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CYTOKINE INVOLVEMENT IN
PULMONARY OXYGEN TOXICITY

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**CYTOKINE INVOLVEMENT IN
PULMONARY OXYGEN TOXICITY**

By

Hugh Jeffrey Lindsey

A THESIS

**Submitted to
Michigan State University
in partial fulfillment of the requirements
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ABSTRACT

CYTOKINE INVOLVEMENT IN PULMONARY OXYGEN TOXICITY

By

Hugh Jeffrey Lindsey

Inspiration of high oxygen concentrations can result in severe pulmonary injury. The cytokines - tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6) - have been implicated in the pathophysiology of various pulmonary inflammatory processes. However, direct evidence of their involvement in oxygen toxicity has been lacking.

Consequently, murine alveolar macrophages were exposed to high concentrations of oxygen both in vitro and in vivo. Increased productive capacities for IL-1 and TNF were demonstrated after exposure to hyperoxia for 1 hour in vitro and 2 hours in vivo, respectively. Further, rats exposed to hyperoxia for 52 hours demonstrated increased concentrations of IL-6 in the bronchoalveolar lavage supernatant.

Pretreatment with pentoxifylline (PTX) followed by immediate prolonged exposure of the rats to hyperoxia resulted in marked attenuation of oxygen-induced lung injury as determined by indices of pulmonary injury and decreased pulmonary supernatant and systemic serum levels of IL-6.

Consequently, TNF, IL-1 and IL-6 appear to be involved in the development of pulmonary oxygen toxicity. Further, the oxygen-induced lung injury which develops as a result of prolonged exposure may be attenuated by pretreatment with PTX.

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Hugh J. Lindsey, MD

1994

This thesis is dedicated to my wife and best friend, Barb,
whose love, support, and encouragement were always present
during the course of this project
and
to my parents, Hugh and Beverly, who instilled in me the
importance of education and the gift of perseverance.

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KEY TO ABBREVIATIONS

ABG	arterial blood gas
ACAS	anchored cell analysis station
ARDS	adult respiratory distress syndrome
ATA	atmosphere
BAL	bronchoalveolar lavage
BW	body weight
Con A	concanavalin A
DAD	diffuse alveolar damage
DMEM	Dulbecco's modified eagle's medium
D-PBS	Dulbecco's phosphate buffered saline
ELAM-1	endothelial-leukocyte adhesion molecule-1
FBS	fetal bovine serum
F _i O ₂	forced inspiratory concentration of oxygen
H	hyperoxia
H ₂ O ₂	hydrogen peroxide
HT	Na-hypothanxine-thymidine
ICAM-1,2	intercellular adherence molecule-1,2
ICU	intensive care unit
IL-1	interleukin-1
IL-6	interleukin-6
IM	intramuscular
IP	intraperitoneal

IV	intravenous
kd	kilodalton
LDH	lactate dehydrogenase
LPM	liters per minute
LPS	lipopolysaccharide
LTB ₄	leukotriene B ₄
MAP	mean arterial pressure
MT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazol blue
N	normoxia
NS	normal saline
O ₂	oxygen
O ₂ ⁻	superoxide anion
OH [·]	hydroxyl radical
PGE ₂	prostaglandin E ₂
PTX	pentoxifylline
RBC	red blood cell
SC	subcutaneous
SDS	sodium dodecylsulfate
SEM	standard error of the mean
SOD	superoxide dismutase
TNF	tumor necrosis factor
VCAM-1	vascular endothelial adhesion molecule-1

INTRODUCTION

"Though pure [oxygen] might be very useful as a medicine, ... as a candle burns out much faster in [oxygen] ... so we might ... live out too fast." - J. Priestly, 1775¹.

CLINICAL SIGNIFICANCE

Inspiration of high oxygen concentrations can result in severe pulmonary damage. Today, this usually occurs when the individual is mechanically ventilated as in the intensive care unit (ICU). Barber et al. demonstrated declining pulmonary function in irreversibly brain-damaged, mechanically ventilated patients exposed to a forced inspiratory concentration of oxygen of 1.0 (FiO_2 1.0) for 40 hours². The deterioration of pulmonary function was evident by decreasing arterial oxygen tension, increased ratio of dead space to tidal volume, and increased intrapulmonary shunt. Chest roentgenograms revealing diffuse multilobar infiltrates and autopsy specimens showing grossly heavier lungs further supported the above evidence for oxygen toxicity.

Further work has demonstrated an early breakdown of the lung's defense system. Mucociliary function becomes depressed which may lead to increased adherence of gram-negative organisms to the lower respiratory tract epithelium³. Neutrophil chemotaxins are released by alveolar macrophages, which ultimately results in pulmonary damage by the neutrophils⁴. Also, migration of the alveolar macrophages is inhibited, which may impair movement into and within the bronchoalveolar spaces⁵.

Over the years, the time course of pulmonary oxygen toxicity in otherwise healthy humans after exposure to 100% oxygen has been delineated⁶:

- 6 hrs - decreased tracheal mucus velocity
- 14 hrs - symptoms of tracheobronchitis
- 24-48 hrs - physiologic changes such as decreased
forced vital capacity
- 30 hrs - gas exchange abnormalities
- 72-96 hrs - morphologic abnormalities
- >96 hrs - pulmonary fibrosis.

Moreover, other factors can lower the toxic threshold of oxygen. Consequently, lesser concentrations can lead to pulmonary injury. For example, the duration of respiratory support rather than the duration of illness correlated better with the degree of pulmonary fibrosis upon a review of the pathologic specimens obtained from patients receiving

conventional therapy or extracorporeal membrane oxygenation⁷. Further, histology demonstrated that the pattern of injury was quite similar to that seen with oxygen toxicity despite attempting to minimize inspired oxygen concentrations.

Administration of certain pharmacologic agents, particularly bleomycin, in combination with oxygen may result in pulmonary toxicity. Bleomycin is a chemotherapeutic agent used to treat several neoplasms including squamous cell cancer, lymphoma, and testicular cell cancer⁸. Its mode of action is through generation of free radicals that have a deleterious effect on DNA⁸. Generation of these free radicals is thought to induce pulmonary toxicity^{6,8-10}. Bleomycin, alone and coupled with various inspired concentrations of oxygen ranging from 33%-70%, has been reported to cause severe pulmonary injury in patients^{8,11}. Animal studies have confirmed that bleomycin exacerbates pulmonary oxygen toxicity secondary to hyperoxia^{10,12}.

Animal research has also demonstrated that concurrent acute pulmonary inflammation lowers the toxic threshold of oxygen and exacerbates pulmonary inflammation. Haschek and Witschi have shown this in mice¹³. Immediately after inducing acute alveolitis by butylated hydroxytoluene, the mice were exposed to 70% oxygen for 24 hours. Extensive interstitial fibrosis developed in two weeks. Oxygen or butylated hydroxytoluene administered alone resulted in no or minimal fibrosis, respectively. Moreover, after inducing alveolitis as stated above, administration of 50% oxygen for six days

resulted in increased fibrosis. Apparently, oxygen and butylated hydroxytoluene act synergistically.

Finally, the morphologic changes of pulmonary oxygen toxicity, termed diffuse alveolar damage (DAD), and the mechanism of oxygen toxicity, believed to be the overwhelming production of reactive oxygen species, are present in other conditions. DAD has been described following sepsis, hemorrhagic shock, severe trauma, complicated intraabdominal surgery, ARDS (adult respiratory distress syndrome) and other insults^{1,14}. The production of oxygen-derived free radicals has been implicated in the development of the acute lung injury secondary to ischemia-reperfusion, thermal injury, pancreatitis, and sepsis or TNF infusion¹⁵⁻²². Further, ARDS may be due to free radical production²³. Therefore, despite the fact that pure pulmonary oxygen toxicity is a relatively rare event in the clinical setting, delivering any increased concentration of oxygen may be deleterious to the lungs when other factors and insults are involved.

THE PATHOLOGY OF PULMONARY OXYGEN TOXICITY

The development of pulmonary oxygen toxicity can be divided into stages. When lethal doses of oxygen are administered, such as 100% O₂ at 1 atmosphere (ATA), the phases are, in chronologic order, the initiation, inflammatory, and destructive stages²⁴. The same chronologic order is maintained when sublethal doses of oxygen, generally

regarded as $\leq 85\%$ O_2 in rodents, are administered except the destructive stage is followed by a proliferative and possibly fibrotic stage. The order of morphologic changes is quite similar in different species; however, the severity and duration in each stage varies considerably from species to species. Since lethal concentrations of oxygen were administered to the rat and their alveolar macrophages for these studies, the stages of oxygen toxicity will be discussed predominantly from this framework.

Initiation Stage

The first 40-48 hours mark the initiation phase of pulmonary oxygen toxicity in the rat. During this time, the rat demonstrates few, if any, clinical signs of distress²⁵⁻²⁷. This phase was initially thought to be a period characterized by the production of reduced oxygen species associated with no significant morphologic changes²⁶. However, recent histologic work has demonstrated otherwise²⁷. Wistar rats exposed to 100% O_2 1 ATA for 24 hours demonstrated changes in the lung morphology. With light microscopy, alveolar collapse and vascular congestion with accompanying bronchiolar and vascular constriction in scattered areas were demonstrated. Further, the alveolar septae were folded and compressed which increased the local tissue density and caused adjacent alveolae to be overdistended by air. Arteriolar changes included adventitial distention by edema and fibrin deposition within the walls. Electron microscopy confirmed these findings. In addition,

arteriolar changes such as vacuolization of endothelial cells and fibrin deposition within the elastic layers were noted. Also, some capillary endothelial cells were separated from their basement membrane, and some capillaries demonstrated a pericyte reaction. No marked changes occurred in the alveolar epithelium. During the initiation phase, then, the rat appears to be tolerating the hyperoxia rather well upon gross physical examination. However, histologic evidence of oxygen toxicity is already present. Apparently, the initiating events are well underway within 24 hours of continuous exposure to hyperoxia.

Inflammatory Stage

After approximately 40-48 hours of continuous exposure to 100% O₂, the rat begins to exhibit signs of distress^{26,27}. Progressive lethargy, fur bristling, decreasing oral consumption, and respiratory distress develop. Histologically, at 40 hours post-exposure, the capillary endothelium begins to show evidence of damage^{25,27}. This damage to the endothelial cell takes the form of vacuolization, swollen mitochondria, and plasma membrane detachment. Interestingly, platelets and RBCs aggregate at the areas of endothelial damage. Associated interstitial edema is noted. Over the next 20 hours, the alveolar capillary endothelium is reduced in number by 30%, alveolar and interstitial edema worsen, and increased interstitial cellularity, specifically increased numbers of neutrophils and indeterminate cells, is

seen²⁵. No significant morphologic changes in the alveolar Type I and Type II epithelial cells are noted.

Destructive Stage

At 60 hours post-exposure, the physical signs which began in the inflammatory stage worsen. Usually, the rat succumbs within 72 hours of continuous exposure to 100% O₂^{25,26,28-31}. Histologically, the capillary endothelium is further damaged. Capillary endothelial cells are reduced in number by 44%, and the surviving cells demonstrate evidence of further damage^{25,27}. Although the Type I and II epithelial cells demonstrate some ultrastructural changes, no significant change in cell number is noted.

The interstitium continues to increase in cellularity. Inflammatory cells, consisting of neutrophils, macrophages, monocytes, fibroblasts, and lymphocytes, become more predominant. The accumulation of neutrophils in the microvasculature and interstitium has been thought to herald the onset of this phase. The neutrophil influx has been associated with a rapid increase in the severity of lung injury²⁴. Further, evidence exists that this influx is due at least in part to the endothelial damage with resultant increase in neutrophil adherence and subsequent intrapulmonary retention^{32,33}.

However, neutrophils do not appear absolutely necessary for the deleterious effects of hyperoxia. The increase in alveolar permeability and fibrinopurulent intra-alveolar

inflammation which occurs initially appears to be independent of the accumulation of neutrophils^{34,35}. Moreover, induced neutropenia does not attenuate pulmonary damage nor prolong survival³⁶. Finally, a lung, devoid of neutrophils, appears to be "primed" for further damage by exposure to hyperoxia when the neutrophils are subsequently replaced³⁷.

Proliferative Stage

The proliferative stage is present when sublethal concentrations of oxygen, such as $\leq 85\% \text{ O}_2$ 1 ATA, are administered continuously²⁴. When $85\% \text{ O}_2$ is administered to rats, the initiation phase is prolonged to 72 hours, the inflammatory phase to 5 days and the destructive phase to 7 days^{24,25,38}. By day 7, the number of Type II alveolar cells has nearly doubled, and 41% of the capillary endothelial cells are destroyed. However, no further loss of endothelial cells is noted at the end of the following week²⁵. Also by day 7, the proliferative response has begun. This response, which is characterized primarily by an interstitial influx of fibroblasts and monocytes and is devoid of neutrophils, may be responsible for the survival of the animal^{24,25}.

Fibrotic Stage

After lethal and sublethal exposure to hyperoxia, fibrosis has occurred within the interstitium. Fibrin deposition is noted by 48 hours and quite obvious by 72 hours of continuous exposure to $100\% \text{ O}_2$ ²⁷. Also, after exposure to

70-85% O_2 for 7 days, a fibrotic response is observed^{13,25}. Although this stage is thought to occur later in oxygen toxicity, a fibrotic reaction apparently precedes the influx of neutrophils when lethal concentrations of oxygen are administered and follows the acute inflammatory response when sublethal concentrations are inspired^{24,25,27}.

MECHANISM OF PULMONARY OXYGEN TOXICITY

Pulmonary oxygen toxicity is thought to occur due to the profuse production of reactive oxygen species - superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH\cdot$) - which overwhelms the antioxidant defense system^{1,6,39}. During cellular respiration in a normoxic environment, oxygen is metabolized in the mitochondria to water. Oxygen free radicals are produced in small quantities and are metabolized enzymatically - by superoxide dismutase, catalase, and glutathione peroxidase and reductase - and nonenzymatically - by antioxidants such as vitamins E and C and beta-carotene^{1,6,39}. However, as the concentration of inspired oxygen increases, the production of superoxide and hydrogen peroxide, which occur in the mitochondria and microsomes and possibly other intracellular organelles, also increases⁴⁰⁻⁴³. This ultimately overwhelms the antioxidant defense system.

Superoxide and hydrogen peroxide undergo further reaction to hydrogen peroxide and hydroxyl radical, respectively, via

a superoxide-driven Fenton reaction^{6,9,39,44}:



These reactive species may damage the cellular integrity and possibly cause cell death. Consequently, pulmonary damage occurs.

Cellular damage and death by oxygen free radicals occurs by several different means^{6,9}. Oxidation of DNA by superoxide and hydrogen peroxide results in chromosome aberrations, chromatid exchanges, breaks in the DNA and interference with repair and replication. Lipid peroxidation damages the cell membrane by decreasing the fluidity, increasing permeability, injuring the structural integrity, and altering transport. Sulfhydryl-containing enzymes, which include succinate dehydrogenase, xanthine oxidase, papain, glyceraldehyde-3-phosphate dehydrogenase among others, are oxidized during exposure to hyperoxia resulting in inactivation. DNA and protein synthesis are both inhibited as is the synthesis or transport of surfactant phospholipids. Finally, the microsomal enzymes involved in the cytochrome P₄₅₀ system may be induced leading to increased production of reactive oxygen species.

The exact source(s) of the reactive oxygen species is not fully known. Presently, during acute hyperoxia, early significant lung injury is probably due to the increased intracellular generation of free radicals by the endothelial

cells resulting in their own demise^{9,24,25,45}. Neutrophils, macrophages, and platelets have been identified as cells which release superoxide extracellularly⁴⁶. Neutrophils probably augment the hyperoxic pulmonary injury secondary to free radical production, but this occurs in the latter stages of oxygen toxicity and may not be a requirement for lethality^{25,32,35-38,47-49}. Alveolar macrophages do not appear to increase in number, and evidence exists that they inhibit free radical production^{25,50}. However, platelets have been shown to accumulate at the endothelial surface of the pulmonary capillary bed within 40 hours of continuous exposure to 100% O₂ and may, at least in part, be responsible for the pulmonary injury at this point^{38,45}.

CYTOKINES

Cytokines, the generic term that incorporates both lymphokines and monokines, are soluble polypeptide mediators⁵¹. These antigenically nonspecific, intercellular mediators are produced primarily, but not exclusively, by a variety of immune cells, most notably T lymphocytes and macrophages. They are able to recruit other cells and modulate cellular/physiological responses.

Macrophages are an important component of the innate immune system. They have been found to be significant producers of tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6)⁵². All three have been implicated

in the "acute phase response" of the inflammatory process⁵¹. Each will be discussed in general terms and with regard to their involvement in pulmonary inflammation and oxygen toxicity.

Tumor Necrosis Factor

TNF is a 17 kd, 157 amino acid peptide produced by activated macrophages as well as many other cell types following a variety of stimulants⁵¹. A complex cytokine network has become increasingly understood over the years. Specifically, IL-1, as well as other cytokines, stimulates macrophages to produce TNF⁵³. Additionally, TNF induces IL-1 release from macrophages and endothelial cells¹⁵.

TNF is an important inflammatory mediator. Systemically, TNF is involved in the physiologic response to endotoxin shock, induction of fever, and induction of the hepatocytes to produce the acute phase reactants¹⁵⁻¹⁷. Locally, TNF affects endothelial cells, neutrophils, and macrophages. In response to TNF or IL-1, endothelial cells express more intercellular adherence molecule-1 (ICAM-1, ICAM-2), vascular endothelial cell adhesion molecule-1 (VCAM-1), and endothelial-leukocyte adhesion molecule-1 (ELAM-1)⁵⁴. These molecules increase adherence of the endothelial cell for leukocytes and stimulate transmigration of leukocytes. Procoagulant activity is increased and anticoagulant activity is suppressed by the vascular endothelial cells in response to TNF⁵¹. TNF induces

respiratory burst and degranulation in neutrophils and induces proliferation of fibroblasts.

TNF appears to be an important mediator of pulmonary inflammation which may have both local and systemic effects. Serum TNF is increased in children with acute lower respiratory tract infections⁵⁵. Those with elevated TNF concentrations were found to have longer duration of fever and higher levels of C-reactive protein but no increase in the clinical severity of respiratory tract infection. Animal studies have demonstrated that the lung, after inoculation with bacteria, may be a major source of systemic TNF⁵⁶. Warren et al. has demonstrated that TNF participates in the development of immune-complex alveolitis⁵⁷. TNF was shown to originate primarily from alveolar macrophages and was a necessary component for the full development of this pathology. Others have demonstrated a decreased TNF productive capacity of alveolar macrophages in septic patients and mice⁵⁸. However, the TNF assays were performed only after lipopolysaccharide (LPS) stimulation and consequently, the macrophages may have already been maximally stimulated. Levels of TNF in bronchoalveolar lavage (BAL) fluid were found to be elevated in those individuals with ARDS compared to normal subjects⁵⁹.

