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EFFECTS OF PHARMACOLOGIC AND IMMUNOLOGIC INTERVENTION ON THE PSEUDOMONAS PORCINE MODEL OF ARDS.

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Annual Report

July 1, 1991

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-86-C-6168

Medical College of Virginia, Virginia Commonwealth University
Richmond, Virginia 23298-0519

1 July 1991 Annual Report (7/1/90 - 6/30/91)

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Contract No.
DAMD17-86-C-6168

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WUDA310901

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Lab Animals; Acute Respiratory Distress; Pigs; Capillary Permeability; RAII; Swine

Unclassified Unclassified N/A N/A

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Introduction

It is now over twenty years since Ashbaugh and Petty, in a landmark article[1] described a series of patients suffering from a condition characterized by severe dyspnea, hypoxemia refractory to supplemental oxygen, reduced lung compliance and diffuse bilateral pulmonary infiltrates on chest x-ray. Because the clinical features in these patients were remarkably similar to those seen in infant respiratory distress, a condition associated with prematurity and lack of adequate quantities of surfactant, they coined the term Adult Respiratory Distress Syndrome (ARDS) to describe this condition. Since then, ARDS has become recognized as one of the most frequent causes of acute respiratory failure in both medical and surgical patients who, in many cases have no antecedent history of respiratory problems or illness.

In combat soldiers, a condition known as the traumatic wet lung syndrome during World War II was described. This was observed in soldiers who had sustained varying degrees of thoracic trauma, ranging from simple pulmonary contusion to severe penetrating chest wounds. As Burford et al stated in a report[2] at that time concerning the thoracic injuries that they had seen and treated

" *two fundamental physiopathologic phenomena have been observed:*

1. *That in all wounds of the chest to a greater or lesser degree, depending upon the type and severity of the lesion, the lung tissue reacts to produce more than its normal amount of interstitial and intra-alveolar fluid.*
2. *That in all wounds of the chest, the bronchopulmonary tree not only has more fluid to rid itself of, but becomes less capable of doing so."*

Although this report concerned itself only with cases of thoracic trauma, both blunt and penetrating, the problem of "wet lung" was also noted to be present in soldiers who had sustained non-thoracic trauma such as abdominal wounds, fractures of long bones and head injuries as well those with thoracic injuries. In a report[3] on all injuries treated during World War II, Grant and Reeve noted

"*Of all the organs, the lungs were the most frequently abnormal (at autopsy). ....they displayed varying degrees of congestion and oedema, often with petechial or larger hemorrhages and more or less widespread bronchopneumonia in addition.*"

It was thus clearly in the military setting that many of the problems of diagnosis and management of shock, sepsis and organ failure were first noted and commented upon.

By the time the Vietnam war came to be fought, the concept of rapid evacuation of wounded soldiers from the field and early definitive treatment of their injuries had been established. This saved many lives but, as a contemporary observer[4] remarked

"*A new type of surgical patient has appeared in Vietnam which is of great importance to surgical research. This is the critically wounded patient who is suffering rapid blood loss from vascular or organ injury, who under any other circumstances would have died shortly after injury. However he is now delivered to a hospital frequently within 15 minutes of injury. ...his pO_2 may be 40 but there may be no clinical evidence for this and no respiratory injury.*"

The association of pulmonary complications with non-thoracic wounds was clearly established, at least in the minds of the military surgical community. Less than a year later, Ashbaugh and Petty published the first report describing ARDS as a syndrome in civilian patients.

In the intervening twenty or so years, an enormous amount of data has been gathered on this condition, it's clinical course and outcome, the risk factors which predispose to it's onset and the prognosis which may be expected. In the research laboratory many models of acute lung injury have been developed in an effort to more clearly define the pathogenesis and pathophysiology of ARDS and to identify points at which therapeutic intervention may be employed to achieve a successful outcome.

Despite advances in anesthesia and intensive care, the mortality associated with this condition has hardly changed since Ashbaugh and Petty's report, although it is now generally accepted that patients rarely die from respiratory failure due to ARDS, but rather succumb to sepsis, usually accompanied by multiple organ failure. In these settings, the mortality associated with ARDS may reach 60% or more[5].

Direct Injury:
Aspiration
Inhalation Injury
Pulmonary Contusion
Pneumonia
Indirect Injury:
Sepsis
Pancreatitis
Shock
Fat Embolism

1 Predisposing factors to ARDS

Although the pathogenesis and pathophysiology of ARDS remain incompletely understood, it may be said to comprise a final common pathway whereby predisposing factors involving either direct or indirect injury to the lung result in the catastrophic sequence of events which comprise ARDS. Several common predisposing factors are summarized in Table 1. Direct predisposing factors tend to initiate injury from the epithelial side of the alveolar-capillary

membrane while indirect factors tend to initiate injury from the endothelial side of the alveolar-capillary membrane.

Irrespective of the predisposing factor or initiating event, it is alveolar-capillary membrane damage with increased permeability and accumulation of protein-rich fluid in the pulmonary interstitium and later the alveoli that is the end result with consequent derangement of gas exchange and respiratory failure.

As our understanding of the disease process at work in ARDS has improved over the past twenty years, so too have the criteria used to make the diagnosis changed. In their initial report, Ashbaugh and Petty noted the decreasing compliance, a fall in arterial pO_2 despite an FiO_2 of .5 or greater, in the face of an adequate left atrial filling pressure, so characteristic of this condition. Consequently, the criteria to make the diagnosis were defined as respiratory failure requiring intubation and mechanical ventilation, the appearance of bilateral diffuse pulmonary infiltrates on chest x-ray, despite adequate cardiac function and a marked decrease in lung compliance.

With the widespread introduction of bedside cardiac catheterization utilizing Swan-Ganz catheters, many authorities included the presence of a normal pulmonary artery occlusion pressure as one of the criteria necessary to make the diagnosis. In recent years however, this has not been felt to be an absolute requirement[6]. There was no catheterization data available in the original series of patients reported and it is now widely accepted that given adequate experience it is possible to exclude, using clinical criteria, the presence of significant left ventricular dysfunction in these patients. The criteria for the diagnosis of ARDS are summarized in Table II.

More recently, Murray et al[7] have proposed a wider definition of ARDS using a scoring system for acute lung injury based on a 4 point system. Firstly, the defect in oxygenation is quantified as the ratio of arterial pO_2 to the fractional inspired O_2 concentration (p_aO_2:FiO_2 ratio). This should normally be greater than 300. Secondly, the chest x-ray is scored according to the degree of air-space consolidation in the 4 lung zones. Thirdly, respiratory compliance is calculated if the patient is being ventilated, as indeed most ,if not all, of these patients are. The final criterion used is the degree of positive end expiratory pressure (PEEP) required. This last parameter is an important one as it provides an estimate of the severity of compliance loss as well as improving oxygenation.

1.	Underlying illness or injury known to be associated with ARDS.
2.	Respiratory rate > 20.
3.	Severe Hypoxemia; p_aO_2:F_iO_2 ≤ 150 without PEEP.
4.	Diffuse, bilateral infiltrates on chest x ray.

2 Criteria for Diagnosis of ARDS

The criteria used to make the diagnosis are important insofar as they ensure uniform criteria are applied to make the diagnosis of ARDS, allow for accurate prognostication as well as accurate comparisons to be drawn between patients in different institutions, and permit multicenter trials of treatment strategies to be carried out.

In 1983, Fowler et al[5] estimated that between 150,000 and 200.000 patients annually are afflicted with this condition and that mortality ranges from 35-70%, depending on whether sepsis is a prominent feature. It is clearly a condition responsible for much morbidity and mortality particularly among patients in intensive care units.

Although originally described as non-cardiogenic or high permeability pulmonary edema, ARDS is much more than this and is now viewed as the end result of a series of clinical and pathologic events culminating in the final common pathway of acute lung injury. Of the predisposing factors mentioned in Table 1 sepsis, particularly abdominal sepsis, and aspiration of gastric contents are the most important. The mainstay of treatment remains supportive, with maintenance of adequate oxygenation using ventilation and PEEP as required, careful fluid therapy and treatment of the underlying illness or predisposing factor.

Background

In the fully developed state, ARDS is a protein rich inflammatory pulmonary edema with sequestration of neutrophils, initially in the pulmonary vasculature but later in the interstitium and the alveolar space. Endothelial damage, though clearly an important component of the syndrome is only partially responsible for the fully developed condition. There is clearly an epithelial component, as reflected by the presence of hyaline material, neutrophils and cellular debris, in addition to the high protein edema fluid efflux from the vascular compartment[6].

The mechanisms that lead to the development of this protein rich inflammatory edema are extremely complex. Many substances have been implicated as either triggers or mediators of a cascade of events which, once started, spins rapidly out of control. Those substances implicated include endotoxin[8,9], complement[10], eicosanoids[11,12] and cytokines such as tumor necrosis factor-α[13,14] (TNF-α), interleukin-1[15] (IL-1), and interleukin-6[16] (IL-6). It has long been speculated that, of the cells mediating the acute lung injury, the neutrophil is the one most central to the disease process[17-20]. This speculation is supported by the fact that neutrophils

sequester in the lung, are capable of adhering to the endothelium and migrating from the vascular compartment to the interstitium and of producing many of the mediators involved in the pathogenesis of ARDS. Furthermore neutrophils are found in greatly increased numbers in bronchoalveolar lavage samples from ARDS patients.

