed rats were also similar and significant differences did not occur between groups as a result of pretreatment with endotoxin (Table 2). Because of the lack of significant differences in responses between these 3 groups of air exposed rats the data were pooled for later comparison with oxygen exposed rats.

Effect of oxygen and endotoxin: Whereas endotoxin pretreatment had little effect on air exposed rats or their phagocytes (Tables 1 and 2), exposure to oxygen for 3 days (Table 3) caused endotoxin pretreated rats (E-3 rats) to differ significantly from paired oxygen exposed rats pretreated with saline (S-3 rats). The protective effect of endotoxin in this model of lung inflammation was nearly complete. However, six of 11 E-3 rats did show slight pleural effusions (Table 3) which had a protein concentration significantly less than pleural effusions of S-3 rats.

Air exposed rats yielded an average of 15.3 ± 1.5 x 10^6 cells during lavage (Table 4). No significant changes were observed in the
total number of cells harvested from the lungs of oxygen exposed rats (data not shown, Days 1 and 2) until Day 3 when E-3 rats yielded more cells than either air controls or S-3 rats. Table 4 also shows that most of the phagocytes lavaged from air exposed rats were AM (mean = 98.9%) with PMN being only 1.1% of the mixed phagocyte population. Similar to air exposure, these cell type percentages did not change significantly in oxygen exposed rats (data not shown, Days 1 and 2) until Day 3 when both S-3 and E-3 rats showed a large increase in the % PMN of lavaged phagocytes (differences from air control were significant at p < 0.001). The total number of PMN lavaged from S-3 rats increased to 5.2 x 10^6 cells, while lavage of E-3 rats yielded significantly more PMN (14.3 ± 2.7 x 10^6, p < 0.01).

**Chemiluminescence:** Cells from endotoxin pretreated rats breathing oxygen were capable of generating more free radicals than phagocytes from saline injected rats exposed to oxygen (Fig. 1). This difference became greater as the % PMN within the mixed cell population increased. Figure 1 also shows that changes in Peak CL are correlated with changes in % PMN within the mixed phagocyte population (the "r" value correlating these two variables is 0.89 for both saline and endotoxin groups). The CL data is therefore presented as peak CL/10^6 PMN (Fig. 2). Work by others (Allen and Loose, 1976; Williams and Cole, 1981) also suggests that CL is largely a product of PMN activity. It must be pointed out that this method of determining peak CL increased the amount of variability in data from control rats
(Table 2) since the % PMN was typically small in this group. As a result of this variability intergroup differences which appear sizable; i.e. the comparison of peak CL between PMN from saline and endotoxin fasted rats in air (Table 2) and peak CL of cells from endotoxin vs air control (Table 4) were not significant at p < 0.05.

Figure 2 illustrates the tendency for free radical production by PMN from oxygen exposed rats to decrease with time of exposure. This difference was significant for PMN from Day 2 and Day 3 saline pretreated rats compared to Day 0 rats breathing air. PMN from Day 2 and Day 3 endotoxin pretreated rats also produced a significantly larger peak CL than PMN from paired saline pretreated rats. A trend was seen for peak CL in PMN from endotoxin pretreated rats to decrease with time of oxygen exposure but these differences were not significantly different from Day 0.

Table 5 shows that blood phagocytes from endotoxin pretreated rats exposed to oxygen were able to generate a larger peak CL than blood phagocytes from paired rats pretreated with saline. Peak CL by blood phagocytes therefore reflects the same trend found in lavaged phagocytes from these rats (Figures 1 and 2).

Alveolar phagocytes from E-3 rats responded more quickly when generating CL than cells from either S-3 rats or air controls (Figure 3). This trend was also seen in cells lavaged from endotoxin pretreated rats following 1 or 2 days in oxygen. In addition, cells from oxygen breathing rats required more time to generate peak CL than cells from air controls (Figure 4). None of the differences in time
to peak CL between cells from saline and endotoxin pretreated rats breathing oxygen were significant.

There was a tendency for alveolar cells from saline pretreated rats to release fewer total free radicals during the attainment of peak CL (Figure 5). This intergroup difference was significant on Day 2. There was also a trend for alveolar cells from oxygen exposed rats to generate fewer total free radicals to peak CL than cells from air controls though observed differences were not significant.
TABLE 1: Similarity in response\(^1\) of air exposed rats to fasting and endotoxin. Animals were divided into a fed and a fasted group. Rats in the fasted group were then pretreated with saline or endotoxin. All values are mean ± SEM.

\(^1\)None of the differences between groups are significant at \(p < 0.05\).

