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INTRODUCTION

During the period of the past two years, this laboratory has focused on the phenomenon of acute lung injury following the onset of systemic infection. To accomplish this we have used the pig to model acute lung injury. In our laboratory using the model as we have developed it, the pig predictably develops acute lung injury following an intravenous infusion of a predetermined quantity of live *Pseudomonas aeruginosa*. Over the last eight years the model has undergone progressive refinements in the quest for more accurately mimicking bacterial sepsis and subsequent lung injury as it occurs in humans. This research funded by the DAMD has led us to progressively greater degrees of sophistication of our understanding of the acute inflammatory responses which lead to acute lung injury following onset of severe systemic bacterial infection. Included in this midterm report are the results of research performed during the past two years of DAMD sponsorship.

Report Format. We have formatted the report to provide clarity and ease of reading. *Section one* will focus on scientific methodologies employed for study of the animal model, certain in vitro techniques and for analysis of materials generated. *Section two* will focus on studies aimed at pharmacological attenuation or blockade of critical biological mediators known to promote acute lung injury. *Section three* will present the results of studies focused at disrupting neutrophil and endothelial cell adhesion molecules which are critical in promoting the tight binding of activated neutrophils to vascular endothelium. *Section four* will focus on the results of inhaled nitric oxide therapy. *Section five* will focus on the results of in vitro studies of adhesion receptor expression by cultured endothelial cells. *Section six* will give the results of the effects of elevated intra-abdominal pressure on cardiovascular and central nervous systems. The report will end with *Section seven* which will present conclusions.

I. SCIENTIFIC METHODOLOGIES EMPLOYED

Animal Preparation and Conditioning. Yorkshire pigs, 15-20 kg, are obtained from a commercial vendor in the Mid-Atlantic region and housed in the Virginia Commonwealth University vivarium for at least 24 hours prior to study. The experimental protocol used for these studies was approved by the Institutional Animal Care and Use committee of Virginia Commonwealth University and adhered to National Institutes of Health guidelines for the use of experimental animals.

The Porcine Model. Swine were pre anesthetized with intramuscular ketamine hydrochloride (25 mg/kg) and placed supine. Sodium pentobarbital (20-30 mg/kg) is then administered intravenously to induce anesthesia. Tracheostomy is performed and the trachea intubated with a cuffed endotracheal tube (Argyle, Tullamore, Ireland). Mechanical ventilation was employed using a Harvard large animal ventilator (Harvard Apparatus, Boston, MA) at 0.5 FiO_2 and 5 cm H_2O positive end expiratory pressure. The ventilator was set to deliver a tidal volume of 15 ml/kg with a respiratory frequency adjusted in all animals to produce a $PaCO_2$ of 40 torr at the beginning of each experiment. *[Approximately 4 months ago we moved to a more sophisticated form of mechanical ventilation using the Emerson Intermittent Manditory Ventilation (IMV) ventilator (Emerson Incorporated, Cambridge MA, to provide greater flexibility in the performance of mechanical ventilation].* Throughout the period of study, anesthesia was maintained by continuous infusion pentobarbital (2-15 mg/kg/hr). Indwelling vascular catheters are then placed in the left common carotid artery for systemic arterial pressure (SAP) monitoring, blood sampling and arterial oxygen determination. The left external jugular vein is cannulated for infusion of saline and *Pseudomonas* organisms. The left internal jugular vein is cannulated for infusion of study agent or saline for control animals. An indwelling balloon tipped pulmonary arterial catheter is inserted via the right external jugular vein and positioned in the pulmonary artery via pressure monitoring for measurement of pulmonary arterial pressure (PAP), pulmonary arterial occlusion (wedge) pressure (PAOP), central venous pressure (CVP) and thermodilution cardiac output (Gould, Oxnard, CA). Cardiac index (CI) was calculated using the formula $CI=CO/(0.112 \times weight^{0.66})$. Systemic vascular resistance index (SVRI) was calculated as $SVRI=(SAP - CVP) \times 79.92/CI$ (1).

Bronchoalveolar Lavage. Bronchoalveolar lavage (BAL) is performed through the indwelling endotracheal tube using a flexible fiberoptic bronchoscope (Olympus BF 4, Olympus Corp, Tokyo, Japan) The instrument is inserted under direct visualization and the distal end of the bronchoscope guided to the 3rd or 4th order bronchi of the middle and lower lobes respectively and gently wedged. Each lobe is lavaged with two aliquots of 25 ml sterile 0.9% NaCl. BAL fluid is removed and immediately centrifuged at 400 g , 4°C, 10 min, and the supernatant stored at -20°C. *Cell analysis:* Cellular constituents from the BAL procedure are analyzed as follows. Cell pellets are resuspended in Dulbecco's phosphate buffered saline (PBS) containing 0.01% bovine serum albumin (BSA). Cell counts are determined using a hemacytometer and slide directed cytocentrifugation is performed (Shandon Southern Instruments, Sewickley,

PA). Differential counts are performed on 200 cells stained using a modified Wright-Giemsa stain (Diff-Quik®, Baxter Scientific, McGaw Park, Ill). BAL protein was measured in the noncellular fraction by the bicinchoninic acid (BCA) microplate method. The BAL procedure is performed both at 0 and again at 300 minutes in right and left lungs respectively (2).

Total White Blood Cell Counts. Arterial blood samples are drawn into sterile glass tubes containing 0.15% EDTA and are kept at 4°C (Vacutainer, Becton Dickinson, Rutherford, NJ). Small aliquots of blood are set aside for white blood cell counts and blood smear differentials which are performed as described above for BAL differential counting. The blood is centrifuged at 1000 g, 4°C for 20 min and the resulting plasma stored at -20°C.

Polymorphonuclear Neutrophil Isolation (PMN). At 0 and 300 minutes arterial blood samples were drawn into sterile syringes containing 0.15% EDTA. PMNs were immediately isolated by dextran sedimentation and ficoll hypaque density gradient centrifugation as described previously (3). Cells counts and viability were confirmed using hemacytometer and trypan blue exclusion respectively.

Organ Myeloperoxidase (MPO) Content. At 300 minutes, animals are sacrificed by infusion of pentobarbital (100 mg/kg) and the organ of interest is excised immediately. To obtain true homogeneity multiple random samples are obtained from throughout each organ. Tissues are weighed and a total of 2 grams are homogenized (Virtis S-45 homogenizer, Virtis, NY) in 4 ml of 20 mM potassium phosphate buffer (pH 7.4). All homogenates are then centrifuged (40,000 g, 4°C, 30 min, Beckman L5-65 Ultracentrifuge, Fullerton, CA). The pelleted materials are resuspended in 4 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB)(Sigma Chemical Co. St Louis, MO) and frozen at -70°C.

Prior to assay batched samples are thawed, sonicated x 90 sec, incubated for 2 hrs (60°C) and centrifuged (1000 g, 30 min, 4°C). Myeloperoxidase content is assessed by adding 50 µl of each sample to quadruplicate wells of a 96 well microplate. Fifty µl of 0.025% dimethoxybenzidine (DMB, Sigma) in 50 mM potassium phosphate buffer containing HTAB is then added. The reaction is started by addition of 50 µl of 0.01% H_2O_2 and the optical density at 460 nm is measured at 0, 1, 2, and 3 minutes (note: times previously established as the linear interval of the reaction kinetics). The average change in optical density (OD) over the period of observation is compared with OD of 10^6 freshly isolated porcine neutrophils prepared in an identical fashion as noted for tissue samples. Results are expressed as units of equivalent myeloperoxidase activity per gram of study tissue (1).

Immunophenotyping for Neutrophil Integrin and Selectin Expression. Direct immunophenotyping is performed using a monoclonal antibody (MoAb 60.3, Oncogen, Seattle, WA), which recognizes a functional epitope on the CD18 adhesion receptor and monoclonal antibody EL-246 (Gift of Mark Jutila, Veterinary Molecular Biology, Montana State University). Antibodies utilized were previously conjugated with

fluorescein isothiocyanate (FITC). Arterial blood samples from study animals are drawn into polypropylene tubes containing 0.15% EDTA and 0.1% NaN_3 and immediately placed on ice. One hundred microliter aliquots of blood are then incubated with an equal volume of MoAb 60.3 or EL-246 or IgG_{2a} control for 30 min at 4°C such that the final concentrations is at antibody excess (previously established by antibody titration curves). Samples are washed twice with phosphate buffered saline (PBS) containing 0.1% EDTA, 0.1% NaN_3 and 0.2% bovine serum albumin (BSA) at 4°C. Erythrocytes are lysed with NH_4Cl buffer and cells resuspended in PBS. Cells are shielded from light at 4°C prior to analysis. Analysis is performed on a flow cytometer with a 4 decade, 1024 channel, logarithmic amplifier (Becton-Dickinson FACScan). Neutrophils are gated according to forward angle and 90° light scatter characteristics. A minimum of 5,000 events are analyzed for each sample and the mean channel fluorescence of gated neutrophils is calculated. Mean channel fluorescence, a logarithmic function, is converted to a linear scale using fluorecent microbead standards and Quickcal software (Flow Cytometry Standards Corp., Research Triangle Park, NC). Results are expressed as molecules of equivalent soluble fluorochrome (MESF) (1).

Superoxide Anion Assay (O_2^-). Neutrophil superoxide anion production is determined by measuring the superoxide dismutase (SOD) inhibitable reduction of cytochrome *c* using a dual-beam spectrophotometer (Shidmadzu, Columbia, MD). Neutrophils ($1x10^6$/ml) and cytochrome c (100 µM) are combined in a thermostatically controlled stirred cuvette (37°C). Identically prepared reference cells contain reaction products plus SOD (300 U/ml). The reaction is started by adding phorbol myristate acetate (PMA) (200 ng/ml) to each cuvette. The change in absorbance at 550 nm is continuously recorded for 10 minutes. Results are expressed as the rate of O_2^- production (nmol/10^6PMN/min) based on an extinction coefficient of: $\Delta\varepsilon 550nm = 2.10 \times 10^4 M^{-1}cm^{-1}$ (3).

Detection of Pulmonary E-selectin Expression In-Situ Via Immuno-histochemistry. Following animal sacrifice as outlined above, a segment of lung is removed and sliced into 2 x 2 cm cubes and further sliced wafer thin with a fresh scalpel. The lung tissue is fixed in neutral buffered formalin for 24 hours. The tissue is then removed and flooded with Bouin's fixative (0.95 picric acid, 0.9% formaldehyde, 5% acetic acid, Sigma, St Louis, MO) for 6 hours. Following Bouin's fixation the tissue is transferred to 70% ethanol where it is stored until processing. The tissue was placed in paraffin blocks and multiple 5 µm thickness sections made. Prior to antibody staining the sections were deparaffinized using xylenes x 2 minutes followed by graded hydration by immersion in falling concentrations of EtOH (100%, 95%, 80%, 70%) for 3 minutes at each stage to dH_2O. The sections are then placed in Bouin's fixative for 2 minutes followed by immersion in TRIS buffer 100 mM x 10 minutes. Endogenous peroxidase activity is blocked by incubating sections with 0.3% H_2O_2 for 30 minutes at room temperature (RT). Sections are washed d-H_2O and incubated with normal goat serum to reduce background staining. Slides are rinsed with TRIS and then incubated with, primary antibody, (EL-246, 100 µg/ml or 1.2B6, 100µg/ml), overnight at 4°C. Antibody is shaken off and a secondary biotinylated antibody (Goat anti-mouse) is

flooded onto sections and incubated x 30 min at RT. Sections are then exposed to streptavidin-horseradish peroxidase and developed by exposure to diaminobenzidine as per manufacturers recommendations (Histomark Streptavidin-HRP, KPL, Gaithersburg, MD, DAB Reagent Set, KPL, Gaithersburg, MD). Control for non-specific binding of EL-246 and 1.2B6 is accomplished by the identical procedure via addition of non-immune IgG_1 as a substitute for primary antibody (4).

Tumor Necrosis Factor-α (TNF) Assay. The PK-15 (porcine kidney cell) bioassay used is based on the method of Bertoni et al (5). Freshly passaged PK-15 cells (ATCC, CCL33, Rockville, MD) are seeded into 96-well microtiter plates (Costar, Cambridge, MA) in 100 µl Eagle's Essential Medium (Sigma, St Louis, MO) with 7% Fetal calf serum (GIBCO, New York, NY). After culture (37°C in 5% CO_2), the medium is removed and replaced with 50 µl basal Iscove's media (Sigma) supplemented with 0.5% bactopeptone (Difco, Detroit, MI) and 3 µg/ml Actinomycin D. Following a 2 hour incubation, 50 µl of diluted samples are added to each well. The plates are then incubated for an additional 18 hours (37°C, 5% CO_2). Fifty µl per well of a 3 mg/ml solution of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) in d-H_20 is added to each well and plates are incubated for 4 hours at 37°C. The test is terminated by the aspiration of medium and addition of 100 µl cell lysis buffer (0.5% SDS, 36 mM HCl in isopropanol). The plates are shaken for 3 minutes and optical densities determined spectrophotometrically at 570 nm. On each microplate two wells contain medium without TNF (defined as 100% viability) and two wells are washed with d-H_2O to lyse PK-15's before addition of MTT (defined as 100% cytotoxicity). TNF activity is expressed as the percentage of non-viable cells using the formula: % Cytotoxicity = $1-(OD_{sample} \div OD_{control})x100$. Units are defined as the reciprocal of the dilution at which 50% cytotoxicity occurs.

Culture of Endothelial Cells. Endothelial cells are isolated from human umbilical veins (HUVEC). Fresh umbilical veins are cannulated with one-way stopcocks, and the lumen perfused with phosphate buffered saline (PBS). The lumen is then filled with PBS containing 0.1% collagenase (Clostridium hystolyticum, type I Sigma, St. Louis, MO) and incubated at 37°C x 15 minutes. The collagenase solution is flushed into conical tubes with PBS and HUVEC are pelleted by centrifugation (200g x 5 minutes). HUVEC are resuspended in Medium-199 containing 10% fetal bovine serum, 20 µg/ml endothelial cell growth supplement (Chemicon, Temecula, CA), 90 µg/ml heparin (Sigma), and 1% antibiotic/ antimycotic solution and plated in 25 cm^2 gelatinized flasks (Corning). Cells are grown to confluence at 37°C, 5% CO_2. Confluent cultures are passed weekly at 1:5 ratios until an experiment is performed or are frozen in the first or second passages for later use in liquid nitrogen. Endothelial identity of cultured cells is confirmed by immunofluorescent staining for von Willebrand factor (6).

Intercellular Adhesion Molecule-1, E-Selectin Quantification By Enzyme Linked Immunosorbant Assay. E-selectin in media and lysates of HUVEC cultures were measured in a quantitative sandwich ELISA sensitive to 0.1 ng/ml. This assay was developed and performed at the Maryland Research Laboratories of Otsuka America

Pharmaceutical, Inc. ICAM-1 in the same media and lysates was measured in a quantitative sandwich ELISA sensitive to 0.3 ng/ml (T Cell Diagnostics, Inc.). Amounts of adhesion molecules in media and lysates were expressed as ng/cm^2 of culture growth surface. The cell density was approximately 5 x 10^4 cells/ cm^2 (7,8)

Establishing Pseudomonas Aeruginosa Cultures. *P. aeruginosa* cultures were grown in nutrient broth (Difco, Detroit, MI) at 37°C until stationary growth phase. They were then centrifuged, washed once with 0.9% saline and resuspended in 0.9% saline. A sample of this suspension was diluted x 5 and the final concentration of the sample was adjusted using a spectrophotometer (Shimadzu, Japan) to an optical density of 0.2 at 660 nm. The whole bacterial solution was then adjusted similarly to obtain a fixed concentration of organisms and maintained at 4°C until use.

Establishing Acute Lung Injury. Studies will proceed once all animals prepared as noted above are judged to be physiologically stable. Baseline blood sampling, hemodynamic measurements and bronchoalveolar lavages are performed prior to initiation of intravenous infusions (bacteria, agents). For virtually all studies involving the use of pharmacological or immunopharmacologic intervention, three groups of animals are studied. Typically, **Group 1** animals (Controls) receive only a 60 minute intravenous infusion of sterile saline. **Group 2** animals (Untreated Sepsis) receive a 60 minute intravenous infusion of live *Pseudomonas aeruginosa,* PAO strain. *Pseudomonas* organisms are infused at 5 x 10^8 Colony Forming Units (CFUs) per ml at a concentration by body weight of 0.3 ml of organism solution/20 kg/min. **Group 3** animals (Treated Sepsis) receive an infusion of pharmacological study agent at predetermined time points either immediately prior to commencing the infusion of *Pseudomonas* aeruginosa or at delayed time points (e.g., 30 min, 60 min) following organism infusion. In all studies involving the *acute* animal model system animals were studied for a total of 300 minutes.

Statistical Analysis. Unless otherwise stated, data are presented as means ± SEM. Differences between and within groups were analyzed using analysis of variance (ANOVA) with Tukey's Studentized range test. Paired data were analyzed using a Paired T-test. Statistical significance was assumed for a *P* value less than 0.05.

II. ATTENUATION OF SEPTIC LUNG INJURY BY PHARMACOLOGICAL AGENTS WHICH ATTENUATE PROINFLAMMATORY MEDIATORS.

PENTOXIFYLLINE

Introduction. Pentoxifylline (PTX) is a methylxanthine previously utilized in chronic arterial insufficiency because of its ability to affect the fluidity of erythrocytes. The agent displays multiple inhibitory effects on neutrophil function *in vitro* including reduction of oxidant burst (9-11) and down regulation of adhesion receptor ($ß_2$ integrin, $CD11_b$ CD18) expression (9,10,12). Pentoxifylline attenuates tumor necrosis factor-α (TNF-α) output by monocytes *in vitro* (13). The exact mechanisms of neutrophil function attenuation are unknown. The agent is a phosphodiesterase inhibitor and may well exert its effects via increased intracellular cAMP levels (14). Welsh et al first showed that PTX pre-treatment protected the lungs in a canine model of endotoxin induced acute lung injury (15). Other investigators showed beneficial effects of pre-treatment PTX in a variety of models of sepsis and acute lung injury (16-19). Only one study has investigated and demonstrated beneficial effects of PTX administered after the onset of sepsis (20). Pre-treatment with PTX in animal models of septic shock and acute lung injury improves survival and reduces tissue damage in both endotoxin (15,16) and TNF (19) induced lung injury. However, little data on delayed treatment of sepsis using PTX, a more clinically relevant situation, exists (20). We therefore sought to: (a) study the effects of pre- and post-treatment PTX in a porcine model of bacterial sepsis and acute lung injury; (b) determine the size of a potential "therapeutic window" for PTX in sepsis and; (c) further elucidate the mechanism of action of PTX *in vivo.*

Experimental Design. Six groups of animals were studied. **Group 1** (Control, n=8) received a 60 minute intravenous infusion of sterile saline. **Group 2** (Sepsis, n=8) received a 60 minute intravenous infusion of live *Pseudomonas aeruginosa*, PAO strain ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min) alone with no further intervention. Three further groups of septic animals were studied, each receiving a 20 mg/kg bolus of Pentoxifylline (Hoechst-Roussel, Somerville, NJ) followed by continuous infusion of (6 mg/kg/hr) until the end of the experiment. **Group 3** (Pretreatment PTX, n=6) commenced the PTX regimen 15 minutes prior to the bacterial infusion. **Group 4** (Delayed-treatment PTX, n=6) received the bolus of PTX immediately following the termination of the bacterial infusion (T=60 mins). **Group 5** (Late-treatment PTX, n=4) began PTX administration 60 minutes after the bacterial infusion (T=120 mins). **Group 6** (Control PTX, n=3) received the PTX regimen only. All animals were studied for a total of 300 minutes.