Evidence for the involvement of TNF and IL-1 in pulmonary oxygen toxicity has been growing. Reactive oxygen species, particularly hydrogen peroxide, have demonstrated the ability to increase the LPS-induced TNF release from macrophages in

vitro. These reactive oxygen species also facilitated the LPS-induced TNF release in the serum⁶⁰. Further, TNF, IL-1, and LPS increased the mRNA levels of manganese-superoxide dismutase (Mn-SOD), the mitochondrial SOD, of pulmonary epithelial cells with only minor increases noted in the fibroblasts⁶¹. Hyperoxia appeared to have no effect within 24 hours on the Mn-SOD mRNA levels of the pulmonary epithelial cells or fibroblasts. Also of note is that LPS has been shown to induce SOD levels in endothelial cells and monocytes^{62,63}. Immune-complex activated alveolar macrophages demonstrated increased superoxide production following addition of IL-1 and, to a lesser extent, TNF⁶⁴. Pretreatment with TNF and/or IL-1 prior to exposure to hyperoxia appears to attenuate pulmonary injury. White et al. has shown in rats that administration of both TNF and IL-1 intravenously and intraperitoneally prior to administration of >99% O₂ decreased lung injury and increased survival which was associated with a small increase in antioxidant enzymes early on (4-16 hours after exposure) and a much larger increase at 72 hours^{65,66}. White and Ghezzi have also shown in a similar model that the beneficial effects of pretreatment could be partially blocked by lysine acetylsalicylate and, to a lesser extent, ibuprofen. This suggests that cyclooxygenase products may be responsible for the induction of antioxidant enzymes³¹. Others have administered TNF alone via tracheal insufflation prior to exposure to hyperoxia and demonstrated a protective effect as evidenced by decreased lung injury and also increased

antioxidant enzymes levels⁶⁷. This is in contrast to White's work in which evidence was presented that pretreatment with IL-1 played a more important role in protecting against hyperoxia than TNF. Finally, sera from endotoxin-treated rats conferred protection in recipients exposed to hyperoxia⁶⁸. TNF and IL-1 levels were elevated in the sera of the recipients 60 hours post-exposure whereas the antioxidant enzymes were not. Consequently, TNF and IL-1 by themselves appear to have protected against pulmonary oxygen toxicity.

Interleukin-1

IL-1 is a 17 kd, 159 amino acid peptide produced primarily by macrophages but also by endothelial cells and other cell types^{51,69}. As mentioned previously, IL-1 and TNF are capable of inducing macrophages to produce each other^{51,69}. Further, IL-1 may induce the cell that produced it to make more IL-1 in a positive feedback loop^{51,69}. Although TNF and IL-1 have many similar properties and often act synergistically, IL-1 is distinctly different from TNF in that it participates directly in the activation of lymphocytes.

Like TNF, IL-1 is an important inflammatory mediator. Systemically, IL-1 has been shown to induce shock, fever and production of acute phase proteins by the liver^{51,69,70}. IL-1 administered intravenously has been able to induce a shock-like state in rabbits^{71,72}. Further, when a low dose of both IL-1 and TNF were administered together, but not separately, a shock-like state was induced. This synergistic property

also resulted in marked accumulation of protein, red blood cells (RBCs), and neutrophils in the alveolar space secondary to the disruption of the pulmonary vascular endothelium⁵⁵. However, TNF has been found to induce greater lung injury than IL-1⁷¹. This may be why TNF and not IL-1 appeared to be the primary mediator of acute pulmonary dysfunction in a canine model challenged with TNF and IL-1⁷³. On the other hand, IL-1 appears to play a greater role than TNF in the neutrophil accumulation despite similar abilities to induce increased adherence to endothelial cells⁷⁴. Other studies have demonstrated that IL-1 initiates vascular congestion, cellular infiltration, and endothelial leakage⁶⁹. Finally, bleomycin-stimulated alveolar macrophages release IL-1 in the early phases of the fibrogenic response⁷⁵. Possibly, IL-1 plays a critical role in the initial development of pulmonary fibrosis.

Interleukin-6

IL-6 is a 22 kd, 190 amino acid peptide produced primarily by macrophages and activated T lymphocytes as well as fibroblasts and endothelial cells^{51,76}. IL-6, along with TNF and IL-1, are all involved in inducing fever and production of acute phase proteins, part of the acute phase response of the inflammatory process^{51,77}. Moreover, activated macrophages can release IL-6, along with IL-1 and TNF, in a coordinated fashion. Specifically, TNF and IL-1 induce production of IL-6 in fibroblasts, and IL-1 induces production of IL-6 in

endothelial cells, fibroblasts, and monocytes⁷⁸⁻⁸⁰. Elevated serum levels of IL-6 have been identified in lung transplant patients undergoing rejection or experiencing pulmonary infection⁸¹. The source of the IL-6 was presumably considered to be the activated alveolar macrophages.

PROTECTION AGAINST PULMONARY OXYGEN TOXICITY

To date, other than to decrease the FiO_2 , no effective method to protect against oxygen toxicity exists in the clinical setting. However, this is often not a practical consideration. A number of experimental methods have protected against oxygen toxicity in animals. These methods have been aimed at the mechanism and inflammatory processes of oxygen toxicity. The antioxidants, vitamin E and butylated hydroxyanisole, and antioxidant enzymes, polyethylene glycol-conjugated superoxide dismutase and catalase, have been given prior to exposure to hyperoxia and found to decrease lung injury in rabbits^{29,82}. Particularly, a decrease in the alveolar-capillary permeability was noted. Others have administered cimetidine to lambs resulting in attenuated oxygen-induced lung injury, decreased oxidant stress, and increased ratio of reduced to oxidized glutathione concentrations⁸³. Cimetidine's mechanism of action is through competitive and noncompetitive inhibition of the cytochrome P_{450} system and subsequent decrease production of reactive oxygen species. In infant mice exposed to 80% oxygen,

dexamethasone was administered and found not to decrease lung collagen, thickness of the air-blood barrier, change the epithelial or basement membrane components, or neutrophils in the lung^{28,84,85}. However, dexamethasone was found to decrease lung wet weight and lung water. Low-dose endotoxin administered prior to exposure to hyperoxia has also been found to extend survival in adult mice⁸⁶. Similarly, endotoxin conferred protection from oxygen toxicity in adult rats^{68,87}. This is believed to be due to the production of TNF and IL-1 after induction by endotoxin. While some have suggested that the protection against oxygen toxicity from TNF and IL-1 is due to the induction of antioxidant enzymes, particularly Mn-SOD, others have demonstrated that induction of these enzymes is not necessary for protection^{66-68,87}. Studies have shown that leukotriene B₄ (LTB₄) protects rats from hyperoxic lung injury. One possible explanation is that TNF and IL-1 stimulate prostaglandin E₂ (PGE₂) production which subsequently leads to decreased production of LTB₄ and formation of free radicals from neutrophils⁸⁸.

Unfortunately, many of these treatments are not practical. Administration of antioxidants needs to take place approximately 2 days prior to exposure to hyperoxia in order to confer protection. One does not usually desire to give steroids or endotoxin to an individual who may already be suffering from an infection or sepsis. Finally, administration of antioxidant enzymes seems effective but its clinical applicability is unknown. Consequently, no effective

treatment is available at this time.

PURPOSE

The purpose of this project was to determine if cytokines were involved in oxygen-induced lung injury. To date, TNF and IL-1 have been implicated in the development of pulmonary inflammation due to a variety of etiologies but not oxygen. Further, though TNF and IL-1 appear to play a role indirectly in the pathogenesis of pulmonary oxygen toxicity, it is difficult to determine from previous work whether it is a protective or detrimental one. Finally, little is known with regard to the role of IL-6 in pulmonary inflammation, let alone oxygen toxicity, despite its involvement in the acute inflammatory process and close interactions with TNF and IL-1. The aims of this project, therefore, were to determine if TNF, IL-1, and/or IL-6 were involved in oxygen-induced lung injury utilizing the following methods in the rat model:

1. Expose alveolar macrophages in vitro to lethal hyperoxia for 30 minutes and 1 hour followed by assessment of their ability to produce TNF, IL-1, and IL-6 in response to LPS challenge.
2. Expose the animals in vivo to hyperoxia for 1, 2, and 4 hours. The alveolar macrophages will then be harvested and their ability to produce TNF, IL-1, and IL-6 in response to LPS challenge determined.
3. Obtain serum samples from animals exposed to

hyperoxia for 1, 2, and 4 hours to determine the TNF, IL-1, and IL-6 concentrations.

4. Assess membrane-associated levels of the particular cytokine(s) that demonstrated increased alveolar macrophage productive capacity.
5. If the cytokine(s) is elevated, block or downregulate the production and determine if this has protective or detrimental effects with regard to pulmonary oxygen toxicity.

MATERIALS AND METHODS

ANIMAL MODEL

The unmanipulated, adult male Sprague-Dawley rat was chosen as the animal model for studying the involvement of cytokines in pulmonary oxygen toxicity. The first and foremost reason is that the rat demonstrates a pattern of pulmonary injury similar to primates but over a shorter period of time^{9,43}. Also, the animal is small, easy to house, and relatively inexpensive. Adult animals were chosen over neonates because neonates appear to have induction of their antioxidant enzymes which results in longer survival times compared to the adult animals⁸⁹. Additionally, adult rats, as opposed to adult mice, allow harvesting of an adequate number of alveolar macrophages via bronchoalveolar lavage⁹⁰.

GENERAL

Pathogen and viral antibody free male Sprague-Dawley rats (from Charles River Labs., Portage, MI) weighing 275-350 g were used throughout the studies. They were housed and acclimatized for at least 72 hours at the University Laboratory Animal Research (ULAR) building prior to experimentation. The rats were kept on a 12 hour light on and off cycle. Prior to experimentation, they were fasted overnight (approximately 16 hours) but allowed water ad libitum. All protocols were carried out in accordance with the guidelines set forth in the Animal Welfare Act and as outlined in the Guide for Care and Use of Laboratory Animals by the National Institutes of Health Publications.

IN VITRO EXPOSURE OF ALVEOLAR MACROPHAGES
TO HYPEROXIA AND NORMOXIA

Alveolar macrophages were harvested by bronchoalveolar lavage (BAL) immediately after each rat was euthanized by overdose of ether. This was accomplished by cannulating the exposed trachea with a dulled 18 gauge needle under direct vision and lavaging the lungs with ice-cold Dulbecco's Modified Eagle's Medium (DMEM), supplemented with glutamine (GIBCO Labs., Grand Island, NY) and gentamicin (GIBCO Labs.). Three separate, consecutive 5 ml lavages were carried out, and

the effluents combined in a 15 ml sterile conical centrifuge tube (Falcon 2095, Becton Dickenson and Co., Lincoln Park, NY) kept on ice. Though precise amounts of each total collected effluent were not recorded, this was usually 12 ± 1 ml. Once all the effluents were collected for each animal, they were then centrifuged at $320 \times g$ for 15 minutes at 4°C (Sorvall RT 6000B and D models, DuPont Biotechnology Systems, Wilmington, DE). The cells were resuspended in 1 ml DMEM. The number of viable macrophages were determined by morphologic criteria and trypan blue exclusion. The cell suspension was then diluted to 2 ml. One ml of the each cell suspension was placed in one of two 24 well plastic plates, and the other ml was placed in the remaining plate. The macrophages were allowed to adhere on plastic for 2 hours by placing them in an incubator (Steri-Cult 200 Incubator, Forma Scientific Inc., Marietta, OH) at 37°C , 5% CO_2 , and >90% humidity. The cells were washed with DMEM, and 1 ml DMEM was then added to each well. One plate with adherent cells was placed in an air-tight container (Modular Incubator Chamber, Voss Ind. Inc., Cleveland, OH) with equal sized inlet and outlet ports and exposed to 95% O_2 and 5% CO_2 for the selected time period (30 or 60 minutes). The other plate was placed back in the incubator and exposed to 21% O_2 and 5% CO_2 for the same length of time. The O_2 concentration was verified by an oxygen gas analyzer (5525 Oxygen Analyzer, Hudson Ventronics Division, Temecula, CA) and the CO_2 concentration, by a Fyrite carbon dioxide gas analyzer (Bacharach Inc., Pittsburgh, PA). After the time of exposure

was complete, the plates were removed from their respective environments and the DMEM in each well was then replaced with 1 ml of DMEM containing 10% by volume heat inactivated fetal bovine serum (FBS, Biologos Inc., Naperville, IL) and lipopolysaccharide (LPS, 10 μ g LPS/ml DMEM) (LPS from *Escherichia coli* 055:B5, Difco Labs., Detroit, MI). The macrophages were then incubated for 24 hours. Following the incubation period, the supernatants were collected, aliquoted, and stored at -70° C until cytokine determination by bioassay was to be performed.

IN VIVO EXPOSURE OF ALVEOLAR MACROPHAGES
TO HYPEROXIA AND NORMOXIA

Alveolar macrophages were exposed to hyperoxia and normoxia in vivo by placing unmanipulated rats into one of two equal compartments of a specially designed 3 x 1 x 1' plexiglass chamber. One rat was placed in each compartment which contained bedding with wood chips. Both compartments had inlet and outlet ports of 5/16" diameter. After flushing the compartments for 3 minutes at 15 liters per minute (LPM) with either room air or medical grade O₂ (AGA Medical Gas, 99.5% purity, AGA Gas Inc., Cleveland, OH), the animals were then exposed to the appropriate gas, as determined by prior computer randomization, at 8.5 LPM for the selected time period (1, 2, or 4 hours). This allowed simultaneous

exposures to be carried out. The humidity and temperature of each compartment were constantly monitored and ranged from 48-70% and 19.4-24.4° C, respectively. The atmospheric pressure was checked immediately prior to the beginning of an experiment. Due to the fact that the inlet and outlet ports were of the same size, the pressure within the chamber would not be expected to be vastly different from the atmospheric pressure. The O₂ concentration on the hyperoxic side was continuously monitored and was $\geq 98\%$ at all times. The O₂ concentration on the normoxic side was measured at selected intervals and found to be 21% at all times. The CO₂ concentration was determined at 2 and 4 hours post-exposure and was always $\leq 0.2\%$. During the period the animals were exposed to hyperoxia or normoxia, they were allowed food (standard rat chow) and water ad libitum.

Immediately upon completion of exposure, euthanasia of each rat was accomplished by overdose of ether within 4 minutes. A midline neck incision was extended through the thorax. The thoracic cavity and pulmonary parenchyma were grossly inspected. A 1.0 ml blood sample was obtained by cardiac aspiration, transferred to sterile plastic Microtainer tubes containing a serum separator (Becton Dickenson and Co., Rutherford, NJ), and placed on ice. BAL was then carried out in the fashion described earlier. Briefly, the trachea was cannulated with a dulled 18 gauge needle which was secured in place with 3-0 silk. Three consecutive 5.0 ml lavages were performed with ice-cold DMEM. The combined effluents were

transferred to a 15 ml sterile conical centrifuge tube. Though precise amounts of the total effluent were not recorded, it was usually 12 ± 1 ml.

Blood samples and BAL effluents were prepared in the following manner. The blood was separated by centrifugation at $12,000 \times g$ for 15 minutes at 4°C (Model 235C Micro-Centrifuge, Fisher Scientific, Chicago, IL). The serum samples were aliquoted and stored at -70°C until cytokine bioassay could be performed.

The BAL effluents were centrifuged at $320 \times g$ for 15 minutes at 4°C . The cells were then resuspended in 1 ml supplemented DMEM. The number of viable macrophages were determined by morphologic criteria and trypan blue exclusion. Counts $>7 \times 10^5$ cells/ml or $<1 \times 10^5$ cells/ml were excluded. Large counts indicated that the animal likely had an underlying respiratory infection which was not apparent when the animal was examined by ULAR personnel. Small counts were likely secondary to inadequate lavage. Viable macrophages were diluted to 1×10^5 cells/ml in supplemented DMEM, and 1 ml of the macrophage suspension was placed in a 24 well plastic plate. The cells were allowed to adhere on plastic for 2 hours by placing them in an incubator (37°C , 5% CO_2 , $>90\%$ humidity). The cells were washed with DMEM and then checked for adherence microscopically. One ml 10% FBS/DMEM with LPS ($10 \mu\text{g}$ LPS/ml DMEM) was added to each well. The macrophages were then placed in an incubator for 24 hours. At the conclusion of this time period, the supernatants were

collected, aliquoted, and stored at -70°C until assayed.

To verify further that the animals were breathing the predetermined concentration of oxygen, a group of rats underwent arterial blood gas (ABG) analysis. This was accomplished in the following manner: Under light ether anesthesia, the right carotid artery was catheterized via a midline neck incision with a hybrid catheter consisting of a 3.0 cm sialastic tip (size 0.02" I.D.x 0.037"O.D, Baxter Healthcare Corporation, Scientific Products Division, McGraw Park, IL) secured to PE 50 tubing (Becton Dickenson and Co., Parsipanny, NJ). The tip was secured to the artery with 6-0 nylon suture. The tubing was tunnelled subcutaneously posteriorly and brought through a separate small skin incision at the nape of the neck. The catheter was then threaded through a miniature single channel fluid swivel (Harvard Apparatus, South Natick, MA). This swivel was sutured to the musculature with 4-0 nylon, and the skin was approximated with the same material. After an adequate recovery period from the anesthesia, the animal's temperature and hematocrit were determined, and baseline arterial blood gas analysis obtained. Utilizing a Harvard pump (Model 2400003, Harvard Apparatus, South Natick, MA), heparinized normal saline (2U heparin/ml NS) (bovine heparin from Upjohn Company, Kalamazoo, MI) was infused continuously at 0.358 ml/hr to replace the blood loss due to blood draws at a 3:1 ratio of solution infusion: blood loss and to keep the catheter patent. The animal was then exposed to either a normoxic or hyperoxic condition as

described above. At 1-hour post-exposure, another ABG analysis was performed.

DETERMINATION OF CELL-ASSOCIATED TNF

Since the TNF productive capacity of the alveolar macrophage from the in vivo exposure to hyperoxia was increased at 2 hours (please see RESULTS section), cell-associated TNF was assessed near this time period according to the methods of Hogan et al.⁹¹. Following exposure of the animals to normoxia or hyperoxia for 2, 3, or 4 hours and BAL performed with subsequent centrifugation of the effluents as described previously, the number of viable alveolar macrophages were determined by morphologic characteristics and trypan blue exclusion. Cells from each sample were then diluted to 5×10^5 cells/ml supplemented DMEM, and 100 μ g of each were transferred to a single well of a 96-well plastic microculture plate. The plate was then placed in an incubator for 2 hours to allow the cells to adhere. Nonadherent cells were removed by repeated washing with DMEM. The cell monolayers were further washed with ice-cold D-PBS-BSA [Dulbecco's Phosphate Buffered Saline (GIBCO Labs.), containing 1% bovine serum albumin (Sigma Chemical CO., St. Louis, MO) and 0.1% Na Azide (Sigma Chemical Co.)] repeatedly and then incubated with 50 μ g of mouse IgG μ g/ml at 4° C for 30 minutes. The cell monolayers were then washed repeatedly

again with D-PBS-BSA. Addition of 50 μ g of primary antibody (10 μ g/ml of anti-mouse TNF [Genzyme, Cambridge, MA]) was followed by incubation for 45 minutes at 4° C. The cells were again repeatedly washed with D-PBS-BSA. Secondary antibody (50 μ g of 20 μ g anti-hamster IgG-FITC [CAPPEL Research Products, Durham, NC]/ml PBS-BSA) was added to each well and incubated for 45 minutes at 4° C. The wells were washed repeatedly with D-PBS-BSA and fixed with 100 μ g of 1% paraformaldehyde-PBS. The cells were stored at 4° C until analyzed.

The average amount of fluorescence per cell was then determined using an anchored cell analysis station (ACAS 470, from Meridian Instruments Inc., Okemos, MI). The ACAS contained a 5 W argon laser and high-speed data acquisition (laser power 200mW; scan strength 10-25%; PMT 1 sensitivity 50-70%). Data acquisition then translated fluorescent emissions pixel by pixel into a 15 pseudo-color image. This image had been previously assigned in accordance with the fluorescent intensity pattern for each cell. By integrating the fluorescent intensity values from the pixel image of each analyzed cell, the average fluorescence per cell was determined⁹¹. To exclude the fluorescence produced from nonspecific secondary antibody (FITC) binding, the minimum fluorescence threshold was set at 250-300 arbitrary fluorescence units. This resulted in excluding 90-95% of the fluorescence from nonspecific binding.