Three patterns of neutrophil-endothelial adherence have been described to date[20]. These are spontaneous neutrophil adherence, activated neutrophil adherence and activated endothelial adherence. Of these mechanisms, the first causes margination of neutrophils in the lung and is thought to be due to weak attractive forces generated by a divalent cation process[21]. The physics of particulate flow also contribute to the marginating process whereby neutrophils are pushed out of the area of axial flow in the blood vessel in favor of the smaller erythrocytes[22]. A dynamic equilibrium exists between those neutrophils in the circulation and those in the marginated pool.

The latter two patterns of neutrophil-endothelial adherence are active processes and lead to sequestration of neutrophils in the pulmonary vasculature. In contradistinction to margination, sequestration of neutrophils occurs due to strong adhesive forces generated between the neutrophil and endothelial cell, often mediated by glycoprotein adhesion molecules on the neutrophil and on the endothelial cell[23].

The adhesion molecules on the surface of the neutrophil are collectively known as the CD11/CD18 adhesion receptor complex. These molecules are known to become active within a very short time of exposure to substances such as chemotactic peptides, activated complement fragments, leukotrienes and platelet activating factor. Adhesion complexes present on the endothelial cell surface tend to differ from those present on the neutrophil in that they take a

period of hours, rather than minutes, to become activated and appear to respond to different stimuli such as thrombin and the cytokines, TNF-α and IL-1[24]. These receptors may promote neutrophil adhesion in the absence of activation of the CD11/CD18 receptor complex on the neutrophil.

It has been shown that this pulmonary leukosequestration is greatly increased in the presence of complement activation following cardiopulmonary bypass or renal hemodialysis[25]. In this setting, neutrophils actively attach themselves to the endothelium of the pulmonary vasculature by means of adhesion receptors. The outpouring of proteinases and toxic oxygen species that accompanies neutrophil activation occurs with the neutrophil tightly attached to the endothelium and thus, in a position to wreak greatest havoc on the endothelium and, as they migrate, to the interstitium and ultimately the epithelial side of the alveolar-capillary membrane.

This hypothesis of acute lung injury holds that neutrophil activation is preceded by neutrophil adherence to endothelium and migration. Neutrophil activation is characterized by an increased respiratory burst, production of toxic oxygen products and activation of the hexose monophosphate shunt. The products of neutrophil activation are released into a protected microenvironment generated by the adhesion of the neutrophil to the endothelium, thus preventing the oxidant scavengers and proteases from being as effective as they might otherwise be. This results in increased local concentrations of toxic oxygen species and increased endothelial damage

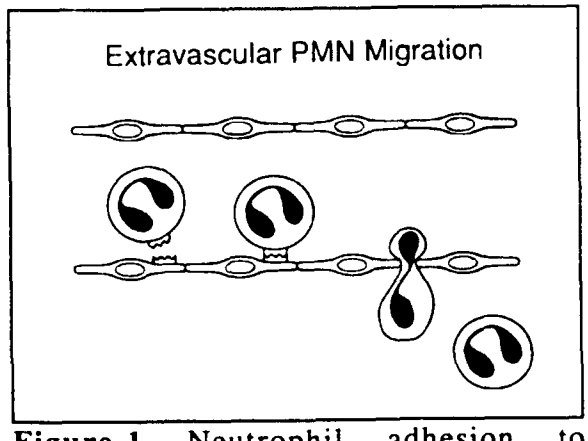

Figure 1 Neutrophil adhesion to Endothelium with subsequent migration

as depicted in Figure I. In this setting, neutrophil adhesion to the endothelium is clearly a pivotal step.

In addition to oxygen free radicals, neutrophils and monocytes also release vasoactive substances such as eicosanoids and leukotrienes which directly affect pulmonary and systemic vascular tone. Along with alveolar macrophages, these cells also generate large amounts of cytokines such as TNF-α, IL-1 and IL-6. In recent years, research has shown that cytokine networks play a central role in the initiation and propagation of the physiologic and pathologic events seen in acute lung injury.

The Model

The porcine model is used in all experiments. Swine weighing between 15-25 kgs are anesthetized with intramuscular ketamine hydrochloride 25 mg/kg and placed supine. Anesthesia is currently induced with sodium pentobarbital (10 mg/kg) and maintained with an infusion of pentobarbital and fentanyl at a rate of 5-10 mg/kg/min and 2.5-7.5μg/kg/min respectively. Previous studies were performed with paralyzed animals, since alveolar-capillary protein leak was measured with a computerized gamma camera in which the animals could not be allowed to move. Paralysis made evaluation of adequate anesthesia difficult. Following intubation with a cuffed endotracheal tube, the animals are ventilated with an F_IO_2 of 0.5 at a positive end expiratory pressure (PEEP) of 5 cm H_2O and a tidal volume of 12-15cc/kg at a rate to produce a $PaCO_2$ of approximately 40 torr at the beginning of the experiment.

Live *Pseudomonas aeruginosa* (PAO strain, 5 x 10^8 CFU/ml at 0.3 ml/20kg/min) is then administered for 1 hour. In pseudomonas (Ps) control animals this has been shown to produce a marked physiological deterioration, representative of acute ARDS, resulting in an immediate significant increase in pulmonary artery pressure (PAP) which persists throughout the entire duration of the experiment. Systemic arterial pressure (SAP) shows a progressive decline as does cardiac index (CI) and PaO_2. Extra-vascular lung water (EVLW) becomes significantly elevated when compared to saline controls.

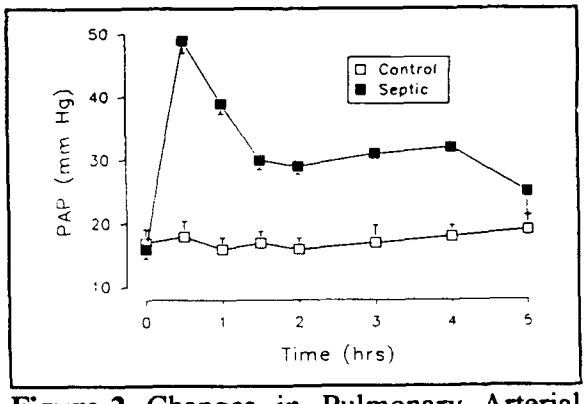

Figure 2 Changes in Pulmonary Arterial Pressure

Catheters are inserted into the left common carotid artery for monitoring of SAP and arterial blood gas determination, and into the right and left external jugular veins for infusion of *Pseudomonas* and the therapeutic agents to be studied. A Swan-Ganz catheter is passed from the right jugular vein into the pulmonary artery and wedged in a small branch with the balloon inflated, using pressure monitoring. It is thus possible to record pulmonary artery pressure (PAP), pulmonary capillary occlusion pressure (PCOP) and cardiac output, using a thermodilution technique. Cardiac output is converted to cardiac index (CI) by the formula:

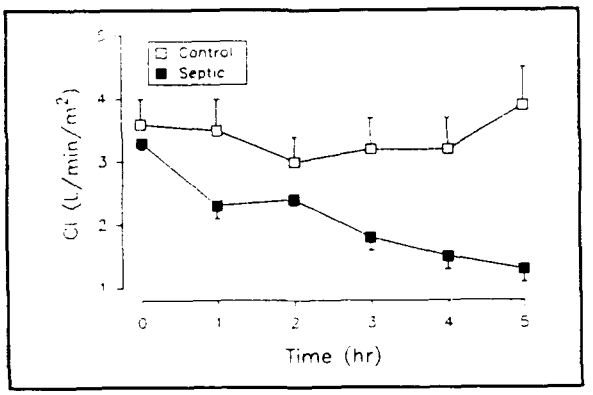

Figure 3 Changes in Cardiac Index

$$CI = \frac{CO}{0.112\ BW^{2/3}}$$

where BW is the body weight in kg.

Arterial and mixed venous blood gases are analyzed using a blood gas analyzer (Instrumentation Laboratories, Model 113).