In Group 5 animals we observed a 50% mortality and therefore the study in this group was terminated at n=4 animals. The two non-surviving animals developed profound hypotension following initiation of PTX infusion. Neither animal survived beyond 3 hours. However, in the two animals surviving to 300 minutes, we noted similar measurements of hemodynamics and pulmonary function as compared to the post-

treatment group. The small numbers in the Group 5 did not permit statistical conclusions. Group 6 animals showed no differences in any measured variable when compared to Group 1 (Controls).

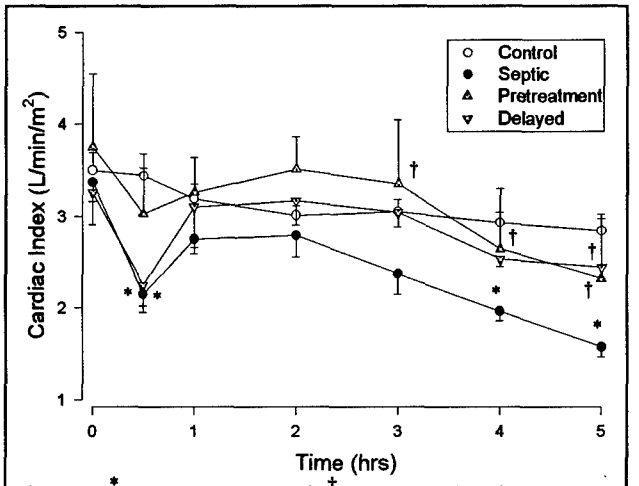

Figure 1 *p<0.05 vs Control, †p<0.05 vs Septic.

Hemodynamic Measurements. Pre-treatment and post-treatment administration of PTX improved declining cardiac index (CI) observed in septic unprotected animals. Animals in the septic and post-treatment groups showed a significant reduction in cardiac index at 30 minutes, coinciding with infusion of *P. aeruginosa*. Animals pretreated with PTX were protected from this initial acute decline in CI. Following cessation of *P. aeruginosa* infusion, CI improved in all groups and from 1 hour onwards both PTX treatment groups showed no significant difference from control animals. Septic unprotected animals, underwent a progressive decline in CI which was significantly lower than control and pretreatment animals at 4 hours, and lower than all groups at 5 hours (Figure 1).

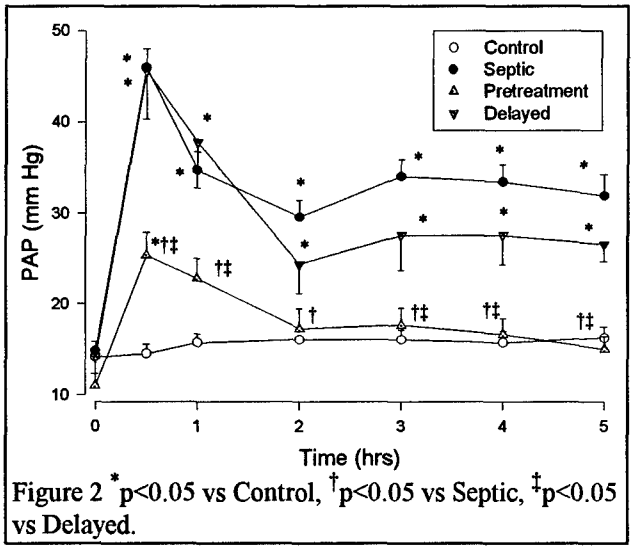

Figure 2 *p<0.05 vs Control, †p<0.05 vs Septic, ‡p<0.05 vs Delayed.

Pulmonary artery pressure (PAP) rose significantly at 30 minutes in all groups compared to controls. The rise in PAP in pretreated animals was significantly attenuated compared to post-treated and unprotected animals (Figure 2). In animals pretreated with PTX, PAP fell to control levels by 2 hours. In septic and post-treated animals PAP remained significantly elevated compared to both control and pretreated animals.

Systemic arterial pressure (SAP) rose sharply in the first 30 minutes of the *P. aeruginosa* infusion in all groups, although it was significantly elevated only in the septic unprotected animals (Figure 3). Following the initial 30 minutes we observed progressive systemic arterial hypotension in all septic animals, becoming significantly worse than controls

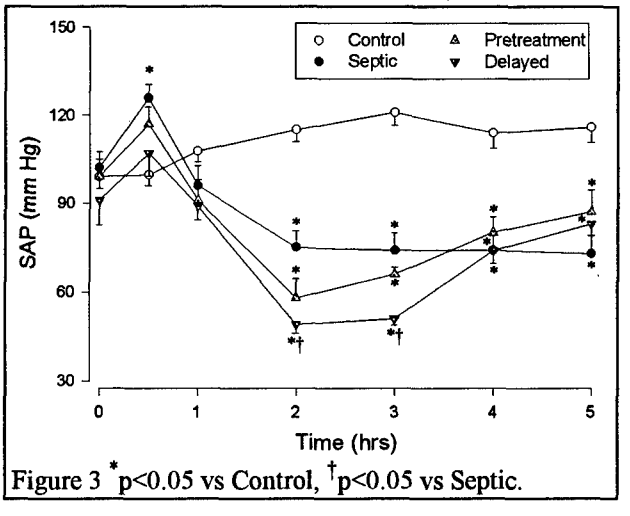

Figure 3 *p<0.05 vs Control, †p<0.05 vs Septic.

from 2 hours onwards. At 2 and 3 hours both pre and post-treatment PTX animals exhibited more severe hypotension than untreated septic animals. Hypotension was significantly worse in the post-treatment animals at 2 hours, compared to the septic unprotected group. SAP in both PTX treated groups recovered and returned to levels similar to those of untreated septic animals by 4 hours.

Systemic vascular resistance index (SVRI) mirrored the changes seen in SAP. By 30 minutes SVRI was significantly raised in septic and post-treated animals compared to controls. Rising SVRI in pretreated animals was significantly attenuated compared to untreated septic animals. At 1 hour all levels returned to that of controls. By 2 hours SVRI was significantly lower in all groups compared to controls. Those animals treated with both pre- and post- PTX exhibited a significantly lower SVRI than the untreated septic group at this time point. By 4 hours onwards, all groups had recovered to control levels (data not presented).

Blood Gas Measurements. PTX treatment attenuated the decline in arterial PaO_2 levels while oxygen tension rapidly and progressively deteriorated in the septic unprotected animals, being significantly worse than controls from 30 minutes onwards.

		TIME (hrs)						
		0	*0.5*	*1*	*2*	*3*	*4*	*5*
Arterial pH	Control	7.46±0.01	7.45±0.02	7.46±0.01	7.45±0.01	7.46±0.01	7.46±0.01	7.46±0.01
	Septic	7.46±0.01	7.41±0.02*	7.35±0.02*	7.33±0.02*	7.31±0.01*	7.27±0.02*	7.21±0.03*
	Pentox	7.47±0.02	7.43±0.02	7.42±0.01	7.40±0.02	7.37±0.03*	7.35±0.03*†	7.34±0.02†
	Delayed	7.50±0.01	7.45±0.02	7.41±0.02	7.37±0.02*	7.36±0.03*	7.37±0.03*†	7.38±0.02*†
PaO_2	Control	250±8	246±10	262±7	251±7	245±8	254±9	252±8
	Septic	262±7	182±14*	136±20*	113±13*	118±18*	96±11*	87±7*
	Pentox	251±10	200±14	206±16*†	175±18*†	185±21*†	159±23*†	154±19*†
	Delayed	262±5	194±28	176±34*	190±41†	177±38*†	159±28*†	164±26*†

Table 1 *p<0.05 vs Control, †p<0.05 vs Septic

(Table 1). Untreated porcine sepsis was characterized by a severe, progressive acidosis. Administration of PTX partially protected the animals from acidosis with pre-treated animals exhibiting a gradual decline in arterial pH which was significantly lower than controls from 3 hours onwards. Post-treated animals had significantly lower pH by 2 hours compared to controls. Both PTX groups, however, showed a significantly improved pH compared to the septic untreated group at 4 and 5 hours.

BAL Analysis. BAL protein levels (Figure 4) at 5 hours were significantly elevated compared to baseline levels in the septic unprotected group. In both the PTX treated groups and in the control group, 5 hour levels were not significantly increased compared to baseline.

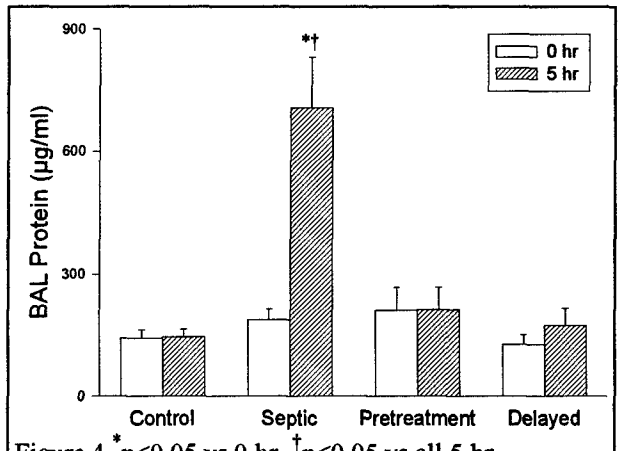

Figure 4 *p<0.05 vs 0 hr, †p<0.05 vs all 5 hr treatments.

		TIME (hrs)					
		0	*1*	*2*	*3*	*4*	*5*
CD18	Control	45±6	46±6	47±7	47.0±7	46.3±6	46±6
(MESF x10^3)	Septic	43±5	57±7	94±12*	87.6±15*	80.8±13*	74±12
	Pentox	54±7	69±3	112±15*	116±17*	105±22*	92±16*
	Control	21.0±2	16.4±1	17.4±2	19.8±2	20.4±3	22.0±3
Total WBC	Septic	18.0±2	5.3±1*	2.6±1*	2.4±1*	2.4±1*	3.0±1*
(x10^3/μl)	Pentox	15.2±3	2.5±1*	1.9±1*	2.2±1*	3.4±1*	4.0±2*
	Delayed	22.7±4	5.8±2*	4.8±2*	7.7±5*	6.9±5*	8.5±7*

Table 2 *p<0.05 vs Control

BAL neutrophil counts at 5 hours were significantly elevated in the septic unprotected group compared to baseline levels. Both PTX treated groups and the control group showed no significant rise in BAL PMN count at 5 hours compared to baseline. The BAL PMN count at 5 hours was significantly elevated in the septic group compared to all other groups at this time point (data not shown).

Neutrophil Adhesion Receptor Expression and Kinetics. Neutrophil CD18 receptor expression was studied at hourly intervals in control, septic and pretreatment groups. In septic unprotected animals, receptor expression rose sharply from one hour to peak at two hours (Table 2). A gradual decline then occurred for the remainder of the study. Pretreated animals showed a similar pattern of increased receptor expression with maximal expression at 3 hours. Both groups showed significantly increased receptor expression compared to controls from 2 hours onwards.

Peripheral neutrophil counts fell in unprotected septic animals, being significantly lower than controls by 1 hour and reaching a nadir by 2 hours, remaining depressed for the remainder of the study (Table 2). Both PTX groups also showed a similar fall in neutrophil counts, significantly lower than controls by 1 hour and not different from each other or unprotected septic animals throughout the entire study.

Plasma Tumor Necrosis Factor Assay. Both septic and pretreatment groups exhibited a rapid, significant rise in TNFα by one hour whereas the control group exhibited almost undetectable levels throughout the experiment (Table 3). TNF levels in the septic and pretreatment groups peaked between 1-2 hours and then rapidly declined, becoming similar to controls by 4 hours. There was no significant reduction in TNF production in the pretreatment group compared to untreated septic animals. TNF production was not measured in the post-treatment group.

Neutrophil Superoxide Generation. Neutrophils isolated from control animals at the conclusion of the study showed no evidence of significant priming for increased

		TIME (hrs)					
		0	*1*	*2*	*3*	*4*	*5*
Plasma TNF	Control	0.2±0.1	0±0	0±0	0±0	0.4±0.2	0.6±0.4
(U/ml)	Septic	0.7±0.5	175±45*	180±49*	48±11*	24±7	15±4
	Pentoxifylline	0.4±0.4	184±52*	176±56*	48±14*	20±5	11±2

Table 3 *p<0.05 vs Control.

		0 hour	*5 hours*
Superoxide Generation	Control	6.6 ± 0.8	8.9 ± 1.4
(nmol/10^6 PMN/10 mins)	Septic	7.1 ± 1.1	14.0 ± 2.7*
	Pre treat PTX	6.6 ± 1.9	17.5 ± 2.0*
	Delayed	2.4 ± 1.6	16.2 ± 5.6*
Lung Myeloperoxidase	Control	---	9.4 ± 1.4
(10^6 PMN / gram tissue)	Septic	---	57. 4 ± 6.4†
	Pre Treat PTX	---	39.6 ± 8.2†

Table 4 *p<0.05 vs 0 hr, †p<0.05 vs Control.

superoxide production compared to neutrophils isolated at baseline (Table 4). However, neutrophils isolated at five hours in septic unprotected animals and both PTX treatment groups exhibited significant priming for superoxide production compared to neutrophils isolated at baseline.

Lung Myeloperoxidase Content. Septic and pretreated animals showed significant elevation of lung myeloperoxidase content at 5 hours compared to controls. There was no significant reduction of myeloperoxidase in the pretreated group compared to untreated septic animals (Table 4). Lung myeloperoxidase was not measured in the post PTX group.

Summary

Pentoxifylline pre-treatment in our model of sepsis-induced acute lung injury (ALI) was effective. This study also helps define a "therapeutic window" for PTX in the septic model. In our initial studies, we found that administration of PTX in a pre-treatment protocol greatly improved cardiac index, attenuated pulmonary artery hypertension and significantly reduced lung injury. These findings are in agreement with other studies of sepsis-induced lung injury showing protection by PTX pre-treatment protocols (16,19). Animals receiving PTX 60 minutes after the onset of sepsis also exhibited similar improvement in cardiac index and lung injury, although it failed to prevent development of pulmonary artery hypertension. We have previously shown pulmonary artery hypertension due to the release of thromboxane which occurs within minutes of the onset of sepsis (21). Therefore, although PTX has been shown, *in vivo*, to inhibit thromboxane release (22) , post-treatment PTX at 60 minutes would not be expected to have any significant inhibitory effect on the development of pulmonary artery hypertension in this model.

Pre-treatment PTX attenuated early systemic arterial hypertension but, interestingly, by two hours both pre- and post-treated groups showed a lower systemic pressure than that observed in unprotected septic animals. Both PTX treated groups then showed a later recovery in SAP with no significant difference between all septic groups by 4 hours. These findings are mirrored by the systemic vascular resistance index; which showed a significant fall in pre and post-treated animals at two hours compared to unprotected septic animals with all groups recovering to normal levels by 4 hours. However, cardiac index at 2 hours is not significantly different from control values in septic, pre- or post-treated PTX animals, indicating that worsened systemic

arterial hypotension observed in PTX treated animals likely resulted from a reduction in the systemic vascular resistance. These results suggest that administration of PTX to septic animals worsens systemic arterial hypotension. Hypotension induced by PTX has previously been reported (20,23,24) although the mechanisms are not clearly defined. However, in our model there was no significant reduction in SAP by PTX administration in the PTX control group (data not shown). Administration of PTX 2 hours after the onset of sepsis produced a 50% mortality in 4 animals studied. Both animals which died, did so within one hour of the PTX infusion when profound systemic hypotension occurred. The 2 animals which survived to 5 hours in this group exhibited very similar hemodynamic and pulmonary protection to that observed in animals treated at 60 minutes (data not shown).

The hemodynamic data presented for PTX treated animals helps to define a therapeutic window for its use in sepsis. Our results show that PTX can be safely administered early in the septic process and confers significant hemodynamic and pulmonary protection. If PTX is administered to animals in uncorrected septic shock, the agent may produce further fatal systemic arterial hypotension, thereby limiting the size of the potential therapeutic window. The effects of delayed PTX administration in a resuscitated model of sepsis needs to be studied. If systemic hypotension is corrected by the administration of intravenous fluids or vasopressors, prior to PTX administration, the resulting size of the therapeutic window for PTX administration may possibly expand.

In the model utilized in these studies, septic unprotected animals exhibited a progressive, severe metabolic acidosis. PTX (pre and post sepsis administration) attenuated the severity of acidosis following onset of sepsis, significantly improving arterial pH by 4 hours compared to untreated animals. Improved pH correlated with the improved cardiac index in PTX treated animals. Thus, although PTX administration lowered SAP and peripheral resistance, improved cardiac index appeared to improve tissue perfusion deficits.

The three markers of lung injury, (arterial oxygen tension, bronchoalveolar lavage protein levels and neutrophil counts), used to assess alveolar capillary membrane injury showed that all variables were improved by PTX treatment. Following the onset of sepsis, arterial oxygen tension rapidly and progressively fell in unprotected animals. In both pre- and post-treated animals, the observed fall in $Pa0_2$ was attenuated by 1 hour and remained significantly improved compared to untreated septic animals. No significant difference in arterial $Pa0_2$ between PTX animals was observed. BAL protein and neutrophil count at 5 hours in septic unprotected animals was significantly increased when compared to baseline levels, indicating alveolar - capillary membrane injury had occurred. At 5 hours in both pre- and post-treatment groups no significant increase in BAL protein or neutrophil counts occurred showing protection by PTX. Our results agree with other studies where pre-treatment PTX has been shown to reduce pulmonary permeability and PMN migration in models of endotoxin or TNF-induced lung injury (15,19) as well as in septic lung injury (16,25). However, post-treatment PTX has not previously been shown to reduce PMN migration despite protecting against increased permeability to proteins (26).

The mechanism by which PTX confers protection on our model of sepsis and acute lung injury is not clear. Septic unprotected animals exhibit a precipitous drop in peripheral white blood count due to neutrophil sequestration in the lung. Pulmonary sequestration of PMN can be indirectly assessed by lung myeloperoxidase levels. In this study PTX failed to prevent the development of neutropenia or reduce lung myeloperoxidase levels, indicating continuing lung sequestration of neutrophils.

Several studies have shown reduced superoxide generation by neutrophils treated with PTX *in vitro* (9,11,27) , whereas our results show no reduction in neutrophil priming for superoxide generation in either PTX treated group. With large numbers of apparently activated neutrophils sequestered in the lungs of PTX treated animals we would expect similar levels of pulmonary injury and dysfunction as observed in unprotected septic animals, rather than the significant protection demonstrated.

Another measure of neutrophil activation which PTX has been shown to reduce *in vitro*, is upregulation of CD11/CD18 adhesion receptors. These receptors are required for firm adherence to endothelium to permit migration across the basement membrane. Sepsis significantly upregulated the receptors within 2 hours and this was not inhibited by PTX treatment. This is in agreement with an *in vivo* (28) and an *ex vivo* (9) study where PTX failed to down regulate adhesion receptor expression, despite doing so, *in vitro*, in the same studies.

Tumor necrosis factor-α is an important mediator in the inflammatory process. Studies have shown a reduction in monocyte TNF production by PTX *in vitro* (13,29). These prior studies demonstrated a reduction in TNF levels (measured by ELISA), bioactivity and mRNA transcription. We, however, demonstrated no significant reduction in TNF production *in vivo* in PTX treated animals, compared to untreated septic animals. This appears to be in direct conflict with other *in vivo* studies which have reported a significant reduction in TNF levels by PTX treatment in a variety of models of sepsis (30-32). These studies, however, all used an ELISA technique to measure TNF rather than a bioassay which measures bioactivity of TNF rather than absolute concentrations.

In summary pre-treatment and early post-treatment pentoxifylline exerts significant beneficial effects on pulmonary and systemic hemodynamics in a model of sepsis-induced lung injury. However, if the administration of pentoxifylline is delayed until the animal is in established septic shock, the agent may induce further fatal hypotension. These findings help to delineate a "therapeutic window" for the use of pentoxifylline in sepsis. The effects of pentoxifylline on a resuscitated model of septic shock remain to be determined. One small, non-randomized study (26) has demonstrated that pentoxifylline may reduce mortality in humans suffering from sepsis syndrome.