CYTOKINE ASSAYS

Purpose and Cell Line Maintenance of Cytokine Assays

In order to assess TNF, IL-1, and IL-6 activity in alveolar macrophage supernatant, serum, or BAL effluent, a WEHI-clone 13 cytotoxicity assay, a D10.G4.1 Th2 proliferative assay, and a 7TD1 B-cell hybridoma proliferative assay were performed, respectively.

The WEHI 164 clone 13 cell line utilized for TNF determinations was a gift from Dr. Steven Kunkel, University of Michigan, Ann Arbor, MI. The maintenance of this mouse fibrosarcoma cell line was carried out as described by Espevik and Nissen-Meyer⁹².

The IL-1 sensitive mouse D10.G4.1 Th2 cell clone was a gift from Dr. Charles Janeway, Yale University, New Haven, CT. The maintenance of this mouse Th cell clone was carried out as described by Kaye et al.⁹³.

The IL-6 sensitive murine 7TD1 B-cell hybridoma assay was a gift from Dr. Jacques Van Snick. The maintenance of this cell line was performed as described previously⁹⁴.

TNF Assay

TNF activity was determined by assessing the supernatants and sera for WEHI-clone 13 cytotoxicity as previously described by Ayala et al.⁹⁵. Briefly, serial dilutions of supernatant (1:3) and serum (1:20, then 1:2 thereafter) in RPMI-1640 media (GIBCO Labs., Grand Island, NY) with 10% FBS for a final volume of 100 μ g were performed in duplicate in a 96-well microtiter plate. WEHI-clone 13 cells were harvested and resuspended in 10% FBS-RPMI-1640 at a concentration of 5×10^5 cells/ml. Actinomycin D (Calbiochem, San Diego, CA) was added to the cell suspension for a final Actinomycin D concentration of 0.5 μ g/ml. The cell suspension/Actinomycin D (100 μ g) was added to each well and incubated for 20 hours. The cell suspension was then pulsed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;thiazolyl blue - from Sigma Chemical Co., St. Louis, MO) 20 μ g/well for 4 hours. The supernatant (150 μ g) was aspirated and discarded. One hundred-fifty μ g/well of 10% SDS (sodium dodecylsulfate from Sigma Corp.)/PBS was then added. The plates were wrapped in cellophane and aluminum foil. After approximately 16 hours, the plates were examined under the microscope to ensure that the crystals had completely dissolved. The absorbance was read at 620 nm on the Microplate reader (Microplate Autoreader EL311, Biotek Instruments Inc., Winooski, VT). The units of TNF activity/ml were determined by comparison of the curve produced from serial dilution of mouse TNF-alpha standard (200

U/ml; Amgen Corp., Thousand Oaks, CA). This assay for TNF reacts in a specific way to TNF- α ^{95,96}.

IL-1 Assay

IL-1 activity was determined by assessing the capacity of the supernatants and sera to induce the proliferation of D10.G4.1 (D10) cells as described by Ayala et al.⁹⁷. Briefly, serial dilutions of supernatant (1:3 initially) and serum (1:20 initially and then 1:2 thereafter) in 10%FBS-DMEM resulting in a total volume of 100 μ g were performed in duplicate in a 96-well microtiter plate. The D10 cells were harvested and resuspended in 10%-DMEM at a concentration of 4×10^5 cells/ml. Concanavalin A (Con A; from Pharmacia, Gaithersburg, MD) in 10% FBS-DMEM at a concentration of 10 μ g/ml was made. The cell suspension and Con A solution were mixed together, and 100 μ g of this resultant suspension was transferred to each well yielding a final volume of 200 μ g/well. The cells were incubated for 48 hours. Following this, 20 μ g of ^3H -thymidine (DuPont NEN, Boston, MA) were pipetted to each well and incubated overnight (approximately 14-16 hours). The suspension was then harvested onto glass fiber filter mats with the aid of a Skatron Micro 96-well Semiautomatic Cell Harvester (Skatron Instruments Inc., Sterling, VA) and the entrapped radioactivity counted utilizing a Model 1205 Beta-plate liquid scintillation counter (Wallac Inc., Gaithersburg, MD). The units of IL-1

activity/ml were determined by comparison of the curve produced by purified human IL-1 standard (5 U/ml; Genzyme, Boston, MA) and mouse IL-1 standard according to the methods of Mizel⁹⁸.

IL-6 Assay

IL-6 activity was determined by assessing the ability of the supernatants and sera to induce the proliferation of a 7TD1 B-cell hybridoma as described by Hultner et al.⁹⁹. Briefly, serial dilutions of supernatant (1:3 initially) and serum (1:20 initially and then 1:2 thereafter) in 10% FBS-HT[NA-hypothanxine (10 mM)-thymidine (1.6mM) from American Type Culture Collection, Rockville, MD]-RPMI-1640 were carried out in a 96-well microtiter plate with a total resultant volume of 100 μ g in each well. The 7TD1 cells were harvested and resuspended in 10 % FBS-HT-RPMI-1640 to a concentration of 4×10^4 cells/ml. After adding Polymyxin B 10 μ g/ml media (Polymyxin B from Sigma Chemical Co.) to the cell suspension, 100 μ g of the cell suspension/Polymyxin B was then pipetted into each well bringing the total volume of each well to 200 μ g. After incubating the cells for 72 hours, the suspension was pulsed with MTT, the supernatant aspirated, and 10% SDS-PBS added as described for the TNF assay. The plates were wrapped with cellophane and foil overnight, and absorbance read at 620nm also as stated previously in the TNF assay section. The units of IL-6 activity/ml were determined by

comparison of the curve produced with a recombinant mouse IL-6 standard (200 U/ml; Amgene Corp., Thousand Oaks, CA) according to the methods of Mizel⁹⁸.

DETERMINATION OF THE EFFECTS OF PRETREATMENT WITH PENTOXIFYLLINE (PTX) ON LETHAL HYPEROXIA

The Effects of PTX on Mean Arterial Pressure (MAP)

Under light ether anesthesia, PE 90 catheter (Becton Dickenson and Co., Parsippany, NJ) was inserted 2.5 cm into the right carotid artery via midline neck incision. Sodium pentobarbital 30-35 mg/kg body weight (BW) was then administered intravenously in increments over the next 70 minutes to maintain anesthesia. Once the MAP had equilibrated as evidenced by a constant reading over a 3-5 minute period, 1 ml of PTX (Sigma Chemical Co.) 50 mg/kg BW in normal saline (NS) was administered subcutaneously (SC). MAP readings were recorded every minute for the first 15 minutes post-PTX injection. Subsequent readings were then taken at 20, 30, 45, and 60 minutes after PTX administration.

The Effects of Hyperoxia on Rats Pretreated with PTX

To investigate the effects of PTX with regard to oxygen toxicity, the rats were divided into 4 groups: 1) those that

received 1 ml NS SC and exposed to normoxia (N/NS), 2) those that received 1 ml of PTX 50 mg/kg BW SC and exposed to normoxia (N/PTX), 3) those that received 1 ml of NS SC and exposed to hyperoxia (H/NS), and 4) those that received 1 ml of PTX 50 mg/kg BW in NS SC and exposed to hyperoxia (H/PTX). The injections were administered subcutaneously in the nape of the neck without the aid of anesthesia. Within 30 minutes of the first injection, the animals were placed in the appropriate environment. An inflatable glove bag (Model X-37-27H, Instruments for Research and Industry, Cheltenham, PA) was utilized for both exposure to room air or hyperoxia for 52 hours. While in the glove bag, the animals were housed in plastic cages lined with wood chip bedding and allowed food and water ad libitum.

For hyperoxic exposures, medical grade O₂ was administered through a 3/16" inlet port and exited through a port of the same diameter. After flushing the glove bag for 5 minutes at 15 LPM, the oxygen was administered at 8.5 LPM. The O₂ concentration was checked initially within 2 hours post-exposure and then at 12, 24, 36, 48, and 52 hours utilizing the oxygen analyzer mentioned earlier. The concentration ranged from 95-100% except for the brief periods when the indicating anhydrous calcium sulfate (Drierite, W.A. Hammond Drierite Co., Xenia, OH) was changed. Upon each change of the Drierite, the glove bag was flushed for 5 minutes at 15 LPM with the rate decreased back to 8.5 LPM. The Drierite was changed 3-4 times over the 52 hour period at

12 hour intervals in order to keep the humidity within acceptable range (59-75%). The CO₂ concentration was measured at 12, 24, 36, 48, and 52 hours post-exposure utilizing the Fyrite CO₂ analyzer and was < 0.4% at all time points. The temperature ranged from 22.2-23.3° C.

For normoxic exposures, a similar inflatable glove bag was again utilized. The rats were housed in the glove bag as described earlier. Room air was administered through a 3/16" diameter port and allowed to exit through the open front of the glove bag. After flushing the glove bag for 5 minutes at 15 LPM, room air was administered at 8.5 LPM. The O₂ and CO₂ concentrations were determined at 12, 24, 36, 48, and 52 hours post-exposure and found to be 21% and < 0.2%, respectively. The humidity ranged from 49-77% and the temperature, from 21.7-23.9° C.

Beginning at 52 hours, the animals were removed from their experimental environment, weighed, and euthanized by overdose of ether. A midline neck incision was extended through the thorax. The lungs were grossly inspected. Pleural fluid, if any, was measured by careful aspiration. A blood sample was obtained by cardiac aspiration. A small amount was placed in a heparinized capillary tube for hematocrit determination. The remainder was transferred to sterile Microtainer tubes with a serum separator and placed on ice. The trachea was then exposed and cannulated as described earlier in preparation for BAL. Two consecutive bronchoalveolar lavages were performed with D-phosphate

buffered saline (PBS) at room temperature. The volume of PBS utilized was the same for a given group of animals and was determined by multiplying the mean body weight of the group in kg by 23 ml/kg¹⁰⁰. The effluent obtained was recorded, transferred to a sterile conical centrifuge tube, and placed on ice. The midline thoracic incision was then extended through the abdomen, and the abdomen inspected for ascites. The amount of ascites, if any, was carefully aspirated and recorded. All animals were removed from their experimental environments by 54 hours post-exposure. Following the procedures on the animals, the lavage effluent was centrifuged at 600 x g for 10 minutes at 4° C. The supernatant was aliquoted with a 1 ml sample stored at 4° C, and the remainder stored at -70° C. The blood samples were centrifuged at 12,000 x g for 15 minutes at 4° C. The serum was aliquoted with a small sample stored at 4° C and the rest at -70° C. The LDH content was determined in those samples stored at 4° C.

ASSAYS FOR INDICATORS OF PULMONARY INJURY

Animals undergoing 52 hour exposures were assessed for pulmonary injury by determining the supernatant lactate dehydrogenase (LDH) and protein levels. Elevated LDH and protein concentrations in BAL supernatant have been utilized as markers of pulmonary injury¹⁰¹⁻¹⁰³. Serum levels were also measured to determine if changes in the supernatant concentrations were related to changes in the serum.

Lactate Dehydrogenase Assay

LDH levels were determined in a quantitative kinetic fashion spectrophotometrically (U-3110 Spectrophotometer, Hitachi Instruments, Inc., Danbury, CT). BAL supernatant concentrations were assessed utilizing the method of Bergmeyer and Bernt adapted for a commercially available LDH kit (Lactate Dehydrogenase [LD] Procedure No. 340-UV, Sigma Diagnostics, St. Louis , MO)¹⁰⁴. Serum concentrations of LDH were measured by following the manufacturer's specifications.

Protein Assay

Protein concentrations in the BAL supernatants and sera were assessed according to the methods of Lowry et al.¹⁰⁵.

STATISTICAL ANALYSES

All results are presented as the mean $\pm$ SEM. Statistical significance was set at $p < 0.05$. The types of statistical analyses were the following:

1. For the in vitro exposures of the alveolar macrophages, paired Student's t-tests were performed on the cytokine levels. N=8/group.
2. At each time period, the number of viable alveolar macrophages harvested during the in vivo exposures to normoxia and hyperoxia were determined. A Student's independent t-test was utilized. N=8/group.
3. ABG analyses were determined at time 0 and 1 hour post-exposure. A Student's paired t-test was utilized. N=5/group.
4. To assess significance of the cytokine productive capacities of the alveolar macrophages exposed to normoxia and hyperoxia in vivo, Student's independent t-tests were utilized. N=7-8/group.
5. Student's independent t-tests were performed on serum cytokine levels obtained at the selected time intervals from the in vivo exposures. N=7-8/group.
6. To assess significance for the parameters determined at the conclusion of the 52 hour exposures to normoxia and hyperoxia with and without PTX, a one-way ANOVA followed by Tukey's test was utilized.

These parameters included supernatant and serum cytokine, LDH, and protein levels; % weight change; % lavage fluid recovered; amount of pleural fluid; amount of ascites; and hematocrit. For each parameter, N=5-6/group.

RESULTS

ALVEOLAR MACROPHAGE CYTOKINE PRODUCTIVE CAPACITIES AFTER EXPOSURE TO HYPEROXIA OR NORMOXIA IN VITRO

Exposure of alveolar macrophages to hyperoxia in vitro for 60 minutes (Figure 1) resulted in a significant increase in the IL-1 productive capacity when compared to its control (62 ± 16 vs 33 ± 9 U/ml/ 10^5 cells, $p < 0.05$). Though the IL-1 productive capacity of those alveolar macrophages exposed to hyperoxia trended upward at 30 minutes (Figure 2), no significant difference was present (623 ± 114 vs 506 ± 81 pg/ml/ 10^5 cells). The TNF (Figure 3) and IL-6 (Figure 4) productive capacities of those cells exposed to hyperoxia, also noted to trend upward in some instances, were not significantly different from their controls.

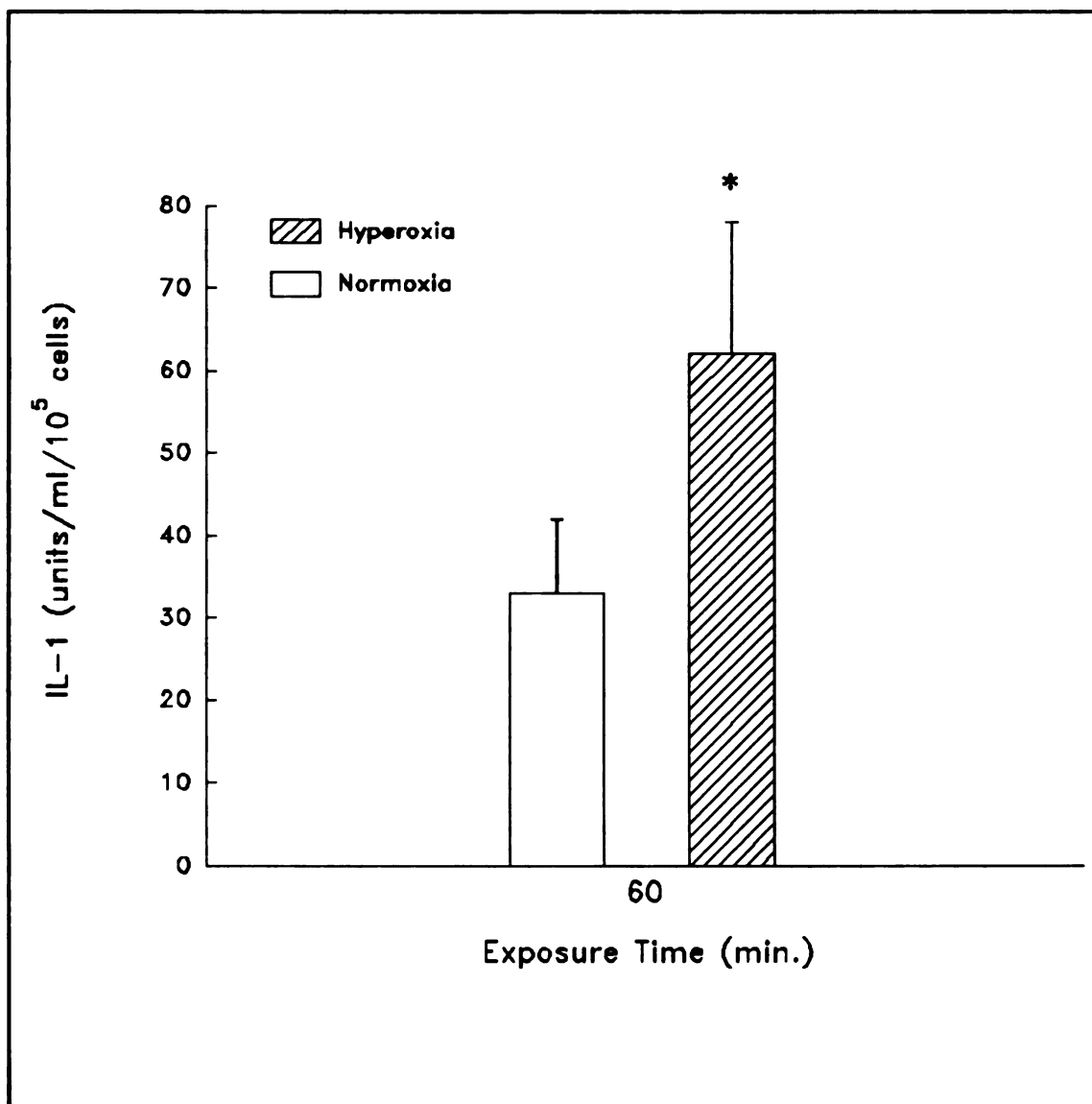


Figure 1: IL-1 productive capacities of alveolar macrophages after 60 minute exposure to normoxia or hyperoxia in vitro. Mean $\pm$ SEM, N=8/grp, Student's paired t-test, *p<0.05 vs control.

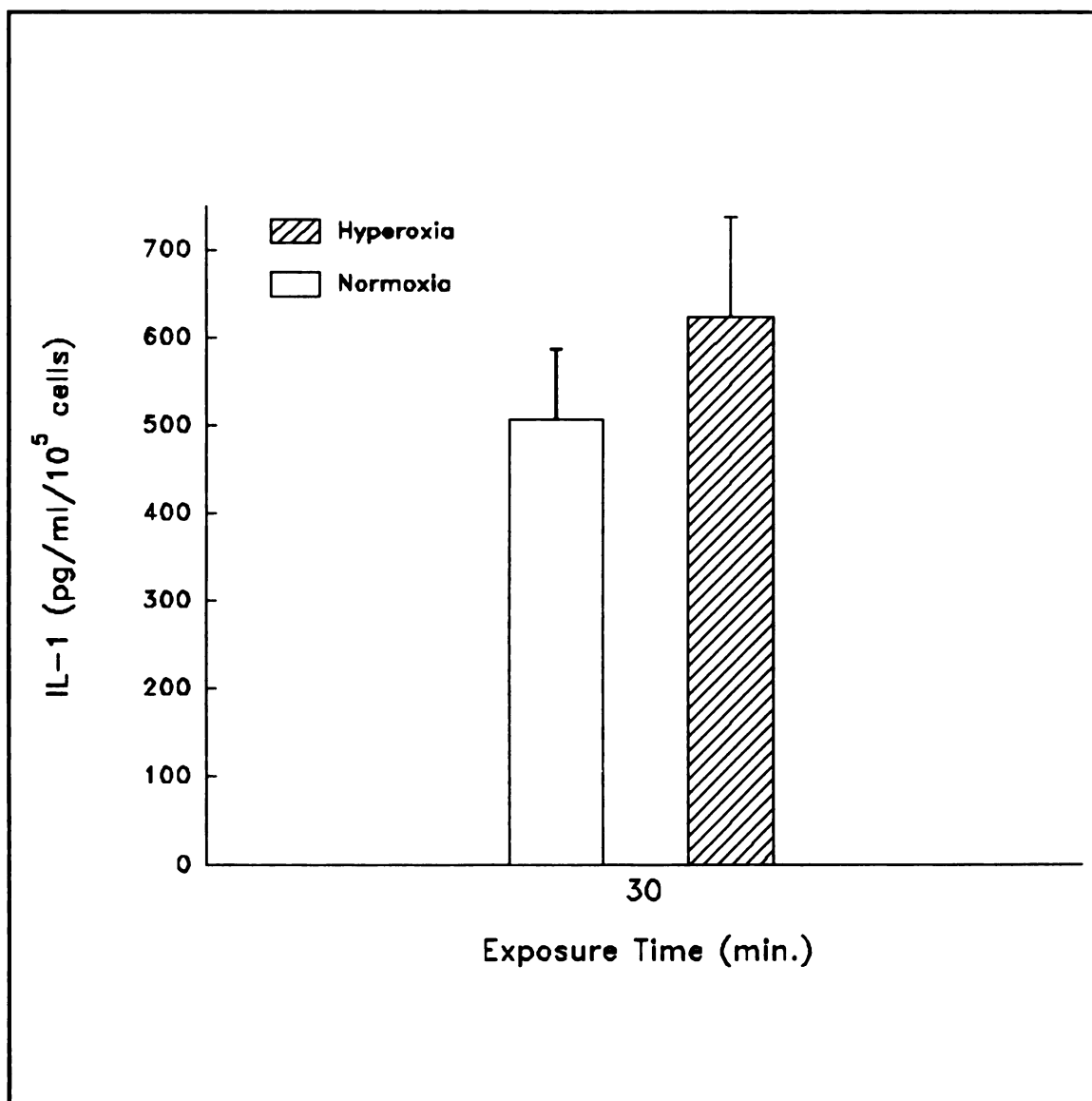


Figure 2: IL-1 productive capacities of alveolar macrophages after 30 minute exposure to normoxia or hyperoxia in vitro. Mean $\pm$ SEM, N=8/grp, Student's paired t-test, significance set at $p<0.05$.