A 5 French femoral artery lung water catheter (American Edwards Laboratories, Model 96-020-5F) is passed into the lower abdominal aorta for measurement of extravascular lung water (EVLW) using a thermal dye dilution technique[26]. In this technique, 10 ml of iced, green dye solution (2 mg indocyanine green dye in 10 ml 5% dextrose) are injected as a bolus through the proximal port of the Swan-Ganz catheter as blood is simultaneously withdrawn through the thermistor-tipped femoral artery catheter connected to a densitometer cuvette (Waters

Instruments In.. Model 402A) which is linked to a lung water computer (American Edwards Laboratories, Model 9310). The computer measures the mean transit times of the intravascular dye (MTD) and freely diffusible thermal component (MIT) as well as the cardiac output (CO). EVLW is calculated by the formula:

$$EVLW = \frac{CO\ (MTD-MIT)}{BW\ (kg)}$$

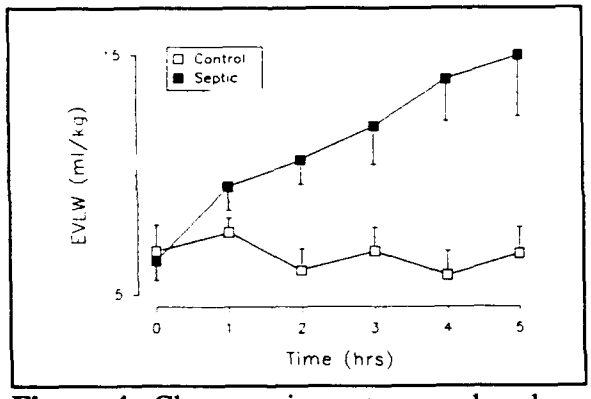

Figure 4 Changes in extravascular lung water during sepsis

Bronchoalveolar Lavage and Protein Assay

Using a fiberoptic bronchoscope (Machita VT-5100C, 4mm) bronchoalveolar lavage (BAL) is performed at 0 and 300 min. The middle and lower lobes of the right lung are lavaged (3 x 25 ml aliquots of sterile 0.9% NaCl in each lobe) at 0 min. This is repeated in the corresponding lobes of the left lung at 300 min.

Lavage returns are consistently high (>75%) in all animals. BAL protein content, expressed as micrograms protein per milliliter of recovered lavage fluid, is performed on non-cellular fractions of the BAL by a modification of the Lowry technique[27].

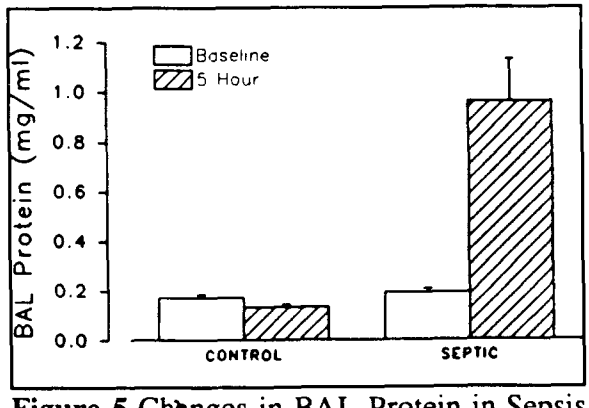

Figure 5 Changes in BAL Protein in Sepsis

Peripheral blood samples for isolation of neutrophils and subsequent assay of short and long-lived toxic oxygen metabolites are withdrawn at baseline (zero timepoint), and at 5 hours (end-stage sepsis). The neutrophils are isolated using dextran sedimentation and Ficoll-sodium diatrizoate density gradient centrifugation. Assays of phorbol ester stimulated production of oxygen-dependent neutrophil products are performed as outlined in a previous report. Similarly, arterial blood samples are drawn at hourly intervals for estimation of neutrophil counts, thromboxane B_2 levels, TNF-α and IL-6 and also for measurement of CD18 receptor expression on peripheral neutrophils.

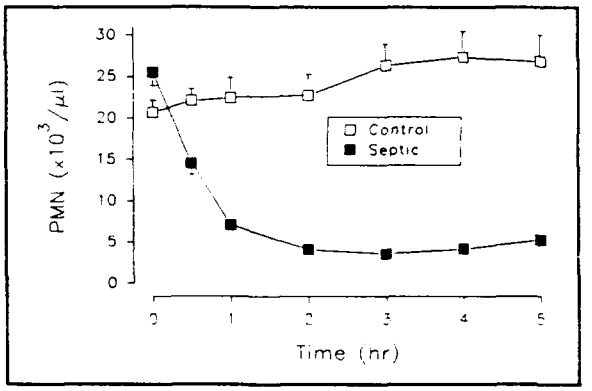

Figure 6 Effect of sepsis on peripheral neutrophil Count

Measurement of CD11/CD18 Receptor Expression

Saturating concentrations of fluorescein-conjugated monoclonal antibodies specific for the ß-subunit of the CD18 glycoprotein are incubated with the neutrophils after incubation with plasma (20 min at 4°C). To control for non-specific binding, the same concentration of fluorescein-conjugated murine IgG_{2a} is incubated with equally treated neutrophils. Cells are washed thoroughly and fixed in 1ml paraformaldehyde.

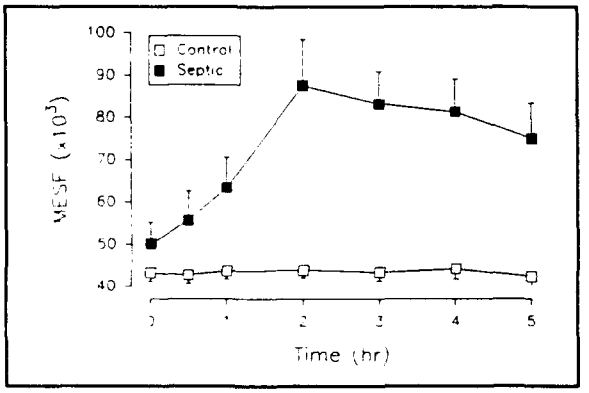

Figure 7 Effect of sepsis on CD11/CD18 adhesion receptor expression

Immunofluorescence intensity is analyzed with a flow cytometer equipped with a logarithmic amplifier[28] . The channel number (log scale) representing the peak fluorescence intensity of 5,000 cells is determined. Linear fluorescence-intensity is calculated from a logarithmic-linear calibration curve[29]. Quantitation of the expression of CD18 is calculated by subtracting the linear fluorescence intensity of the bound non-specific IgG_{2a} from the linear fluorescence intensity of the bound MoAb 60.3.

Tumor Necrosis Factor Activity

Arterial blood samples are collected at baseline, 30 min and then at 60 min intervals for measurement of plasma TNF activity. The mouse L929 fibroblast bioassay is used to quantify TNF activity[30]. Arterial blood samples are drawn into sterile glass tubes containing 0.15% EDTA and kept at 4°C. Specimens are centrifuged at 500 g for 20 min at 4°C and the resulting plasma frozen at -20°C until time of assay. L929 cells are seeded into flat-bottomed 96 well microtiter plates (Corning, NY) at a density of 4 x 10^4 cells/well and grown to confluence overnight in Dulbecco's minimal essential medium (GIBCO) containing 1% penicillin-streptomycin and 5% fetal calf serum (DMEM). Medium is then removed from confluent monolayers and 100 μl of DMEM containing Actinomycin-D (Merck, Sharp and Dohme, Westpoint, PA, final concentration = 5ug/ml) are added to each well. One hundred μl of each of the following are then added to selected duplicate wells containing L929 cells: 1) DMEM

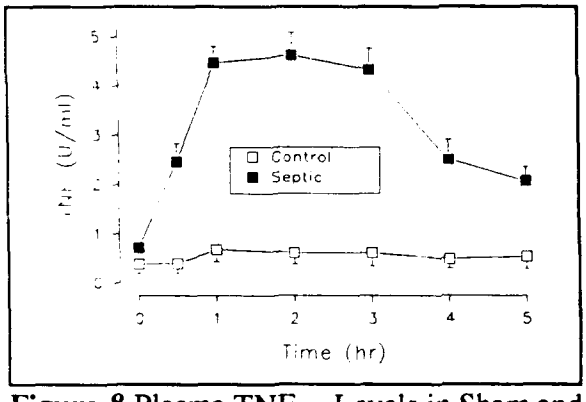

Figure 8 Plasma TNF-α Levels in Sham and Septic Animals

(0% cytotoxicity): 2) serial dilutions of recombinant TNF (5×10^{-3} to 6×10^{-4} U/ml) (Cetus Corp., Emeryville, CA); 3) plasma samples from different groups; 4) DMEM in blank wells without cells (100% cytotoxicity). Plates are then incubated for 20 hr at 37°C in 5% CO_2. Following incubation, the medium is removed and the L929 cells were stained for 10 min with 0.5% crystal violet in 20% methanol, rinsed in water and air dried. Optical density (OD) of each well was determined by a microplate reader (Bio-Tek EL 309) and calibrated to non-cellular reagent blanks at a wavelength of 550 nm. The percent cytotoxicity of L929 cells was calculated by

$$\% \text{ Cytotoxicity} = \frac{\text{OD wells with 0\% cytotoxicity} - \text{OD experimental sample well}}{\text{OD wells with 0\% cytotoxicity}}$$

TNF activity is expressed in units per milliliter (U/ml), where one unit of TNF activity is defined as 50% L929 cytotoxicity.

Plasma Thromboxane B_2 Activity

Plasma thromboxane (TxB_2) levels are measured by radioimmunoassay[31]. Arterial blood samples are obtained at 0, 30, 60, 180 and 300 min. Samples are collected in heparinized glass tubes containing indomethacin (10 μg/ml). Following centrifugation, plasma is stored at -70°C until time of assay. Aliquots of plasma are extracted using Baker SPE 10 reverse phase columns and analyzed for TxB_2 using specific TxB_2 antiserum (Advanced Magnetics, Boston MA). All assays are run in duplicate and the results expressed as pg per ml of plasma.