LIPID A ANALOG, B464

Introduction. Many pathophysiological derangements associated with gram-negative sepsis result from the release of lipopolysaccharide (LPS, endotoxin), an integral component of the cell walls of gram negative bacteria (33,34). It is now well established that the **lipid A** portion of LPS mediates the deleterious effects of LPS (35). Lipid A initiates the release of proinflammatory mediators from monocytes, such as tumor necrosis factor and interleukin-1. These mediators promote activation of neutrophils and vascular endothelium (36,37). As noted above, sequestration of activated neutrophils in lung and other organs, is a critical primary event in the genesis of sepsis associated lung injury and in the genesis of other nonpulmonary organ failure (38).

Certain lipid A precursors, such as the monosaccharide, lipid X, inhibit the effects of LPS both *in vitro* and *in vivo* (39,40). LPS from some nonenterobacterial species, such as *Rhodobacter capsulatus* (41) and LPS or lipid A from *Rhodobacter sphaeroides* (42,43) block LPS-induced cytokine release from monocytes and macrophages. The structures of these *non-toxic lipid A molecules* have been determined and molecules with equivalent activity synthesized. B464, a synthetic, stabilized analog of *R. capsulatus* lipid A demonstrates potent *in vivo* antagonism of LPS in murine endotoxin challenge models as well as in human *in vitro* systems. We hypothesized that a lipid analog such as B464 may be protective against gram-negative sepsis and sought to determine the effects of pretreatment with the agent in our porcine model of sepsis-induced acute lung injury.

Experimental Design Of B464 Studies. Four groups of animals were studied. **Group 1** (Control, n=8) received a 60 minute intravenous infusion of sterile saline. **Group 2** (Sepsis, n=8) received a 60 minute intravenous infusion of live *Pseudomonas. aeruginosa,* PAO strain ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min). **Group 3** (Pretreatment B464, n=6) received a B464 bolus (100 µg/kg in 20 ml 0.9% w/v saline) immediately prior to commencing the infusion of *P. aeruginosa* followed by a 60 minute infusion of B464 (100 µg/kg) during the bacterial infusion. **Group 4** (Control B464, n=2) received B464 only. All groups were studied for a total of 5 hours after which the animals were sacrificed in the usual fashion.

Hemodynamics. Untreated septic animals exhibited similar significant increases in systemic arterial pressure (SAP) during the bacterial infusion as that observed in pentoxyfilline studies. Systemic arterial hypertension was followed by progressive fall in SAP becoming significantly lower than controls after 2 hours. B464-treated animals (Group 3) exhibited a similar pattern in SAP until 2 hours following onset of sepsis. From two hours onwards septic B-464 treated animals developed a progressive increase in SAP, becoming significantly elevated above untreated septic animals at 5 hours (Table 5).

		TIME (hrs)						
		0	*0.5*	*1*	*2*	*3*	*4*	*5*
SAP	Control	99±4	100±4	109±4	115±4	121±4	112±5	113±5
	Septic	102±5	120±6*	93±5	67±6*	68±6*	73±5*	72±5*
	B464(S)	90±8	109±6	93±5	75±8*	81±10*	93±9	98±3†
	B464(C)	105±7	100±3	105±4	100±4	100±5	100±6	103±4
PAP	Control	15±1	15±1	16±1	17±1	16±1	16±1	16±1
	Septic	16±1	47±2*	36±2*	31±2*	35±2*	35±2*	33±2*
	B464(S)	14±1	49±3*	39±3*	33±2*	34±1*	36±2*	34±3*
	B464(C)	17±3	18±3	17±3	18±2	16±1	20±3	22±3
CI	Control	3.5±0.2	3.5±0.2	3.2±0.1	3.0±0.1	3.0±0.1	2.9±0.1	2.9±0.2
	Septic	3.3±0.2	2.0±0.2*	2.7±0.2*	2.5±0.3*	2.2±0.2*	1.9±0.1*	1.6±0.1*
	B464(S)	3.1±0.2	2.2±0.2*	2.4±0.2*	2.8±0.2	2.5±0.2	2.2±0.2*	2.1±0.2*
	B464(C)	3.0±0.3	3.1±0.3	3.1±0.1	3.4±0.2	3.0±0.2	2.8±0.3	2.7±0.2
SVRI	Control	2366±177	2277±133	2659±173	2956±172	3128±219	2994±173	3046±162
	Septic	2523±214	4688±387*	2501±210	1967±168*	2258±149*	2741±312	3202±196
	B464(S)	2303±166	4025±252*	3089±257	2154±223*	2614±223	3431±867	3807±674*
	B464(C)	2410±199	2289±200	2800±220	2765±300	2989±221	2763±312	2899±243

Table 5 (S)=Septic, (C)=Control, *p<0.05 vs Control, †p<0.05 vs Septic

Both treated and untreated septic animals exhibited significant pulmonary arterial hypertension within 30 minutes of initiation of sepsis compared to control animals. PAP fell following cessation of *Pseudomonas* infusion, however, significant pulmonary arterial hypertension persisted in treated and untreated septic groups. Cardiac index (CI) exhibited a biphasic pattern in both treated and untreated animals. CI declined abruptly by 30 minutes in both groups corresponding to increased PAP. A partial recovery occurred in CI by 2 hours, followed by a second progressive decline until 5 hours. A biphasic pattern also occurred in SVRI in untreated septic animals. During the bacterial infusion there was a significant increase in SVRI. However, by 2 hours SVRI had decreased significantly compared to controls. We then observed a progressive recovery of SVRI reaching control measurements by 4 hours. In B464-treated animals we observed an initial pattern similar to that of untreated septic animals. However, from 2 hours onward SVRI recovered rapidly and we observed a rebound increase so that at 5 hours, in B464-treated animals, SVRI was significantly elevated above that observed in control animals.

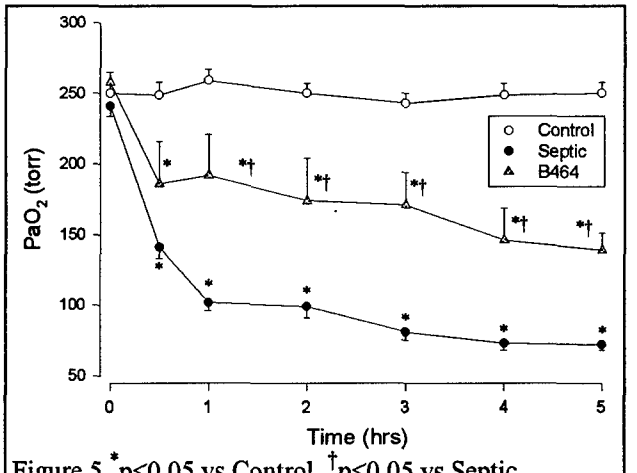

Figure 5 *p<0.05 vs Control, †p<0.05 vs Septic.

Arterial blood gas analysis. Arterial oxygen tension (Figure 5) in septic animals declined rapidly and progressively, being significantly lower than controls within 30 minutes and remaining so for the duration of the experiment. B464-treated animals also

exhibited a significant decline in arterial oxygen tension compared to controls, however, they exhibited a significantly improved PaO_2 compared to untreated septic animals. In untreated septic animals we observed a severe, progressive acidosis during the experiment with arterial pH significantly lower than controls from 1 hour onwards. Arterial pH also declined in B464-treated animals compared to controls but was significantly improved from 2 hours onwards compared to the untreated group (data not shown).

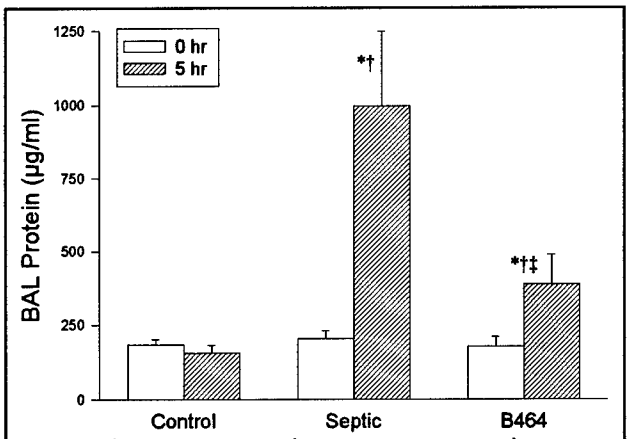

Figure 6 *p<0.05 vs 0 hr, †p<0.05 vs Control, ‡p<0.05 vs Septic.

Bronchoalveolar lavage analysis. Bronchoalveolar lavage (BAL) protein content (Figure 6) in untreated animals showed a significant increase at 5 hours compared to baseline levels indicating a permeability injury to the alveolar-capillary membrane had occurred. B464-treated animals also exhibited a significant increase in BAL protein at 5 hours compared to baseline, however, the protein content was significantly lower than that observed in untreated animals. Both groups exhibited a significant increase in BAL protein content compared to control animals which showed no significant change from baseline levels.

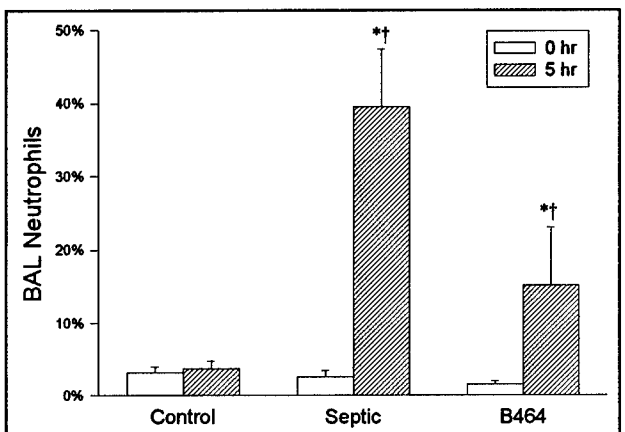

Figure 7 *p<0.05 vs 0 hr, †p<0.05 vs Control.

A significant increase in BAL neutrophil count was observed in untreated septic animals at 5 hours compared to baseline and to control animals (Figure 7). A significant rise in BAL neutrophil count was also observed in the B464-treated animals, and although this was less than that of untreated animals, a statistically significant reduction did not occur.

Neutrophil kinetics. Both untreated septic animals and B464-treated animals developed, significant neutropenias within the first hour of the experiment (Figure 8). Cessation of the bacterial infusion was not associated with significant amelioration of the neutropenia

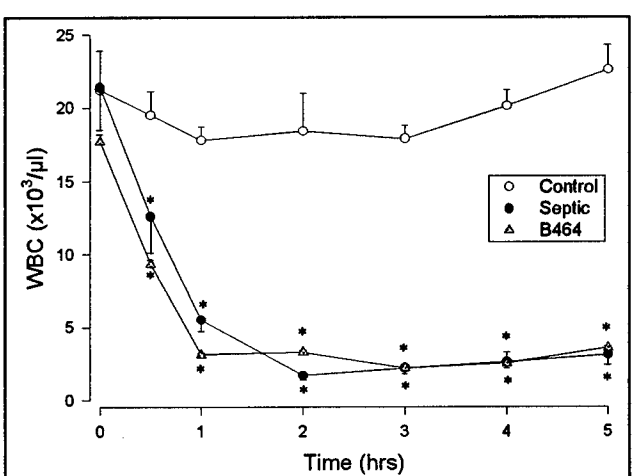

Figure 8 *p<0.05 vs Control.

and there was no significant difference between the two septic groups at any time point.

Lung myeloperoxidase content. (Figure 9), Lung MPO, was significantly elevated at 5 hours in the untreated animals compared to controls indicating significant neutrophil sequestration within the lung. B464 treatment did not significantly reduce lung neutrophil sequestration compared to septic.

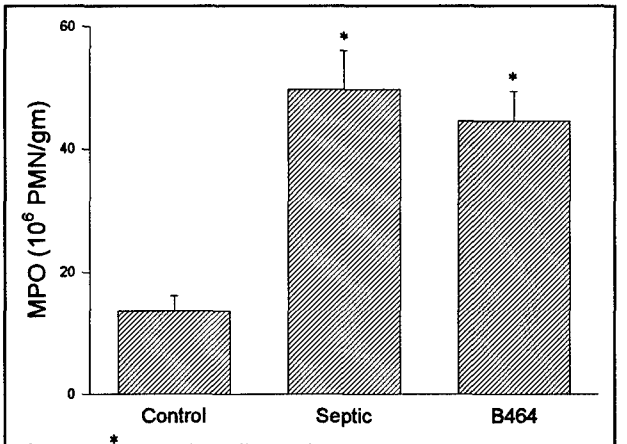

Figure 9 *p<0.05 vs Control.

Systemic Effects of B464 infusion. Table 5 (Group 4) shows that B464 infusion was not associated with any agonistic LPS-like effects. In all the parameters measured (SAP, PAP, CI, arterial pH and PO_2, lung myeloperoxidase and peripheral blood neutrophil count) no significant changes from baseline occurred.

Summary

Administration of LPS to otherwise healthy humans (34) or dogs (44) produces a cardiovascular response indistinguishable from septic shock. LPS consists of a variable polysaccharide domain covalently bound to a phosphorylated and acylated di-glucosamine disaccharide, designated lipid A. Lipid A remains highly conserved across diverse gram-negative bacteria. Purified lipid A preparations exhibit potent LPS-like agonistic activity and considerable evidence suggests that lipid A is responsible for most of the biological effects of LPS (45) (i.e., upregulated neutrophil function: upregulation of CD11/CD18 adhesion receptors, priming for oxidant burst; activation of macrophages/monocytes to release TNF-α, and interleukin-1) (44,46). LPS initiates cytokine release by binding to the plasma protein LPS-binding protein or LBP. The LPS-LBP complex avidly binds to CD14 receptors on monocytes and macrophages and triggers the release of cytokines (47). Lipid A (B464) antagonizes the effects of LPS, reducing LPS-induced cytokine production by macrophages (42) and decreasing mortality in a murine model of endotoxemia (37,41,42). In a recent study B464 reduced LPS-induced synthesis of nitric oxide, TNF and interleukin-6 by macrophages in a dose dependent fashion (48). B464 differs from toxic *E.coli* lipid A in several ways; the fatty acid substituents are shorter in chain length, linked via ether bonds instead of esters, and an unsaturation is present on the alkyloxyacyl moiety. The mechanism of cytokine production inhibition appears to be at the level of LPS binding and/or signal transduction (49). In prior studies we showed significant protection against gram-negative sepsis using anti-TNF antibodies (50,51). We therefore hypothesized that a reduction in TNF production, and other mediators, by an agent such as B464 may also prove beneficial.

The results of this study confirmed that pretreatment with B464 modestly, but significantly, attenuated the effects of porcine gram-negative sepsis. B464 improved

systemic hemodynamics and reduced acute lung injury. B464 attenuated second phase systemic arterial hypotension by increasing systemic vascular resistance, but did not prevent the initial phase drop in blood pressure. The mediators responsible for the development of hypotension in this model have not been fully determined. The likely candidate causing persistent hypotension is nitric oxide. B464 inhibits LPS-mediated induction of nitric oxide synthase and the production of nitric oxide *in vitro* (48). A reduction by B464 of nitric oxide production may well be the mechanism behind the improvement seen in systemic arterial pressure in these studies. In contrast, pulmonary arterial hypertension was not attenuated. We have previously shown that pulmonary arterial hypertension is due to the generation of arachidonic acid metabolites (e.g. thromboxane A_2) (52). Release of these metabolites appears to be independent of cytokine release and therefore may not be reduced by B464 treatment (50).

Bronchoalveolar lavage protein content and arterial oxygen tension were both significantly improved by B464 treatment. BAL protein content measures lung permeability increases in pulmonary microvasculature which in the setting of gram negative sepsis is mediated by oxygen radicals and proteolytic enzymes released by neutrophils closely adherent to pulmonary vascular endothelium (21,53). The reduction in lung permeability injury was associated with an overall improvement in $Pa0_2$ in the B464-treated animals. During the first hour of the experiment, however, in this group PaO_2 still fell significantly compared to controls. The initial rapid decline in $Pa0_2$ observed in both treated and untreated septic animals is likely due to acute ventilation-perfusion mismatch associated with pulmonary arterial hypertension (52,54). In the untreated septic animals PaO_2 is then further worsened by neutrophil mediated oxidant injury.

In conclusion, pretreatment with a synthetic lipid A analog, B464, provided significant protection against cardiovascular and pulmonary derangements in a porcine model of gram-negative sepsis. The protection observed was incomplete, however, indicating that other bacterial products apart from endotoxin, such as exotoxin A and elastase, may also be partially responsible for the injuries observed. These preliminary data indicate that B464 may be a useful adjunct to the armamentarium used in the treatment of gram-negative sepsis. Further studies however, are required to elucidate the mechanisms of action of this agent in this and other models of sepsis.

BRADYKININ ANTAGONIST (NPC 17731)

Introduction. Lipopolysaccharide (LPS) released into the circulation as a consequence of systemic bacterial infection produces complex interaction between cellular and humoral mediators. The roles of newly discovered cytokines, such as tumor necrosis factor-α and the interleukins, have been a major focus of recent scientific research. In septic patients, however, activation of the plasma kallikrein-kinin (KK) system, which produces bradykinin (BK), has been recognized for 20 years (55,56). Bradykinin's precise role in sepsis, beneficial or pathologic, remains unknown. Measurements of the components of this system, many of which are unstable and short-lived *in vivo*, have proven difficult and the lack of specific inhibitors to bradykinin have impeded precise definition of its actions in sepsis. More recent studies in septic patients have strongly implicated a role for bradykinin in the development of septic shock (57-59) and adult respiratory distress syndrome (60,61) and some studies have correlated the degree of activation of the kallekrein-kinin system with overall survival (58,59). In animal models pretreatment with newly available specific BK antagonists have demonstrated attenuated shock in response to endotoxin administration (62-64) and improved survival (64,65). In other models inhibition of the KK system with other inhibitors also improved septic shock (62,66,67). We sought to determine the effects of both pre- and early post-treatment with a specific BK inhibitor, NPC17731 (Scios-Nova), on the development of sepsis and acute lung injury in a porcine model of gram negative sepsis.

Bradykinin Antagonist. The bradykinin antagonist, NPC17731, (Scios-Nova, Mountain View, CA) is a synthetic peptide similar in structure to bradykinin ([D-Arg^{10}, D-Hype (trans-propyl)^{7}, Oic^{8}]-bradykinin); where D-Hype (trans-propyl) is D-trans-4-n-propoxyproline and Oic is octahydroindole carboxylic acid.

Prostacyclin Assay. The production of prostacyclin (PGI_2) was determined by measurement of its stable metabolite, 6-keto prostaglandin $F_{1\alpha}$, in plasma using an enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). Briefly, 6-keto $PGF_{1\alpha}$ was purified from plasma by elution with ethyl acetate through a reverse phase C-18 column (Peninsular Lab, Belmont, CA). The ethyl acetate was evaporated under vacuum and the substrate solubilized in EIA buffer (Cayman Chemical). The assay was then carried out according to the manufacturer's instructions and samples from controls, septic and pretreated animals analyzed at various time points.