\(^2\)\(T_b\): post-fast colonic temperature minus initial colonic temp.
TABLE 1

<table>
<thead>
<tr>
<th>Pre-treatment (i.p.)</th>
<th>Fed</th>
<th>Fasted 66 hours</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Saline</td>
</tr>
<tr>
<td>(n)</td>
<td>(8)</td>
<td>(4)</td>
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<tr>
<td>% Body weight loss</td>
<td>...</td>
<td>14.9 ± 1.0</td>
</tr>
<tr>
<td>Change in T_b (°C)^2</td>
<td>...</td>
<td>-2.1 ± 0.4</td>
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<tr>
<td>% Hematocrit</td>
<td>44.0 ± 0.9</td>
<td>46.0 ± 1.4</td>
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<tr>
<td>Plasma protein (g/dL)</td>
<td>7.2 ± 0.3</td>
<td>6.6 ± 0.3</td>
</tr>
</tbody>
</table>

AIR

- Fed Fasted 66 hours
- Pre-treatment (i.p.)
- % Body weight loss
- Change in T_b (°C)^2
- % Hematocrit
- Plasma protein (g/dL)
TABLE 2: Similarity in response\(^1\) of alveolar phagocytes from air exposed rats to fasting and endotoxin. Cells were harvested by bronchoalveolar lavage, counted microscopically and assayed for chemiluminescence (CL). All values are mean ± SEM.

\(^1\)None of the differences between groups are significant at p<0.05.

\(^2\)Peak CL: peak chemiluminescence (CPM x 10\(^6\)/10\(^6\) PMN) following phagocyte challenge with excess zymosan in \textit{vitro}. 
<table>
<thead>
<tr>
<th>Pre-treatment (i.p.)</th>
<th>Fed</th>
<th>Fasted 66 hours</th>
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<tr>
<td>(n)</td>
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<td>(8) (4) (4)</td>
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<table>
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<th></th>
<th>Fed</th>
<th>Saline</th>
<th>Endotoxin</th>
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<tr>
<td>Total cell # (x10^6)</td>
<td>15.5 ± 2.1</td>
<td>14.4 ± 3.3</td>
<td>15.8 ± 3.9</td>
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<td>% Cell viability</td>
<td>92.2 ± 1.5</td>
<td>96.2 ± 1.1</td>
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<tr>
<td>Total AM # (x10^6)</td>
<td>15.4 ± 2.1</td>
<td>14.3 ± 3.3</td>
<td>15.3 ± 3.6</td>
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<td>% PMN</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.3</td>
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<td>Total PMN # (x10^6)</td>
<td>0.12 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.5 ± 0.4</td>
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<tr>
<td>Peak CL^2</td>
<td>23.2 ± 11.2</td>
<td>28.8 ± 9.9</td>
<td>10.9 ± 5.5</td>
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</table>
TABLE 3: Protective effect of endotoxin on rats exposed to > 99% oxygen for up to 3 days. Data was collected, when possible, shortly after death in the saline pretreated rats which died before 60 hours. All values are mean ± SEM (n).

$T_b$: post-fast colonic temperature minus initial colonic temp.

Levels of significance between saline and endotoxin:
(*, p < 0.05; †, p < 0.01; ‡, p < 0.001).
<table>
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<tr>
<th>Pre-treatment (i.p.)</th>
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<th>Endotoxin</th>
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<tr>
<td>60 hour survival</td>
<td>53 % (10/19)</td>
<td>100 % (11/11)†</td>
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<td>% Body weight loss</td>
<td>13.0 ± 0.5 (8)</td>
<td>15.4 ± 0.8 (8)*</td>
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<td>Change in Tb (°C)</td>
<td>-5.4 ± 0.6 (12)</td>
<td>-1.1 ± 0.3 (10)‡</td>
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<td>% Hematocrit</td>
<td>70.8 ± 2.2 (13)</td>
<td>48.8 ± 4.0 (8)†</td>
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<td>Pleural effusion vol. (ml)</td>
<td>6.4 ± 0.7 (14)</td>
<td>0.3 ± 0.1 (11)‡</td>
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<td>Effusion protein (g/dL)</td>
<td>6.3 ± 0.2 (10)</td>
<td>4.9 ± 0.5 (6)‡</td>
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<td>Effusion protein / Plasma protein</td>
<td>0.92 ± 0.04 (10)</td>
<td>0.68 ± 0.06 (6)‡</td>
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TABLE 4: The effect of saline or endotoxin pretreatment on alveolar phagocytes from rats breathing >99% oxygen for 3 days. Cells were harvested by bronchoalveolar lavage, counted microscopically and assayed for chemiluminescence (CL). All values are mean ± SEM.

1Peak CL: peak chemiluminescence (CPM x 10^6/10^6 PMN) following phagocyte challenge with excess zymosan *in vitro*.