Superoxide anion kinetics assay

Spontaneous and phorbol myristate acetate (PMA) stimulated generation of superoxide anion is measured in freshly harvested neutrophils, by continuously monitoring superoxide

dismutase (SOD) inhibitable reduction of ferricytochrome C[32,33] using a dual beam spectrophotometer (Shimadzu UV-160). Briefly, 650 μl of neutrophil suspension (4.0 x 10^6 cells/ml, in PBS) is added to a reaction mixture in a flat bottomed cuvette (Fisher Scientific). The reaction mixture contains 200μl of stock ferricytochrome C solution (16 mg/ml, Sigma chemical company) in a volume brought to 2.6 ml with PBS. Reference cuvettes contain cells plus cytochrome C plus superoxide dismutase (100μg/ml). Cuvettes are permitted to equilibrate in the spectrophotometer at 37°C under continuous stirring for ten minutes. PMA (2.6 μl of 100 ng/ml stock) is then added to give a final cuvette concentration of 200 nmol/ml. The change in optical density is continuously recorded at 550 nm for a 10 minute period. Assays are performed in triplicate. The nanomolar extinction coefficient of 0.0211 for the reduction of ferricytochrome C is used to quantify superoxide anion production (24). Superoxide anion production, expressed as nmols of O_2^-/min/10^6 PMN, is calculated for each minute and plotted against time (Fig 9).

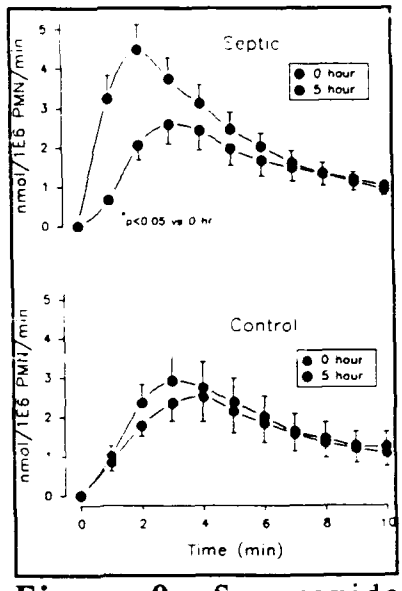

Figure 9 Superoxide Production in the Porcine model

Interleukin-6 Assay

The activity of IL-6 is measured by the proliferation of the IL-6 dependent hybridoma, designated 7TD1. Serial dilutions of filtered plasma are incubated in triplicate with 4 x 10^4 7TD1 cells/ml for 72 hours in 96 well microculture plates. At the end of this period, MTT tetrazolium is added and the plates incubated for a further 4 hours. The wells are then aspirated and isopropanol added to lyse the cells. The degree of cell proliferation is assessed

colorimetrically using a Biotek plate reader and the amount of IL-6 activity is calculated by reference to a standard curve generated by incubating known amounts of recombinant human IL-6. Results are expressed in ng/ml.

Experiments

Monoclonal Antibody 60.3 Studies

As we have seen, the neutrophil (PMN) is implicated in the pathogenesis of the adult respiratory distress syndrome[34]. Experimental models of septic acute lung injury (ALI) are associated with PMN activation[35], acute neutropenia[36], PMN sequestration in the lung[37] and migration into the alveolus[38]. These events coincide with injury to the alveolar capillary membrane with alveolar protein leak[39] and increased extravascular lung water[40].

It is established that the CD11b/CD18 glycoprotein adhesion receptor on the PMN cell surface is of primary importance for the adhesion of activated PMNs to vascular endothelium[41]. It has been demonstrated *in vitro* that cell-cell adhesion is necessary for endothelial injury by activated PMNs[42]. Monoclonal antibody 60.3 (MoAb 60.3) is a murine IgG2a monoclonal antibody that recognizes a functional epitope on the CD18 subunit of the CD11/CD18 adhesion complex[43]. It is of proven benefit in attenuating PMN induced reperfusion injury in experimental hemorrhagic shock[44]. We have previously demonstrated that this antibody recognizes the CD18 receptor on porcine PMNs and that, in the presence of Pseudomonas endotoxin, PMN adhesion to porcine pulmonary endothelium is predominantly CD18 dependent[45]. Further, we have demonstrated that the marked neutropenia which occurs following infusion of live *Pseudomonas aeruginosa* in pigs is associated with increased CD18 expression by circulating PMNs[46].

MoAb 60.3 was used in this study to examine the hypothesis that neutropenia and pulmonary PMN sequestration, with subsequent transvascular migration into the alveolar space

during septic ALI, are CD18 dependent phenomena. The effect of blocking CD18 dependent leukocyte adhesion on Pseudomonas induced alveolar capillary membrane injury was assessed.

Control animals (n=8) received an infusion of sterile NaCl (0.9%) for 60 min. Septic animals (n=9) were infused for 60 min with live *Pseudomonas aeruginosa* PAO strain ($5x10^8$ organisms/ml at 0.3ml/20 kg/min). A third group of animals (n=7) were pretreated with monoclonal antibody 60.3 (MoAb 60.3) prior to Pseudomonas infusion. Fifteen minutes following MoAb 60.3 infusion the 1 hr infusion of *Pseudomonas aeruginosa* was commenced. Physiologic and blood gas parameters (pH, PaO_2, $PaCO_2$) were measured before and after MoAb 60.3 infusion, after 30 and 60 min of sepsis and hourly thereafter. EVLW was measured at baseline and then hourly until the end of the study. All groups were studied for a total of 300 min.

Purified MoAb 60.3 was provided (Bristol Myers Squibb, Oncogen Division, Seattle, WA.) at a concentration of 2.6 mg/ml in 50 mM sodium phosphate buffer, pH 7.0. Samples were stored at -70°C after aliquoting to avoid freeze thaw cycles. Prior to each experiment MoAb 60.3 was thawed and reconstituted in 25 ml sterile saline. The antibody (2mg/kg) was infused over 15 min via the left internal jugular vein. Serum antibody concentration was measured by an enzyme linked immunosorbent assay (ELISA)[47] immediately following infusion and again at 180 and 300 min. Serum antibody activity was also assessed at these time points. To measure serum antibody activity, serum samples from MoAb 60.3 treated animals were incubated with normal porcine PMNs as the first part of an indirect immunofluorescent staining procedure. Following a second incubation with a fluorescein conjugated $F(ab')_2$ anti IgG, MoAb 60.3 serum activity was represented by the fluorescence profile for that serum sample, as

detected by flow cytometry. Normal porcine PMNs were isolated by standard dextran sedimentation/ Ficoll Hypaque centrifugation techniques. Isolated PMNs were resuspended in phosphate buffered saline at a concentration of 10 x 10^6/ml and 10^6 cells, in 100μl PBS, were incubated for 20 min at 4°C with 100μl of pig serum. Following incubation samples were washed twice in PBS containing 0.1% sodium azide, 0.1% EDTA and 0.2% fetal bovine serum, prior to a second incubation (20 min, 4°C) with fluorescein conjugated Fab_2' anti IgG. Following the second incubation cells were washed twice and resuspended in 1% paraformaldeyde. The fluorescence profiles of the fixed cells were examined by flow cytometry (Becton Dickinson FACScan) and the mean channel fluorescence of samples at 0, 180 and 300 min compared.

Monoclonal anti-TNF-α Studies

Tumor necrosis factor (TNF), a proinflammatory cytokine elaborated predominantly by cells of macrophage / monocyte lineage in response to bacterial endotoxin is now recognized as a primary mediator of the host response to gram negative sepsis[48-50]. Pretreating mice[51], rabbits[52] and baboons[53,54] with anti-TNF MoAb reduced animal mortality following administration of endotoxin or lethal numbers of gram negative organisms. The mechanisms which acted to reduce mortality following pretreatment with anti-TNF MoAb have not been fully elucidated.

This study examined the effects of anti-TNFα MoAb pretreatment on the course of gram negative sepsis and acute lung injury produced by infusion of live *Pseudomonas aeruginosa* in the pig. The pretreatment design of this study permitted the pathogenic effects of TNF to be examined. Mounting evidence suggests that many of the effects of TNF occur via cyclooxygenase metabolites[49]. We set out to examine these relationships between TNF and cyclooxygenase metabolites in porcine sepsis by studying the effects of anti-TNFα MoAb pretreatment on plasma thromboxane levels.

The IgG1 anti-TNFα monoclonal antibody (anti-TNFα MoAb) used in this study was provided by Cutter Biological, Miles Inc. Berkley CA. The antibody was purified from murine hybridoma culture harvests via cell separation, polyethylene glycol precipitation, anion exchange and size exclusion chromatography. Purified MoAb was 99% pure with fully functional binding to human TNF. Endotoxin levels were less than 2 U/mg protein (Limulus assay). Stabilization was performed with glycine and maltose prior to lyophilization. Lyophylized MoAb was kept at 4 C until time of experiment, at which stage it was reconstituted with 20 mls of sterile water. Anti-TNFα MoAb (15mg/kg) was administered over 10 min via the left internal jugular vein.