Experimental Design. Four groups of animals were studied. **Group 1** (Controls, n=10) received a 60 minute intravenous infusion of sterile saline. **Group 2** (Untreated Sepsis, n=10) received a 60 minute intravenous infusion of live *Pseudomonas aeruginosa*, PAO strain ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min). **Group 3** (Pretreatment NPC17731, n=6) received NPC17731 (5mg/kg in 20 ml 0.9% saline initial bolus followed by an hourly 1mg/kg bolus) immediately prior to commencing the infusion of *Pseudomonas aeruginosa*. **Group 4** (Delayed-treatment NPC17731, n=6) received the

		TIME (hrs)						
		0	*0.5*	*1*	*2*	*3*	*4*	*5*
SAP	Control	96±4	95±4	103±4	105±4	109±5	109±5	105±4
	Septic	100±6	116±8	87±8	65±7*	67±6*	70±6*	66±5*
	B464	91±4	107±5	84±4	54±5*	69±7*	89±8†	97±8†
	Delayed	82±2	120±7	88±9	52±9*	59±3*	78±9*	86±11†
SVRI x 10^3	Control	2.3±0.2	2.3±0.1	2.6±0.1	2.9±0.2	3.1±0.2	3.1±0.2	3.1±0.1
	Septic	2.6±0.2	4.6±0.5*	2.2±0.3	1.9±0.2*	2.2±0.2*	2.7±0.4	3.1±0.2
	B464	2.3±0.2	3.9±0.2*†	2.5±0.1	1.4±0.1*	2.1±0.3*	3.4±0.5	4.3±0.5*†‡
	Delayed	2.6±0.2	3.6±0.3*†	1.9±0.3	1.3±0.2*	1.7±0.3*	2.5±0.3	2.8±0.3
CI	Control	100±0	100±3	96±3	92±4	93±3	89±4	89±4
	Septic	100±0	60±4*	74±5*	79±4	69±5*	55±4*	48±3*
	B464	100±0	61±4*	77±6	87±7	77±7	62±4*	54±4*
	Delayed	100±0	78±8	90±7†	86±9	74±8	66±7*	59±5*
PAP	Control	15±1.2	15±1.3	16±1.0	16±1.1	16±1.2	16±1.2	15±1.2
	Septic	16±0.9	47±2.0*	36±2.7*	32±2.6*	36±2.3*	35±2.3*	33±2.7*
	B464	14±1.1	49±1.9*	42±1.0*	31±1.3*	33±1.3*	32±2.6*	32±1.9*
	Delayed	11±1.9	42±2.1*	35±0.2*	27±1.2*	28±2.1*	28±1.4*	28±1.2*

Table 6 *$p<0.05$ vs Control, †$p<0.0.05$ vs Septic, ‡$p<0.05$ vs Delayed. SAP (mm Hg), SVRI ($dyn^{sec}/cm^5/m^2$), CI (% of baseline), PAP (mm Hg).

same NPC17731 treatment regimen, but commencing 30 minutes after the onset of the *Pseudomonas* infusion. All groups were studied for a total of 5 hours.

Hemodynamics. (Table 6) Systemic arterial pressure (SAP) in untreated septic animals rose slightly at 30 minutes, corresponding to the *Pseudomonas* infusion, followed by a rapid decline. From 2 hours onwards, SAP remained significantly lower than controls. Both pre and post-treatment groups exhibited identical patterns in SAP as that observed in untreated septic animals until 2 hours. From 2 hours onwards, however, both treatment groups showed progressive recovery, rather than further decline in SAP as that observed in untreated septic animals. By 5 hours both treatment groups had returned to baseline SAP with no significant difference compared to controls. Control animals exhibited minor elevations in SAP between 0 and 5 hours although this was not significant.

In the untreated septic animals, systemic vascular resistance index (SVRI) rose significantly at 30 minutes then fell rapidly reaching a nadir at 2 hours (Table 6). There was then a progressive recovery, returning to control levels by 5 hours. Both treatment groups also showed a similar initial pattern in SVRI, however, the pretreated animals exhibited a rebound increase in SVRI from 4 hours, being significantly elevated above all other groups at 5 hours, whereas the post-treatment group returned to control levels.

Pulmonary artery pressure (PAP) in untreated septic animals showed a significant increase by 30 minutes followed by a persistent but less severe pulmonary arterial hypertension until the end of the experiment (Table 6). Both pre and post-treatment NPC17731 exhibited no protection against the development of PA hypertension.

Cardiac index (CI) (Table 6) in septic animals showed a biphasic response. By 30 minutes there was a significant decrease in CI, corresponding to the increase in PAP. There was then partial recovery but a second progressive decline from 2 hours onwards. Both treatment groups also showed a similar biphasic pattern in cardiac index.

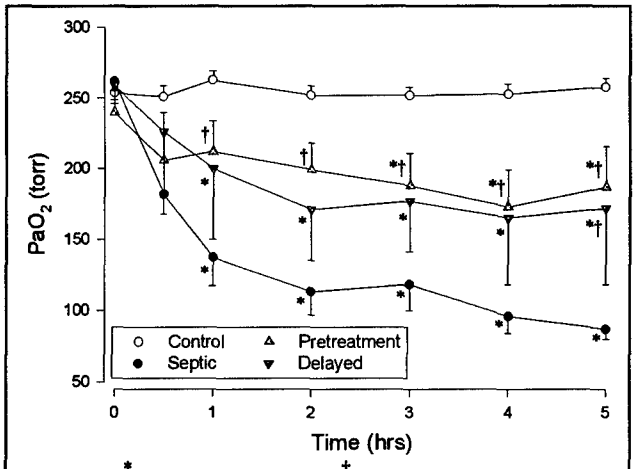

Figure 10 *p<0.05 vs Control, †p<0.05 vs Septic.

Arterial oxygen tension (PaO_2) in untreated septic animals showed a rapid, significant decrease by 1 hour, followed by a further progressive decline for the remainder of the experiment. Both pre and post-treatment groups significantly attenuated this decline although both groups exhibited significantly lower PaO_2 than control animals (Figure 10).

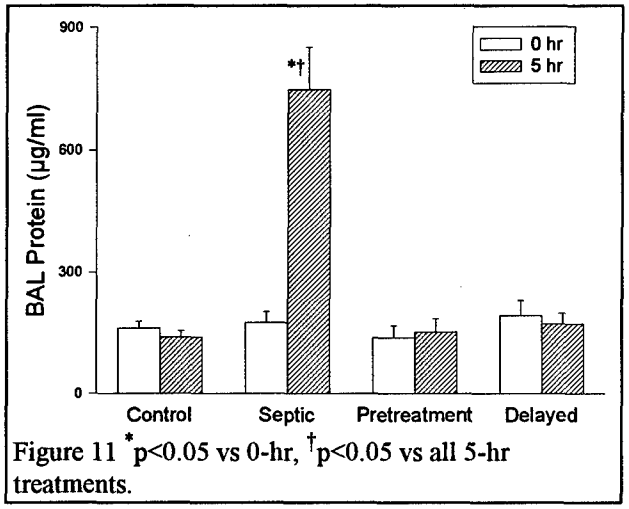

Figure 11 *p<0.05 vs 0-hr, †p<0.05 vs all 5-hr treatments.

Bronchoalveolar Lavage. The recovery of instilled BAL fluid at 0 and 5 hours was consistently high (≅ 70%) and did not differ between groups. Baseline BAL protein content was similar in all groups. At 5 hours in controls and both treatment groups, there was no significant increase in protein content compared to baseline (Figure 11). In contrast, the untreated septic animals exhibited more than a four-fold increase in protein content at 5 hours, compared to baseline. This rise was significant compared to baseline values and 5 hour values of all other groups.

The BAL neutrophil count (Figure 12), expressed as a percentage of the total white cell count recovered, in untreated septic animals showed a ten-fold increase at 5 hours compared to baseline. There was no significant increase from baseline to 5 hours in the pretreatment group and only slight increase in the delayed treatment group. At 5 hours, the PMN counts in both treatment groups were significantly reduced compared to untreated septics and not different from controls.

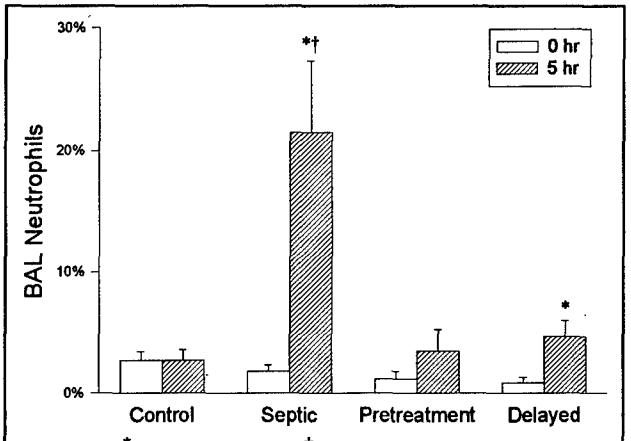

Figure 12 *p<0.05 vs 0-hr, †p<0.05 vs all 5-hr treatments.

		TIME (hrs)						
		0	*0.5*	*1*	*2*	*3*	*4*	*5*
Total WBC	Control	16.4±1.9	17.2±1.1	16.8±1.1	16.0±1.6	19.1±1.3	19.6±2.0	20.2±2.2
($x10^3/\mu l$)	Septic	14.6±0.9	7.2±0.5*	5.0±0.8*	2.1±0.4*	1.6±0.7*	2.0±0.5*	2.7±0.5*
	Pre Rx	11.0±2.1	3.3±0.2*	2.5±0.9*	0.9±0.4*	0.9±0.4*	1.2±0.7*	1.3±0.7*
	Post Rx	12.0±0.8	4.8±1.5*	2.4±0.9*	0.9±0.1*	0.5±0.1*	0.6±0.2*	0.9±0.2*

Table 7 *p.0.05 vs Control

Peripheral white cell counts. Peripheral white cell counts fell precipitously within 30 minutes of the onset of sepsis in untreated animals and they remained severely neutropenic for the remainder of the experiment Both pre and post-treatment NPC17731 failed to attenuate the development of a similar, severe neutropenia (Table 7).

Neutrophil Oxidant Burst. PMNs obtained at 5 hours from untreated septic animals demonstrated a marked priming response for PMA-stimulated superoxide (O_2^-) production when compared to baseline PMNs, as determined by an increase in the total amount of O_2^- produced over 10 minutes (Figure 13). In the septic NPC17731 treated groups there was also significant priming of neutrophils at 5 hours.

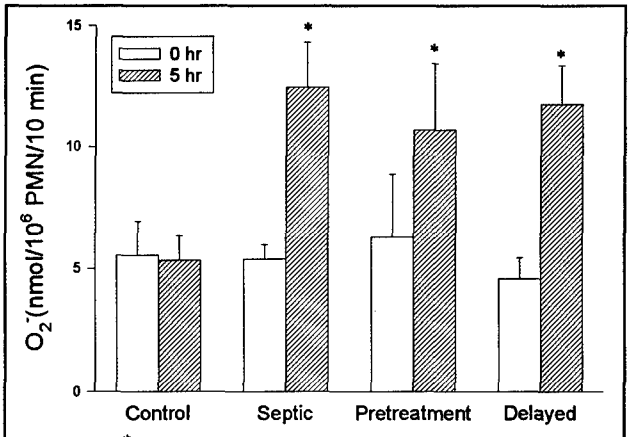

Figure 13 *p<0.05 vs 0-hr.

Neutrophil CD11/CD18 Receptor Expression. Untreated septic animals exhibited significant upregulation of the CD11/CD18 receptor expression from both baseline values and control animals within 2 hours (Figure 14). Peak upregulation was then followed by a gradual decline but receptor expression remained significantly upregulated for the duration of the study. Pretreatment animals also exhibited similar, significant upregulation of CD11/CD18 with no evidence of inhibition by NPC17731. This assay was not performed in post-treatment group.

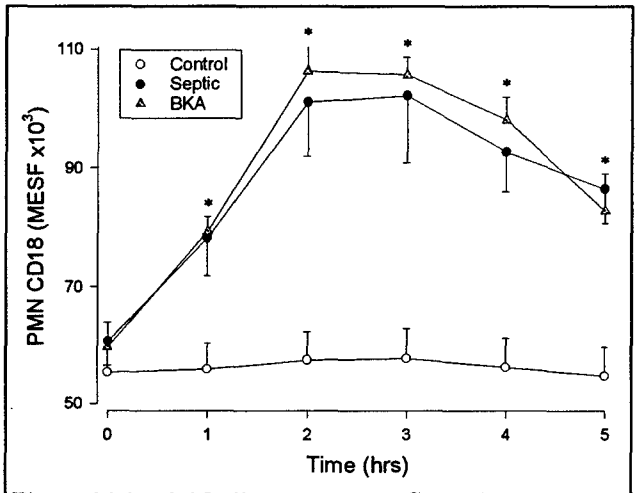

Figure 14 *p<0.05 all treatments vs Control.

Lung Myeloperoxidase Content. Untreated septic animals underwent significant neutrophil sequestration into the lung as measured by a significant increase in myeloperoxidase content compared to control animals (Figure 15). Both NPC17731 treated groups showed a significant reduction in lung PMN sequestration, although the myeloperoxidase content was significantly increased compared to controls in both these groups.

Prostacyclin Assay. Control animals had minimal detectable levels of 6-keto $PGF_{1\alpha}$ during the whole experiment. In untreated septic animals however, we detected progressively increasing levels indicating increased production of prostacyclin (Table 8). In the pretreatment group we observed a similar increase in 6-keto $PGF_{1\alpha}$ production which was significantly increased compared to controls with no statistical difference from untreated septic animals.

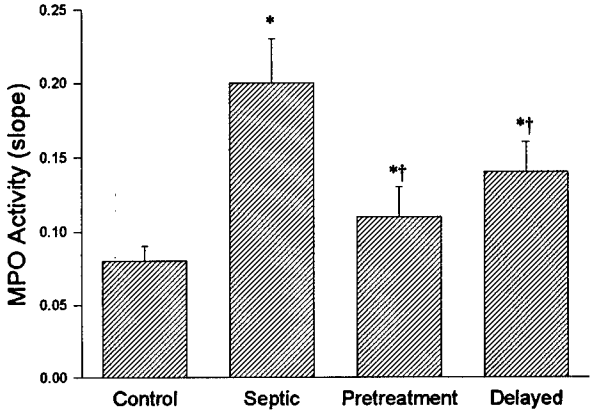

Figure 15 *p<0.05 vs Control, †p<0.05 vs Septic.

		TIME (hrs)					
		0	*1*	*2*	*3*	*4*	*5*
6-keto $PGF_{1\alpha}$	Control	19.5±72	13.5±6	18.6±5	8.2±3	15.2±4	19.5±8
	Septic	29.6±6	49.6±10	69.6±15	92.7±20*	114.3±36*	128.9±38*
	Pre Rx	8.8±3	34.4±4	123.3±42*	95.3±54*	121.9±57*	147.2±66*

Table 8 *p<0.05 vs Control

Summary

The kallikrein-kinin system is a component of the contact system of plasma proteases, related to the complement and clotting cascades. Factor XII (Hageman factor) is activated by endotoxin, either directly, or indirectly by damage to endothelium and exposure of the basement membrane (68). Activated Factor XII hydrolyzes circulating prekallikrein to form kallikrein, which in turn cleaves high molecular weight kininogen to form bradykinin (68). BK exerts its effects via receptors, B_1 and B_2, which have been classified according to the relative potencies of various agonists or antagonists (69). BK also exerts effects on the cardiovascular system. Intra-arterial infusion of BK in isolated canine limbs promotes vasodilatation and increased blood flow, increased lymph flow and lymph protein concentration (70-72). Changes in lymph flow and protein content are independent of hydrostatic changes indicating a direct effect on vascular permeability by BK (71,72). Systemic administration of BK in dogs caused profound but short lived hypotension without increased vascular permeability (73). The permeability changes are inhibited by catecholamines (71,73). BK's hemodynamic effects are not fully elucidated. *In vitro* studies suggest altered cAMP/cGMP ratios in smooth muscle produce vasodilatation; induced possibly by endothelial nitric oxide (74) or prostacyclin (75). Increased permeability may be due to endothelial cell contraction in post-capillary venules, producing gaps or "pores" through which plasma extravasation occurs (76).

Recent evidence suggests a role for the kallikrein-kinin (KK) system in sepsis based upon detection of activation of the system in patients or animals with sepsis.

Prekallikrein levels are low in sepsis indicating significant activation of the KK system. Several studies have demonstrated extremely low levels in patients who developed septic shock compared to those with uncomplicated sepsis (57-59). Due to the lack of specific BK antagonists determining a role for KK activation in sepsis has been difficult. Recently BK receptor antagonists have been synthesized. In sepsis models pretreatment with BK antagonists significantly improves overall survival and development of shock.(62-64,66). The above studies have documented the effects pre- and post-treatment with a newly synthesized BK antagonist, NPC17731, on porcine gram negative sepsis and sepsis induced acute lung injury.

We found that NPC17731 produced significant protection against lung injury and improved systemic hemodynamics in the model. BAL protein content and arterial oxygen tension, were both significantly improved by pre- and post-treatment with NPC17731. The effects of a BK antagonist on sepsis induced acute lung injury have not been previously studied. One study showed a reduction in respiratory distress with a BK antagonist in rats receiving lipopolysaccharide (64). As noted above the progressive fall in arterial $P0_2$ observed in our model is multifactorial; the initial rapid decline is due to pulmonary arterial hypertension leading to V/Q mismatch. Later neutrophils sequester in pulmonary microvasculature further reducing gas exchange by release of reactive oxygen intermediates producing vascular endothelial cell damage and a further reduction in oxygenation. Failure of NPC17731 to inhibit pulmonary arterial hypertension and neutrophil sequestration led to the early fall in $Pa0_2$ seen in both pre and post-treated animals. The later progressive deterioration in $Pa0_2$ observed in untreated animals was significantly attenuated by both pre and post-treatment. A significant reduction in endothelial damage was further evidenced by reduced BAL neutrophil counts which showed inhibition of PMN transmigration across the vascular endothelium and interstitium into the alveolar space.

These data imply a role for bradykinin in the later development of sepsis induced lung injury in this model. The exact role of BK in the development of lung injury is still speculative. Lungs from pre and post-treated animals the lungs contained large numbers of PMNs (MPO results) which exhibited upregulated CD11/CD18 expression, and priming for superoxide production. Despite the potential for high oxidative stress in the lung, treated animals exhibited attenuation of lung injury. Previous studies have shown high levels of PMN elastase/α_1 protease inhibitor complexes are present in BAL fluid of patients with ARDS, suggest that active degranulation of PMNs as they migrate from the vascular space. In our model there was marked attenuation of PMN migration despite activation and this suggests that BK plays a role in this migration, perhaps by increasing the gaps between pulmonary endothelial cells to promote passage of PMNs and increase the permeability of the pulmonary circulation. Reducing gap junctions between pulmonary vascular endothelium may be one potential mechanism by which NPC17731 acts to attenuate vascular injury in this model. Reduced gap size would account for the reduction in BAL protein content and PMN count as well as the improved arterial oxygen tension. The pulmonary effects of BK are less well studied than those on the systemic circulation. BK infusion into the lungs does produce vasodilatation, although the effect is mild, and there is only a minor increase in vascular permeability (77). In the presence of activated PMNs however, even a small

increase in the intercellular gaps between endothelial cells may be sufficient to permit extrusion of PMN pseudopodia between cells and thus the commencement of transmigration.

Bradykinin antagonism significantly improved systemic arterial blood pressure in these studies. Interestingly, the initial period of hypotension observed in septic animals was not inhibited by NPC17731. From 2 hours onwards we observed a progressive improvement in treated animals. Recovery of SAP in post-treatment animals appeared to lag behind that of the pretreated group, but by 5 hours both groups were significantly improved compared to untreated septic animals. Thus, BK clearly plays a role in the latter stages of septic shock in this model, with the initial period of hypotension mediated by other mechanism(s). The mechanism by which NPC17731 restores SAP appears to predominantly result from increased systemic vascular resistance, rather improved cardiac output which continues to decline in treated animals. Our results suggest that BK is intimately involved in control of vascular tone during periods of sepsis. The primary function of BK is as a vasodilator and therefore inhibition produces increased vasoconstriction and increased vascular resistance. The actual mechanism by which BK causes vasodilatation is not fully elucidated. BK binds to B_2 receptors on endothelium and produces "factors" which alter the cyclic GMP/AMP ratio in underlying vascular smooth muscle. This alteration in the cyclic GMP/AMP ratio likely causes the vascular effects of BK (74,75). There is evidence which suggests that prostacyclin (PGI_2) and subsequently nitric oxide production act as effector factors produced by endothelium in response to BK binding (74,75,78). We found progressive accumulation of the stable derivative of PGI_2 , 6-keto $PGF_{1\alpha}$ during the latter stages of the model indicating its partial responsibility for persistent hypotension. Although there was considerable variability in 6-keto $PGF_{1\alpha}$ in the pretreated group, there was no evidence of production inhibition by NPC17731 and therefore the recovery in systemic arterial pressure observed does not appear to be due to inhibition of prostacyclin production. It is therefore likely that a reduction in nitric oxide production is the mechanism by which BK antagonism is effective in protecting against septic shock in this model, although this is yet to be confirmed.