Levels of significance between saline and endotoxin (*, p < 0.05; †, p < 0.01; ‡, p < 0.001); between the oxygen group and control rats in air (a, p < 0.05; b, p < 0.01; c, p < 0.001).
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<td>(16)</td>
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<tr>
<td>Total # cells (x10^6)</td>
<td>15.3 ± 1.5</td>
<td>12.4 ± 0.9</td>
</tr>
<tr>
<td>% cell viability</td>
<td>94.4 ± 0.9</td>
<td>87.8 ± 0.8 c</td>
</tr>
<tr>
<td>Total AM # (x10^6)</td>
<td>15.1 ± 1.5</td>
<td>7.2 ± 0.7 b</td>
</tr>
<tr>
<td>% PMN</td>
<td>1.1 ± 0.4</td>
<td>41.8 ± 4.2 c</td>
</tr>
<tr>
<td>Total PMN # (x10^6)</td>
<td>0.2 ± 0.1</td>
<td>5.2 ± 0.6 c</td>
</tr>
<tr>
<td>Peak CL1</td>
<td>21.5 ± 6.2</td>
<td>0.9 ± 0.1 a</td>
</tr>
</tbody>
</table>
Figure 1: Plot of peak chemiluminescence by mixed alveolar phagocytes from oxygen exposed rats pretreated with endotoxin or saline vs % PMN in the mixed cell population. Least squares regression analysis of CPM vs % PMN yielded:

Endotoxin; $\text{CL} = (29,990.4 \times \text{PMN}) + 253,929.5$, $r = 0.893$, $p < 0.001$.
Saline; $\text{CL} = (11,525.0 \times \text{PMN}) + 46,293.8$, $r = 0.894$, $p < 0.001$. 
Pretreatment
- Saline
+ Endotoxin
Figure 2: Peak chemiluminescence (CL) by alveolar PMN from air exposed (Day 0) and oxygen exposed rats injected with endotoxin or saline prior to exposure for 1 to 3 days. Levels of significance between saline and endotoxin groups (*, p < 0.05; †, p < 0.01); from Day 0 (a, p < 0.05). Values are mean ± SEM (n).
TABLE 5. Peak chemiluminescence by blood phagocytes (CL/10^6 cells) from saline and endotoxin pretreated rats breathing > 99% oxygen for 1 - 3 days.
<table>
<thead>
<tr>
<th>DAY</th>
<th>SALINE</th>
<th>ENDOTOXIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75,086</td>
<td>162,839</td>
</tr>
<tr>
<td>2</td>
<td>112,597</td>
<td>539,590</td>
</tr>
<tr>
<td>3</td>
<td>--------</td>
<td>770,272</td>
</tr>
</tbody>
</table>

Peak CL by blood phagocytes (CL/10^6 cells) from air control rats = 101,786.
Figure 3: A comparison of the time required for mixed phagocytes from saline and endotoxin pretreated rats to initiate CL. Values are mean ± SEM and represent the response of $1 \times 10^6$ cells following maximal challenge with zymosan. Levels of significance between groups ($T$, p < 0.01); from Day 0 ($C$, p < 0.001).
Figure 4: A comparison of the time required for mixed phagocytes from endotoxin and saline pretreated rats to generate peak CL. Values are mean ± SEM and represent the response of 1 x 10^6 cells following maximal challenge with zymosan. Levels of significance from Day 0:
(a, p < 0.05; b, p < 0.01; c, p < 0.001).
Pretreatment

- Saline
- Endotoxin

**TIME TO PEAK CL (Minutes)**

**DAYS IN OXYGEN**

0 1 2 3

- (16) (8) (5) (8) (7)
Figure 5: A comparison of total free radical production to peak CL by alveolar PMN following maximal challenge with zymosan. Values are mean ± SEM and represent the total CL production by PMN lavaged from saline or endotoxin pretreated rats following air or oxygen exposure for 1-3 days. Level of significance between saline and endotoxin; *, p < 0.05.
TOTAL FREE RADICAL PRODUCTION (CPM × 10⁸ / 10⁶ PMN)

DAYS IN OXYGEN

Pretreatment

- Saline
- Endotoxin

(16) (8) (8) (5)
Discussion

Previous work on neutropenic rabbits has established an edemagenic role for lung phagocytes in oxygen toxicity (Fox et al., 1981b; Shasby et al., 1982). This study uses chemiluminescence (CL) to quantitate free radical release by phagocytes lavaged from rats breathing >99% oxygen. We found that mixed phagocytes from endotoxin pretreated rats generated significantly greater CL than mixed cells from saline pretreated rats (Fig. 1). This trend remains when CL is expressed as a function of the % PMN in the mixed phagocyte population (Fig. 2). Assuming that PMN contribute to edema formation (Fox et al., 1981b; Shasby et al., 1982 and 1983) the results of this study raise two important questions: 1) How can PMN which are capable of releasing free radicals to a degree not significantly less than PMN of control rats accumulate in large numbers in the lungs of E-3 rats without causing major edema? and 2) Why do massive pleural effusions (Table 3) develop in S-3 rats when their PMN are relatively deficient in the ability to generate free radicals when challenged maximally in vitro? Further work is necessary and many possibilities exist; however, the following, somewhat speculative hypothesis will be offered.