Three groups of animals were studied. Two groups received a 1 hour infusion of live *Pseudomonas aeruginosa* ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min. One of these groups was pretreated with intravenous monoclonal antibody against TNFα (anti-TNFα MoAb, 15mg/kg) 15 min prior to commencing Pseudomonas infusion. The remaining group received sterile saline only. All animals were studied for 300 min.

TGF-β_1 and Pulmonary Intravascular Macrophage (PIM) Studies

Mononuclear phagocytes are known to play a central role in lung repair through release of various cytokines and growth factors. Pulmonary intravascular macrophages (PIMs) may modulate repair mechanisms following septic lung injury from within the pulmonary capillaries. We hypothesized that PIMs influence the course of angiogenesis and repair of the interalveolar septum following septic acute lung injury through local production of growth factors.

In order to test this hypothesis, we set out to demonstrate that PIMs are capable of producing TGF-β_1 mRNA, that MRNA for TGF-β_1 increases following exposure to LPS and finally to assess the effect of coincubation with TGF-β_1 and other cytokines on PIM mRNA production.

PIMs were harvested as previously described and the effluent centrifuged having been layered over 60% isosmotic Percoll. The cells at the interface were then collected, counted and checked for viability. Following culture with serum-free media and incubation with LPS, IL-1 and TGF-β_1 for a 24 hour period, the cells were lysed and RNA extracted. Northern Blot analysis was performed with a[^{32}P]-labelled TGF-β_1 probe

Soluble Complement Receptor Blocker (sCR1)

Complement depletion in a porcine model using cobra venom factor has been shown to exert a protective effect on the alveolar-capillary membrane as evidenced by reduced extra vascular lung water[36]. In addition the rise in pulmonary artery pressure and fall in arterial pO_2 were also attenuated. Complement receptor blockade represents another way of studying the effects of complement inactivation in animal models of acute lung injury.

The activation of C3 and C5 constitute a critical step in the activation pathway of complement and accordingly is tightly regulated *in vivo*. Of the five proteins that inhibit the activation of C3 and C5, two are in plasma and the remaining three are membrane bound. Of these latter three proteins, one is called complement receptor 1 (CR1) and is able to act as a highly specific inhibitor of complement activation through both the classical and alternative pathways. The distribution of this receptor, however, is relatively limited, being found primarily on erythrocytes and leukocytes. This drawback has been overcome by synthesis of a truncated form of CR 1 (sCR1), lacking the transmembrane and cytoplasmic domains of the molecule. With these modifications, it is highly soluble and capable of suppressing complement *in vivo* and has been shown to reduce inflammatory tissue damage in an ischemia-reperfusion model of myocardial injury[55].

The current study was performed in order to ascertain whether selective blockade of the complement cascade utilizing sCRI is capable of attenuating the acute lung injury induced by Pseudomonas infusion in a porcine model.

Five animals were studied. Data on hemodynamic responses, leukocyte counts, cytokine profiles and histological specimens were collected.

The sCR1, was administered in equally divided dosages prior to and following induction of sepsis. Three animals were given 15 mg/kg and the remaining two animals were given 30 mg/kg.

Statistics

All results are expressed as mean $\pm$ sem. Differences were tested for significance using analysis of variance (ANOVA). Differences between means were analyzed using Tukey's Studentized range test. The level of statistical significance was set at $p<0.05$.

Results

Monoclonal Antibody 60.3

Following IV infusion, the serum concentration of MoAb 60.3 was 3.26 $\pm$ 0.09 μg/ml. Serum concentrations remained very constant and there was no significant change from the post infusion levels over the 300 min study period (Fig 10). The activity of MoAb 60.3 in the serum also remained constant over time.

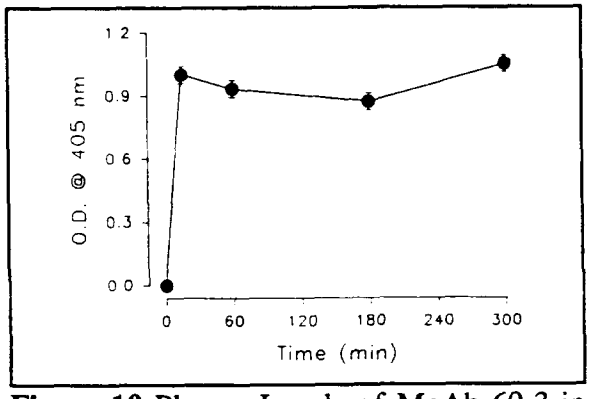

Figure 10 Plasma Levels of MoAb 60.3 in septic treated animals

Hemodynamic Measurements

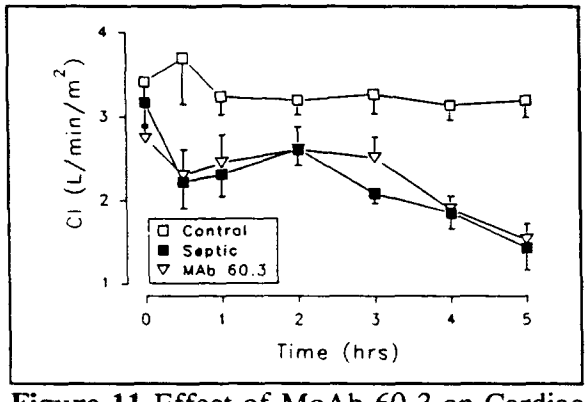

Figure 11 Effect of MoAb 60.3 on Cardiac Index

Prior to the onset of sepsis in the MoAb 60.3 treated group, hemodynamic parameters were measured before and after infusion of the antibody. There was no significant effect of MoAb 60.3 infusion on PAP, SAP, CI or arterial pO_2 prior to commencing the Pseudomonas infusion (Figs 11-13). After the onset of sepsis, MoAb 60.3 treated animals developed the same hemodynamic derangements as untreated animals,

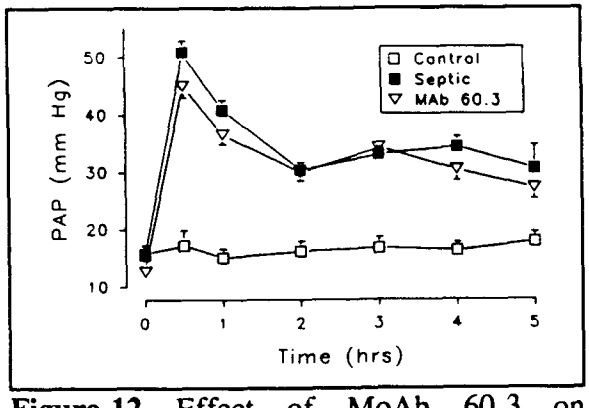

Figure 12 Effect of MoAb 60.3 on Pulmonary Pressure

namely pulmonary arterial hypertension, systemic hypotension, decreased cardiac index and relative arterial hypoxemia (Fig 2). These changes, not seen in the control animals, are typical of this model and have been described in detail previously[39].

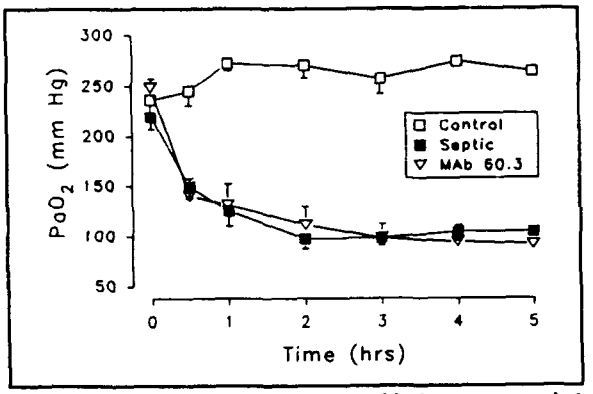

Figure 13 Effect of MoAb 60.3 on Arterial pO_2

Extravascular Lung Water (EVLW)

EVLW was maintained at baseline levels in control animals but was significantly elevated by 180 min in the septic group (Fig 10). There was no increase in EVLW from baseline in the MoAb 60.3 treated group until the final hour of the study at which time a non significant upward trend was observed when compared with baseline or with 300 min values in control animals (Fig 14).

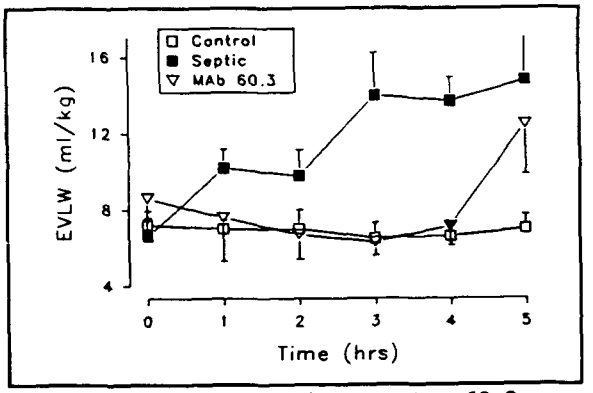

Figure 14 Effect of MoAb 60.3 on Extravascular Lung Water.