Thus our final study focused on mediator inhibition using a novel competitive bradykinin antagonist (NPC1773) shows that the agent protects against sepsis-induced acute lung injury and also significantly attenuates the intensity of septic shock when given in either a pretreatment or early post-treatment regimen. Further studies are required to determine whether a greater delay from the onset of sepsis would also be beneficial, but this study does indicate that bradykinin antagonism may be a useful addition to the armamentarium against Gram negative sepsis.

III. DISRUPTION OF NEUTROPHIL AND ENDOTHELIAL CELL SELECTIN BINDING AS A THERAPEUTIC TOOL TO ATTENUATE SEPTIC INDUCED LUNG INJURY

MONOCLONAL ANTIBODY AGAINST NEUTROPHIL L-SELECTIN AND ENDOTHELIAL E-SELECTIN

Introduction. As noted above, the importance of activated neutrophils as critical cellular mediators in producing acute microvascular injury of the lung is becoming increasingly recognized in sepsis syndrome(79). Sequestration of PMNs in the lung and other non-pulmonary organs followed by extracellular release of reactive oxygen intermediates and potent lytic enzymes, are believed to be critical events in the genesis of multiorgan failure (38). A critical first stage in this complex sequence of events is the adhesion of PMNs to endothelium (80). The adhesion phenomenon is a multistage process which results from expression of adhesion molecules on both endothelial cells and PMNs. The leukocyte $ß_2$-integrin ($CD11_b$/CD18) interacts with intercellular adhesion molecules one and two (ICAM-1, ICAM-2). This interaction is essential for PMN adhesion and subsequent transendothelial migration (81,82). However, under physiological conditions of flow another class of adhesion molecules, the selectins, must be expressed and engaged to induce leukocyte rolling along the endothelial surface before integrin/ICAM-1 binding can occur (83,84). Selectins are characterized by a lectin-type domain at the N-terminal region, and consist of L-selectin, constituitively expressed on leukocytes, P-selectin, an inducible endothelial antigen and E-selectin, a second inducible endothelial antigen. Monoclonal antibodies against E-selectin (85) or L-selectin (86) reduce PMN adhesion to activated endothelial cells *in vitro*. In this study we hypothesized that E- and L-selectin play a critical role in the pathophysiology of sepsis-induced lung injury. We further hypothesized that an antibody capable of binding to these selectin molecules may therefore alter events early in PMN/endothelial adhesion processes and play a protective role in sepsis. For these studies we utilized antibody EL-246 (kindly provided by Dr. Mark Jutila, Montana State University) which binds to both porcine L-selectin and E-selectin to investigate the effects of selectin blockade in our model of sepsis induced acute lung injury.

The EL-246 Antibody. EL-246 is a mouse IgG_1 that reacts with both E-selectin and L-selectin in a variety of animal species (87). Specifically, it has been shown to cross-react in pigs staining peripheral blood leukocytes (L-selectin) and is shed following leukocyte activation. Further, EL-246 also binds to TNFα-stimulated cultured porcine endothelial cells (M.A. Jutila, unpublished observations). Domain mapping studies have localized the EL-246 epitope to the short consensus repeat (SCR) domains of L-selectin. Functional and molecular analyses have been performed in humans and ruminants because of availability of assays and cDNAs. Intact EL-246 blocked leukocyte-endothelial interactions at the level of the leukocyte and the endothelial cell using human or animal cells. The whole molecule does not cross-link the leukocyte to the endothelial cell. No difference in the blocking activity of EL-246 has

been detected in *in vitro* assays using cells of different animals. In bovine and human cell assays, EL-246 is highly effective at blocking leukocyte-endothelial interaction in assays performed under shear force conditions to reflect blood flow (88).

Experimental Design. Five groups of animals were studied. Baseline blood sampling, hemodynamic measurements and bronchoalveolar lavage were performed prior to any intravenous infusions. **Group 1** (Control, n=10) received a 60 minute intravenous infusion of sterile saline. **Group 2** (Untreated Sepsis, n=10) received a 60 minute intravenous infusion of live *Pseudomonas aeruginosa,* PAO strain ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min). **Group 3** (Pretreatment EL-246, n=8) received EL-246 (1 mg/kg in 20 ml 0.9% saline) immediately prior to commencing the infusion of *Pseudomonas aeruginosa*. **Group 4** (Delayed-treatment EL-246, n=4) received EL-246 (2 mg/kg in 20 ml 0.9% saline) 30 minutes after commencing infusion of organisms. **Group 5** (Control EL-246, n=2) received EL-246 only. All groups were studied for a total of 5 hours.

Hemodynamic Measurements. Pulmonary artery pressure (PAP) rose significantly at 30 minutes in all septic groups compared to controls (Table 9). Although PAP then decreased partially in these groups, pulmonary arterial hypertension persisted until the end of the experiment compared to controls.

Systemic arterial pressure rose sharply in the first 30 minutes following onset of *Pseudomonas* infusion in septic and EL-246 treated animals (Table 9). From 30 minutes onward progressive systemic arterial hypotension was observed in all septic groups becoming significantly worse than control animals from 2 hours onwards. At baseline, EL-246 treated animals had significantly lower systemic arterial pressures than septic animals, although no significant differences were detected at any other time point between these three groups.

Pretreatment EL-246 animals showed significant improvement in early phase decline of cardiac index compared to that observed in untreated septic animals at 30 minutes (Table 9). This did not hold true for delayed treatment EL-246 animals.

		TIME (hrs)						
		0	*0.5*	*1*	*2*	*3*	*4*	*5*
CI	Control	3.5±0.7	3.5±0.7	3.2±0.5	3.0±0.3	3.0±0.4	2.8±0.3	2.8±0.1
	Septic	3.2±0.2	1.9±0.1*	2.7±0.2	2.5±0.2*	2.1±0.1*	1.9±0.1*	1.5±0.1*
	EL246	3.2±0.6	3.2±0.5†	3.2±0.5	2.9±0.2	2.4±0.3	1.9±0.2*	1.8±0.1*
	Delayed	3.0±0.1	2.0±0.3*	2.2±0.3	2.9±0.3	2.6±0.3	2.0±0.3*	1.8±0.3*
PAP	Control	14.8±1.2	15.2±1.3	16.3±1.0	16.5±1.1	16.5±1.2	15.9±1.2	15.8±1.2
	Septic	16.4±1.0	47.5±2.4*	36.1±2.1*	31.1±2.2*	35.3±2.1*	34.3±2.3*	32.8±2.6*
	EL246	13.8±1.0	45.4±2.1*	37.8±1.5*	29.6±1.2*	28.0±1.2*†	26.4±2.1*†	24.0±3.2
	Delayed	12.5±1.0	47.2±2.9*	37.3±3.0*	30.2±4.2*	32.1±1.1*†	29.4±2.2*†	30.2±3.8
SAP	Control	96±4	97±4	105±4	111±5	116±6	110±5	110±5
	Septic	104±6	121±5*	92±7	66±6*	59±6*	72±6*	69±6*
	EL246	80±4†	114±5	91±8	62±8*	56±2*	56±10*	46±11*
	Delayed	82±4†	113±5	78±5	63±6*	73±7*	69±7*	89±5*

Table 9 *$p<0.05$ vs Control, †$p<0.05$ vs Septic. CI ($L/min/m^2$), PAP (mm Hg), SAP (mm Hg).

However, as in the untreated septic group, EL-246 treated animals exhibited a gradual decline in CI which was significantly lower than controls after 3 hours.

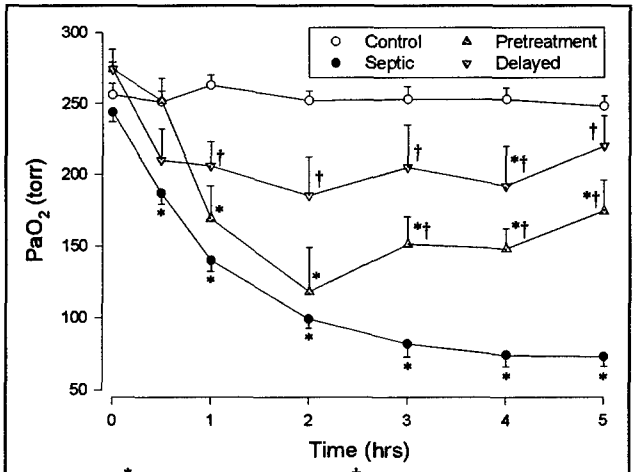

Figure 16 *p<0.05 vs Controls, †p<0.05 vs Septics.

Blood Gas Measurements. Untreated porcine sepsis was characterized by severe, progressive acidosis which was significantly worse than controls by one hour. Pretreatment with EL-246 attenuated the severity of the acidosis but a significant, progressive decline in arterial pH occurred when compared to control animals. At three and five hours arterial pH was significantly improved by EL-246 treatment compared to untreated septic animals (data not shown).

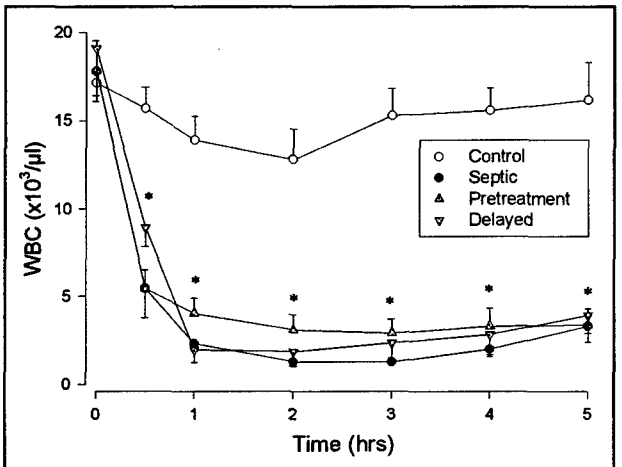

Figure 17 *p<0.05 all treatments vs control.

Arterial oxygen tensions rapidly and progressively fell in untreated septic animals, being significantly worse than controls from 30 minutes onward (Figure 16). The pretreatment EL-246 animals also exhibited a similar initial decrease in PaO_2. Beyond two hours no further decline occurred and this group maintained a significantly improved PaO_2 compared to the untreated septic animals for the remainder of the experiment. The higher dose delayed treatment EL-246 group approached control levels for nearly the entire experimental period and was always significantly improved when compared to the untreated septic group.

Peripheral Neutrophil Count. Circulating neutrophil counts in both septic and EL-246 treated animals declined rapidly following the onset of sepsis becoming significantly lower than controls within 30 minutes (Figure 17). Neutrophil counts in these three groups reached a nadir by two hours and failed to exhibit any significant recovery during the remainder of the experiment.

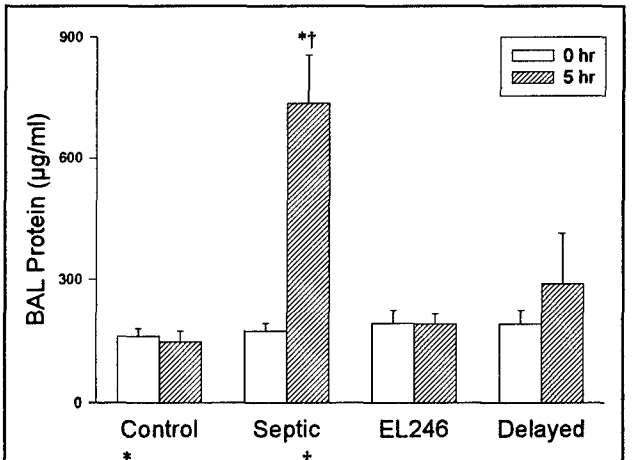

Figure 18 *p<0.05 vs 0 hr, †p<0.05 vs all other groups.

Bronchoalveolar Lavage Analysis. Bronchoalveolar lavage (BAL)

protein content in septic untreated animals at 5 hours was significantly increased compared to baseline values (Figure 18). BAL protein content at 5 hours was elevated in delayed EL-246 treated animals, but was not statistically significant. Five hour BAL protein content in septic, untreated animals was significantly increased compared to all other groups.

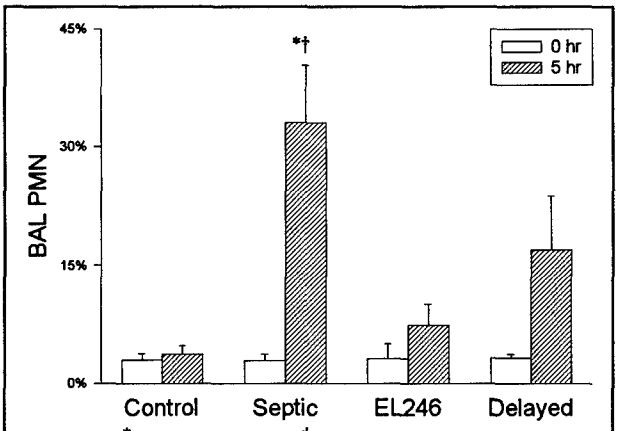

Figure 19 *p<0.05 vs 0 hr, †p<0.05 vs Control, EL246

BAL neutrophil counts in septic, untreated animals were significantly elevated at 5 hours compared to baseline (Figure 19). There was some degree of neutrophil influx at 5 hours in EL-246 treated animals, however, it was not statistically significant. Five hour BAL PMN in septic, untreated animals was significantly increased compared to controls and pretreatment EL-246 animals.

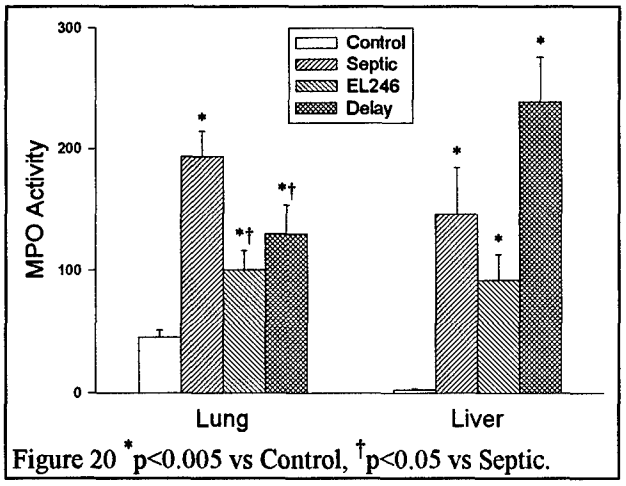

Figure 20 *p<0.005 vs Control, †p<0.05 vs Septic.

Organ Myeloperoxidase Content. At 5 hours lung myeloperoxidase content was significantly increased in untreated septic animals compared to control animals, indicating significant neutrophil sequestration during sepsis (Figure 20). Lung MPO levels in the EL-246 treated groups were also elevated when compared to controls, however, they were significantly reduced compared to untreated septic animals.

Liver myeloperoxidase content was almost undetectable in control animals but showed a significant increase in both the treated and untreated septic groups (Figure 20). Although liver myeloperoxidase content in EL-246 delayed treatment septic animals appeared higher than that observed in untreated septic animals this was not significant.

Neutrophil Oxidant Burst. Neutrophils obtained at 5 hours from untreated septic animals demonstrated a marked priming response for PMA-stimulated superoxide (O_2^-) production when compared to baseline PMNs, as determined by an increase in the total amount of O_2^- produced over 10 minutes (Figure 21). In both the EL-246 treated

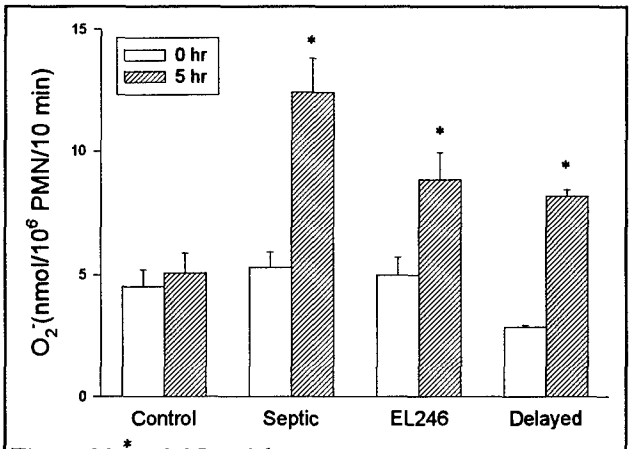

Figure 21 *p<0.05 vs 0 hr.

groups there was significant priming of neutrophils at 5 hours when compared to baseline.

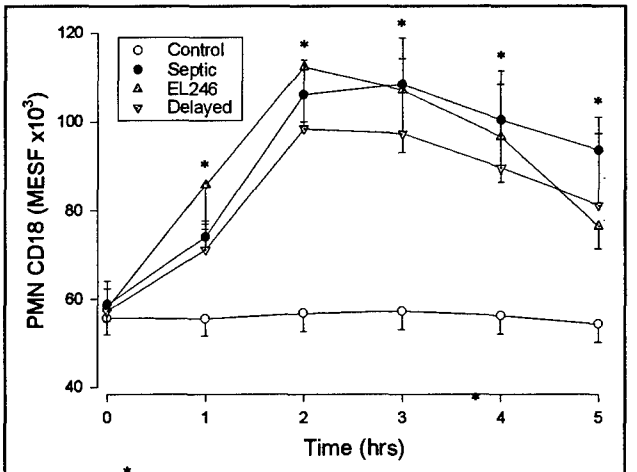

Figure 22 *p<0.05 all treatments vs Controls.

Neutrophil CD11/CD18 Receptor Expression. Untreated septic animals exhibited significant upregulation of the CD11/CD18 receptor expression within 2 hours (Figure 22). Peak upregulation was then followed by a gradual decline but receptor expression remained significantly elevated, when compared to controls, for the duration of the study. Both EL-246 treatment groups showed similar, significant upregulation of CD11/CD18.

Effect of EL-246 Infusion Into Control Animals. The two animals studied as EL-246 controls (Group 5), receiving the antibody only, showed no differences in hemodynamics, arterial blood gases or bronchoalveolar lavage parameters as compared to group 1 controls. EL-246 infusion did however, induce a *transient* neutropenia with peripheral neutrophil counts falling to 40% of baseline values at 1 hour. However counts rapidly increased being 85% of baseline by 2 hours and up to 90% of baseline by 3 hours.

Summary

PMNs, show both adherence to, and migration across vascular endothelial surfaces in humans with ARDS and in a host of animal models including the one utilized for these studies. Only recently have mechanisms of adhesion and transmigration been elucidated. Recent studies confirm that a two-stage mechanism of leukocyte-endothelial interaction in the systemic circulation is emerging. This mechanism has yet to be confirmed to occur in the pulmonary circulation. Following exposure to inflammatory peptides (eg., cytokines) endothelium initiates adhesion by expression of the single chain glycoproteins, P-selectin and later, E-selectin. In post-capillary venules neutrophils bind loosely to endothelium via engagement of selectin molecules and their ligands. At present the ligand for P- and E-selectin is felt to be the fucosylated carbohydrate moiety, Sialyl Lewis^x (86,89). The ligand for L-selectin is yet to be fully determined but may also be a glycoprotein (90). Selectin engagement promotes margination of PMNs by initiating a rolling process along vascular luminal surfaces. Subsequently local exposure to inflammatory factors (e.g. interleukin-8, platelet activating factor) promotes up-regulation of CD11/CD18 receptors on PMNs, arresting rolling cells at sites of activated endothelium, where CD11/CD18 - ICAM-1,2 binding occurs. Integrin molecules on PMN surfaces then form strong bonds with up-regulated ICAM-1,2 on the endothelial surface which is followed by transendothelial migration, a CD11/CD18 dependent phenomenon(82). As activated neutrophils bind

tightly to endothelium and become flattened in shape, a "microenviroment" is produced in the intercellular space. The microenviroment is protected from circulating oxygen radical scavengers and protease inhibitors. Formation of the microenviroment permits released oxygen radicals and lytic enzymes by PMNs bound to endothelium to produce localized endothelial injury, which ultimately results in increased permeability to fluid and plasma proteins (91).