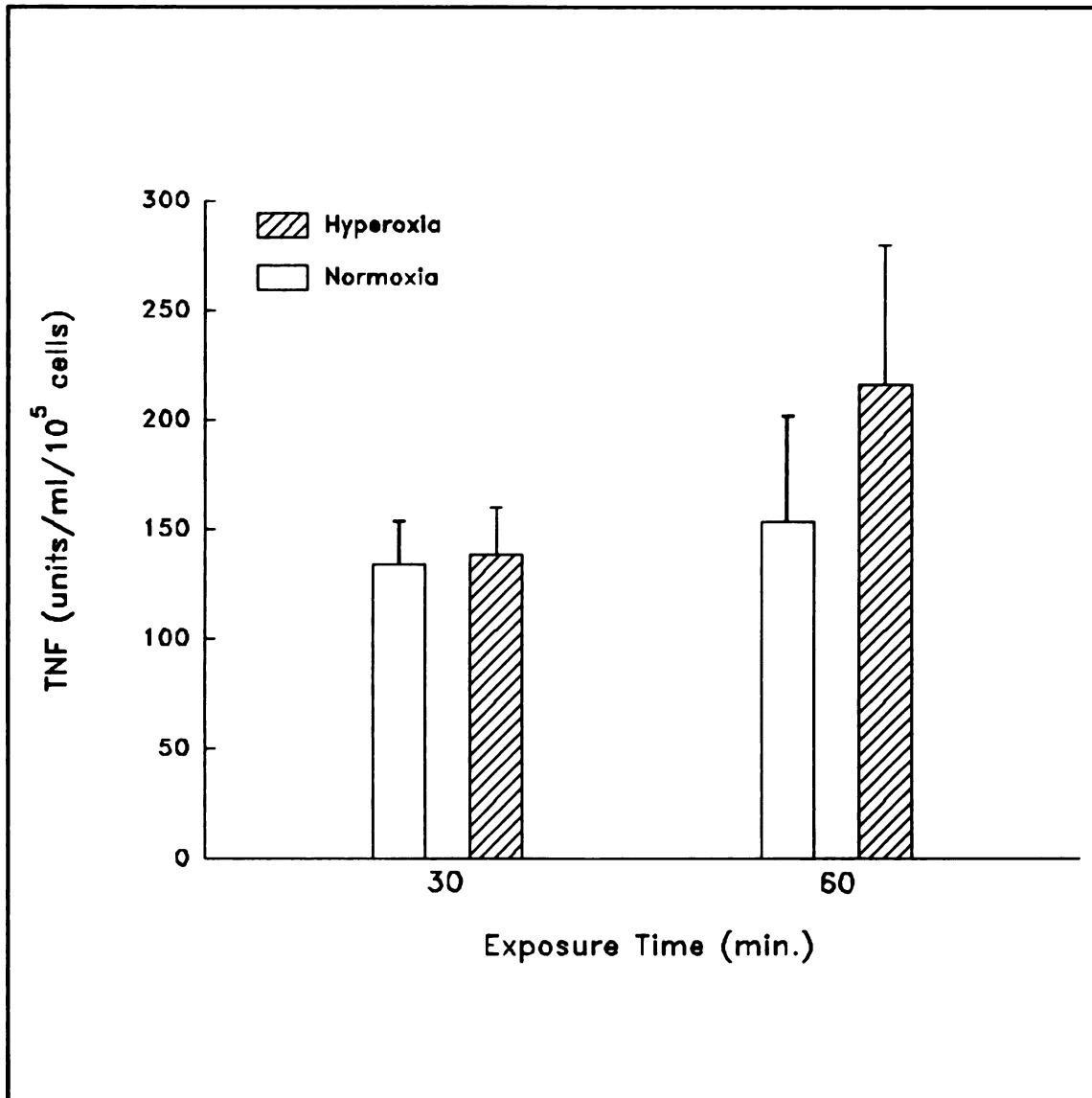


Figure 3: TNF productive capacities of alveolar macrophages after 30 and 60 minute exposures to normoxia or hyperoxia *in vitro*. Mean $\pm$ SEM, N=8/grp, Student's paired t-test, significance set at $p<0.05$.

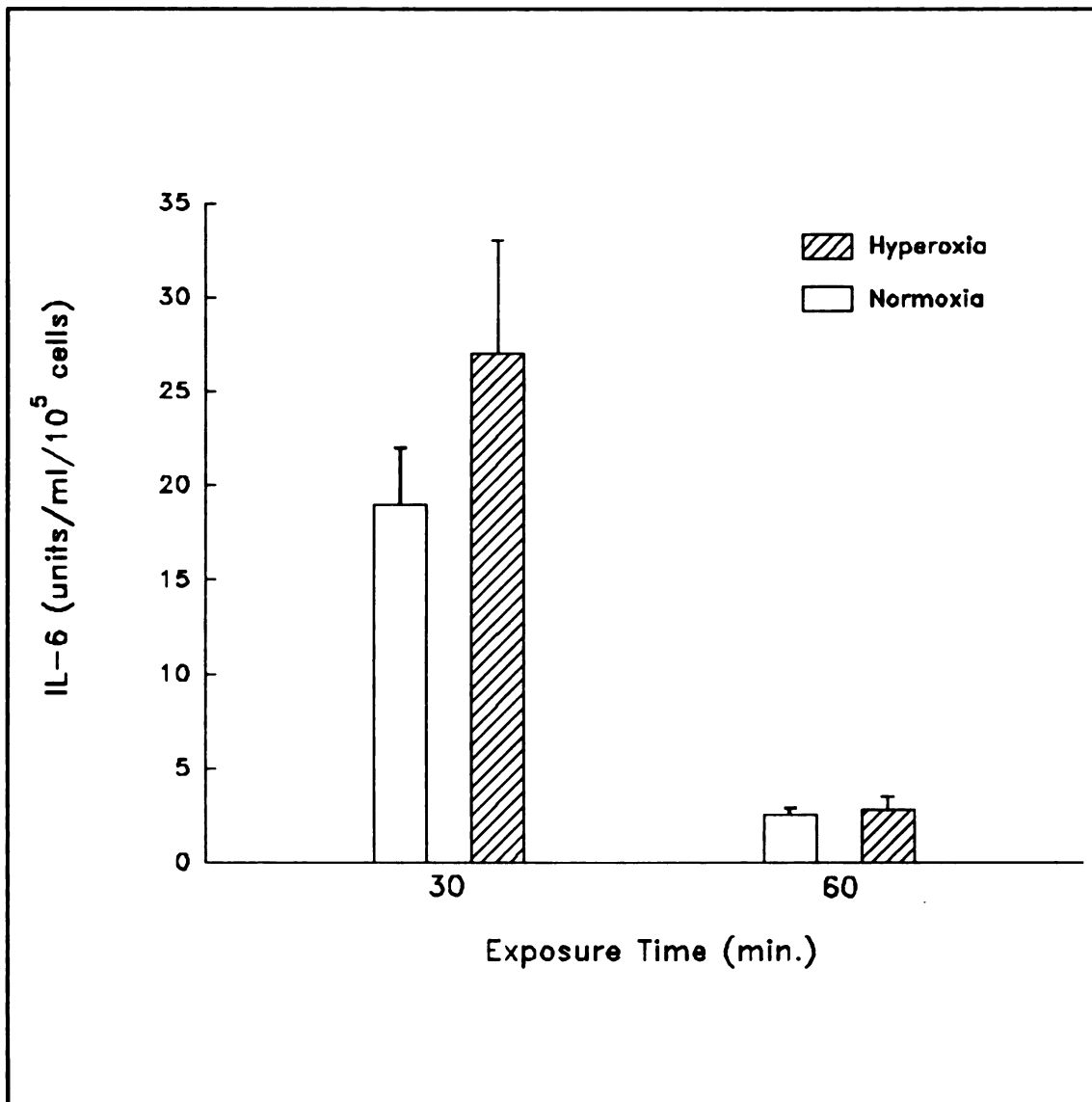


Figure 4: IL-6 productive capacities of alveolar macrophages after 30 and 60 minute exposure to normoxia or hyperoxia in vitro. Mean $\pm$ SEM, N=8/grp, Student's paired t-test, significance set at $p<0.05$.

EXPOSURE OF ANIMALS TO
HYPEROXIA AND NORMOXIA

Quantity of Viable Alveolar Macrophages Obtained by
Bronchoalveolar Lavage:

The number of viable alveolar macrophages obtained from each animal by broncholaveolar lavage was determined according to morphologic characteristics and trypan blue exclusion. At each time point, no significant differences in the number of viable cells obtained from animals exposed to either hyperoxia or normoxia were present (Table 1).

Table 1: Viable alveolar macrophages harvested by BAL after 1, 2, and 4 hour exposures to normoxia or hyperoxia in vivo. Mean $\pm$ SEM, N=8/grp, Student's independent t-test, significance set at $p < 0.05$.

<u>Hours of Exposure</u>	<u>ALVEOLAR MACROPHAGE CELL COUNT</u> <u>(x 10⁵ cells/ml)</u>	
	<u>Normoxia</u>	<u>Hyperoxia</u>
1	3.19 $\pm$ 0.42	2.39 $\pm$ 0.35
2	3.73 $\pm$ 0.45	2.67 $\pm$ 0.37
4	3.22 $\pm$ 0.35	3.31 $\pm$ 0.46

Arterial Blood Gas Values 1 Hour After Exposure to Normoxia or Hyperoxia:

After arterial catheterization, recovery, and exposure to either hyperoxia or normoxia for 1 hour, an arterial blood sample was drawn. Those rats exposed to hyperoxia demonstrated a marked increase in the partial pressure of oxygen (380.7 ± 19.0 vs 108.7 ± 3.2 mm Hg, $p < 0.05$) in the arterial blood (Table 2). No significant differences were found in the pH and PaCO_2 of those animals exposed to hyperoxia compared to those exposed to normoxia.

Table 2: Arterial blood gas values obtained 1 hour after exposure to either normoxia or hyperoxia. Mean $\pm$ SEM, N=5/grp, Student's independent t-test, * $p < 0.05$ vs exposure to normoxia.

	<u>21% O₂</u>	<u>>98% O₂</u>
pH	7.468 ± 0.003	7.461 ± 0.009
PaCO_2 (mm Hg)	36.9 ± 0.9	38.6 ± 1.1
PaO_2 (mm Hg)	108.7 ± 3.2	$380.7 \pm 19.0^*$

Alveolar Macrophage Cytokine Productive Capacities After Exposure to Normoxia or Hyperoxia In Vivo:

The TNF productive capacity of those alveolar macrophages exposed to hyperoxia in vivo for 2 hours and then challenged with LPS in vitro demonstrated a marked increase when compared to normoxic values (141 ± 24 vs 77 ± 29 U/ml/ 10^5 cells, $p < 0.05$) (Figure 5). The TNF productive capacities of those macrophages exposed to high oxygen concentrations were not significantly increased over the normoxic controls at the 1 and 4 hour time points. The IL-1 and IL-6 productive capacities of those cells exposed to hyperoxia, though elevated in some cases compared to normoxic controls, were not significantly different at any of the time points (Figures 6 and 7).

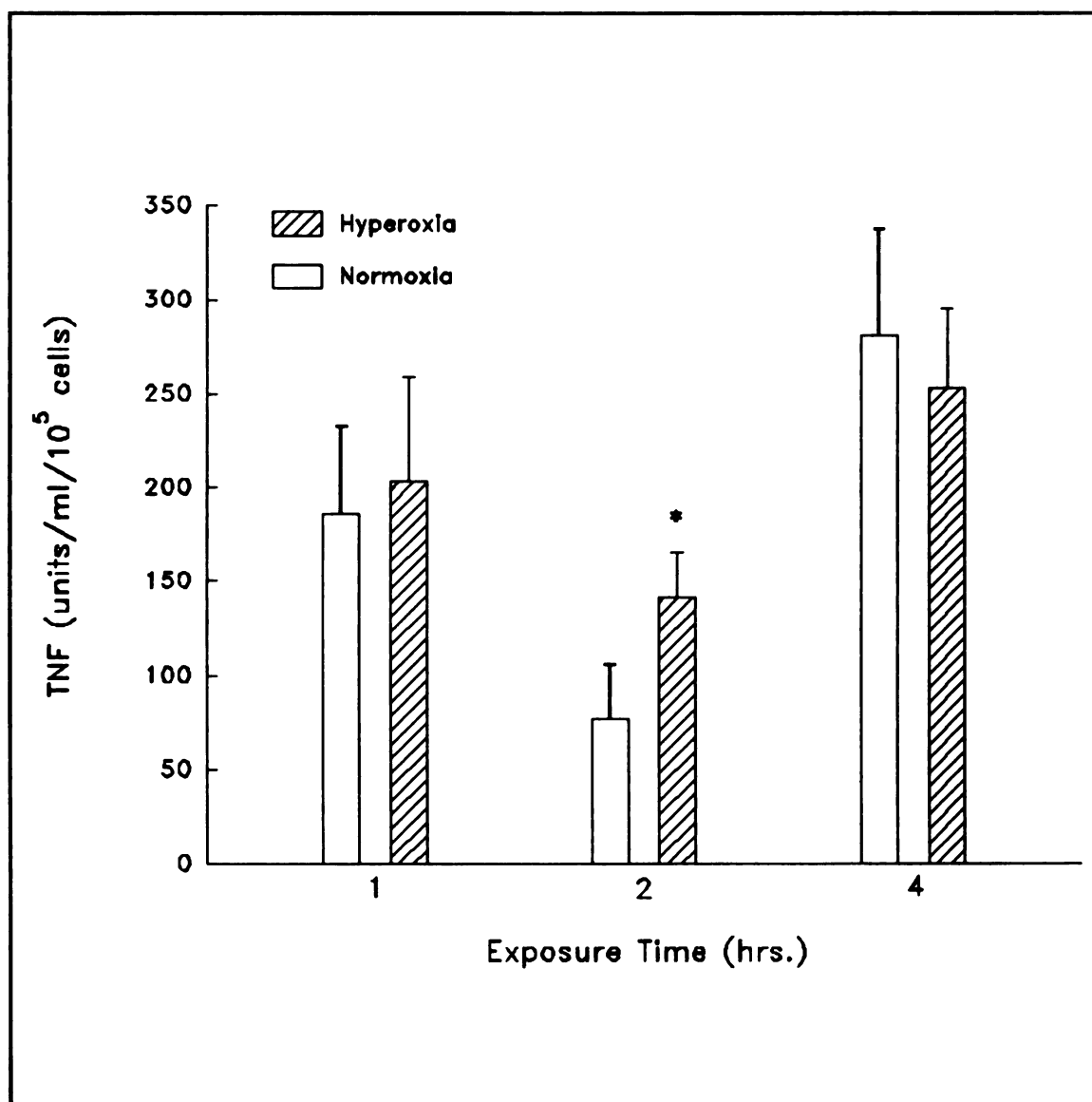


Figure 5: TNF productive capacities of alveolar macrophages after 1, 2, and 4 hour exposures to normoxia or hyperoxia *in vivo*. Mean±SEM, N=7-8/grp, Student's independent t-test, $p < 0.05$ vs normoxic values.

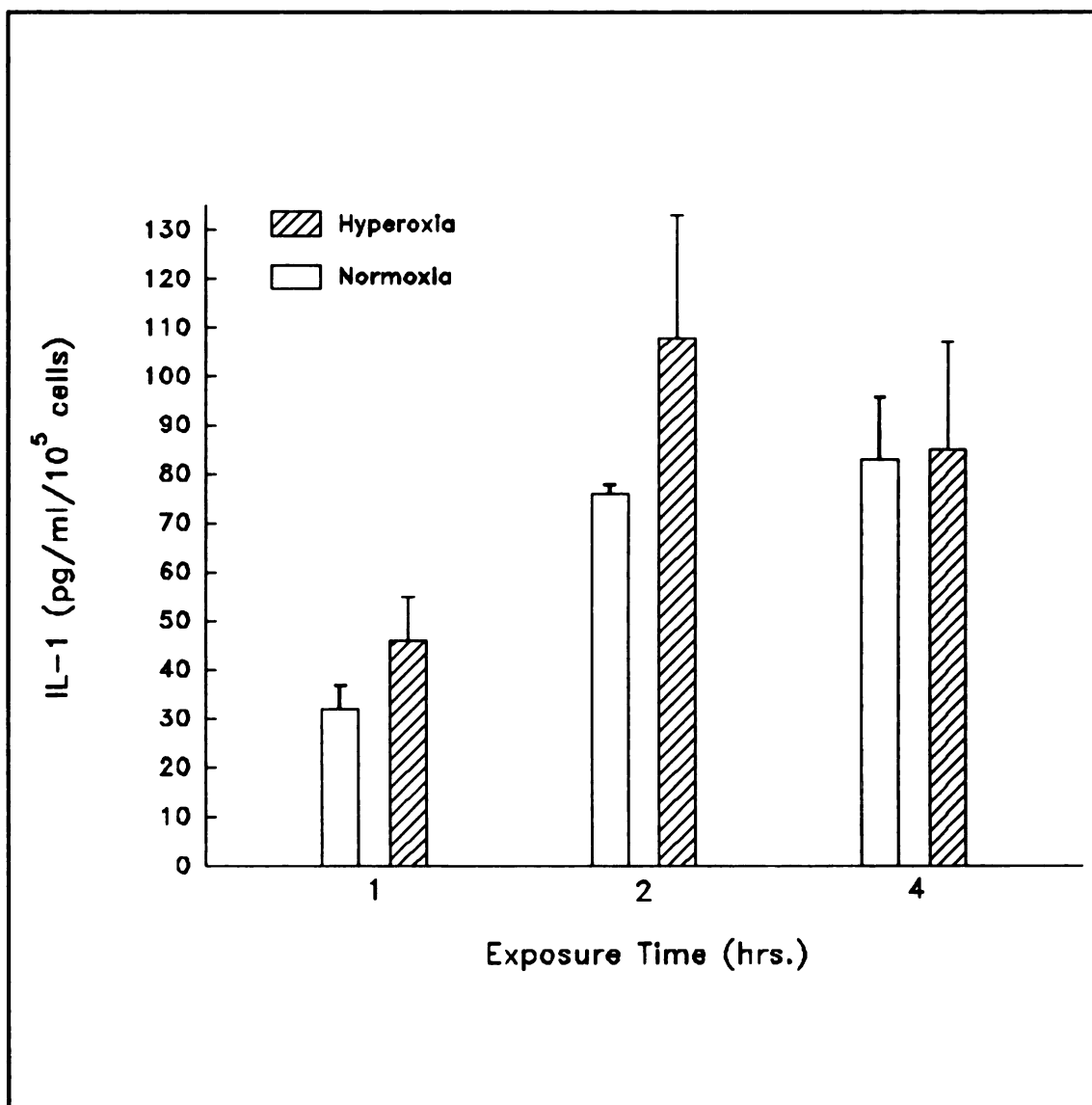


Figure 6: IL-1 productive capacities of alveolar macrophages after 1, 2, and 4 hour exposures to normoxia or hyperoxia *in vivo*. Mean $\pm$ SEM, N=7-8/grp, Student's independent t-test, significance set at $p<0.05$.