The magnitude of peak CL (measured in vitro) may inversely reflect the history of free radical release by lung PMN prior to lavage. For example, PMN from S-3 rats may have been severely stressed in terms of free radical release prior to lavage (i.e.,
during oxygen exposure) and therefore unable to generate substantial CL when challenged \textit{in vitro} with zymosan. PMN from E-3 rats, on the other hand, may have been relatively quiescent in free radical release prior to lavage and therefore able to generate a significantly larger peak CL when challenged with zymosan. The reduction in the ability of PMN from S-3 rats to release free radicals may have occurred through an inhibition of, or damage to, essential glycolytic enzymes (Haugaard, 1968; Balentine, 1982) so that PMN could not metabolize substrate and generate free radicals when challenged; or an actual depletion of substrate or cofactor (eg. glucose, NADPH or glutathione) may have developed during \textit{in vivo} hyperactivity. In support of these interpretations, Jowa et al., (1981) have shown that the magnitude of peak CL in rat alveolar phagocytes is influenced by changes in glucose concentration. In this study the reaction medium was devoid of glucose so cells had to rely on their own substrate reserves during the generation of CL.

The possibility that alveolar PMN from S-3 rats had experienced a higher level of free radical activity than cells from E-3 rats prior to lavage is consistent with the following observations:

1). The effusion to plasma protein ratio in S-3 rats was found to be significantly higher than the ratio in E-3 rats (Table 3). This indicates that the capillary endothelium was more permeable in the unprotected S-3 rats. Free radicals (eg. superoxide) are known to increase endothelial permeability \textit{in vitro} (Del Maestro et al., 1980; Shasby et al., 1982).
2). The magnitude of peak CL by PMN from E-3 rats (Fig. 2) was intermediate between the value for air controls, which exhibited no edema, and the value for PMN from S-3 rats, which suffered severe vascular leakage prior to lavage. This suggests that PMN from E-3 rats were also intermediate in free radical release prior to lavage, a possibility supported by the fact that E-3 rats do exhibit slight edema and other morphological signs of tissue damage (Thet et al., 1983).

3). S-3 rats yielded significantly fewer viable AM than either E-3 rats or air controls (Table 4). This reduction in AM viability and number may reflect cell death (Fox et al., 1980) as a result of an increase in the release of free radicals by neighboring PMN.
Summary

In this study PMN were found in abundance in the lung lavage fluid of endotoxin protected rats following 66 hours exposure to >99% oxygen. This study further reveals that PMN from oxygen breathing rats tend to produce fewer free radicals than PMN from control rats breathing air. In addition, PMN from endotoxin protected rats generated free radicals at over 4 times the rate of PMN lavaged from paired saline pretreated rats which suffered severe lung damage. A hypothesis has been offered to explain this difference in CL response between groups: an inverse relationship may exist between the cell's history of free radical release in vivo and its ability to generate CL in vitro, particularly in low substrate media.
ENDOTOXIN AND COMPLEMENT

Introduction

Endotoxin (1 mg/kg, i.p.) protects rats from the lung damage and edema of oxygen toxicity (Frank, Yam and Roberts, 1978). Both complement and PMN have been shown to play a role in the development of lung damage in several models of lung inflammation (Shaw, 1980; Till et al., 1982 and 1983). Study 1 of this thesis has confirmed the observation of Smith (personal communication) that PMN are present in abundance in the lung lavage fluid of endotoxin protected rats following exposure to > 99% oxygen for 66 hours. In addition, Study 1 demonstrated that these cells generated free radicals at over 4 times the rate of PMN lavaged from paired saline pretreated rats (which exhibited severe lung damage). Hence, endotoxin does not deplete PMN in oxygen exposed rats or significantly lessen the ability of lavaged PMN to generate free radicals when challenged in vitro with zymosan.

Endotoxin is known to activate complement and thereby generate complement fragments (Fantone and Ward, 1983). Complement fragments are also known to act as opsonins and activate PMN (Sacks et al., 1978). These facts suggested that endotoxin may protect rats from pulmonary oxygen toxicity by activating and thereby depleting complement. If this is true PMN will not be exposed to complement fragments and will remain quiescent and not damage surrounding lung tissue during oxygen exposure.
This study was undertaken to determine:

1) Does pretreatment with endotoxin deplete complement in rats exposed to oxygen?

2) Does depletion of complement 24 hours prior to oxygen exposure protect rats from the lung injury of oxygen toxicity?

Materials and methods

Wistar albino rats of either sex (200 - 450 gm) were donated by the University of Hawaii, Department of Nutrition, Animal Care Facility. Animals were maintained on standard laboratory chow and water ad libitum.