Under conditions of normal physiological flow, selectin engagement is a pre-requisite for CD11/CD18 - ICAM-1 interaction (82,83). L-selectin is constituitively expressed on PMN surfaces but is rapidly shed following activation, a time when CD11/CD18 expression is concurrently increased (92). Endothelial P-selectin molecules, stored in Weibel-Palade bodies of endothelial cells, is expressed on endothelial surfaces within minutes of endothelial cell activation (93). E-selectin expression depends upon transcription and *de novo* synthesis of the molecule with peak expression occurring 4-6 hours after *in vitro* stimulation (94) followed by a rapid decline (85). *In vivo* models of sepsis exhibit evidence of widespread upregulation of E-selectin in many organs (95) including the lungs where it may be detected in pulmonary venules within 2 hours following onset of sepsis (96). Following exposure to pro-inflammatory mediators (e.g. TNF, LPS, IL-1, IL-8) endothelial ICAM-1 upregulation occurs within 6-12 hours *in vitro*. Maximal expression of ICAM-1 occurs at 24 hours (97). Because of this information we postulated that disruption of selectin engagement or integrin binding might have relevance as a treatment modality where PMN-mediated injury is present.

In these studies we found significant protection against lung injury in both pre and post treatments protocols. BAL protein content showed no increase at 5 hours compared to baseline in EL-246 treated animals. In untreated septic animals as in other studies a 5-fold increase in BAL protein occurred at 5 hours indicating significant increase in alveolar-capillary membrane permeability. Significant improvements in arterial oxygen tension occurred compared to septic in both EL-246 treatment groups.

From our findings, we speculate that EL-246 treatment blocks accumulation of neutrophils in lung by diminishing adhesion at one or both of two critical points. First, EL-246 binds L-selectin receptors constituitively expressed on porcine PMNs (87). Previous *in vitro* studies show that binding of EL-246 to human neutrophils blocks L-selectin dependent rolling and it may be assumed to do so in a porcine model although this has not been directly tested. A key factor in sustaining PMN adhesion to endothelium results from expression of E-selectin by endothelial cells. Endothelial E-selectin expression requires transcription and protein synthesis and therefore may not play a significant role in adhesion until up to 2 hours following induction of sepsis (96). In the model employed in these studies, PMNs become activated by 2 hours with increasing CD11/CD18 expression (98) and, L-selectin shedding (see below). However, E-selectin binds to ligands on PMNs other than L-selectin (i.e. Sialyl Lewis^x) (86). We speculate that binding of EL-246 to newly synthesized E-selectin may be responsible for the protective effects we observed in both protocols.

Pretreatment with a monoclonal antibody to porcine E-selectin and L-selectin, EL-246, in the current study failed to significantly alter the hemodynamic derangements of *Pseudomonas* sepsis. Animals treated prior to the onset of sepsis exhibited identical

decrements in systemic arterial pressure and cardiac index as that observed in untreated septic animals. These alterations in hemodynamics are likely mediated by cytokines and nitric oxide which would not be expected to be altered by administration of this antibody. Further, EL-246 failed to attenuate pulmonary arterial hypertension which we have previously shown to result from thromboxane release (99). In the model utilized in these studies, septic animals develop severe, progressive acidosis which is primarily metabolic (i.e., lactate) resulting from reduced tissue perfusion. EL-246 partially attenuated the severity of acidosis, however, as cardiac index later declined arterial pH declined, albeit to a significantly lesser extent than that observed in septic untreated animals.

Although EL-246 treatment produced a reduction in lung PMN sequestration, peripheral PMN counts in treated animals declined precipitously with no significant difference observed when compared to untreated septic animals. Our organ MPO data suggests that EL-246 prevents lung PMN sequestration but does not reduce accumulation into abdominal visceral organs such as liver. This shifting of PMN sequestration to predominately non-pulmonary organs results from hypoperfusion of non-pulmonary organs. As perfusion to organs such as liver falls during shock associated with sepsis selectin-independent binding occurs. This did not occur in a high flow organ such as the lung in these studies.

This work represents the first study of anti-selectin antibodies in a model of sepsis. Mulligan et al using other models of neutrophil-dependent lung injury (IgG immune complex injury, cobra venom factor infusion) showed significant attenuation of lung injury using monoclonal antibodies directed against selectin molecules (100,101). The use of antibodies to adhesion molecules in sepsis may prove clinically useful by reducing tissue damage associated with sepsis, however, further research is required to determine the exact effects and limitations of such therapy.

Thus this phase of the research has used a porcine specific antibody to both E-selectin and L-selectin in a porcine model of sepsis and acute lung injury. Although the antibody appeared to provide no protection against the development of septic shock, we observed significant protection against sepsis induced lung injury. Studies are underway to further elucidate the mechanisms of protection and to evaluate potential clinical application.

INVESTIGATIONS OF A PROTECTIVE ROLE OF SIALYLATED OLIGOSACCHARIDES IN SEPSIS-INDUCED ACUTE LUNG INJURY

Introduction. Under physiological conditions of flow, selectins must be expressed and engaged to induce leukocyte rolling along the endothelial surface (83,84). The *lectin domain* of selectin molecule appears to be of prime importance for selectin-mediated binding (102,103) with non lectin domains possessing predominately modulatory roles (101). Recent research has focused on determining the physiological ligands for each of the selectins. The main body of opinion indicates that the ligand for E- and P-selectin is an oligosaccharide which contains fucose and sialic acid termed ***Sialyl Lewis^x*** (SLex) (104-107). SLex is expressed on neutrophil L-selectin and also on other glycoprotein structures (86,108,109). The endothelial ligand for neutrophil L-selectin is not yet determined but may also be a similar glycoprotein (90).

We hypothesized that infusion of a synthetic SLex analogue, CY-1503 (Cytel Corp.), in order to block selectin interaction by binding to the lectin domain, may reduce neutrophil dependent lung injury. We tested the hypothesis in our porcine model using pretreatment and post treatment protocols.

Experimental Design. Four groups of animals were studied. Baseline blood sampling, hemodynamic measurements and bronchoalveolar lavage were performed prior to any intravenous infusions. **Group 1** (Control, n=10) received a 60 minute intravenous infusion of sterile saline. **Group 2** (Untreated Sepsis, n=10) received a 60 minute intravenous infusion of live *Pseudomonas aeruginosa,* PAO strain ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min). **Group 3** (Pretreatment CY-1503, n=7) received a 60 mg/kg bolus of CY-1503 immediately prior to commencing the infusion of *Pseudomonas aeruginosa* followed by a 60 mg/kg infusion over 5 hours. **Group 4** (Delayed-treatment CY-1503, n=5) received a 60 mg/kg bolus of CY-1503 30 minutes following onset of sepsis followed by a 60 mg/kg infusion over the remaining 4.5 hours. All groups were studied for a total of 5 hours.

Hemodynamics. Systemic arterial pressure in untreated septic animals increased significantly compared to controls during the bacterial infusion. SAP then declined rapidly becoming significantly lower than control animals by 2 hours and hypotension persisted for the duration of the experiment. Treatment of septic animals with CY-1503 failed to prevent the development of significant systemic arterial hypotension (Table 10). Untreated septic animals exhibited rapid development of pulmonary arterial hypertension following the onset of sepsis. After termination of the bacterial infusion PAP declined but remained significantly elevated above control animals throughout the duration of the experiment. CY-1503 treated animals also developed similar pulmonary arterial hypertension which was not significantly different from untreated septic animals at any time point. Control animals exhibited no increases in PAP during the experiment.

		TIME (hrs)						
		0	*0.5*	*1*	*2*	*3*	*4*	*5*
SAP	Control	95±4	95±3	104±4	110±5	114±5	108±6	109±5
	Septic	101±5	119±5*	89±7	64±6*	63±6*	71±5*	768±6*
	CY1503	83±5	113±6*	96±6	68±5*	69±5*	73±5*	82±6*
	Delayed	97±10	110±6*	97±5*	62±5*	70±7*	80±7*	91±6*
PAP	Control	14±1	15±1	16±2	16±1	16±2	16±2	16±2
	Septic	16±2	47±4*	35±4*	31±2*	35±4*	35±4*	32±3*
	CY1503	14±1	50±3*	41±3*	33±3*	33±2*	33±3*	31±2*
	Delayed	16±2	46±3*	36±3*	32±4*	34±4*	33±3*	33±5*
CI	Control	3.1±0.2	3.3±0.2	3.1±0.1	3.0±0.1	2.9±0.2	2.8±0.1	2.9±0.1
	Septic	3.2±0.3	2.0±0.2*	2.8±0.2*	2.6±0.3	2.2±0.2*	1.9±0.1*	1.6±0.3*
	CY1503	3.4±0.3	2.3±0.3	2.6±0.3	2.8±0.2	2.3±0.2	1.8±0.3*	1.6±0.2*
	Delayed	4.0±0.3*	2.1±0.3*	2.0±0.3*	2.5±0.3	2.4±0.3	2.0±0.3*	1.9±0.3*
PVRI	Control	202±19	225±27	248±38	290±37	301±35	306±46	320±28
	Septic	247±28	1536±233*	830±128*	766±63*	942±24*	1108±139*	1455±171*
	CY1503	189±13	1180±243*	1009±12*	656±214*	899±211*	1207±83*	1382±123*
	Delayed	191±22	1592±435*	1657±207*	1040±121*	930±90*	1034±112*	1211±23*
SVRI	Control	23±157	23±141	25±178	28±168	31±205	31±179	29±135
	Septic	26±218	47±328*	25±198	19±182*	23±152*	27±235	32±213
	CY1503	17±64	34±271*	25±142	18±181*	23±243	31±445*	40±212*
	Delayed	18±117†	43±666*	26±242	17±198*	22±323*	29±645	40±856

Table 10 *p<0.05 vs Control, †p<0.05 vs Septic. SAP-systemic arterial pressure (mm Hg), PAP-pulmonary arterial pressure (mm Hg), CI-cardiac index (L/min/m^2), PVRI-pulmonary vascular resistance index (dyn^{sec}/cm^5/m^2), SVRI-systemic vascular resistance index (dyn^{sec}/cm^5/m^2).

In untreated septic animals cardiac index exhibited a bi-phasic response to sepsis. Following the onset of sepsis CI significantly declined corresponding to the acute rise in PAP (Table 10). CI then recovered towards baseline levels but from 2 hours after the onset of sepsis a second progressive, significant decline was observed. Again, animals treated with CY-1503 exhibited an identical pattern in CI as untreated septic animals.

Arterial Oxygen Tension. Control animals showed no changes in arterial oxygen tension (PaO_2) during the experiment (Figure 23). In contrast PaO_2 declined rapidly within 1 hour following the onset of sepsis in the untreated animals and continued to fall throughout the experiment. CY-1503 treated septic animals also exhibited a significant decline in PaO_2 compared to control animals from 1 hour onward. However, CY-1503 treated animals exhibited a significant improvement in oxygen tension

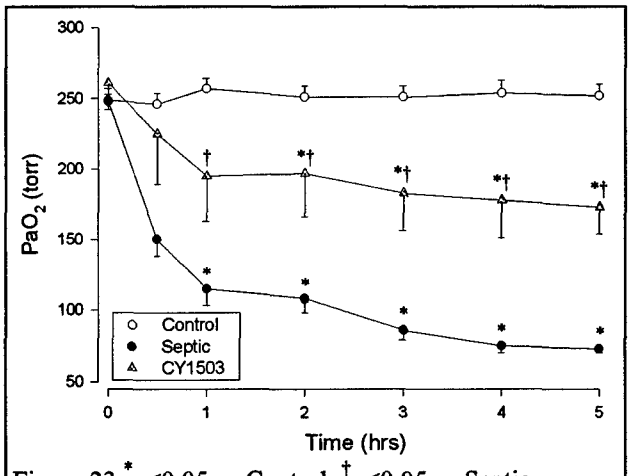

Figure 23 *p<0.05 vs Control, †p<0.05 vs Septic.

compared to untreated septic animals from 30 minutes onward. Unlike the pretreated group, delayed treatment with CY-1503 did not significantly improve arterial oxygen tension. Animals in this group demonstrated the identical rapidly progressive decline in $Pa0_2$ as that observed in the untreated septic animals.

Lung Permeability Injury. Bronchoalveolar lavage (BAL) protein content in the untreated septic group was increased more than 5-fold at 5 hours relative to baseline and compared to control animals, indicating that lung permeability injury had occurred (Figure 24). Septic animals pretreated with CY1503, however, exhibited no increase in BAL protein content at 5 hours compared to both baseline and the control group. A significant reduction in BAL protein content was also observed compared to untreated septic animals. In the delayed treatment group, BAL protein was markedly elevated at 5 hours, indicating the development of a significant permeability injury during sepsis. The 5 hour lavage protein content was also significantly elevated above controls and the CY1503 pretreatment group at the same time point. Although the delayed treatment group BAL protein content was lower than the untreated septic group at 5 hours, this was not a significant reduction.

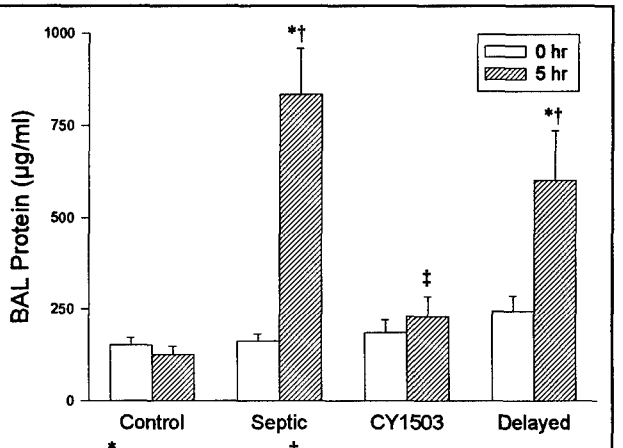

Figure 24 *p<0.05 vs 0-hr, †p<0.05 vs Control 5-hr, ‡p<0.05 vs Septic 5-hr.

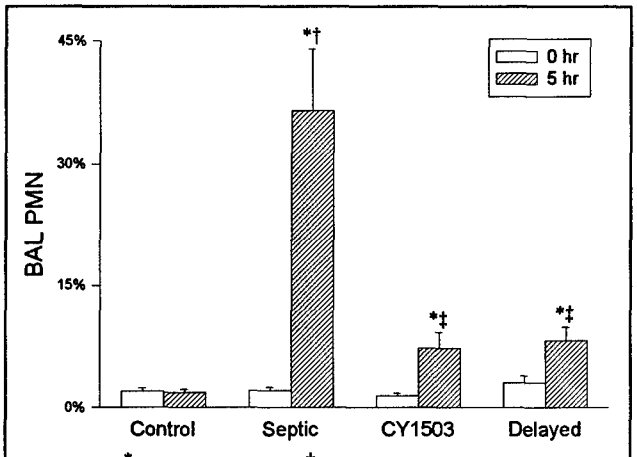

Figure 25 *p<0.05 vs 0-hr, †p<0.05 vs Control 5-hr, ‡p<0.05 vs Septic 5-hr.

Neutrophil Transendothelial Migration. In untreated septic animals a 17-fold increase in BAL neutrophil count was observed at 5 hours compared to baseline, indicating significant migration of neutrophils across the alveolar-capillary membrane into the alveolar spaces (Figure 25). Treatment with CY1503 significantly attenuated this transmigration compared to septic untreated animals. Bronchoalveolar lavage neutrophil counts at 5 hours were modestly, but significantly elevated in the treatment animals compared to the baseline lavage.

Peripheral Neutrophil Count. In both untreated and CY1503 treated septic groups, peripheral neutrophil counts declined dramatically following the onset of sepsis, indicating organ sequestration (Figure 26). The neutrophil counts were

significantly lower than controls within 30 minutes and reached a nadir at 2 hours. No significant recovery in neutrophil counts was observed from this point onward in any of the septic groups and no differences were observed between these groups at any time point. There was no evidence of a recovery in circulating neutrophil counts following CY-1503 administration in the delayed treatment group.

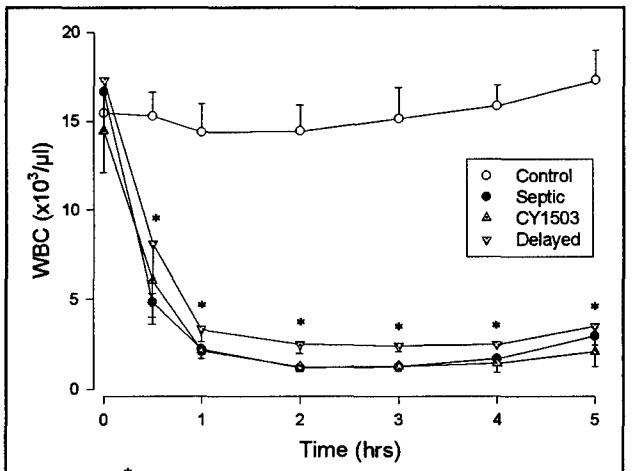

Figure 26 *p<0.05 all treatments vs Control.

Organ Myeloperoxidase Content. Myeloperoxidase activity, a measure of neutrophil sequestration, was significantly increased in the lungs of untreated septic animals compared to controls (Figure 27). Animals pretreated with CY1503 also demonstrated a significant increase in lung myeloperoxidase activity compared to controls but, this was significantly reduced relative to untreated septic animals. In control animals liver myeloperoxidase activity was barely detectable, whereas, both the septic and pretreatment groups showed significant liver neutrophil sequestration at 5 hours (Figure 27). Post treatment CY-1503 animals demonstrated a significant increase in lung myeloperoxidase content compared to both controls and the pretreatment group and a *similar MPO content compared to untreated septic animals*. This demonstrates no protection against neutrophil sequestration into the lung by delayed administration of CY-1503. MPO analysis of delayed treatment liver was not performed.

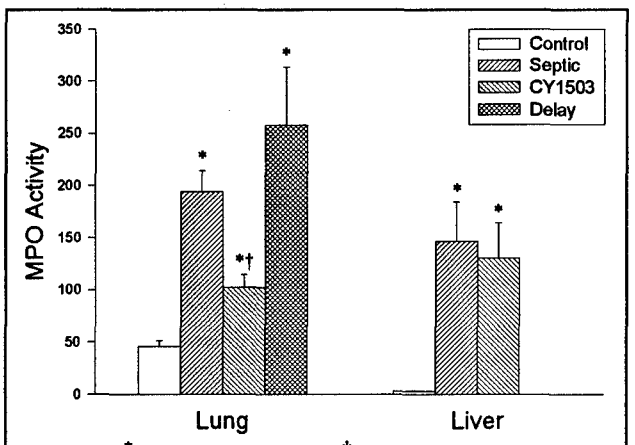

Figure 27 *p<0.05 vs Control, †p<0.05 vs Septic.