Bronchoalveolar Lavage

BAL protein concentration was significantly elevated after 300 min of sepsis (1059 ± 216μg/ml vs 222 ± 42μg/ml at baseline) (Fig 5). Control animals showed no significant change in BAL protein concentration. Compared with the septic group, pretreatment with MoAb 60.3 caused a significant reduction (Fig 15) in BAL protein concentration at 300 min (388 ± 75μg/ml). However, this represented a significant elevation compared with baseline (141 ± 21μg/ml) within the MoAb 60.3 group. i.e. there was a significant, albeit markedly attenuated,

increase in BAL protein. The number of PMNs migrating into the alveolar space (percent PMNs in BAL fluid) at 300 min was unchanged from baseline in control animals but was significantly elevated in Pseudomonas infused animals (29 ± 8% vs 7.3 ± 3% at baseline). MoAb 60.3 reduced PMN migration at 300 min by nearly half (16 ± 4%) but this value failed to reach statistical significance ($p > .05$). Equally the increase in percent PMN concentration over 300 min within the MoAb 60.3 treatment group was not significant (Fig 16).

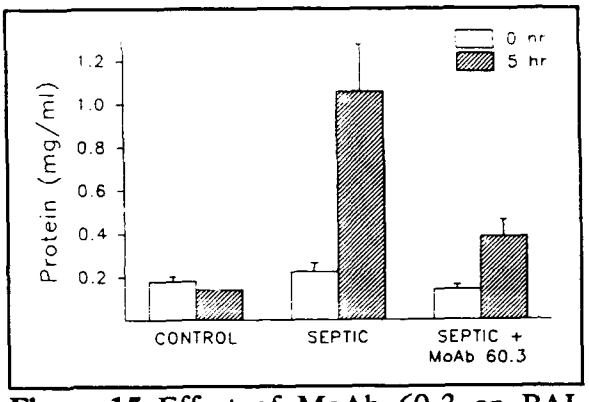

Figure 15 Effect of MoAb 60.3 on BAL Protein

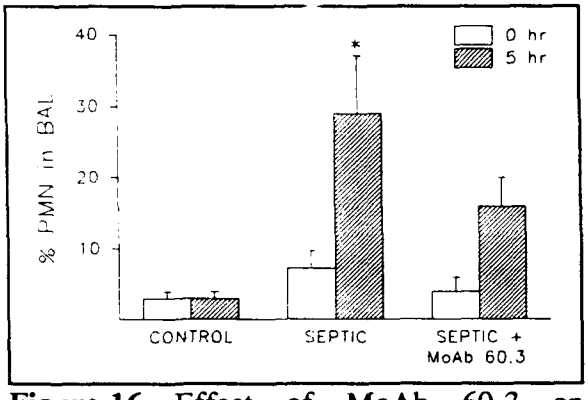

Figure 16 Effect of MoAb 60.3 on neutrophil Migration across the Alveolar-capillary membrane

Blood Neutrophil Count

Control animals maintained a stable peripheral PMN count throughout the 300 min study period (Fig 17). There was an upward trend in the last 3 hours though this was not significant. In contrast the Pseudomonas group exhibited marked neutropenia which occurred predominantly within the first hour of the study ($5.1 \pm 0.5 \times 10^3$ cells/μl at 60 min vs $24.4 \pm 2.0 \times 10^3$ cells/μl at baseline). Neutropenia

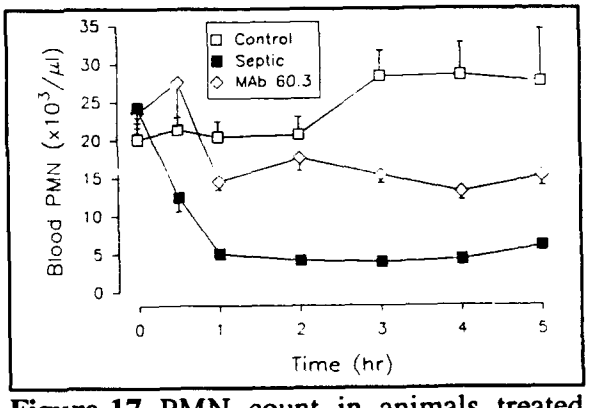

Figure 17 PMN count in animals treated with MoAb 60.3

persisted in the septic animals and there was no significant change from the 60 min values over the next 4 hr of the study. In MoAb 60.3 pretreated animals there was a non significant upward trend in PMN count over the first 30 min ($27.6 \pm 5.6 \times 10^3$ cells/μl vs $23.6 \pm 2.7 \times 10^3$ cells/μl at baseline), followed by a dramatic decrease over the next 30 min ($14.6 \pm 1.0 \times 10^3$ cells/μl at 60 min). However the PMN count was still significantly higher than that observed in septic animals at 60 min ($p < .01$). There was no further fall in PMN count in the MoAb 60.3 group, and PMN counts stayed within 20% of the 60 min values, being significantly higher ($p < .01$) than septic animals at all time points (Fig 17). From 180 min onwards the PMN counts in this group were also significantly lower ($p < .05$) than in controls.

Monoclonal Anti-TNF Studies:

Tumor Necrosis Factor and Thromboxane B_2 Activity

Infusion of Pseudomonas produced significant and rapid increases in plasma TNF levels (Fig 18). Plasma TNF was elevated at 30 min in septic (group 1) animals and was significantly greater than baseline by 120 min (4.8 ± 0.7 U/ml vs 0.4 ± 0.2 U/ml). Plasma TNF levels declined thereafter but remained significantly greater than baseline until 240 min. Pretreatment with anti-TNFα MoAb abolished the surge of TNF observed in the plasma of septic animals with plasma levels exhibiting no significant difference from baseline or saline control animals (Fig 18).

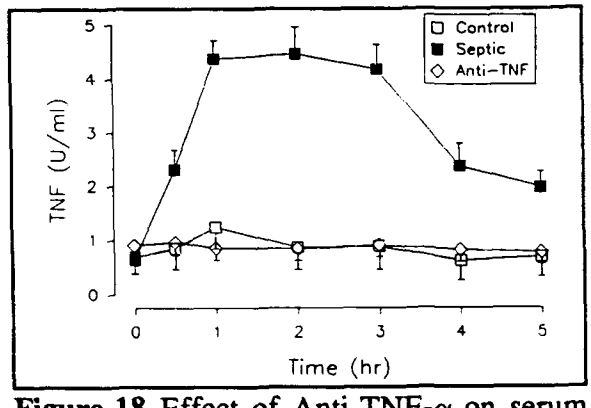

Figure 18 Effect of Anti-TNF-α on serum TNF-α

Plasma thromboxane (TxB_2) levels increased sharply following the onset of Pseudomonas infusion and peaked at 30 min (2877 ± 283 pg/ml). TxB_2 levels remained significantly elevated, compared to baseline, throughout the study in septic animals (Fig 19). Pretreatment with anti-TNFα MoAb failed to attenuate the initial increase in plasma TxB_2. Plasma TxB_2 levels in antibody treated animals equalled those observed in septic animals at 30 min and continued to rise until 60 min. Plasma TxB_2 levels subsequently declined in antibody treated animals and were not significantly different from baseline values at 300 min. Saline control animals exhibited no increase in plasma TxB_2.

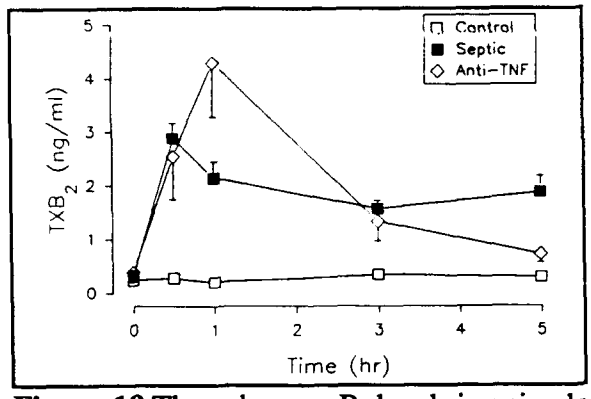

Figure 19 Thromboxane B_2 levels in animals treated with anti-TNF-α

Hemodynamic Measurements

Anti-TNFα MoAb significantly improved the diminished cardiac index (CI) observed in septic animals (Fig 20). Animals in both septic groups (treated and untreated) initially exhibited significant reduction in CI over the first 60 min, coinciding with the infusion of Pseudomonas. However, after 60 min, CI continued to decline in septic animals (1.3 ± 0.2 L/min/m^2 at 300 min), while a significant ($p<0.05$) recovery of CI was observed in antibody treated septic animals following cessation of the Pseudomonas infusion (3.3 ± 0.4 L/min/m^2 at 300 min). From 180 min onward, CI in septic animals (group

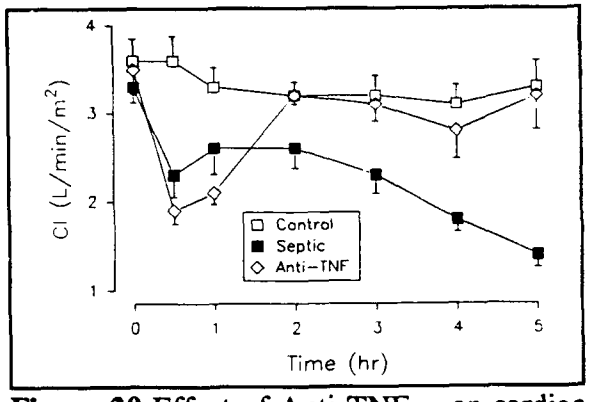

Figure 20 Effect of Anti-TNF-α on cardiac index

I) was significantly lower than that observed in controls. Antibody treated septic animals exhibited no significant difference in CI when compared to saline controls from 180 min onward.