Gas exposure, bronchoalveolar lavage, differential cell counts and the measurement of pleural effusion volume were performed as described in Study 1. Briefly, animals were pretreated with saline or endotoxin (1 mg/kg body weight, i.p.) or cobra venom factor (25 units, i.v.) and housed individually in a plexiglas exposure chamber lined with soda lime during the 60 hour exposure period. Animals were fasted during the exposure period but each had separate access to water. Oxygen was maintained at a concentration >99% at 1 ata (sea level) and CO₂ levels were below 0.25% as measured by mass spectrometry (Perkin-Elmer, model 1100). Air breathing control rats were housed in the same chamber and fasted for 60 hours with filtered, compressed air replacing medical oxygen.
**Complement depletion:** Cobra venom factor (CVF) was obtained from Cordis Laboratories (Miami, Florida). Animals were injected with 25 units dissolved in 0.25 ml distilled water via the penile or tail vein 24 hours before oxygen exposure. This dose abolished complement hemolytic activity in serum samples collected at 4, 20 and 24 hours post injection and in serum collected at the end of the 60 hour exposure period.

**Measurement of complement:** A standard functional assay (Gewerz and Suyehira, 1980) was used to determine the dilution of serum required to produce 50% hemolysis of sensitized sheep erythrocytes (the reciprocal of the serum complement dilution which produces 50% hemolysis of rbc's is reported as CH₅₀ values). Blood was collected from the abdominal aorta and serum formed by clotting on ice for 75 minutes. All serum samples were frozen at -20 °C and assayed within 1 week (freezing did not affect the constancy of CH₅₀ values in samples stored for 2 weeks). Fresh sheep erythrocytes in Alsever's solution (donated by Dr. T. R. Sawa, Veterinary Laboratory, Department of Agriculture, Honolulu, Hawaii) were refrigerated and used within 1 week following sensitization with rabbit anti-sheep antibody (donated by Dr. Albert Benedict, Department of Microbiology, University of Hawaii). The specificity of the assay for complement mediated hemolysis was checked according to the method of Dr. G. O. Till (personal communication): serum from fed control rats was incubated with inulin (25 mg/ml) at 37 °C for 45 minutes and a total absence of hemolytic activity was noted.
All values are expressed as mean ± SEM (n). Statistical analyses were done using the unpaired, two-tailed Student's t-test with a significance level of $p < 0.05$.

Results

Lack of effect of endotoxin and fasting on complement: Fasting and endotoxin did not produce significant changes in $CH_{50}$ values for serum collected from rats breathing air or oxygen (Table 6). Serum from rats pretreated with saline prior to oxygen exposure showed a significant reduction ($p < 0.01$) in hemolytic activity in comparison to serum from paired endotoxin pretreated rats. No hemolytic activity was measured in serum from CVF treated rats confirming that this group was 100% depleted of functional complement activity at the end of the exposure period.

Lack of protection from oxygen toxicity following complement depletion: All rats pretreated with saline and endotoxin survived the 60 hour exposure to >99% oxygen while only 3 of 7 CVF treated rats survived a similar exposure period. Rats pretreated with saline or CVF also developed severe lung injury as indicated by massive pleural effusions (Table 6). The pleural effusions in CVF rats developed as a result of oxygen exposure since edema was not seen in CVF treated rats breathing air for 60 hours. Endotoxin also did not injure the lungs of fasted rats exposed to air for 60 hours. Slight pleural effusions
TABLE 6. Effect of fasting, endotoxin and cobra venom factor (CVF) on CH50, lung damage and % lavaged neutrophils in rats exposed to air or >99% oxygen at 1 ata for 60 hours. The CH50 and % neutrophil values for the CVF group in oxygen were obtained from rats which survived the 60 hour exposure period. The pleural effusion volume in this group was determined by measuring the pleural fluid in both survivors and rats which died in oxygen. All values are mean ± SEM (n).

Level of significance compared to Fed/Air (Student's t-test):
(a, p < 0.01; b, p < 0.001; c, p < 0.05).
<table>
<thead>
<tr>
<th>GROUP</th>
<th>CH₅₀</th>
<th>PLEURAL EFFUSION VOLUME (ml/kg B.W.)</th>
<th>BRONCHOALVEOLAR NEUTROPHILS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed/Air</td>
<td>32.1 ± 2.5(7)</td>
<td>None</td>
<td>0.3 ± 0.1(5)</td>
</tr>
<tr>
<td>Fasted/Air Saline</td>
<td>29.5 ± 4.6(4)</td>
<td>None</td>
<td>0.6 ± 0.25(5)</td>
</tr>
<tr>
<td>Endotoxin CVF</td>
<td>31.9 ± 5.5(4)</td>
<td>None</td>
<td>1.4 ± 1.0(3)</td>
</tr>
<tr>
<td></td>
<td>0(3)</td>
<td>None</td>
<td>0.83 ± 0.46(3)</td>
</tr>
<tr>
<td>Fasted/Oxygen Saline</td>
<td>17.4 ± 2.6(5)ᵃ</td>
<td>32.1 ± 4.8(5)</td>
<td>43.7 ± 9.5(4)ᵇ</td>
</tr>
<tr>
<td>Endotoxin CVF</td>
<td>33.0 ± 2.9(3)</td>
<td>1.1 ± 1.1(5)</td>
<td>26.3 ± 23.0(3)</td>
</tr>
<tr>
<td></td>
<td>0(3)</td>
<td>32.0 ± 2.1(6)</td>
<td>24.6 ± 9.5(3)ᶜ</td>
</tr>
</tbody>
</table>
did develop in 2 of 5 endotoxin pretreated rats following 60 hours in oxygen.