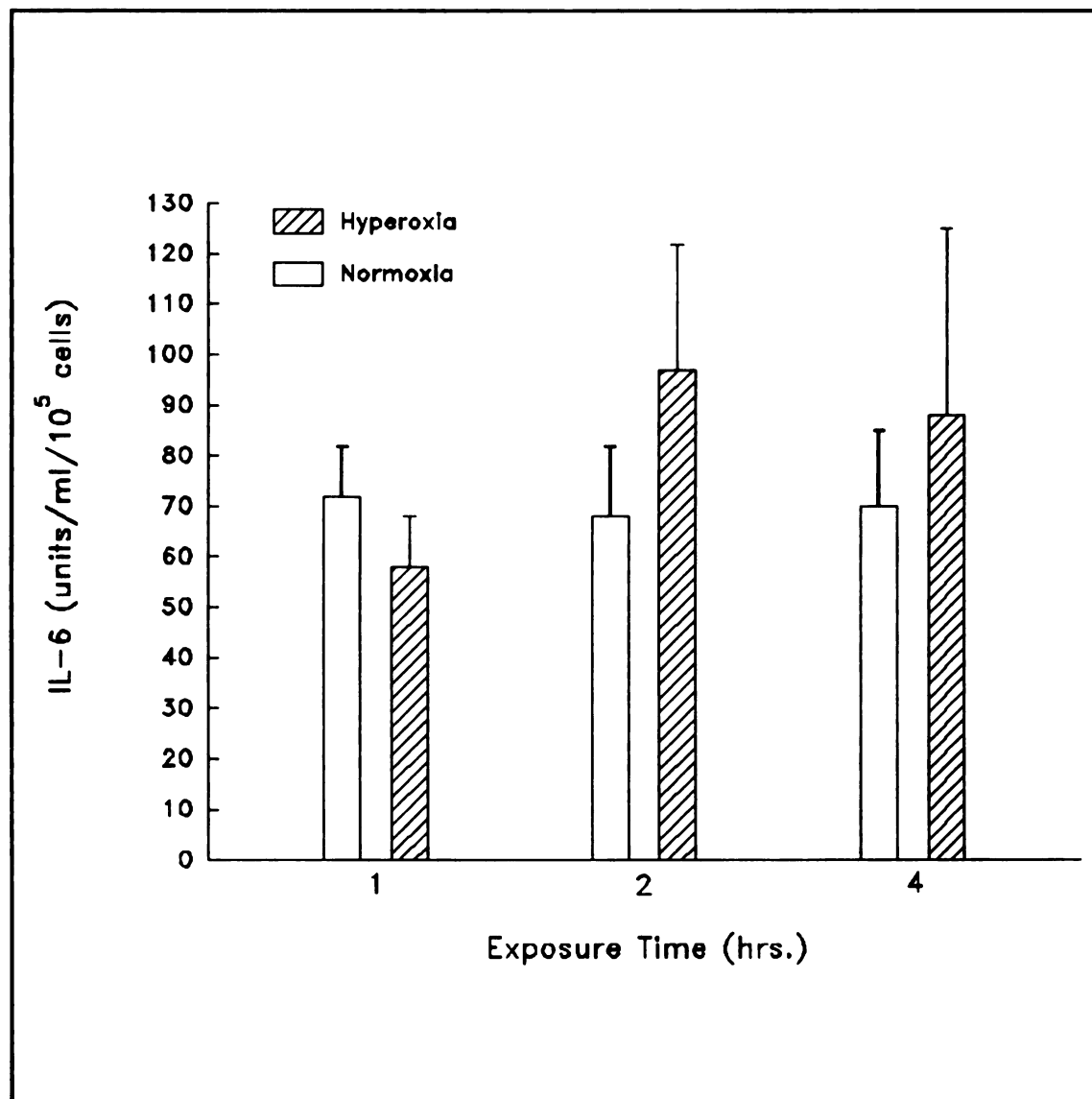


Figure 7: IL-6 productive capacities of alveolar macrophages after 1, 2, and 4 hour exposures to normoxia or hyperoxia *in vivo*. Mean $\pm$ SEM, N=7-8/grp, Student's independent t-test, significance set at $p<0.05$.

Serum Cytokine Levels After Exposure to Normoxia or Hyperoxia In Vivo:

No TNF was detected in the serum of either group at any of the time points. The serum IL-1 and IL-6 levels of those animals that had been exposed to hyperoxia were not significantly different from their respective controls (Table 3). While the same cytokine bioassay was utilized for the 1 and 4 hour determinations, a separate cytokine assay was performed for the 2 hour values. Consequently, the seemingly increased IL-1 levels at 2 hours cannot be compared to the 1 and 4 hour values. Further, the comparison was intended to be made to its respective control not to other values at another time point.

Table 3: Serum cytokine levels after 1, 2, and 4 hour exposures to hyperoxia and normoxia in vivo. Mean $\pm$ SEM, N=7-8/grp, Student's independent t-test, significance set at $p < 0.05$. ND=nondetectable.

<u>SERUM CYTOKINE LEVELS</u>						
<u>TIME (hrs)</u>	<u>TNF (U/ml)</u>		<u>IL-1 (U/ml)</u>		<u>IL-6 (U/ml)</u>	
	<u>21% O₂</u>	<u>>98% O₂</u>	<u>21% O₂</u>	<u>>98% O₂</u>	<u>21% O₂</u>	<u>>98% O₂</u>
1	ND	ND	46 $\pm$ 16	96 $\pm$ 37	14 $\pm$ 4	19 $\pm$ 4
2	ND	ND	658 $\pm$ 87	577 $\pm$ 55	8 $\pm$ 2	8 $\pm$ 2
4	ND	ND	7 $\pm$ 2	20 $\pm$ 9	18 $\pm$ 3	23 $\pm$ 7

RESULTS OF THE CELL-ASSOCIATED TNF BIOASSAY AFTER
EXPOSURE TO NORMOXIA OR HYPEROXIA IN VIVO

Preliminary data revealed no marked increase in macrophage/cell-associated TNF following 2, 3, and 4 hours of *in vivo* exposure to hyperoxia compared to their respective normoxic controls. Moreover, the amount of fluorescence/cell was barely above background for each group. Consequently, no further studies utilizing this technique were performed. Presented below are the results of two typical experiments on alveolar macrophages harvested following 3 hour in vivo exposures (Table 4).

Table 4: Cell-associated TNF values after 3 hour exposures to hyperoxia or normoxia. Results are presented as amount of fluorescence/cell from each random reading and as mean $\pm$ SEM with N=2.

<u>N</u>	<u>Average Fluorescence/Cell</u>	
	<u>21% O₂</u>	<u>>98% O₂</u>
1	29.11	29.30
	23.49	14.85
	42.82	27.55
2	2.84	15.63
	3.84	8.32
	4.96	8.03
MEAN $\pm$ SEM	17.84 $\pm$ 7.40	17.28 $\pm$ 4.12

THE EFFECTS OF HYPEROXIA ON RATS AFTER
PRETREATMENT WITH PENTOXIFYLLINE

Since PTX has been shown to downregulate macrophage production of TNF and superoxide anion, inhibit macrophage chemotaxis, decrease the inflammatory effects of TNF and IL-1, and attenuate sepsis/TNF-induced lung injury, it was chosen as the treatment agent in this project involving cytokines and pulmonary oxygen toxicity¹⁰⁶⁻¹¹¹. However, PTX has been primarily administered intravenously, and Wang et al. have demonstrated that PTX can cause marked hypotension¹¹². Consequently, an acceptable administration route and dosage had to be determined - one which would not appreciably change the healthy rat model nor result in marked hypotension while, at the same time, hopefully retaining the salutary effects of PTX.

The Effects of PTX on MAP

Prior to and after subcutaneous administration of PTX 50 mg/kg BW - a route which would not appreciably alter the healthy rat model, and a dosage which has been shown to have salutary effects, serial determinations of the MAP were made^{108,112-114}. Readings were taken every minute for 15 minutes initially - the time interval around the lowest pressure, and then periodically until the MAP returned to its original baseline level. No significant differences were found among

the MAP determinations at any of the time points with this dosage and route of administration (Figure 8). The lowest average MAP was 95.8 mm Hg which occurred 5 minutes after administration of PTX. This represented a decrease of 11.5 mm Hg from the pre-injection reading. The MAP progressively returned to its baseline level within 1 hour of PTX administration. Also at 1 hour following PTX administration, the subcutaneous weal which had been raised was barely appreciable.

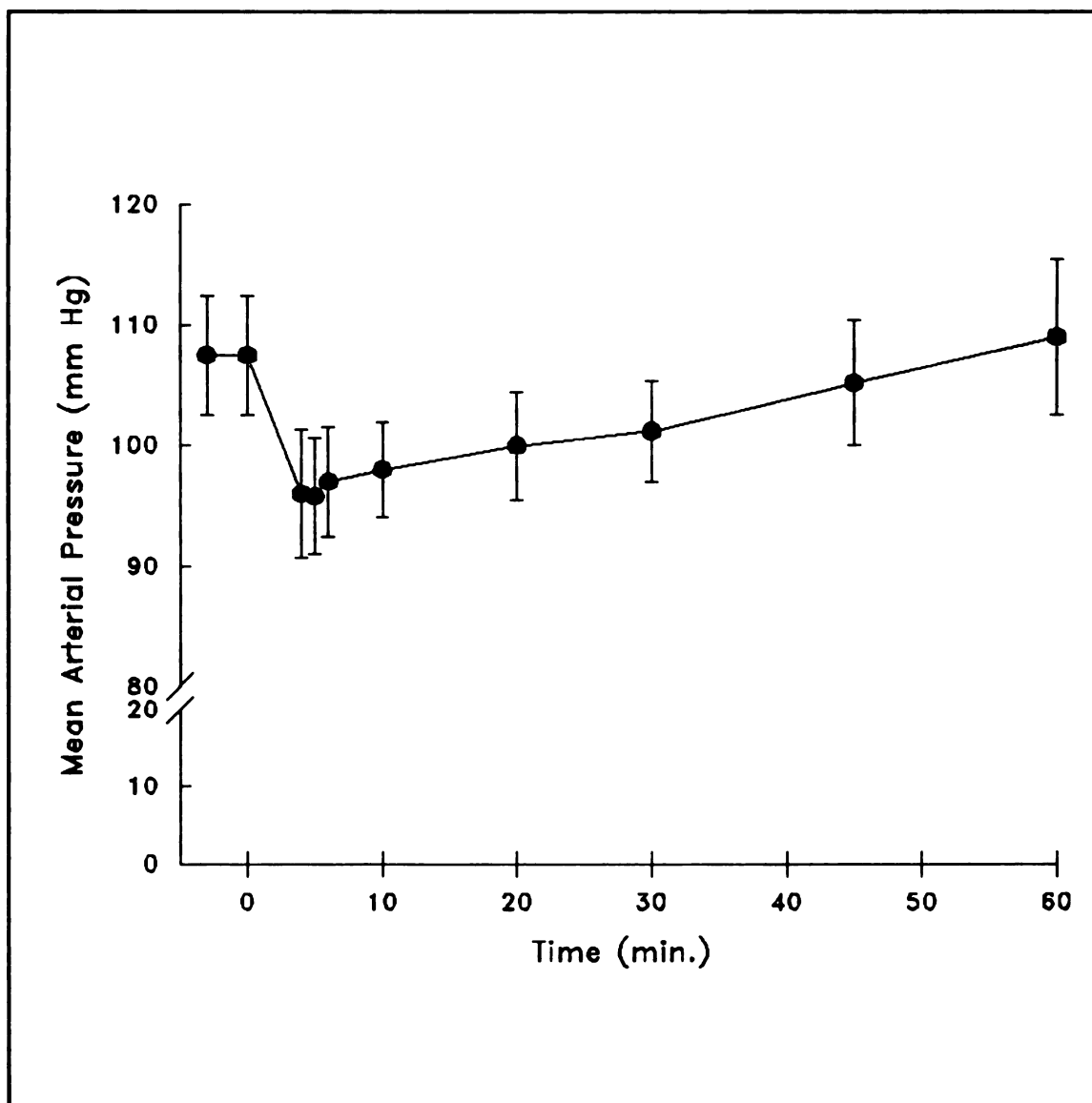


Figure 8: Time course of changes in the MAP following subcutaneous injection with PTX (50 mg/kg BW). Mean $\pm$ SEM, N=4/grp, one-way ANOVA, significance set at $p<0.05$.

The Results of Prolonged Hyperoxia After Pretreatment With PTX

In this study, the animals were divided into 4 groups: 1) those that were exposed to normoxia and were administered NS (N/NS), 2) those that were exposed to normoxia and were administered PTX subcutaneously (50 mg/kg BW) (N/PTX), 3) those that were exposed to hyperoxia (>95% O₂) and were administered NS (H/NS) and 4) those that were exposed to hyperoxia and received PTX (H/PTX).

Pretreatment with PTX appeared to attenuate the pulmonary injury that occurred with sustained lethal hyperoxia. The BAL supernatant LDH concentration of the H/PTX group was significantly decreased compared to the level of the hyperoxic/NS treated group but significantly increased compared to normoxic controls [3.67 ± 0.40 (H/PTX) vs 4.97 ± 0.40 (H/NS), 2.32 ± 0.20 (N/NS), and 2.33 ± 0.19 (N/PTX) IU/100 ml, $p < 0.05$, respectively] (Figure 9). As expected, the untreated H/NS group demonstrated markedly elevated levels compared to all groups [4.97 ± 0.40 (H/NS) vs 3.67 ± 0.40 (H/PTX), 2.32 ± 0.20 (N/NS), and 2.33 ± 0.19 (N/PTX) IU/100 ml, $p < 0.05$, respectively]. The supernatant protein concentration of the H/PTX group was markedly decreased compared to the concentration of the H/NS group, and was not significantly different from the levels of the normoxic controls [2.456 ± 0.531 (H/NS) vs 0.477 ± 0.136 (H/PTX), 0.105 ± 0.016 (N/NS), and 0.097 ± 0.013 (N/PTX) mg/ml, $p < 0.05$, respectively] (Figure 10). Further, the supernatant IL-6 concentration of

the H/PTX group was significantly decreased compared to the level of the H/NS group and, moreover, not appreciably different when compared to the normoxic controls [8.90 ± 3.58 (H/NS) vs 2.07 ± 0.71 (H/PTX), 0.0192 ± 0.0142 (N/NS), and 0.0250 ± 0.0189 (N/PTX) U/ml, $p < 0.05$, respectively] (Figure 11). Additionally, the amount of pleural fluid present in the H/PTX group was not significantly different from the normoxic controls and significantly decreased from the level of the H/NS group [1.90 ± 0.47 (H/NS) vs 0 (N/NS and N/PTX) and 0.14 ± 0.01 ml (H/PTX) ml, $p < 0.05$, respectively] (Table 5). The percentage of the lavage fluid recovered was $>80\%$ for all groups (Table 5). The percentage recovered from the H/PTX group was not appreciably different from the normoxic controls, but the percentage of effluent recovered from the H/NS group was significantly greater when compared to all other groups [86.77 ± 0.74 (H/NS) vs 82.32 ± 0.98 (H/PTX), 82.62 ± 1.22 (N/NS), and 82.17 ± 0.94 %, $p < 0.05$, respectively]. Finally, of note, no TNF or IL-1 was detected in the supernatant of any of the groups at this time point.

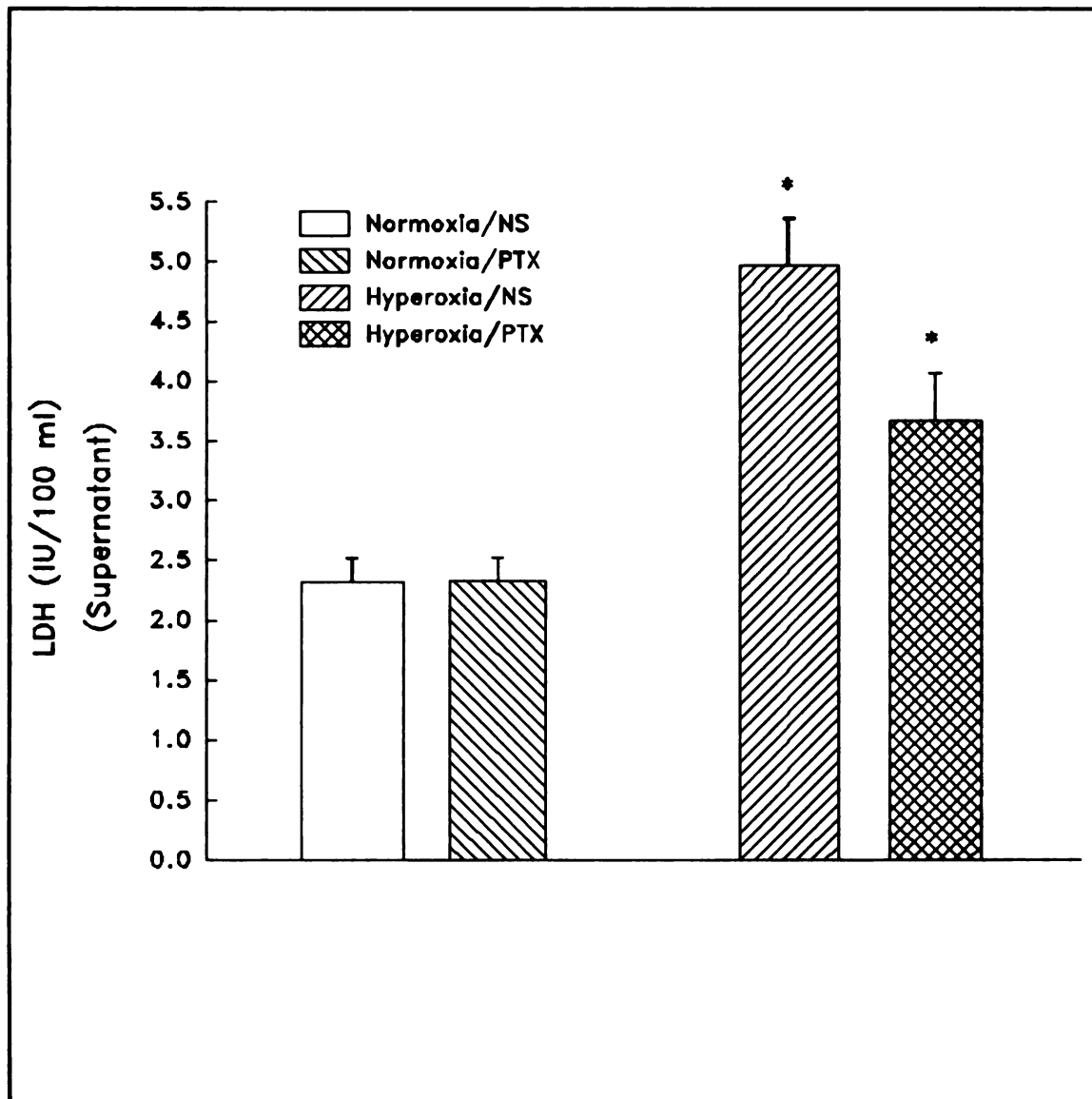


Figure 9: Supernatant LDH concentrations after 52 hr exposures to normoxia or hyperoxia with or without PTX pretreatment. Mean±SEM, N=5-6/grp, one-way ANOVA then Tukey's test, *p<0.05 vs all groups.