Systemic arterial hypertension occurred in the first 30 min following infusion of Pseudomonas in both septic groups. In septic, untreated animals, systemic arterial hypertension was followed rapidly by hypotension which reached a nadir at 120 min (82 ± 6 mm Hg). From 120 min onwards, a trend towards recovery of mean systemic arterial pressure was observed. Anti-TNFα MoAb prevented the systemic arterial hypotension observed in the untreated septic group (Fig 21).

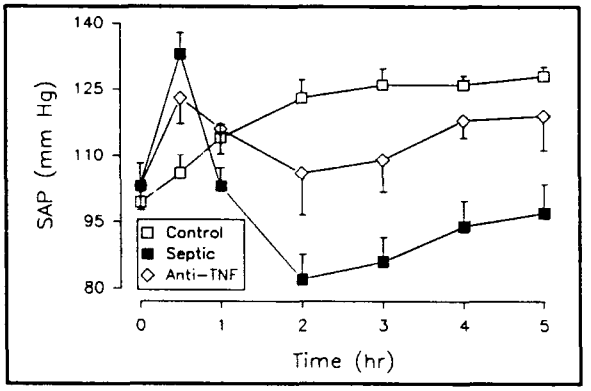

Figure 21 Effect of Anti-TNF-α on Systemic Arterial Pressure

Septic animals developed acute pulmonary arterial hypertension within 30 min of commencing Pseudomonas infusion (Fig 22). The early pulmonary arterial hypertension was not attenuated by pretreatment with anti-TNFα MoAb. However, pulmonary artery pressures were significantly lower at the end of the study in antibody treated animals compared to septic untreated animals.

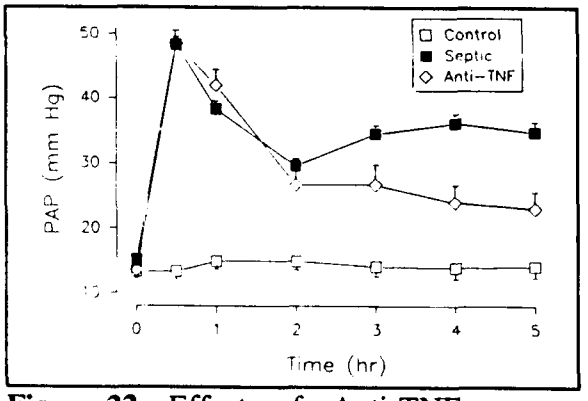

Figure 22 Effect of Anti-TNF-α on Pulmonary Artery Pressure

Blood Gas Measurement

Arterial oxygen tension (PaO_2) fell dramatically in both septic and antibody treated animals over the course of the Pseudomonas infusion. From 60 min onwards, PaO_2 continued to decline in septic animals, whereas; a gradual

increase was observed in antibody treated septic animals. Both groups, however, had significantly lower PaO_2 at the end of the study than saline controls (Fig 23). We observed a gradual 20% decline in mixed venous oxygen tension (PvO_2) over the course of the study in Pseudomonas infused animals. Anti-TNFα treated animals exhibited a rapid 20% drop in PvO_2 during the Pseudomonas infusion; however, this was followed by recovery, such that PvO_2 returned to baseline levels by the end of the study.

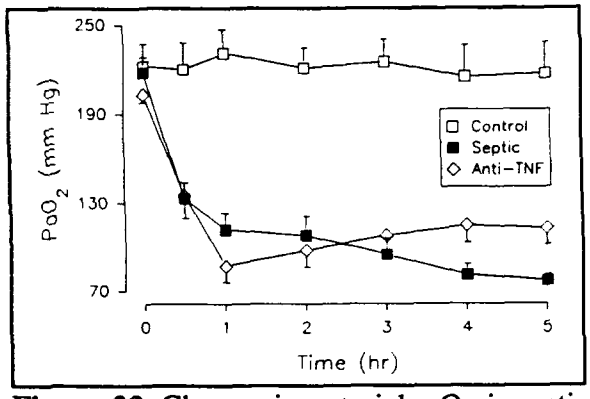

Figure 23 Change in arterial pO_2 in anti-TNF-α treated animals

Porcine sepsis was invariably characterized by the appearance of a progressive, unremitting metabolic acidosis which intensified toward the end of the study period (arterial pH = 7.16 ± 0.03 at 300 min in the untreated septic group). Anti-TNFα antibody failed to prevent the appearance of metabolic acidosis during Pseudomonas infusion (0-60 min). However, from 60 min onward, antibody infused animals exhibited no further intensification of the acidosis (Fig 24). At 300 min, arterial pH in group II was 7.39 ± 0.01 and was not significantly different from saline control animals.

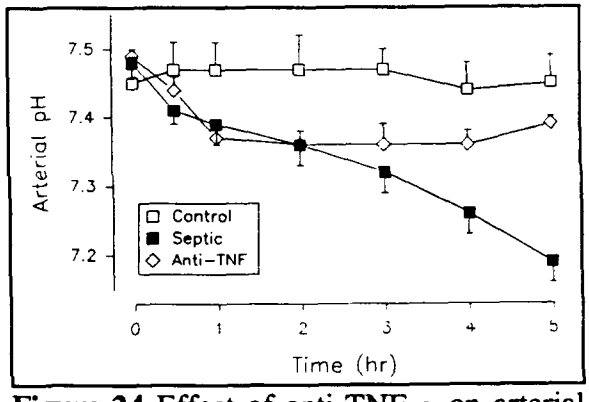

Figure 24 Effect of anti-TNF-α on arterial pH

Peripheral Neutrophil Count

Control animals maintained a stable neutrophil count throughout the entire study period. In the septic untreated animals, a profound neutropenia was observed which developed mainly in the first hour of the study and persisted to the end of the study at 300 mins. The septic treated animals on the other hand, did not develop a neutropenia and cell counts remained at normal or near normal levels throughout the study period (Fig 25).

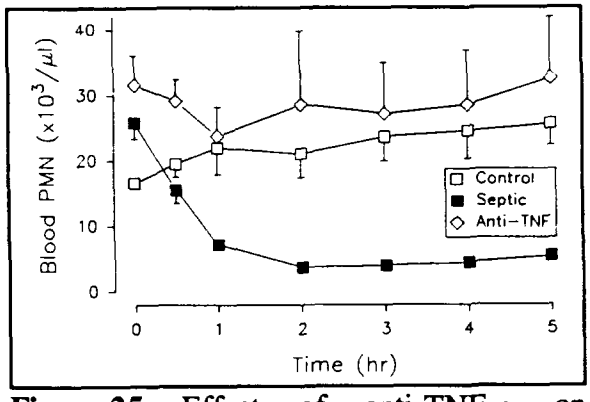

Figure 25 Effect of anti-TNF-α on peripheral neutrophil count

Extravascular Lung Water (EVLW)

EVLW was unchanged in saline controls throughout the study. Septic animals developed significant increases in EVLW and at 300 min EVLW reached 13.2 ± 1.5 ml/kg compared to 6.2 ± 0.9 at baseline. We observed no significant increase in EVLW in the antibody treated septic group over the course of the study (EVLW = 5.9 ± 0.7 ml/kg at 300 min) (Fig 26).

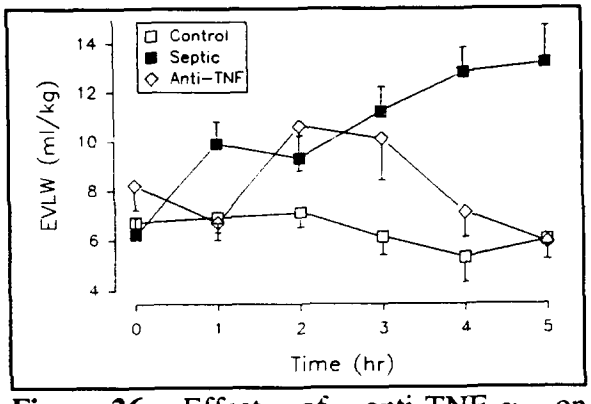

Figure 26 Effect of anti-TNF-α on extravascular lung water

Interleukin-6 Activity

TNF-α which is known to be an important early mediator of gram-negative sepsis activates a complex cytokine cascade, of which the most important elements are probably interleukin-1 and interleukin-6. These cytokines are produced by, and act on, a wide range of tissues and are pleiotropic. Although interleukin-6 has been shown to be elevated in many forms of tissue injury, the precise role of this hormone remains essentially unknown. Some workers regard it as an acute phase protein, as it shares many of the actions of this family of proteins. In this, however, it is no different from many other cytokines including TNF-α and IL-1, both of which have been shown to cause pyrexia and act on the bone marrow to increase hemopoiesis. IL-6 is also a very potent inducer of acute phase protein synthesis in hepatocytes and is capable of shutting down, almost totally, albumin production by hepatocytes in favor of increased globulin production. It clearly has the capability to influence cellular metabolism in a major way.