Saline and CVF pretreated rats showed a significant increase in the percent of alveolar PMN following oxygen exposure in comparison to the % PMN lavaged from fed/air rats. An increase in the number of alveolar PMN occurred in only 1 of 3 endotoxin pretreated rats following hyperoxic exposure for 60 hours.

Discussion and summary

Complement fragments are formed following activation of plasma complement by numerous agents including endotoxin and cobra venom factor (CVF) (Fantone and Ward, 1983). These fragments have been shown to cause acute lung damage when formed systemically following i.v. injection of CVF in rats (Till et al., 1982) or when introduced intratracheally in rabbits (Shaw et al., 1980). Prior depletion of complement proteins or PMN prevents the development of lung injury in these models.

In the oxygen toxic rat model used in this study CVF was used at a dose sufficient to deplete complement without producing visible lung edema or pleural effusion. Endotoxin will also produce lung injury when administered i.v. at a dose 15-20 fold greater than the dose used in this study (Brigham et al., 1979). In this study endotoxin was therefore injected i.p. at a dose which protected the animal from pulmonary oxygen toxicity without damaging the lung.
This study also found that endotoxin did not deplete complement when administered at a dose sufficient to protect rats from pulmonary oxygen toxicity. Depletion of complement with CVF prior to oxygen exposure did not provide protection and generally hastened the development of lung injury as shown by the increase in mortality following the 60 hour exposure period in this group. In addition, endotoxin pretreatment prevented the 50% reduction in serum CH₅₀ which was seen in saline pretreated rats exposed to hyperoxia. These findings support the concept that, unlike some other models of lung inflammatory disease (Shaw et al., 1980; Till et al., 1982 and 1983), the mechanism of endotoxin protection from oxygen toxicity does not involve alterations in plasma complement levels.
GENERAL DISCUSSION AND CONCLUSIONS

The role of free radicals in mediating tissue injury has been firmly established during the past decade. This involvement has been shown in several ways:

1) Edema: A hamster cheek pouch preparation was used (Del Maestro et al., 1980) to demonstrate that endothelial leakage will develop following the introduction of free radical generating enzymes and substrate (the hypoxanthine-xanthine oxidase system). The observation that fluid leak and edema were blocked by the introduction of SOD or other free radical quenchers adds support to the possibility that free radicals play a role in the production of edema in this model.

2) Membrane lysis: Alternate methods have been used to generate free radicals and produce tissue damage; i.e. azoester will generate free radicals only in the presence of oxygen and will not cause rupture of red blood cells unless oxygen is available (Kosower and Kosower, 1978).

3) Protein structure: A change in viscosity will occur following the addition of hypoxanthine-xanthine oxidase to solutions of hyaluronic acid (Del Maestro et al., 1980). Apparently the generation of free radicals disrupts intermolecular bonding between
molecules and the solution becomes less viscous. The addition of SOD to this system will block the change in viscosity; the addition of heat-denatured SOD will not. Similar changes have been shown in the viscosity of synovial fluid following the introduction of free radicals (Matsumara et al., 1966; McCord, 1974).

4) Free radicals have also been assigned major roles in the pathology of post-ischemic injury (Fridovich, 1979; McCord, 1985); aging (Sun and Sun, 1982; Cutler, 1984) and DNA damage (Myers, 1980).

The possibility that phagocytes play a central role in free radical mediated tissue injury in some models of edema has gained support from several observations:

1) Neutrophil depletion will prevent the development of edema in some models of tissue injury (Shasby et al., 1982). Complement depletion will also prevent tissue injury in these models (Shaw et al., 1980; Till et al., 1982 and 1983). Complement fragments are opsonins which attract and activate phagocytes, and it appears that the protection provided by complement depletion is related to its effect on neutrophils.