Neutrophil CD18 Expression. Circulating neutrophils exhibited significant upregulation of CD18 expression during the experiment in both the CY-1503 treated and untreated septic groups from one hour onward compared to controls (Figure 28). Peak upregulation occurred at 3 hours and was followed by a slight decline although both treated and untreated septic groups exhibited

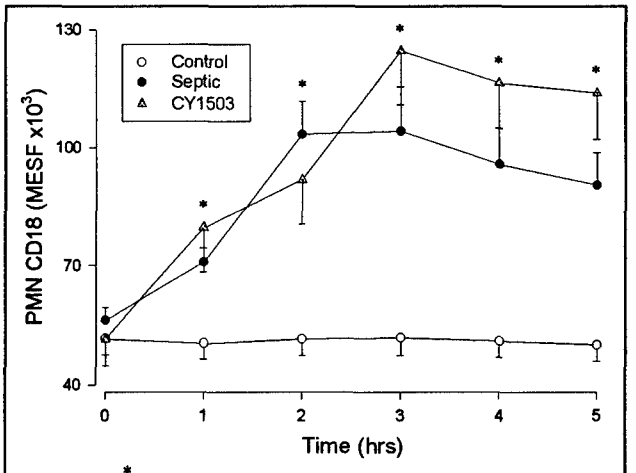

Figure 28 *p<0.05 all treatments vs Control.

significant upregulation until the end of the experiment. No significant differences were observed at any time point between treated and untreated septic animals.

Neutrophil Oxidant Burst. Neutrophil isolated at 5 hours from the septic group showed significant priming for the oxidant burst as shown by PMA-stimulated superoxide anion production (Figure 29). Pretreatment with CY1503 did not alter the priming effect of sepsis. In neutrophils isolated at 5 hours from both these groups a significant increase in the peak rate of superoxide anion generation was observed compared to neutrophils isolated at baseline. Neutrophil oxidant assays were not performed in the delayed treatment group.

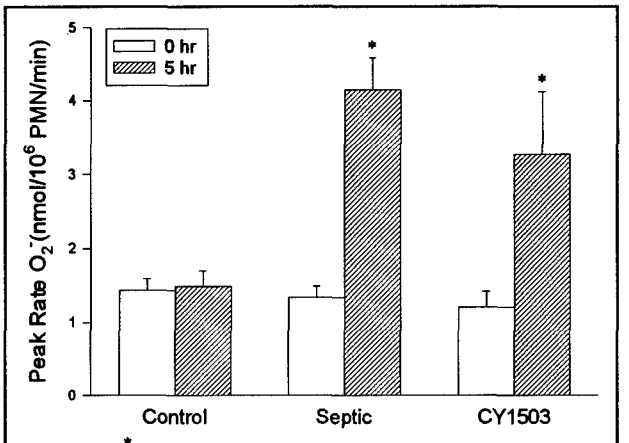

Figure 29 *p<0.05 vs 0-hr.

Summary

Infusion of the SLex analog, CY-1503, did not prevent hemodynamic derangements observed in our porcine model. CY-1503 treated septic animals developed a diminished cardiac output and systemic arterial hypotension similar to that observed in untreated septic animals. CY-1503 did not prevent the development of significant pulmonary hypertension. The derangements in systemic hemodynamics are likely mediated by cytokines, such as tumor necrosis factor and products of the kallikrein-kinin system and possibly nitric oxide. We would not have expected that decreased production of these mediators would have occurred as a result of selectin blockade. Similarly pulmonary arterial hypertension primarily in our model results from thromboxane release (99) and would not be attenuated by CY-1503 infusion.

The striking feature of these experiments was the significant protection against lung injury which occurred in pretreatment protocols. Bronchoalveolar lavage protein content, and arterial oxygen tension were significantly improved following CY-1503 infusion. These results suggest that CY-1503 treatment produced a measurable reduction in lung damage.

Attenuation of pulmonary injury in the model may be produced by one or more mechanisms such as a reduction in lung neutrophil burden, attenuated neutrophil oxidant production, or an alteration of microenvironment formation at the endothelium-neutrophil interface. Results of our lung myeloperoxidase assays confirmed that CY-1503 infusion reduced lung neutrophil sequestration. This agrees with previous studies which showed that similar oligosaccharides reduced neutrophil sequestration in an immune-complex induced model of lung injury (110). However, CY-1503 treatment in the current study (as observed with EL-246) did not prevent significant neutrophil sequestration in the liver. The reasons for the differential effects of CY-1503 on neutrophil sequestration in the liver and lung are not clear. As with the EL-246 studies,

we hypothesize that failure of CY-1503 to prevent sequestration in liver is likely attributable to a flow related phenomenon. Unlike systemic organ perfusion the lungs during sepsis in our model maintain high flows and vascular resistances. The presence of CY-1503 in circulation at the outset of sepsis effectively binds up all selectin sights. In organs where flow remains high (lung) neutrophils will not be removed from axial flow. Abdominal visceral organs like liver are subjected to hypoperfusion. In this environment selectin independent integrin dependent binding can occur.

Circulating neutrophils obtained form CY-1503 exhibited similar levels of activation to that observed in septic animals thus maintaining their ability to produce endothelial injury but failed to do so. Our findings suggest that CY-1503 treatment prevents the formation of a microenvironment interface in between PMN and endothelium in the pulmonary microvasculature and that this represents a major mechanism of protection observed in these experiments. The creation of the microenvironment is dependent on tight neutrophil/endothelial adhesion mediated by CD11/CD18 and ICAM-1 (82,111). As noted above, effective integrin/ICAM binding requires prior selectin engagement. Selectin engagement only serves to slow the passage of circulating neutrophils through capillaries and venules by initiating the rolling phenomenon. The dramatic reduction in velocity produced by selectin engagement permits integrin/ICAM mediated binding. Thus the mechanism responsible for the pulmonary protection afforded by CY-1503 treatment is the disruption of pulmonary vascular rolling which sharply reduces tight binding of neutrophils to pulmonary endothelium, resulting in a decreased microenvironment formation between PMN and vascular endothelium.

Neutrophil migration across endothelium is dependent upon CD11/CD18 receptor expression (82,112,113). Neutrophils in CY-1503 treated animals (despite upregulated CD11/CD18 receptors) failed to migrate. These findings also suggest that close neutrophil/endothelial apposition was prevented.

Our post treatment protocols clearly suggest that a very narrow "window of time" exists during which the agent is effective. As noted above virtually all protection afforded by the agent was lost when infused following onset of sepsis.

Thus, this study has investigated the effects of infusion of sialylated oligosaccharides (ligands for selectin interactions) in a model of acute lung injury. Significant protection against the development of lung injury was observed. No protection, however, was observed against the hemodynamic derangements associated with sepsis. These data indicate a significant role for selectins in the genesis of sepsis-induced lung injury.

IV. PRETREATMENT WITH INHALED NITRIC OXIDE TO ATTENUATE SEPSIS-INDUCED ACUTE LUNG INJURY

Introduction. Inhaled nitric oxide gas is a new therapy which shows promising results in the treatment of acutely injured lungs. Inhaled nitric oxide is a selective pulmonary vasodilator. Pison and colleagues demonstrated that inhaled nitric oxide at 20 ppm reversed hypoxic pulmonary artery vasoconstriction in anesthetized sheep. Frostell et al documented that inhaled nitric oxide reversed acute pulmonary vasoconstriction produced by infusion of a stable thromboxane analogue or hypoxia (114,115). Also, Pepke-Zaba and colleagues found that inhalation of nitric oxide at 40 ppm produced selective pulmonary vasodilation in patients with severe idiopathic pulmonary hypertension (116). Lastly, Roissant et al showed that inhaled nitric oxide at 10 to 20 ppm increased the PaO_2/ FiO_2, reduced venous admixture, and attenuated mean pulmonary artery pressure in patients with ARDS (117). These studies support the hypothesis that inhaled nitric oxide promotes redistribution of pulmonary blood flow away from non-ventilated lung units toward ventilated regions, thus, diminishing right-to-left shunt, and improving arterial oxygenation.

Inhaled nitric oxide may improve lung function in patients with ARDS by other mechanisms. Sequestration of neutrophils in lung and nonpulmonary organs, followed by extracellular release of reactive oxygen species and lytic enzymes is a primary event in the pathogenesis of ARDS and multiple organ failure (118,119). In this paradigm sequestered, activated neutrophils adhere tightly to endothelium creating an intercellular cleft or microenvironment (120). Reactive neutrophil products (i.e., elastase, superoxide anion) are protected from circulating antiproteases and antioxidants permitting damage to occur (121). Recent evidence suggests that nitric oxide may attenuate endothelial injury by inhibiting leukocyte/endothelial adhesive interactions (122,123).

We hypothesized that inhaled nitric oxide may reduce neutrophil-dependent acute lung injury by interfering with adhesive interactions between pulmonary endothelium and activated neutrophils, in addition to its effect upon pulmonary hemodynamics and ventilation-perfusion matching. This hypothesis was tested in a porcine model of sepsis-induced acute lung injury in which the pathophysiology is highly dependent upon adhesion and sequestration of activated neutrophils within the lung (124-126).

NO Delivery System. Nitric oxide gas (BOC Group, Inc., Murray Hills, NJ) at 20 ppm was introduced into the afferent limb of a dual heated limb universal ventilator circuit (Hudson RCI, Temecula, CA). The concentration of the source gas was 808 ppm. The final concentration of NO administered to the animal was obtained by mixing the nitric oxide gas with the compressed air and O_2 being continuously administered by the ventilator. The nitric oxide gas entered the system through a port in the afferent limb 40 cm proximal to the endotracheal tube in order to limit combination with molecular oxygen and thus decrease subsequent formation of nitrogen dioxide (NO_2). This method of ventilation is analogous to that being employed in current clinical trials. The concentrations of nitric oxide and NO_2, and the FiO_2 were monitored continuously using a nitric oxide monitor (National Draeger, Inc. Pittsburgh, PA), a NO_2 monitor

(National Draeger, Pittsburgh, PA), and a MiniOx 1 oxygen analyzer (Catalyst Research, Owings Mills, MD). All monitors employed an electrochemical sensing system and were calibrated at the start of each experiment using known standard gas concentrations. Blood samples were drawn at time 0, 2, and 5 hours for measurement of circulating methemoglobin (metHb) levels using a CO-oximeter (Instrumentation Laboratories, Lexington, MA).

Scanning Electron Micrographic Studies. Immediately following terminal thoracotomy, several wedges of lung tissue, each including two pleural surfaces and approximately 5 mm in thickness, were taken from the middle lobe of the right lung using iris scissors. The lung wedges were immersed in fixative containing 2.0% paraformaldehyde, 2.5% glutaraldehyde, and 0.05% $CaCl_2$ in 0.08 M cacodylate buffer for one hour at room temperature. The wedges were then trimmed to a thickness of about 2 mm by cutting perpendicular to the pleural surfaces with a razor blade and fixation was continued overnight at 4°C. The tissue wedges were then washed twice and stored in 0.1 M cacodylate buffer containing 7% sucrose at 4°C until postfixation. The wedges were post-fixed with 1% osmium tetroxide in 0.1M cacodylate for one hour, dehydrated with increasing concentrations of ethanol, and air dried from hexamethyldisilazane (Polysciences, Warrington, PA). Following flat-mounting to aluminum stubs with conductive paint and sputter coating of the exposed cut surface with gold palladium, the lung wedges were examined using a JSM-820 scanning electron microscope (JEOL, Tokyo, Japan) at a 15° tilt.

Experimental Design. Four groups of animals were studied. Baseline blood sampling, hemodynamic measurements and bronchoalveolar lavage were performed prior to any intravenous infusions. Control (n=10) animals received a 1 hour intravenous infusion of sterile saline only. Control-NO (n=5) animals received a 1 hour intravenous infusion of sterile saline and received inhaled NO at 20 ppm. Septic (n=10) animals received a 1 hour intravenous infusion of live *Pseudomonas aeruginosa,* PAO strain ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min). Septic-NO (n=5) animals, in addition to receiving an identical infusion of *Pseudomonas aeruginosa*, received inhaled nitric oxide at 20 ppm throughout the experiment. Nitric oxide administration was begun 15 minutes prior to infusion of either *Pseudomonas aeruginosa* or saline to ensure that a nitric oxide concentration of 20 ppm was present within the lung at time zero. Animals in the different groups were run in random order. All groups were studied for a total of 5 hours.

Peak Airway Pressures. Septic untreated animals exhibited a marked increase in peak inspiratory pressure evident at 30 min following onset of sepsis which continued to rise throughout the five hours of study (figure 30). Nitric oxide treatment in

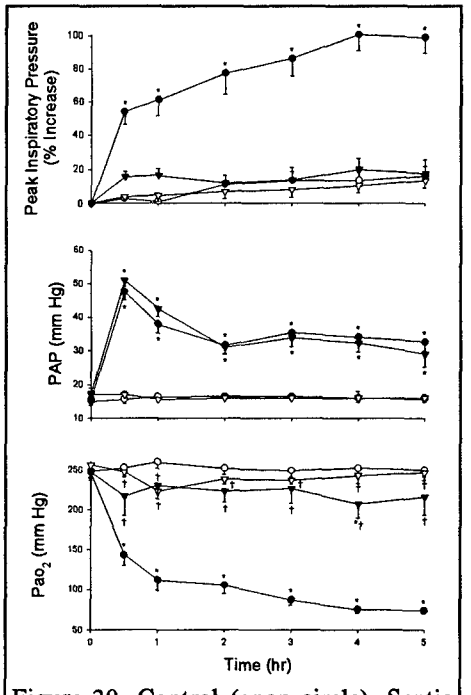

Figure 30. Control (open circle), Septic (closed circle), Control-NO (open triangle), Septic-NO (closed triangle), *p<0.05 vs Control, †p<0.05 vs Septic

septic animals dramatically attenuated the rise in peak airway pressures. Peak airway pressure in both control groups were not significantly elevated (Figure 30). No alterations in inspiratory:expiratory ratios were observed in any animal.

Pulmonary Hemodynamics. Pulmonary arterial pressure in both the septic-NO and untreated septic animals increased significantly ($p<0.05$) within 30 minutes following onset of sepsis. Following termination of the bacterial infusion pulmonary artery pressure declined in both septic groups. However, both septic groups exhibited persistent pulmonary arterial hypertension for the remainder of the experiment compared to controls (Figure 30). There was no statistically significant difference in pulmonary artery pressure between septic-NO and untreated septic animals. Pulmonary artery pressure remained unchanged compared to baseline throughout the experiment in both control and control-NO groups.

Pulmonary vascular resistance index increased significantly in both septic-NO and untreated septic animals within 30 minutes (Table 1). PVRI then declined in both groups but remained significantly ($p<0.05$) elevated compared to baseline and control in both groups. However, administration of inhaled nitric oxide significantly ($p<0.05$) attenuated the increase in PVRI at thirty minutes compared to untreated septic animals. Beyond 30 minutes there was no significant difference in PVRI between the septic-NO and untreated septic animals. The PVRI remained unchanged compared to baseline throughout the experiment in both the control and control-NO groups.

	0 hr	0.5 hr	1 hr	2 hr	3 hr	4 hr	5 hr
CI							
Control	3.5 ± 0.2$^{\#}$	3.4 ± 0.2$^{!\#}$	3.4 ± 0.2$^{\#}$	3.1 ± 0.1$^{!\#}$	3.0 ± 0.1$^{!\#}$	2.8 ± 0.1$^{!\#}$	2.8 ± 0.1$^{!}$
Control-NO	4.9 ± 0.2$^{*!}$	4.9 ± 0.2$^{*!}$	4.4 ± 0.1$^{*!}$	3.9 ± 0.1$^{*!}$	3.6 ± 0.1$^{*!}$	3.6 ± 0.1$^{*!}$	3.2 ± 0.1$^{!}$
Septic	3.1 ± 0.2$^{\#}$	2.0 ± 0.1$^{*\#}$	2.8 ± 0.1$^{\#}$	2.6 ± 0.1$^{*\#}$	2.2 ± 0.1$^{*\#}$	1.9 ± 0.1$^{*\#}$	1.6 ± 0.1$^{*\#}$
Septic-NO	3.0 ± 0.2$^{\#}$	2.1 ± 0.1$^{*\#}$	2.8 ± 0.1$^{\#}$	2.8 ± 0.1$^{\#}$	2.4 ± 0.1$^{*\#}$	1.8 ± 0.1$^{*\#}$	1.5 ± 0.1$^{*\#}$
SVRI							
Control	2200 ± 159	2250 ± 109$^{!}$	2417 ± 109	2717 ± 143	2943 ± 218	3019 ± 184	2958 ± 129
Control-NO	1566 ± 203$^{!}$	1607 ± 187$^{!}$	1812 ± 180	2014 ± 200	2165 ± 112	2074 ± 133*	2204 ± 118
Septic	2536 ± 481$^{\#}$	4290 ± 344$^{*\#}$	2476 ± 177	1782 ± 194*	2104 ± 173*	2585 ± 157	3041 ± 203$^{!}$
Septic-NO	2248 ± 128	4212 ± 306$^{*\#}$	2901 ± 308$^{\#}$	1541 ± 107*	1835 ± 186*	2806 ± 387	3579 ± 373$^{\#}$
PVRI							
Control	188 ± 14	223 ± 20$^{!}$	257 ± 37$^{!}$	281 ± 34$^{!}$	255 ± 29$^{!}$	300 ± 38$^{!}$	319 ± 37$^{!}$
Control-NO	188 ± 15	162 ± 10$^{!}$	165 ± 18$^{!}$	178 ± 14$^{!}$	189 ± 10$^{!}$	189 ± 17$^{!}$	209 ± 20$^{!}$
Septic	254 ± 29	1662 ± 348$^{*\#}$	782 ± 163$^{*\#}$	813 ± 62$^{*\#}$	966 ± 18$^{*\#}$	1158 ± 107$^{*\#}$	1412 ± 169$^{*\#}$
Septic-NO	235 ± 32	1193 ± 124$^{*\#}$	918 ± 61$^{*\#}$	607 ± 159$^{*\#}$	1030 ± 152$^{*\#}$	1366 ± 128$^{*\#}$	1300 ± 357$^{*\#}$
SAP							
Control	94 ± 4	97 ± 5	105 ± 4	110 ± 5	113 ± 5	109 ± 5	107 ± 4
Control-NO	97 ± 10	102 ± 10	102 ± 8	101 ± 7	102 ± 4	96 ± 5	92 ± 4
Septic	98 ± 5	114 ± 8*	91 ± 5	60 ± 6$^{*\#}$	60 ± 5$^{*\#}$	67 ± 5$^{*\#}$	67 ± 5*
Septic-NO	88 ± 4$^{!}$	120 ± 5	104 ± 9	58 ± 5$^{*\#}$	58 ± 4$^{*\#}$	64 ± 6$^{*\#}$	61 ± 11$^{*\#}$

Table 1. Hemodynamic Parameters. Cardiac index (CI, L/min/m^2), systemic vascular resistance index (SVRI,dynes/m^2/cm^5), pulmonary vascular resistance index (PVRI, dynes/m^2/cm^5), and mean systemic arterial pressure (SAP, mm Hg). Values are mean ± SEM. $^{*}p < 0.05$ vs. control, $^{!}p < 0.05$ vs. septic, $^{\#}p < 0.05$ vs. control-NO.

Systemic Hemodynamics. Systemic arterial pressure in the septic-NO and untreated septic animals exhibited a transient increase during the bacterial infusion (Table 1). Following cessation of the infusion, systemic arterial pressure declined rapidly in both groups becoming significantly ($p<0.05$) lower than control animals by 2 hours. Systemic arterial pressure in untreated septic and septic-NO animals remained lower than control animals for the remainder of the experiment. Inhalation of NO alone (control-NO) produced no significant change in systemic arterial pressure.

Cardiac index exhibited a biphasic response in both septic-NO and untreated septic animals (Table 1). Cardiac index declined significantly during the first 30 minutes, corresponding to the acute rise in pulmonary artery pressure. Between 30 minutes and 2 hours these groups demonstrated a partial recovery in cardiac index which was then followed by a second progressive, significant decline over the remainder of the experiment. No differences existed between septic-NO and septic animals. Although cardiac index was elevated in control-NO animals, the degree of change over the course of study was similar to that observed in control animals.