In some studies looking at IL-6 levels in septic patients, extremely high levels have been shown to be correlated strongly with mortality and it has been suggested that this is a reflection of high TNF-α levels in these patients. In the model we have demonstrated an increase in IL-6 levels in septic animals which is not seen in sham-control animals (Fig 27). In animals given monoclonal antibody to TNF-α we demonstrated a marked attenuation of IL-6 levels, but these still remained slightly above levels

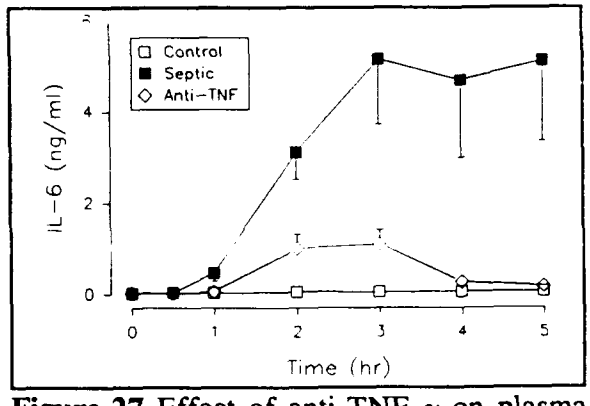

Figure 27 Effect of anti-TNF-α on plasma IL-6

observed in sham animals, suggesting that there are TNF-α dependent and independent pathways for release of IL-6. The significance of this finding will require further investigation.

TGF-β_1 And Pulmonary Intravascular Macrophages

These experiments showed that endotoxin (LPS) induced a four fold increase in static TGF-β_1 mRNA levels and that this response was maximal after two hours (Fig 28). RNA production had returned to baseline levels by 24 hours. TGF-β_1 itself induced a 5-8 fold increase in static TGF-β_1 mRNA levels produced by PIMs (Fig 29) and these were synergistic in increasing mRNA production by PIMs. These experiments suggest that PIMs are indeed capable of expressing the TGF-β_1 gene and that exposure of these cells to endotoxin increases steady state production of TGF-β_1 by these cells. In addition we have also shown that TGF-β_1 exerts an autocrine effect on it's own secretion by PIMs.

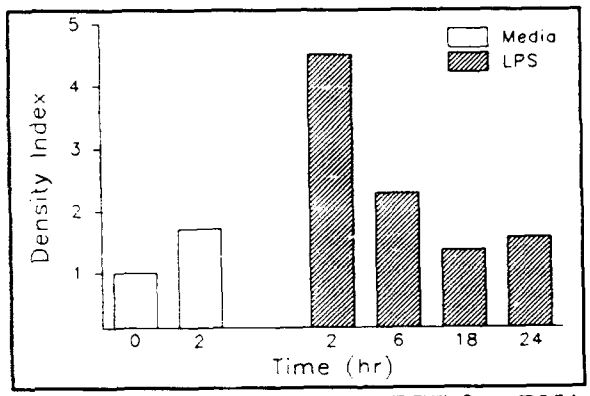

Figure 28 Effect of LPS on TGF-β_1 mRNA by PIMs over time

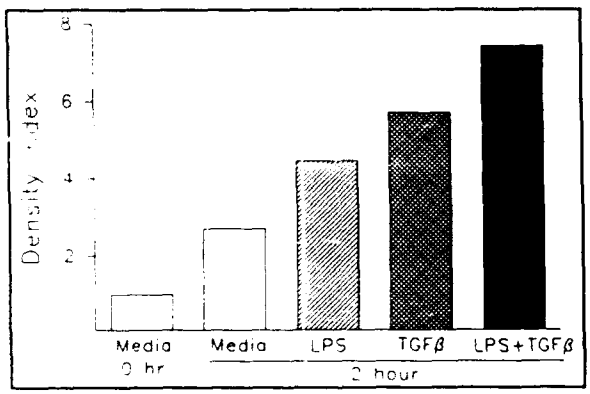

Figure 29 Effect of LPS on TGF-β_1 mRNA secretion by PIMs

These results support the hypothesis that PIMs may act to modulate lung repair mechanisms and that PIMs themselves are influenced by cytokine networks activated by acute lung injury

Soluble Complement Receptor (sCR1)

The cohort of animals treated with sCR1 were compared with results obtained in control and septic animals, previously studied. There were no significant differences between the septic animals and those treated with sCR I with respect to any parameter measured, including systemic arterial pressure, pulmonary arterial pressure, cardiac index, arterial pO_2, arterial pH and extra-vascular lung water.

We have not yet had an opportunity to study neutrophil adhesion and migration to the interstitium in samples of lung tissue obtained from these animals. As complement activation has clearly been shown to be of importance in initiating adhesion of neutrophils to endothelium without necessarily causing tissue injury, we believe that it would be of interest to see if neutrophil adhesion to endothelium was in any way limited by infusion of sCR1 used in this study.

Future Plans

The search for an effective treatment for ARDS is as old as recognition of the condition itself. In the early period after Ashbaugh and Petty had initially described the syndrome as a clinical entity, expiration against a fixed resistance was found to be of benefit in improving patient oxygenation. They coined the term PEEP for this therapeutic modality and it was initially hoped that it would prove to be an effective cure for ARDS. Although it has indeed proved to be effective, it is not a cure but rather a supportive measure and may be associated with significant barotrauma and at high levels, with impairment of cardiac output.

As our understanding of the pathogenesis and pathophysiology of this condition has improved it has been held that prevention is the best approach - a point of view fostered by it's high morbidity and mortality and the overall lack of success of any existing treatment modality. This situation may now be changing as increased knowledge of the disease process may permit physicians to arrest the cascade of destruction, including the cyclooxygenase and/or the cytokine systems. As we have shown in a previous report from this laboratory, ibuprofen is a highly effective compound in this regard, attenuating the neutrophil respiratory burst and blocking the formation of cyclooxygenase metabolites. We have also shown in a previous report that the platelet activating factor antagonist, designated SRI 63-675 is an effective means of blocking the septic cascade but that it causes hemolysis in the course of exerting an effect. The search for an effective therapeutic approach will inevitably be marked by obstacles such as this.

Our plans in the immediate future are to perform a further study utilizing the monoclonal antibody to TNF-α, to see if it is possible to exert a therapeutic effect at a lower dose of anti-TNF-α (5 mg/kg) similar to that already seen at a dose of 15 mg/kg. In addition we also wish

to focus in this study on the protective effect , if any, of anti-TNF-α on the alveolar-capillary membrane. We plan to do this by morphometric studies of tissue from the lungs and other organs of other animals. We also plan to assay tissue myeloperoxidase in septic treated and untreated animals to see whether or not pretreatment with anti-TNF-α has a protective effect.

Following this study, we then plan to study the effects of combined cyclooxygenase and cytokine blockade in the model. We have previously demonstrated the benefits of cyclooxygenase blockade, using ibuprofen in the model. Although many of the derangements seen in this model of sepsis-induced were blocked or attenuated using this agent, the characteristic leukopenia and pulmonary leukosequestration were not. Monotherapy in this model using anti-TNF-α alone however, did attenuate the fall in peripheral neutrophil count, and also the profound metabolic acidosis characteristic of sepsis but did not prevent the pulmonary hypertension and fall in oxygenation noted with cyclooxygenase blockade.

Following the combined cyclooxygenase-cytokine blockade study, we plan to commence a post-treatment study, looking at the effectiveness of anti-TNF-α alone and in conjunction with with ibuprofen administered sixty minutes following the pseudomonas infusion. We believe this to be a particularly important study as it more closely reproduces the clinical setting in which the physician so often finds himself - treating a patient in the early stages of sepsis.

As a follow-up to the study in which no beneficial effect was noted with sCR1, a selective inhibitor of the complement cascade, we plan to study a series of septic animals following cobra venom factor complement depletion to determine if a protective effect is found, as noted by Dehring et al in a similar model.

During the past year we have acquired an assay for nitrate, a stable derivative of endothelium derived relaxing factor (EDRF), a vasodilator which may be involved in the septic process and which may prove to be one of the substances mediating the profound hypotension induced by high levels of circulating TNF-α. In addition to the assay for nitrate, we have also acquired an assay for endothelin, an endogenous antagonist of EDRF, levels of which have been demonstrated recently to be elevated in humans with the sepsis syndrome. We plan to evaluate these mediators in the porcine sepsis model following treatment with ibuprofen and anti-TNF-α, alone and in combination.

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