2) Inserting a piece of filter paper between a layer of cultured endothelial cells and activated phagocytes is sufficient to prevent the leakage which occurs when direct contact is allowed between these
two cell types (Shasby et al., 1983). In addition, phagocytes which lack the ability to release free radicals (CGD-phagocytes) will not produce edema in this model even when direct contact is allowed between phagocytes and the endothelial cell layer.

3) A lack of oxygen will prevent bactericidal activity by phagocytes (Selvaraj and Sbarra, 1966). These cells can phagocytose bacteria and other particles but are unable to kill. Free radicals can not be generated unless oxygen is present. The inability of cells to kill in the absence of oxygen may therefore be related to an absence of free radicals.

The part that phagocytes play in initiating tissue injury during oxygen toxicity is less well understood. At the present time considerable controversy exists concerning their possible role. J.D. Crapo (Barry et al., 1982); Aron Fisher et al., (1984) and Small (1984) favor the hypothesis that phagocytes are not responsible for early lung injury during hyperoxia and appear in the lung as part of the more generalized inflammatory response subsequent to the initial insult. They feel that organelles in pulmonary endothelial or epithelial cells such as mitochondria, endoplasmic reticulum and the nuclear membrane produce increased quantities of free radicals during hyperoxia as demonstrated by Crapo et al., 1983. Fisher presents the following observations as evidence favoring a secondary role for phagocytes in the early phase of lung injury during oxygen toxicity:
1) The development of tissue injury appears to precede the influx of phagocytic cells (Barry, Freeman and Crapo, 1982).

2) Rats which have been preexposed to 2 ata oxygen or pretreated with disulfiram die in 100% oxygen without an influx of PMN into the lung (Glass et al., 1985).

3) In vitro systems have indicated that hyperoxia can damage lung in the absence of PMN (Martin et al., 1981).

4) Free radical release is depressed in phagocytes following in vitro hyperoxia (Forman et al., 1982; Rister and Baehner, 1977), not increased as would be expected if they played an active role in producing tissue injury.

5) The presence of PMN in lungs with pre-existing tissue injury has been associated with decreased rather than increased sensitivity to subsequent oxygen toxicity (Fisher et al., 1984).

In addition, McCord et al., (1980) have isolated a chemotactic factor in rats following in vivo exposure of lung tissue to O$_2^-$. This finding supports the concept that phagocytes may arrive in the lung subsequent to the generation of free radicals and do not produce the initial insult.
Evidence favoring a role for phagocytes in the early stages of lung injury during oxygen toxicity has come from several laboratories and is summarized as follows (see Tate and Repine, 1984 for a review of this topic):

1) Phagocytes accumulate in the lungs of oxygen exposed animals before, or in association with, the development of tissue injury (Fox et al., 1980; Baehner et al., 1981; Ohta et al., 1983).

2) Granulocyte depletion protects rabbits from the lung injury of oxygen toxicity. A correlation of 0.894 was found between the % of granulocytes in the lavage fluid and the concentration of albumin in this fluid (Shasby et al., 1982).

3) Chemotactic factors have been isolated in the lavage fluid of rats following oxygen exposure (Fox et al., 1980 and 1981b).

4) Oxygen-derived free radicals have been shown to alter endothelial cell function in isolated perfused rat lung (Steinberg et al., 1982). Hyperoxia has also been shown to cause increased adherence for PMN on cultured bovine endothelial cells (Bowman, Butler and Repine, 1983). Interestingly, Del Maestro et al., (1980) noted phagocyte margination in the microvasculature of the hamster cheek pouch following the generation of free radical with xanthine oxidase and hypothanithine as substrate.
5) Increased cytotoxicity has been demonstrated in PMN exposed to \textit{in vitro} hyperoxia (Suttorp and Simon, 1982).

6) Neutrophils from the lungs of patients suffering from adult respiratory distress syndrome (a disease very similar to pulmonary oxygen toxicity in pathophysiology) appear to be in an activated state when analyzed for CL, \( O_2^- \) release and chemotactic responsiveness (Zimmerman et al., 1983).

It is interesting that proponents of both sides of the argument use the time of appearance of phagocytic cells in the lungs to support their view. Crapo, Fisher and Small report that tissue injury develops before the arrival of phagocytes in the lungs while workers in the laboratories of Ohta, Repine and Baehner report the opposite. A recent study by Raj and Bland (1983) is significant though it certainly does not settle the question of early phagocyte involvement. These investigators reevaluated the effect that granulocyte depletion had on oxygen exposed rabbits and were unable to confirm earlier claims of protection (Shasby et al., 1982). From these conflicting observations one conclusion is obvious; phagocytes are in the sequence(s) of events leading to tissue injury during oxygen toxicity but the question of possible early involvement can not be settled with the data presently available.