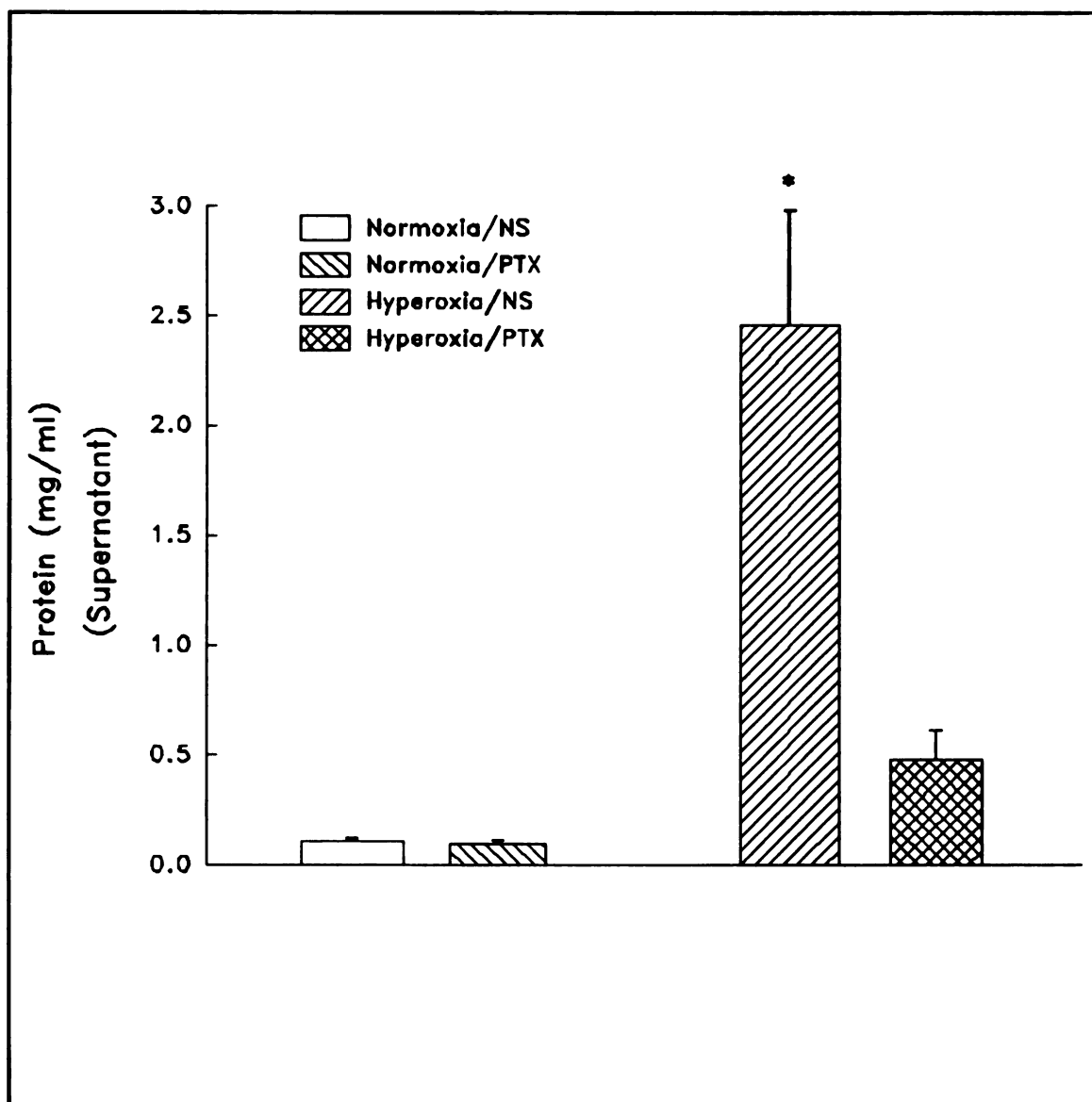


Figure 10: Supernatant protein concentrations after 52 hr exposures to normoxia or hyperoxia with and without PTX pretreatment. Mean $\pm$ SEM, N=5-6/grp, one-way ANOVA then Tukey's test, *p<0.05 vs all groups.

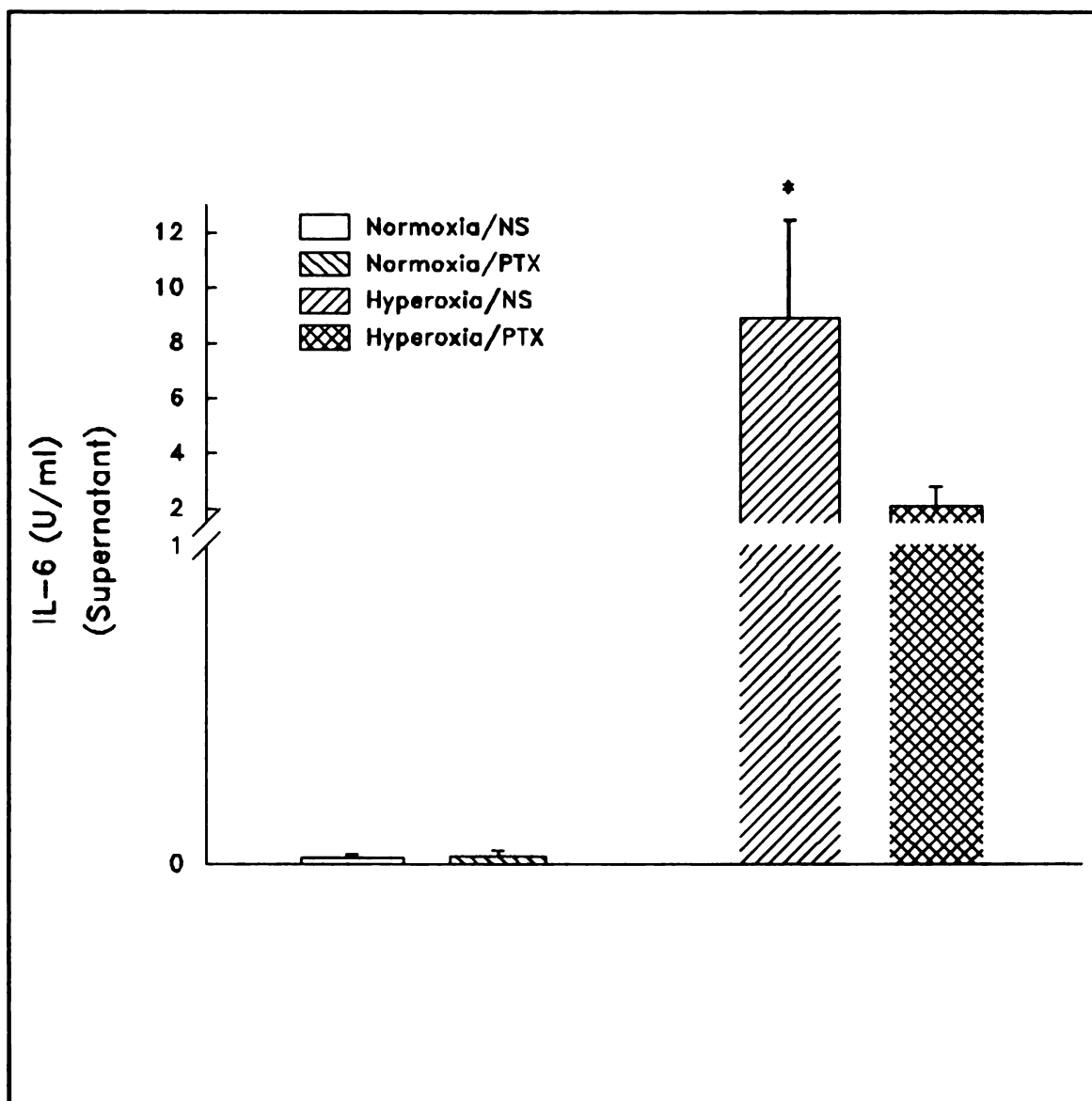


Figure 11: Supernatant IL-6 concentrations after 52 hr exposures to normoxia or hyperoxia with and without PTX pretreatment. Mean $\pm$ SEM, N=5-6/grp, one-way ANOVA then Tukey's test, *p<0.05 vs all groups.

Table 5: Parameters determined after 52 hr exposures with and without PTX. Mean $\pm$ SEM, N=5-6/grp, one-way ANOVA then Tukey's test, *p<0.05 vs all groups and †p<0.05 vs normoxic controls.

	<u>21% O₂</u>		<u>>96% O₂</u>	
Treatment	<u>NS</u>	<u>PTX</u>	<u>NS</u>	<u>PTX</u>
Hematocrit	50.2 $\pm$ 0.7	49.3 $\pm$ 0.6	59.0 $\pm$ 2.2*	53.3 $\pm$ 1.5
Pleural fluid (ml)	0	0	1.9 $\pm$ 0.5*	0.6 $\pm$ 0.6
Ascites (ml)	0	0	0	0
% change in wt	9.4 $\pm$ 0.8	9.5 $\pm$ 0.7	1.6 $\pm$ 1.1†	2.4 $\pm$ 0.6†
% lavage fluid recovered	82.6 $\pm$ 1.2	82.2 $\pm$ 0.9	86.8 $\pm$ 0.7*	82.3 $\pm$ 1.0

Pretreatment with PTX also appeared to attenuate some of the systemic effects of sustained hyperoxia. The serum IL-6 concentration of the H/PTX group was significantly decreased compared to the level of H/NS group and was not appreciably different from the values of the normoxic controls [12.33 $\pm$ 4.61 (H/NS) vs 3.34 $\pm$ 1.40 (H/PTX), 0.0680 $\pm$ 0.0446 (N/NS), and 0.860 $\pm$ 0.397 (N/PTX) U/ml, p<0.05, respectively] (Figure 12). The serum IL-1 level of the H/PTX group [701 $\pm$ 125 U/ml] was not significantly elevated compared to the values of the normoxic controls [143 $\pm$ 73 (N/NS) and 237 $\pm$ 162 (N/PTX) U/ml] nor was it significantly decreased compared to the concentration of the H/NS group [910 $\pm$ 256 U/ml]. However, the serum IL-1 concentration of the H/NS group was significantly greater compared to the normoxic controls [910 $\pm$ 256 (H/NS) vs 143 $\pm$ 73 (N/NS) and 237 $\pm$ 162 (N/PTX) U/ml, p<0.05, respectively].

Further, the hematocrit of the H/PTX group was markedly decreased and closer to within normal limits compared to the value of the H/NS (Table 5). Moreover, the hematocrit of the H/PTX was not significantly different from the normoxic controls while the hematocrit of the H/NS group was dramatically increased compared to all groups [59.0 ± 2.2 (H/NS) vs 53.3 ± 1.5 (H/PTX), 50.2 ± 0.7 (N/NS), and 49.3 ± 0.6 (N/PTX) %, $p < 0.05$, respectively]. However, pretreatment with PTX failed to demonstrate an increase in weight similar to controls [1.58 ± 1.12 (H/NS) and 2.38 ± 0.56 (H/PTX) vs 9.42 ± 0.77 (N/NS) and 9.47 ± 0.66 (N/PTX) %, $p < 0.05$, respectively] (Table 5). No differences in the serum LDH and protein concentrations were found among the groups (Figures 14 and 15). No TNF was detected in the serum of any of the animals, and no ascites was present in any of the animals.

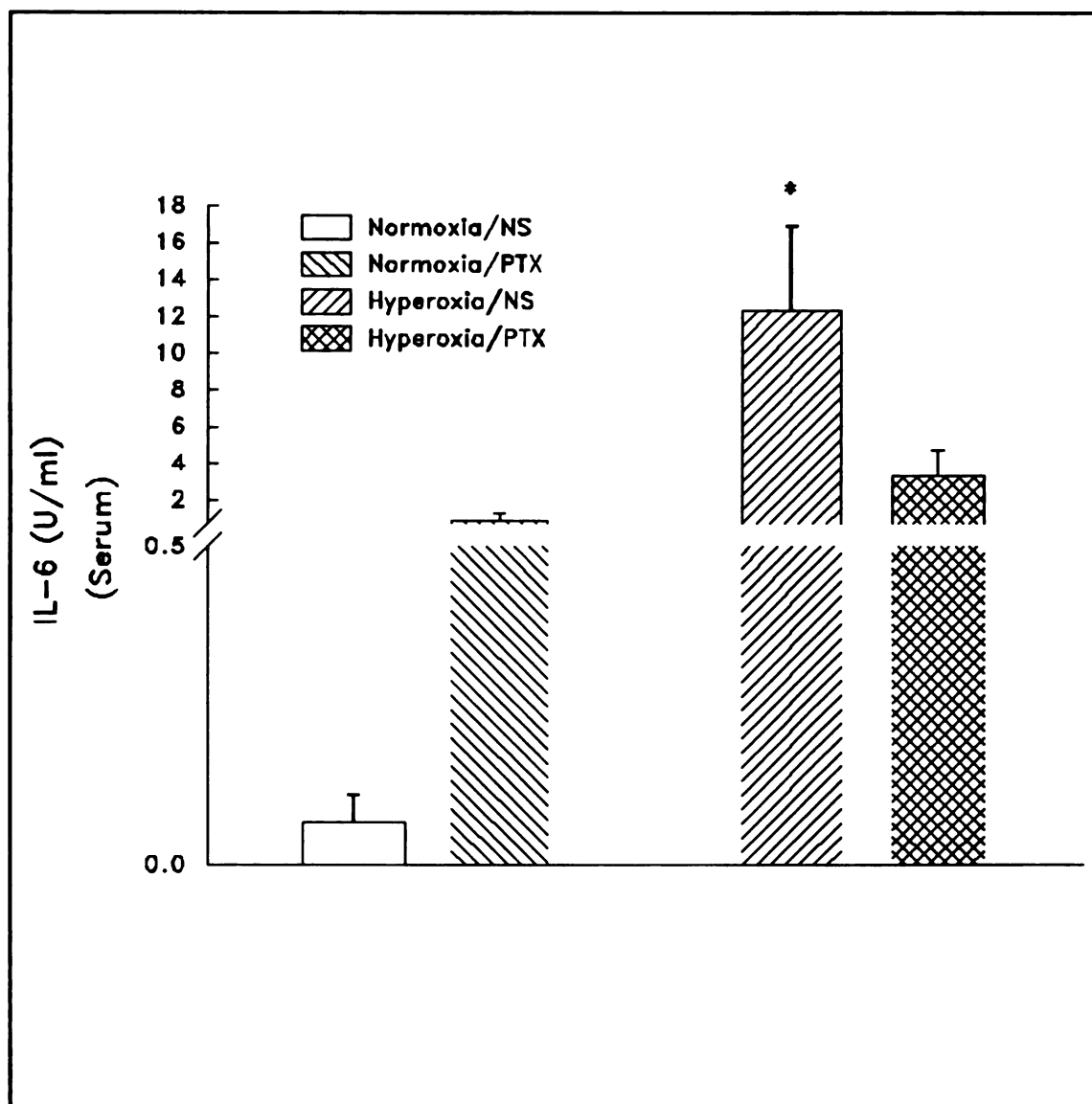


Figure 12: Serum IL-6 concentrations after 52 hr exposures to normoxia or hyperoxia with and without PTX. Mean $\pm$ SEM, N=5-6/grp, one-way ANOVA then Tukey's test, *p<0.05 vs all groups.

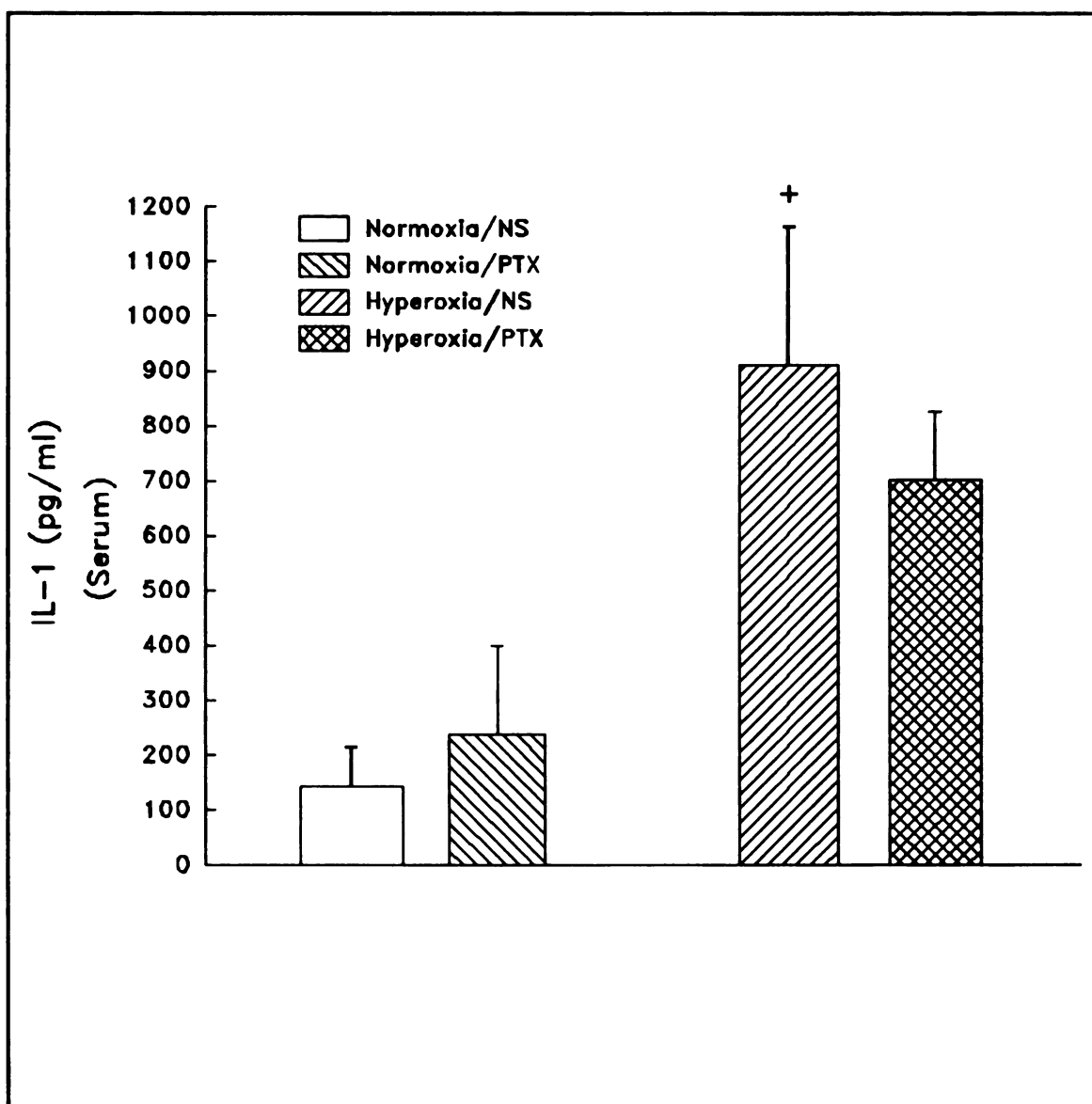


Figure 13: Serum IL-1 concentrations after 52 hr exposures to normoxia or hyperoxia with and without PTX. Mean $\pm$ SEM, N=5-6/grp, one-way ANOVA then Tukey's test, * p <0.05 vs normoxic controls.

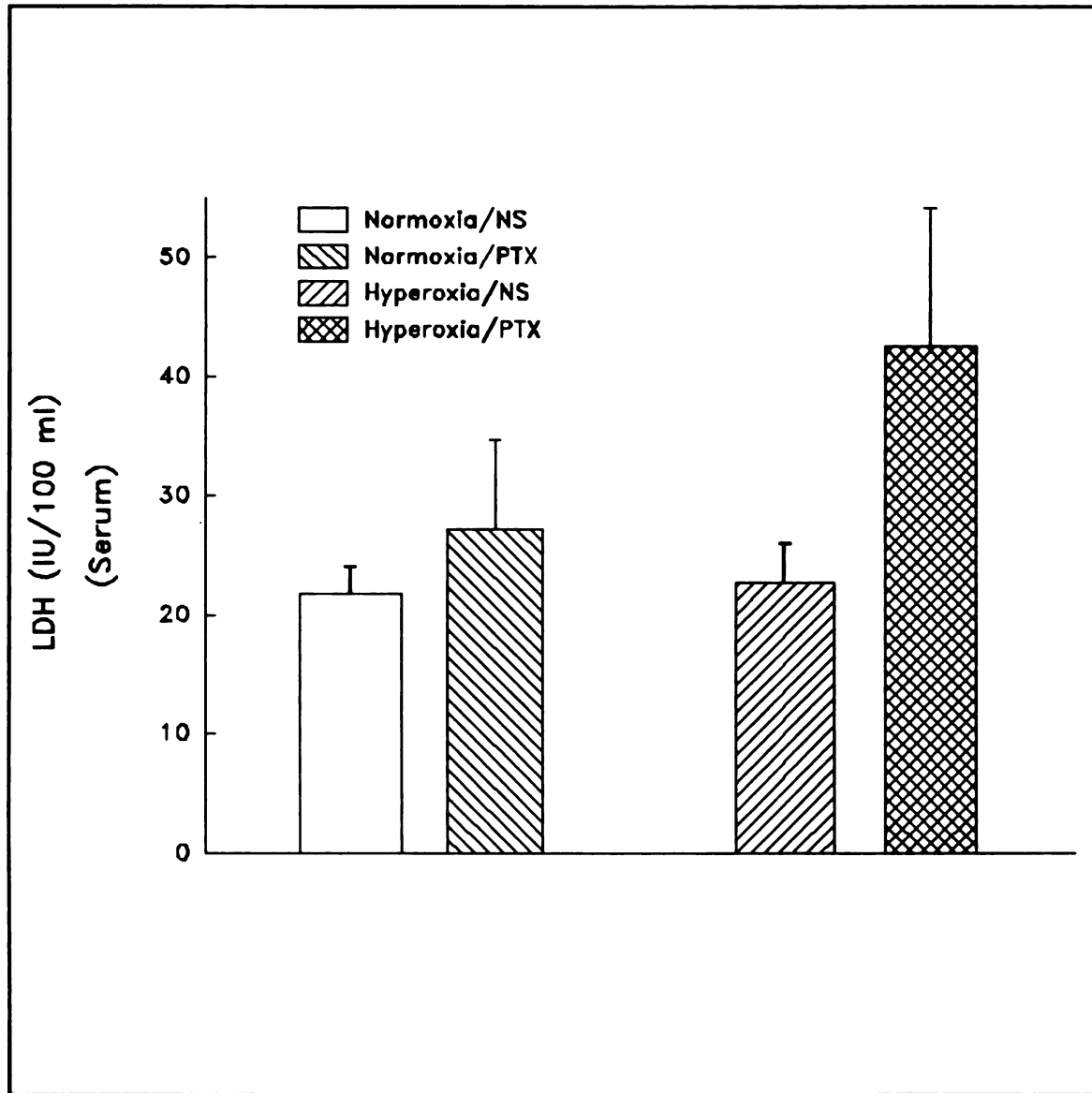


Figure 14: Serum LDH concentrations after 52 hr exposures to normoxia or hyperoxia with and without PTX. Mean±SEM, N=5-6/grp, one-way ANOVA then Tukey's test, significance set at $p<0.05$.

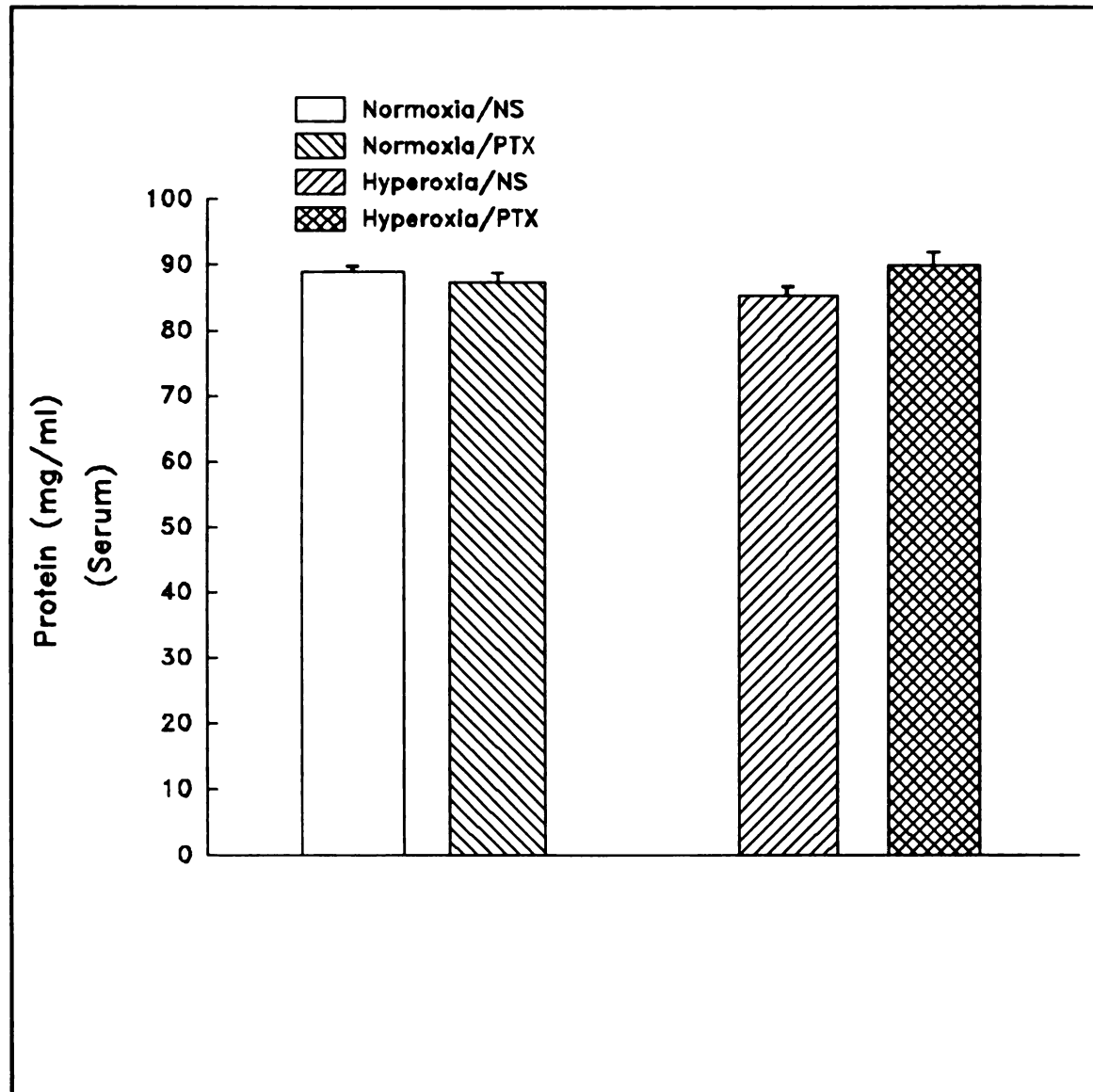


Figure 15: Serum protein concentrations after 52 hr exposures to normoxia and hyperoxia with and without PTX. Mean $\pm$ SEM, N=5-6/grp, one-way ANOVA then Tukey's test, significance set at $p<0.05$.

DISCUSSION

GENERAL DISCUSSION OF OXYGEN-INDUCED LUNG INJURY

Delivery of 100% oxygen to a patient via the mechanical ventilator for 48 hours was considered safe as no significant pulmonary morphologic changes were thought to occur. However, experimental work in healthy humans without pulmonary pathology has demonstrated that injury to the airway does appear within this time period². Moreover, recent work involving healthy rat lungs has revealed that pulmonary parenchymal injury can occur within 24 hours of exposure to 100% O₂²⁷.

Additionally, maintaining the FiO₂ below 50-60% was believed to protect against oxygen-induced lung injury in those requiring mechanical ventilation^{14,23,44,115,116}. However, concurrent inflammation or administration of pharmacologic agents lowers this toxic threshold^{8,11,13}. Indeed, administering an FiO₂ of only 33% may result in severe pulmonary damage in those previously exposed to bleomycin.

The histopathology and mechanism of pulmonary oxygen toxicity is present in many of the acute lung injuries seen in

the general surgery population. Those individuals suffering from sepsis, hemorrhagic shock, severe trauma, complicated intraabdominal surgery, and ARDS may develop an acute lung injury with the same histopathology that is apparent with pulmonary oxygen toxicity^{1,14}. Moreover, the acute lung injury that may develop in patients experiencing pancreatitis, ischemia-reperfusion injury, thermal injury, and sepsis implicates oxygen free radicals as the mechanistic agent - the same mechanism which is believed to be responsible for oxygen-induced lung injury¹⁵⁻²². These individuals, then, are apparently quite at risk for developing a pulmonary injury very similar, if not identical, to that seen in pulmonary oxygen toxicity. Consequently, delivering any increased oxygen concentrations - particularly to the general surgery population - may be deleterious under certain circumstances.

The initiation stage of pulmonary oxygen toxicity, the first 40-48 hours in the healthy rat model, is characterized by the production of oxygen free radicals²⁶. Little morphologic changes were thought to occur during this time period. However, recent work has demonstrated that indeed atelectatic changes, vascular congestion, edema accumulation, and fibrin deposition have been observed within 24 hours²⁷. Over the next 24 hours, these changes worsen and, after 48 hours, are accompanied by a marked inflammatory response^{25,27,37}. Within 72 hours the rat is thought to die from respiratory failure^{36,39,83,85,117}.

The culprit of these pathologic changes is believed to be

the overwhelming production of reactive oxygen species^{1,6,39}. More recently, experimental work has investigated the interrelationships among hyperoxia, oxygen free radicals, and cytokines, particularly TNF and IL-1. Reactive oxygen species and TNF and IL-1 have been shown to augment production of each other by stimulated macrophages^{60,61}. Furthermore, pretreatment with TNF or IL-1 and subsequent exposure to hyperoxia led to decreased lung injury and increased survival^{65,66}. Finally, pretreatment with IL-6 is known to attenuate the effects of acute pulmonary inflammation¹¹⁸. However, to date, no one has demonstrated that hyperoxia directly results in the increased production or cellular productive capacities of the cytokines TNF, IL-1, or IL-6. If increased production or productive capacities of the cytokine(s) is demonstrated, then, perhaps treatment aimed at downregulating their production would be efficacious - as attempts at augmenting the antioxidant defense system has not been overwhelmingly successful.

ALVEOLAR MACROPHAGE CYTOKINE PRODUCTIVE CAPACITIES
AFTER EXPOSURE TO HYPEROXIA

This project is the first to demonstrate that hyperoxia directly results in the increased macrophage productive capacities of particular cytokines. The cytokines, TNF, IL-1, and IL-6, were studied as they are involved in the "acute phase response" of inflammation and have been demonstrated to be involved in pulmonary inflammation^{51,119}. We chose to examine the alveolar macrophage since cells of the macrophage lineage are one of the primary sources of TNF and IL-1. More recently, IL-6 has also been shown to be produced in significant amounts by macrophages. Additionally, macrophages are a component of the innate immune system and consequently, should be among the first cells to respond to injury^{52,119}.

Alveolar macrophages exposed to hyperoxia demonstrated marked increases in their cytokine productive capacities. Those alveolar macrophages exposed to hyperoxia in vitro demonstrated a significant, nearly 2-fold increase in their IL-1 productive capacity after exposure to 95% O₂ for 1 hour. Though trending upward at 30 minutes, the IL-1 productive capacity was not significantly increased at this time. Further, those alveolar macrophages exposed to hyperoxia in vivo for 2 hours resulted in marked significant increase in their TNF productive capacity. This increased cytokine productive capacity of the alveolar macrophage means that the cell is "primed" or upregulated to produce increased amounts

of the particular cytokine should a sufficient stimulus occur while the cell remains in this state. In these studies, IL-1 and TNF productive capacities were the ones upregulated. These results appear to be consistent with those of Chaudri and Clark in which reactive oxygen species resulted in increased TNF production from stimulated peritoneal macrophages⁶⁰. This early enhanced alveolar macrophage TNF and IL-1 productive capacity may be the mechanism responsible for the primary effect on the lung which predisposes it to further injury as noted by Kreiger et al.³⁷. It also appears that after short exposures to either hyperoxia or hypoxia, upregulation of the alveolar macrophages to produce TNF and IL-1 occurs¹²⁰. Increased production of these cytokines may contribute to further pulmonary inflammation^{57,75}.

Exposure of the animals to hyperoxia or normoxia for 1, 2, and 4 hours did not demonstrate any significant differences in the serum cytokine levels between a particular hyperoxic group and its control. In fact, no TNF was detected in the serum of any of the animals. This is consistent with the data which demonstrated that the macrophages exposed to hyperoxia in vivo were primed but not innately producing more TNF. The serum IL-1 and IL-6 levels were not significantly different between each particular hyperoxic group and its control at each time point.

No significant differences in the cell counts of the viable alveolar macrophages were demonstrated between the hyperoxic group and its normoxic control at any of the time

points of the in vivo exposed macrophages. However, the number of cells obtained were approximately half or less compared to the number others obtained^{28,120,121}. The increased number of macrophages obtained by these individuals may be secondary to the fact that the excised lungs were lavaged, the bronchus of the lung was selectively cannulated and the lung subsequently lavaged, or the number of lavages was increased. All of these methods may lead to increased yield of macrophages due to more thorough lavage.

The arterial blood gas determinations revealed a marked increase in the arterial oxygen tension of those animals exposed to $\geq 98\%$ O_2 for 1 hour. No significant differences were demonstrated in the arterial CO_2 tension or pH. These results are important in that they confirm the external O_2 saturation as measured by the oxygen analyzer, and that the animal's arterial oxygen tension is markedly elevated within 1 hour of exposure to hyperoxia. These ABG values are fairly consistent with those obtained by Garner et al. who also utilized Sprague-Dawley rats, but differ from studies utilizing lambs in that the PaO_2 was higher in the lamb model^{83,122}.

DETERMINATION OF CELL-ASSOCIATED TNF AFTER
EXPOSURE TO EITHER HYPEROXIA OR NORMOXIA

In an effort to further demonstrate that the alveolar macrophage was upregulated to produce TNF, determination of the plasma membrane-associated TNF was carried out near the time point which revealed an increased TNF productive capacity, i.e., 2 hours. An increase in cell-associated TNF might explain the primarily local effect hyperoxia has on the lungs. Studies have demonstrated that Kupffer cells have an increase in cell-associated TNF and IL-1 during sepsis; similarly, alveolar macrophages have an increase in cell-associated TNF and IL-1 after induction of immune-complex alveolitis^{57,123,124}. Preliminary results failed to demonstrate any significant differences between the hyperoxic groups and their controls at 2, 3, and 4 hours in the amount of cell-associated TNF expressed. The fluorescence values obtained were of low intensity and barely above that of background levels. The inability to detect an increase in the amount of alveolar macrophage-associated TNF is most likely due to the lack of a LPS challenge. This is not surprising since the macrophages were upregulated or primed and not innately producing more TNF. Consequently, in order to simulate the previous hyperoxic and normoxic exposures more closely, the alveolar macrophages should have been challenged with LPS after exposure to the particular concentration of oxygen and prior to determination of cell-associated TNF levels.

EFFECTS OF EXPOSURE TO LETHAL HYPEROXIA WITH AND WITHOUT
PRETREATMENT WITH PENTOXIFYLLINE

Since the alveolar macrophage TNF and IL-1 productive capacities were shown to be upregulated early after exposure to hyperoxia by the previous studies, treatment aimed at the down-regulation of these cytokines may attenuate the effects of oxygen-induced lung injury. Pentoxifylline (PTX), a methylxanthine derivative, was chosen as the treatment agent for a variety of reasons.

PTX, also known by the trade name Trental, has been widely utilized in the treatment of intermittent claudication secondary to peripheral vascular disease¹²⁵. The usual dose for this condition is 400 mg taken orally three times a day or, assuming a 70 kg adult, 16.5 mg/kg/24 hr¹²⁶. The pharmacokinetics of the parent compound and Metabolite I, one of two primary active metabolites, are dose-related and nonlinear - with half-life and area under the blood-level time curve increasing with dose. The elimination kinetics of Metabolite V, the other primary active metabolite, is not dose-dependent. The plasma half-lives of PTX and its metabolites at the usual doses and assuming no hepatic or renal insufficiency are 0.4-0.8 hrs and 1-1.6 hrs, respectively. The mechanism of action is considered to be the increased deformability of the red blood cells and decreased blood viscosity resulting in increased oxygenation. However, in humans, beneficial effects usually are not seen until at

least 2 weeks after the initiation of therapy.

Over the last few years, PTX has been shown to have significant anti-inflammatory effects. Unlike the clinical situation for peripheral vascular disease, however, these effects are immediate. Further, the route of administration and the dosage and dosing interval are different.

In a murine model of trauma-hemorrhage shock, PTX has been shown to decrease serum levels of TNF and IL-6 and restore depressed hepatocellular function within 4 hours of resuscitation¹¹². PTX has also been shown to increase survival and decrease TNF formation in a murine model of endotoxin shock^{108,114}. Decreased levels of TNF in the serum, decreased TNF productive capacities of peritoneal macrophages, and decreased pulmonary sequestration of neutrophils in those treated with PTX were demonstrated.

Others have also demonstrated that PTX attenuates the acute lung injury that occurs with sepsis, endotoxin, and TNF^{110,113,127}. They have attributed the decreased pulmonary sequestration of neutrophils as being responsible for the attenuated lung injury; however, neutrophils appeared to be necessary for PTX to be beneficial¹²⁸. PTX may exert its effects by decreasing neutrophil phagocytic function, decreasing neutrophil adhesiveness to endothelial cells, or reducing neutrophil superoxide anion and hydrogen peroxide generation¹²⁹⁻¹³¹. Studies have demonstrated that PTX may attenuate endotoxin-induced lung injury as well as other pulmonary inflammatory processes. PTX accomplishes this by

decreasing TNF production via inhibiting the rate of TNF mRNA production (transcription) by peritoneal exudate cells or macrophages, inhibiting superoxide anion generation by alveolar macrophages, and inhibiting macrophage chemotaxis in a dose-dependent fashion^{106,107,132,133}.