The first study in this thesis found that PMN from the lungs of oxygen breathing rats showed a reduced ability to release free
radicals following challenge with zymosan. This observation may reflect the fact that lung phagocytes in oxygen breathing rats were hyperactive in free radical release in vivo. If this is true alveolar PMN may play a major role in producing lung injury during oxygen toxicity. This possibility can not be resolved at the moment and will require additional studies. For example, the measurement of intracellular levels of reduced glutathione (GSH) in alveolar phagocytes from oxygen breathing rats will provide a sensitive indication of the level of free radical activity in these cells prior to lavage and should assist in determining the correct way to interpret the data.

Fox et al., (1981a) pretreated rats with endotoxin (50 μg/kg) 3 days prior to oxygen exposure and observed the disappearance of PMN in the lungs following exposure to oxygen for 65 hours. They proposed that the mechanism of endotoxin protection may be related to the loss of these cells. Study 1 of this dissertation pretreated rats with endotoxin (1 mg/kg) immediately prior to oxygen exposure (mean exposure period = 67.4 hours) and was unable to confirm Fox's observation; PMN were found in great numbers in the lavage fluid of endotoxin pretreated rats (Table 3). The results in this study, therefore, clearly indicate that, with the protocol used in Study 1, endotoxin protection does not result from a lack of PMN in the lung. The disparity between the observed loss of PMN reported by Fox et al. (1981a) and the massive influx of PMN observed in study 1 following hyperoxia may be due to differences in protocol; Fox pretreated his
rats with endotoxin 3 days prior to oxygen exposure and used a much lower dose of endotoxin than was used in this study. In addition, Fox's work was undertaken in Denver, Colorado so the large difference in altitude between the two laboratories may have affected the degree of hyperoxia to which the rats were exposed. Study 2 of this dissertation exposed endotoxin pretreated rats to oxygen for 60 hours while the exposure period for this group of rats was 67 hours in Study 1. Interestingly, the influx of PMN seen in Study 1 occurred in only 1 of 3 rats during Study 2 (Table 6). This observation may have bearing on the inability of Fox et al. to find PMN in the lavage fluid of their rats. Apparently the migration of PMN into the lungs of oxygen breathing rats in our laboratory occurs during the 60-67 hour period of exposure. It maybe that the protocol and/or altitude differences in Fox's study somehow delayed the appearance of PMN in the alveoli of his rats so that they were not present during lavage. It is also interesting that the appearance of PMN in the lungs of Study 2 rats coincided with the initiation of lung injury and pleural edema in these rats (Tables 3 and 6).

The possibility that endotoxin protects rats from oxygen toxicity by inhibiting in vivo free radical release by phagocytes has not been evaluated prior to this dissertation. In vitro systems have been used (Proctor, 1979; Davis et al., 1980) to assess the ability of human phagocytes to release free radicals following incubation with endotoxin. These studies by others have shown that endotoxin reduces the ability of phagocytes to generate free radicals. The results of
the present study confirm that endotoxin produces significant changes in the ability of lavaged phagocytes to release free radicals (Figures 1 and 2). Again, a correct interpretation of what this difference in peak CL means in terms of previous \textit{in vivo} free radical release by alveolar phagocytes can not be made until accurate measurements of phagocyte glutathione levels have been made. The measurement of intracellular glucose or NAD(P)H levels would also be useful in determining if the observed changes in peak CL are a result of differences in substrate availability.
In summary, the following conclusions can be drawn from this study:

A). Effects of hyperoxia:

1. Hyperoxia greatly increases the total number of PMN/lavage.
2. Hyperoxia decreases the viability and number of alveolar macrophages obtained by bronchoalveolar lavage.
3. Hyperoxia greatly decreases the magnitude of peak CL in PMN.
4. Hyperoxia increases the time to peak CL.

B). Effects of endotoxin:

1. Endotoxin alone, in the absence of hyperoxia, did not affect the numbers of PMN or peak CL in lavaged cells. Endotoxin pretreatment, in association with hyperoxia, did result in changes in the number of lavaged PMN. This influx was insignificant compared to fed/air controls after 60 hours exposure (Table 6) but became highly significant after 67 hours in oxygen (Table 4).
2. Alveolar phagocytes from endotoxin protected rats retain the ability to generate free radicals when challenged with zymosan. This ability is reduced significantly in
lung phagocytes from oxygen exposed rats which were pretreated with saline.

3. The time required to initiate C1 decreases in alveolar phagocytes from endotoxin pretreated rats.

C). Role of complement:

1. Exposure to oxygen for 3 days depletes complement by about 50% in saline pretreated rats; endotoxin pretreatment prevents this decrease.

2. Depletion of serum complement did not protect rats from the vascular leakage and pleural effusions of oxygen toxicity.

3) Endotoxin (1 mg/kg, i.p.) does not deplete complement in rats exposed to air or hyperoxia. The mechanism of endotoxin protection from oxygen toxicity therefore does not involve changes in complement.

4) Endotoxin pretreatment prevents the decrease in CH$_{50}$ during oxygen.
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