In order for PTX to protect against sepsis/endotoxin/TNF-induced lung injury, large doses must be administered. Many have utilized 20-25 mg/kg BW IV bolus followed by 6-20 mg/kg/hr continuous infusion for 1.5-8 hrs^{110,112,113,128,134,135}. This is in contrast to the 16.5 mg/kg/24 hrs recommended for treatment of peripheral vascular disease in humans. Others have given a more moderate dose consisting of a single 50 mg/kg IP (intraperitoneal) injection or, yet, even less, a 17 mg/kg IM (intramuscular) injection daily^{106,108,136}.

Consequently, for this project, PTX was administered as a 50 mg/kg BW subcutaneous bolus just prior to exposure to hyperoxia. The timing of our injection was based on our earlier observation that the TNF productive capacity of the alveolar macrophages were upregulated shortly following exposure to hyperoxia in vivo. Therefore, pretreatment just prior to exposure may attenuate oxygen-induced lung injury. Further, while PTX has been shown to reduce serum TNF in a dose-dependent fashion, its salutary effects on pulmonary damage were seen only if given within 30 minutes following onset of injury^{106,114,127,135}. The dose of 50 mg/kg BW was chosen as this appeared to be a moderate, mid-range concentration which the literature implied would still provide protection

against TNF-induced lung injury (see above). Subcutaneous injections were chosen as the route of administration because of the desire to avoid excessive manipulation of the healthy rat model (eg., intravenous catheterization necessitating anesthesia and surgery) and avoid intraabdominal injury which may occur with IP injections. Further, our preliminary results revealed that the rodents demonstrated profound hypotension when 50 mg/kg BW of PTX was injected intramuscularly.

As PTX had not been delivered by the subcutaneous route, and as PTX has vasodilatory effects which might induce profound hypotension, it was necessary to establish that this was a safe dose and route of administration¹¹². The MAP was not significantly different at any of the time points and was noted to decrease an average of 11.5 mm Hg. This is consistent with others' work in which PTX was administered intravenously^{112,128,134}. Further, the MAP was noted to return to baseline levels within 1 hour of PTX administration.

To determine if PTX would attenuate oxygen-induced lung injury, a specific time of exposure to hyperoxia would have to be chosen. An exposure time of 52 hours was selected as this is within the inflammatory stage of oxygen toxicity, i.e., the period during which the rat begins to show evidence of distress and well-demonstrated evidence of pulmonary damage has occurred²⁵⁻²⁷. Further, others have utilized 48-55 hour time period in their work^{28,66,67,101,137}.

Clearly, animals exposed to $\geq 95\%$ O₂ suffered significant

pulmonary damage as evidenced by increased BAL supernatant concentrations of LDH and protein and increased accumulation of pleural fluid. These indices have been utilized by others as evidence of pulmonary injury secondary to hyperoxia as well as other various insults^{28,29,31,32,37,67,68,73,101-103,137,138}. Additionally, markedly increased BAL supernatant concentrations of IL-6 were present in those animals that were exposed to hyperoxia indicating that this cytokine probably is associated with oxygen-induced lung injury.

Pretreatment with PTX led to marked attenuation of pulmonary injury. Those rats pretreated with PTX and exposed to hyperoxia showed a marked decrease in the supernatant concentrations of LDH and protein as well as a decline in the amount of pleural fluid present compared to those who were exposed to hyperoxia and did not receive treatment. Further, except for the LDH supernatant level, these decreased values were not significantly different from the normoxic controls. Moreover, the supernatant concentration of IL-6 of those exposed to hyperoxia and pretreated with PTX was markedly reduced compared to those which suffered exposure to hyperoxia without treatment. This reduced level, too, was not significantly different from the normoxic controls. This further supports the involvement of IL-6 in the development of pulmonary oxygen toxicity. To our knowledge, this is the first direct evidence that IL-6 may be associated with this type of injury.

Exposure to hyperoxia also led to other systemic changes.

Hemoconcentration as evidenced by increased hematocrit values in those exposed to hyperoxia was present in this study and has been recognized by others^{25,67}. This most likely was secondary to extravasation of intravascular fluid into the thoracic cavity and probably the pulmonary parenchyma as evidenced by the accumulation of pleural fluid and lack of ascites. Additionally, hyperoxia led to failure to thrive as evidenced by lack of weight gain; others have observed weight loss¹³⁹. As noted earlier by Roth, hyperoxia did not result in increased serum LDH and protein concentrations¹⁰¹. Perhaps most important from a systemic perspective, hyperoxia led to increased serum IL-1 and IL-6 concentrations. This is the first study showing that elevated levels of these cytokines have been observed in oxygen-induced lung injury.

PTX attenuated some of these systemic effects. Those pretreated with PTX and exposed to hyperoxia developed a hematocrit value and serum IL-6 level that were not significantly different from controls. The serum IL-1 level, though not significantly different from the normoxic controls, was also not significantly decreased from the untreated hyperoxia-exposed animals. Further, PTX did not improve the animals' weight gain amidst exposure to high oxygen concentrations.

These findings raise several interesting questions. First, which cell(s) are responsible for the increased BAL concentrations of IL-6? Secondly, what is the relationship between the earlier findings of increased IL-1 and TNF

productive capacities of alveolar macrophages after a short exposure to hyperoxia and the increased BAL IL-6 concentrations after exposure to hyperoxia for 52 hours? Thirdly, what is the mechanism by which PTX attenuates oxygen-induced lung injury and decreases BAL supernatant IL-6 concentrations? Fourthly, what do the increased serum IL-1 and IL-6 concentrations after exposure to sustained hyperoxia mean? Finally, what does the attenuation of serum IL-6 concentration by pretreatment with PTX tell about the role of cytokines in pulmonary oxygen toxicity?

As the increase in BAL IL-6 concentration occurred within 52 hours, *i.e.*, during the initiation or inflammatory phases, alveolar macrophages and endothelial cells seem to be the most likely sources of IL-6. Alveolar macrophages are components of the innate immune system, and endothelial cells are among the first cells morphologically affected by hyperoxia^{25,27}. Fibroblasts are unlikely as they are thought to be a critical component later on in those situations when lesser concentrations of oxygen are inspired, *i.e.*, during the proliferative phase^{24,25}. Moreover, it seems unlikely that the BAL supernatant IL-6 levels were elevated due to the increased serum IL-6 concentrations diffusing through capillary-alveolar barrier as the comparative elevation of serum IL-1 levels did not result in increased supernatant IL-1 despite the fact that IL-1 is a smaller polypeptide than IL-6⁵¹.

The earlier findings of increased alveolar macrophage productive capacities of IL-1 and TNF shortly after exposure

to hyperoxia also suggest in part that alveolar macrophages or endothelial cells may be responsible for the increased supernatant concentration of IL-6. Others have demonstrated that activated macrophages can release IL-6, along with TNF and IL-1, in a coordinated fashion. More precisely, TNF can induce IL-1 release from macrophages or endothelial cells, and IL-1 can induce production of IL-6 from macrophages and endothelial cells^{15,78-80}. Though TNF or IL-1 were not detected in the supernatant at 52 hours, they possibly could have been produced earlier and are no longer present in significant amounts at that time^{59,119}. Additionally, IL-6 may be the mediator through which neutrophils inflict further damage on the pulmonary parenchyma since IL-6 has been shown to stimulate neutrophilic lysozyme secretion and prime the neutrophils to generate an enhanced respiratory burst¹⁴⁰. Possibly, then, a sequence of cytokine production is involved. This cytokine involvement may be responsible for the priming of the lung for further damage - independent of neutrophils - to which Krieger et al. were referring to in their studies of pulmonary oxygen toxicity³⁷.

This priming may involve interaction between cytokines and LTB₄ with a resultant effect on neutrophils. LTB₄ is known to induce the generation of TNF in BAL supernatant and is thought to be an intermediary responsible for the neutrophil diapedesis¹⁴¹. Moreover, TNF and IL-1 have demonstrated the ability to induce synthesis of an endothelial cell surface factor(s) which promotes increased neutrophil adherence¹⁴². So

possibly the finding by others that LTB_4 is responsible for the accumulation of neutrophils in pulmonary oxygen toxicity actually involves the interaction of LTB_4 and cytokines⁸⁸.

PTX was shown to attenuate oxygen-induced lung injury as evidenced by decreased levels of supernatant LDH and protein and accumulation of pleural fluid. Moreover, PTX resulted in decreased supernatant concentrations of IL-6. As mentioned earlier, this drug is known to have a short half-life and to decrease the TNF production by inhibiting TNF mRNA production^{106,133}. Wang et al. demonstrated decreased serum TNF concentrations as well as IL-6 levels with PTX treatment in a trauma-hemorrhage model¹¹². Since PTX was administered immediately prior to exposure to hyperoxia, and earlier it was demonstrated that the TNF productive capacity of the alveolar macrophage was increased shortly after exposure to hyperoxia, possibly PTX lowered the IL-6 supernatant concentration by inhibiting TNF production. Unfortunately, the precise mechanisms by which IL-6 is involved in oxygen toxicity and PTX attenuates oxygen-induced lung injury cannot be established from this work.

Prolonged hyperoxia also resulted in increased serum concentrations of IL-1 and IL-6. Further, pretreatment with PTX demonstrated a serum IL-6 concentration which was decreased and an IL-1 concentration which appeared to be trending upward though not significantly different from the normoxic controls and untreated hyperoxia-exposed animals. Taken together, this suggests an association and chronology

between IL-1 and IL-6, i.e., IL-6 elevation appears to follow the IL-1 increase. Serum TNF levels possibly could have been elevated prior to 52 hours though this was not demonstrated. Indeed this would not be unexpected in light of studies involving a hemorrhage model in which TNF is increased shortly after hemorrhage is induced¹¹⁹. The TNF level then declines to insignificant levels but is responsible for initiating a cytokine cascade. Further, if PTX inhibits TNF production, as hypothesized earlier, then the decreased IL-6 level would be consistent with the work of Wang et al.¹¹².

The source(s) of the increased serum cytokines were not determined in this project. The lung appears to have the capability to produce TNF - and possibly other cytokines - systemically⁵⁶. However, others have shown that although ARDS may result in increased BAL supernatant TNF and alveolar macrophage production of IL-1, the increased serum concentrations of TNF and monocyte production of IL-1 may be the result of ongoing shock and sepsis as opposed to the ARDS process itself^{59,143,144}. Therefore, possibly another insult was involved which led to the increased serum levels of IL-1 and IL-6.

Certainly in this work, a degree of hypovolemia is suggested in those animals exposed to hyperoxia by the accumulation of intravascular fluid into the thoracic cavity, the increased hematocrit values, and the lack of weight gain. Whether this amount of hypovolemia is sufficient to result in increased serum concentrations of cytokines - as occurs in

hemorrhagic and septic/endotoxin-induced shock - cannot be determined from this work, although it should be investigated^{16,17,71,90,112,119,123,145-150}. Virtually all the rat models utilized, including the one in this project, have been given food and water ad libitum. Certainly as the rat becomes more distressed his oral intake is inadequate as evidenced by the lack of weight gain in those animals exposed to hyperoxia. Further studies should be done with the MAP being continuously monitored and adequate intravascular fluid administered to determine if this hypovolemia is a significant concern and the model heretofore employed adequate.

SUMMARY AND CONCLUSIONS

SUMMARY

This project determined that the cytokines, TNF, IL-1, and IL-6, are involved in pulmonary oxygen toxicity. The IL-1 and TNF productive capacities of the alveolar macrophages were upregulated shortly after exposure to hyperoxia in vitro and in vivo, respectfully. Also, those animals exposed to sustained hyperoxia demonstrated an increased BAL supernatant concentration of IL-6. Pretreatment with PTX led to attenuation of the BAL supernatant IL-6 concentration as well as the indices of pulmonary injury. This suggests that supernatant IL-6 concentration is associated with the severity of pulmonary injury.

Further, those animals exposed to prolonged hyperoxia demonstrated increased serum IL-1 and IL-6 concentrations with associated hemoconcentration and accumulation of pleural fluid. Pretreatment with PTX led to attenuation of serum IL-6 levels and decreased the degree of hemoconcentration and pleural fluid accumulation. All animals exposed to hyperoxia for prolonged periods failed to gain weight despite being

allowed food and water ad libitum. These findings suggest that hypovolemia may be involved. If so, this model of hyperoxia - which is widely utilized - may need to be modified.

In summary, the findings are the following:

1. Alveolar macrophages demonstrated an enhanced IL-1 productive capacity after exposure to hyperoxia in vitro for 1 hour.
2. Alveolar macrophages demonstrated an enhanced TNF productive capacity after exposure to hyperoxia in vivo for 2 hours.
3. Serum TNF, IL-1, and IL-6 concentrations were not elevated after exposure to hyperoxia for 1, 2, or 4 hours.
4. Prolonged hyperoxia resulted in increased BAL supernatant concentrations of IL-6 which may be associated with the severity of pulmonary oxygen toxicity.
5. Prolonged hyperoxia led to increased systemic serum concentrations of IL-6 and IL-1.
6. PTX attenuated oxygen-induced lung injury and decreased the BAL supernatant concentrations of IL-6 which further supports its association with the severity of oxygen-induced lung injury.
7. PTX attenuated the systemic serum concentration of IL-6.
8. The hemoconcentration, leakage of intravascular fluid, and lack of weight gain indicate that inadequate oral intake has occurred and that hypovolemia may be involved.

Consequently, allowing food and water ad libitum only may be inadequate.

CONCLUSIONS

These studies demonstrated that cytokines - specifically TNF, IL-1, and IL-6 - are involved in oxygen toxicity. The alveolar macrophages are primed shortly after exposure to hyperoxia to produce IL-1 in vitro and TNF in vivo. Additionally, both the BAL supernatant and serum IL-6 levels were elevated in those animals exposed to prolonged hyperoxia. Further, PTX pretreatment was shown to markedly attenuate pulmonary oxygen toxicity as well as decrease the BAL supernatant and serum IL-6 concentrations. Though the precise mechanism requires further investigation, this may occur through inhibiting production of TNF with resultant decrease in IL-6 levels. In contrast to other injuries in which the time of insult cannot be predicted and consequently adequate pretreatment is virtually impossible, administration of high oxygen concentrations is a predetermined, controlled event. Therefore, PTX may have a role in protecting patients from oxygen-induced lung injury.

APPENDIX

APPENDIX A

ADDENDUM

This work has been presented in part at the Surgical Forum section of the 78th annual Clinical Congress of the American College of Surgeons (10/13/92) and published in Surgical Forum XLIII ¹⁵¹. At that time, we presented our initial findings, i.e., "Alveolar macrophages exposed to hyperoxia demonstrate early enhanced cytokine productive capacity." During the same year, Jensen et al. demonstrated that TNF, in particular, and possibly IL-1 and IL-6 may play a role in oxygen toxicity ¹⁵². Although their work supports the basic hypothesis that these cytokines are involved in oxygen toxicity, the model and methodology differ significantly from ours.

APPENDIX B

DULBECCO'S MODIFICATION OF EAGLE'S MEDIUM (DMEM)

Amino acids	mg/l
Arginine HCl	84.0
Cystine	48.0
Glutamine	584.0
Glycine	30.0
Histidine HCl.H ₂ O	42.0
Isoleucine	104.8
Leucine	104.8
Lysine HCl	146.2
Methionine	30.0
Phenylalanine	66.0
Serine	42.0
Threonine	95.2
Tryptophan	16.0
Tyrosine	72.0
Valine	93.6
Vitamins	
Choline chloride	4.0
Folic acid	4.0
Nicotinamide	4.0
Calcium d-pantothenate	4.0
Pyridoxal HCl	4.0

Riboflavin 0.4

Thiamine HCl 4.0

Inositol 7.0

Inorganic Salts mg/ml

NaCl 6400.0

KCL 400.0

NaH₂PO₄·2H₂O 125.0

MgSO₄·7H₂O 200.0

CaCl₂ 200.0

NaHCO₃ 3700.0

Fe(NO₃)₃·9H₂O 0.1

Other Components

n-Butyl-p-hydroxybenzoate 0.2

Glucose 4500.0

Penicillin G potassium 5000 units

Phenol red 15.0

Sodium pyruvate 110.0

Streptomycin sulfate, equivalent base . . . 100.0

APPENDIX C

RPMI MEDIUM

L-Amino Acids	mg/ml
Arginine	200.0
Asparagine	50.0
Aspartic acid	20.0
Cystine	50.0
Glutamic acid	20.0
Glutamine	300.0
Glycine	10.0
Histidine	15.0
Hydroxyproline	20.0
Isoleucine (allo-free)	50.0
Leucine (methionine-free)	50.0
Lysine HCl	40.0
Methionine	15.0
Phenylalanine	15.0
Proline (hydroxy-L-proline-free)	20.0
Serine	30.0
Threonine (allo-free)	20.0
Tryptophan	5.0
Tyrosine	20.0
Valine	20.0

Vitamins

Biotin	0.2
Vitamin B ₁₂	0.005
Calcium pantothenate	0.25
Vitamins (continued)	mg/ml
Choline Chloride	3.0
Folic acid	1.0
i-Inositol	35.0
Nicotinamide	1.0
p-Aminobenzoic acid	1.0
Pyridoxine HCl	1.0
Riboflavin	0.2
Thiamine HCl	1.0
Inorganic Salts	
NaCl	6000.0
KCl	400.0
Na ₂ HPO ₄ ·7H ₂ O	1512.0
MgSO ₄ ·7H ₂ O	100.0
NaHCO ₃	2000.0
Ca(NO ₃) ₂ ·4H ₂ O	100.0
Other Components	
Glucose	2000.0
Glutathione (reduced)	1.0
Phenol red	5.0

APPENDIX D

Publications

1. Lindsey, HJ, Kisala, JM, Ayala, A, Lehman, D, Herdon, CD, and Chaudry, IH. Pentoxifylline attenuates oxygen-induced lung injury. J Surg Res 1994;56:543-548.

2. Lindsey, HJ, Kisala, JM, Ayala, A, and Chaudry, IH. Alveolar macrophages exposed to hyperoxia demonstrate early enhanced cytokine productive capacity. Surg Forum 1992;43:66-68.

Presentations and Awards

National

1. "Pentoxifylline attenuates oxygen-induced lung injury" presented November 11, 1993 at the 27th annual meeting of the Association for Academic Surgery, Hershey, PA.

2. "Alveolar macrophages exposed to hyperoxia demonstrate early enhanced cytokine productive capacity" presented October 13, 1992 at the Surgical Forum section of the 78th Clinical Congress of the American College of Surgeons, New Orleans, LA.

State

1. "Pentoxifylline attenuates oxygen-induced lung injury" presented May 6, 1993 at the 42nd annual Collier Day of the Michigan Chapter, American College of Surgeons, Grand Rapids, MI.

2. "Hyperoxia enhances interleukin-1 production by alveolar macrophages" presented May 2, 1993 at the 40th annual Collier Day of the Michigan Chapter, American College of Surgeons, East Lansing, MI.

Local

1. "Pentoxifylline attenuates oxygen-induced lung injury" presented March 11, 1993 at the Ninth Annual Research Day Forum of the Michigan State University Department of Surgery, East Lansing, MI, and won first place in the basic science competition.

2. "Alveolar macrophages exposed to hyperoxia in vivo demonstrate enhanced TNF productive capacity" presented May 10, 1992 at Flint Academy of Surgery, Flint, MI, and won second place in resident research competition.

3. "Alveolar macrophages exposed to hyperoxia in vivo demonstrate enhanced TNF productive capacity" presented March 12, 1992 at the Eighth Annual Research Day Forum of the Michigan State University Department of Surgery, East Lansing, MI, and won first place in the basic science competition.

4. "Hyperoxia enhances interleukin-1 production by alveolar macrophages" presented May 14, 1991 at the Flint Academy of Surgery, Flint, MI, and won first place in resident research competition.

5. "Hyperoxia enhances interleukin-1 production by alveolar macrophages" presented May 2, 1991 at the Seventh Annual Research Day Forum of the Michigan State University Department of Surgery, East Lansing, MI.

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