Systemic vascular resistance index exhibited a gradual progressive increase during the experiment in both the control and control-NO animals, although this did not reach statistical significance compared to baseline (Table 1). In untreated septic animals SVRI increased significantly at 30 minutes during the bacterial infusion. SVRI then declined until 2 hours when it was significantly ($p<0.05$) lower than that observed in control animals. From 2 hours onward SVRI gradually returned towards baseline levels. Septic-NO animals demonstrated an almost identical pattern in SVRI compared to untreated septic animals.

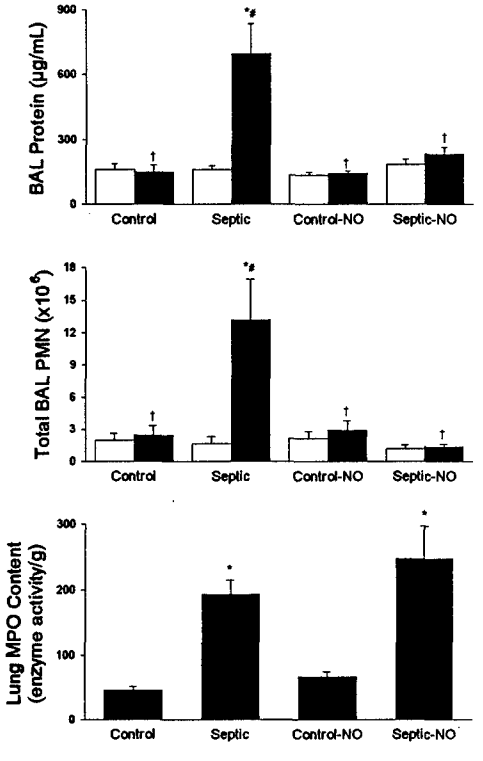

Figure 31. Time points equal 0 hour (open bar) or 5 hours (solid bar), *$p<0.05$ vs Control at 5 hours, †$p<0.05$ vs Septic at 5 hours, #$p<0.05$ vs baseline.

Arterial Oxygen Tension. Untreated septic animals exhibited a rapid, significant decline in arterial PaO_2 compared to baseline within 30 minutes of the onset of sepsis (144 torr vs. 247 torr, $p<0.05$) with further significant decline in the latter phases of study (Figure 30). Septic-NO animals exhibited no significant decline in PaO_2 throughout the experimental period. PaO_2 levels in both control groups remained essentially constant throughout the experiment.

Lung Permeability Injury - Bronchoalveolar Lavage Protein. Bronchoalveolar protein content exhibited a significant ($p<0.05$) increase at 5 hours in untreated septic animals compared to baseline (694±140 vs. 160±18 µg/ml), and to

control animals at 5 hours (149±32 µg/ml), control-NO animals at 5 hours (142±13 µg/ml) and septic-NO animals at 5 hours (230±32 µg/ml) (Figure 31). There was no significant increase at 5 hours compared to baseline in septic-NO, control, or control-NO animals.

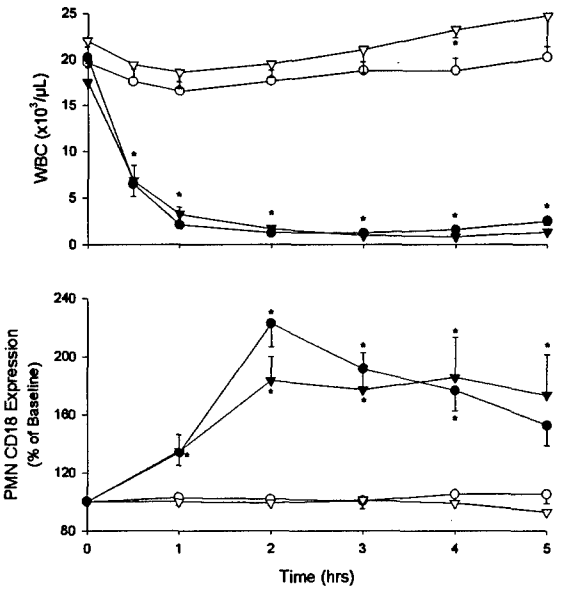

Figure 32. Control (open circle), Septic (closed circle), Control-NO (open triangle), Septic-NO (closed triangle), *p<0.05 vs Control

Neutrophil Kinetics

Peripheral Neutrophil Count. Circulating neutrophil counts in both septic-NO treated and untreated septic animals declined rapidly following the onset of sepsis, becoming significantly lower than controls within 30 minutes (17.5±2.1 vs. 6.8±1.7 and 20.2±1.1 vs. 6.4±1.3 x 10^3/µl, p<0.05). Neutrophil counts in these two groups failed to exhibit any significant recovery during the remainder of the experiment. Neutrophil counts in the two control groups remained at baseline values throughout the experiment (Figure 32).

Neutrophil Transmigration. Bronchoalveolar lavage neutrophil counts in untreated septic animals exhibited a significant (p<0.05) increase at 5 hours compared to both baseline and control animals at the same time point indicating significant transmigration into the alveolar spaces [13.2 ± 3.7 vs. 1.7 ± 0.6 vs. 2.5 ± 0.8 PMN (x 10^6); untreated septic 5 hours vs. baseline vs. control 5 hours] (Figure 31). Bronchoalveolar lavage neutrophil counts in septic-NO animals exhibited no significant increase at 5 hours compared to baseline and control [1.4±0.2 vs. 1.2± 0.4 vs. 2.5± 0.8 PMN (x 10^6); septic-NO 5 hours vs. baseline vs. control 5 hours]. Neither control group exhibited a significant increase in bronchoalveolar lavage neutrophil counts.

Lung Myeloperoxidase Content. At 5 hours lung myeloperoxidase content was significantly (p<0.05) increased in both untreated septic and septic-NO animals compared to both control and control-NO animals (198±32 and 249±48 vs. 41±5 and 67±8, P<0.05), indicating significant neutrophil sequestration during sepsis (Figure 31).

Neutrophil CD18 Expression. Neutrophil integrin expression was unchanged from baseline during the experiment in both control groups (Figure 32). In contrast, both septic groups exhibited significant (p<0.05) upregulation of CD18 expression within two hours following the onset of sepsis with peak expression occurring between 2-4 hours. CD18 expression remained significantly elevated above that observed in controls for the duration of the experiment.

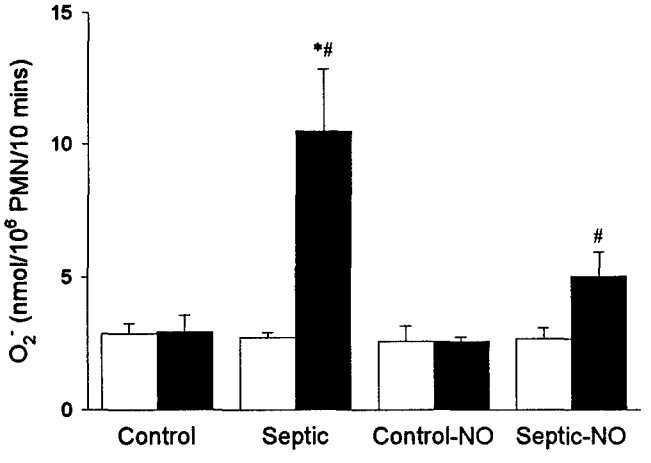

Figure 33. Time points equal 0 hour (open bar) or 5 hours (solid bar), *p<0.05 vs Control at 5 hours, #p<0.05 vs baseline.

Neutrophil Priming. Isolated neutrophils from animals in both control groups exhibited no priming for the oxidant burst during the experiment as indicated by unchanged total superoxide anion production following stimulation by phorbol myristate acetate at baseline and 5 hours (Figure 33). Both septic-NO and untreated septic groups, however, exhibited significant increases in the total amount of superoxide production at 5 hours compared to baseline (5.0±0.9 vs. 2.7±0.4. and 10.5±0.9 vs. 2.7±0.2, $p<0.05$). Priming for the oxidant burst was attenuated by 50% in the septic-NO group compared to the untreated septic group, however this decrease was not statistically significant ($p=0.054$).

Scanning Ultrastructural Studies. Scanning electron micrographic studies of porcine lung obtained from septic-NO animals revealed a qualitative reduction in alveolar and septal edema. Although qualitative in nature, parenchyma of septic-NO animals revealed enhanced aeration as evidenced by prominent alveolar ductular structures and enhanced sized of alveoli in general compared to septic untreated animal. All scanning electron micrographs were taken from peripheral lung areas within 1-1.5 mm of the pleura. The cut surface of the lung from a control animal displayed primarily expanded alveoli with thin walls and a few widely patent alveolar ducts. Compared to the control lung, lung from a septic animal at equal magnification displayed distinctly smaller alveoli, thickened alveolar walls, and relatively constricted alveolar ducts. Lung from a septic-NO animal displayed less constriction of alveoli, less thickening of alveolar walls, and more patent alveolar ducts than did the lung from the septic animal. Nitric oxide treatment alone (control-NO) appeared to have no morphological effects on the lung.

Methemoglobin and Nitrogen Dioxide Levels. Inhalation of nitric oxide at 20 ppm produced no significant increase in methemoglobin levels at 5 hours compared to baseline in either the control-NO (2.5 vs. 1.3 % metHgb) or the septic-NO (2.2 vs. 1.3 % metHgb) animals. It also produced no significant increase in nitrogen dioxide levels at 5 hours compared to baseline in either control-NO (0.6 vs. 0.0 % NO_2) animals or septic-NO animals (0.5 vs. 0.08 % NO_2).

DISCUSSION

Nitric oxide was reported in 1987 to be the previously described endothelium-derived relaxing factor (127,128) and since this report has proven to be an important regulator of pulmonary vascular tone (129,130). Inhaled nitric oxide produces selective vasodilatation in both sheep with hypoxic pulmonary vasoconstriction and humans with

primary pulmonary hypertension (114-116). Inhaled nitric oxide also produces selective pulmonary arterial vasodilation, decreased venous admixture and improved arterial oxygenation in patients with ARDS (117). In addition to its effect upon both systemic and pulmonary vasculature there is evidence which suggests that nitric oxide alters adhesive interactions between leukocytes and endothelial cells. Kubes et al showed that nitric oxide donors (agents capable of generating NO in-vitro) prevented integrin-dependent leukocyte adhesion in post-ischemic venules while nitric oxide inhibitors greatly enhanced neutrophil adherence and emigration in postcapillary venules (122,123). These findings suggest that inhaled nitric oxide improves oxygenation in the setting of acute lung injury by two mechanisms 1) improved ventilation-perfusion matching and 2) changes in pathological leukocyte binding to pulmonary vascular endothelium.

An intimate association exists between the "sepsis syndrome", a hyperdynamic, hypercatabolic state and adult respiratory distress syndrome (118) where significant pulmonary arterial hypertension, elevated pulmonary vascular resistance and exaggerated ventilation-perfusion mismatching are observed. Sepsis and ultimately lung injury are produced by complex interactions between multiple cellular and humoral mediators (e.g., cytokines, eicosanoids).

Polymorphonuclear neutrophils are the primary cellular mediators of the acute lung injury associated with sepsis. Bronchoalveolar lavage fluid obtained from patients with adult respiratory distress during life and the microvasculature of lung tissue examined at autopsy both contain large numbers of neutrophils (131,132). Multiple studies have proven that altering neutrophil adherence or the production of reactive oxygen species and/or lytic enzymes by neutrophils significantly diminishes the acute lung injury characteristic of adult respiratory distress syndrome (124-126).

Recently, a multi-step theory of neutrophil adhesion and transendothelial migration has been proposed based upon the results of a series of in vitro and in vivo experiments (133-136). Following exposure to inflammatory peptides (e.g., cytokines) the endothelium initiates adhesion by rapid expression of stored P-selectin and later, by E-selectin generated de novo. In post-capillary venules neutrophils bind loosely to endothelial cells via engagement of selectin molecules expressed on the endothelium and their ligands expressed on activated neutrophils (137). Selectin engagement promotes margination of neutrophils by causing neutrophils to roll along the activated endothelium. Subsequent local exposure to inflammatory factors (e.g., interleukin-8, platelet activating factor) promotes upregulation of neutrophil β_2-integrin (CD11/CD18) receptors. Formation of tight bonds between these receptors and their upregulated counter-receptor (ICAM-1) on the endothelium arrests neutrophil rolling. Following cessation of rolling adherent neutrophils migrate through intercellular junctions between apposing endothelial cells. There is evidence that this step is dependent upon platelet/endothelial cell adhesion molecule 1 (PECAM-1) and can be blocked by antibodies to PECAM-1 (135,136). Tight binding and transendothelial migration of activated neutrophils to the endothelium permits the formation of a protected "microenvironment" into which neutrophils release reactive oxygen species and lytic enzymes (138). The unchecked action of these substances produces endothelial injury and increased permeability to fluid and plasma proteins.

The results of the current study suggest that administration of inhaled nitric oxide attenuates sepsis-induced acute lung injury by interfering with the process of neutrophil adhesion and emigration. Three directly measured markers of alveolar capillary-membrane injury; bronchoalveolar lavage protein content, bronchoalveolar lavage neutrophil count and arterial oxygen tension (PaO_2) were all significantly improved by nitric oxide inhalation in septic animals. In addition, scanning electron micrographic studies of lungs from septic-NO treated animals qualitatively demonstrates protection against sepsis-induced acute lung injury. Untreated septic animals developed a 4-fold increase in bronchoalveolar lavage protein content at 5 hours compared to baseline and a 3-fold increase compared to septic animals that inhaled nitric oxide gas.

Septic animals administered nitric oxide at 20 ppm exhibited no significant increase in bronchoalveolar lavage protein content at 5 hours compared to baseline and both control groups. Untreated septic animals also exhibited significant increases in bronchoalveolar lavage neutrophil counts at 5 hours compared to baseline and to septic animals administered inhaled nitric oxide. Septic-NO animals exhibited *no significant increase* in neutrophil transmigration compared to baseline and both control groups. As previously reported using this model of porcine sepsis, both static and dynamic lung compliance values decrease significantly correlating with the level of increased lung permeability (139). Although compliance was not measured directly in this study, peak airway pressures in the septic-NO group did not change significantly compared to either baseline or control groups. This occurred during a time when there was a significant and progressive increase in peak airway pressures in septic untreated animals. At no time were there changes in the inspiratory to expiratory ratios indicating any significant increase in airway resistances.

The relentless decline in arterial oxygen tension in untreated septic animals in this model of sepsis is the result of two processes which are separated temporally. Hypoxemia occurring during the first 60 minutes of the study likely results from acute ventilation-perfusion mismatching produced by acute pulmonary arterial hypertension (140). Beyond 60 minutes pulmonary arterial hypertension moderates but remains significantly elevated compared to baseline for the duration of the study. Despite attenuated pulmonary artery pressures arterial hypoxemia continues to evolve in untreated septic animals as sequestration of neutrophils primed by the septic process occurs. Ultimately, vascular injury results secondary to neutrophil mediated injury (141,142). Inhalation of nitric oxide prevented early and later phase arterial hypoxemia maintaining PaO_2 at near baseline levels in septic-NO animals; whereas untreated septic animals exhibited a greater than 3-fold decrease in PaO_2 compared to baseline and controls at 5 hours. Pulmonary artery hypertension was not significantly different in either septic group beyond 60 minutes. These results suggest that arterial oxygen tensions in the later phases of study in the inhaled nitric oxide group resulted from attentuation of neutrophil-induced permeability injury.

The inability of inhaled nitric oxide to attenuate pulmonary artery hypertension in this model of gram-negative sepsis is contrary to the results of prior studies in swine which show small, but statistically significant, reductions in mean pulmonary artery pressure following inhalation of nitric oxide at concentrations ranging from 10 - 80 ppm

(143-145). As previously reported in this and other models of porcine sepsis, high levels of thromboxane A_2 are detectable within 15 minutes of initiating *Pseudomonas* infusion (146, 147). Production of significantly higher plasma levels of vasoconstrictive substances may account for the inability of inhaled nitric oxide to ameliorate the pulmonary artery hypertension observed in this model. Inhalation of nitric oxide at concentrations as high as 80 ppm were examined in these studies and resulted in no significant decline in pulmonary artery pressure (data not shown). We speculate that inhaled nitric oxide's ability to maintain near baseline arterial oxygen tensions during the first hour of study, as in previous models, is likely due to improved ventilation-perfusion matching although changes in ventilation-perfusion matching cannot be detected by a pulmonary artery catheter.

Protection against sepsis-induced alveolar-capillary membrane injury may be attributable to one or more of several mechanisms: (1) reduction of neutrophil sequestration within the lungs, (2) reduction of neutrophil oxidant production, and/or (3) inhibition of effective neutrophil adhesion and transendothelial migration. The first of these possibilities can be discounted since inhalation of nitric oxide by septic animals exerted no effects on neutrophil sequestration, as demonstrated by the significantly elevated lung myeloperoxidase contents of septic-NO animals at five hours. Inhaled nitric oxide, however, attenuated neutrophil oxidant production as proven by decreased priming for the oxidant burst exhibited by neutrophils from septic-NO treated animals compared to untreated septic animals. Although these results failed to achieve statistical significance, priming for the oxidant burst was decreased by nearly half in the septic-NO animals. The decrease in oxidative priming observed is consistent with the results of a previous study by Clancy et al. who demonstrated that nitric oxide significantly inhibited the generation of superoxide by neutrophils exposed *in-vitro* to either FMLP or phorbol myristate acetate (148). The mechanisms by which NO decreases superoxide production is unclear, although Clancy et al. hypothesized that nitric oxide inactivates a still undetermined membrane component required for the assembly and/or activation of the NADPH oxidase which produces superoxide anion. Failure of effective adhesion and transendothelial migration of activated neutrophils is a third explanation for the protective effect of inhaled nitric oxide may be produced. Tight neutrophil-endothelial binding with subsequent transendothelial migration is dependent upon neutrophil β_2-integrin expression (136,137). Inhalation of nitric oxide failed to prevent significant upregulation of neutrophil β_2-integrin receptor expression but significantly reduced neutrophil transmigration into the alveolar airspaces as supported by reduced bronchoalveolar lavage neutrophil counts. One possible explanation for these results is that inhaled nitric oxide attenuates neutrophil transendothelial migration by affecting the expression and/or function of other adhesion molecules expressed on the endothelial cell or the neutrophil such as PECAM-1 or VCAM-1.

This hypothesis is supported by the work of Kubes et al. who documented that nitric oxide donors significantly attenuated ischemia/reperfusion induced migration of firmly adherent neutrophils and that nitric oxide inhibitors greatly increased neutrophil adherence in postcapillary venules (122,123). Kubes et al also demonstrated that nitric oxide donors failed to decrease β_2-integrin (CD11/CD18) expression on activated

neutrophils and exerted no effects on selectin induced neutrophil rolling. These data are consistent with the results of the present experiment which demonstrated significantly increased neutrophil sequestration in the lungs of both septic groups yet significantly decreased bronchoalveolar lavage protein and neutrophil transmigration (bronchoalveolar lavage neutrophil count) in septic animals administered nitric oxide. Increased sequestration is a consequence of selectin mediated rolling and integrin dependent tight adhesion. The exact mechanism by which inhaled nitric oxide inhibits transendothelial migration of activated adherent neutrophils is unclear, but may involve altered expression and/or function of adhesion molecules such as PECAM-1 or VCAM-1. PECAM-1 is expressed on both activated neutrophils and pulmonary endothelium and has been shown to be required for transendothelial migration of leukocytes to sites of inflammation. Nitric oxide donors inhibit VCAM-1 expression on endothelial cells by repressing VCAM-1 gene transcription (149). Nitric oxide may also exert direct effects on both endothelium and/or neutrophils retarding transvascular migration.

In summary, this study has shown that inhaled nitric oxide attenuates alveolar-capillary membrane injury in a porcine model of gram-negative sepsis and acute lung injury. Further, markers of alveolar capillary-membrane injury including bronchoalveolar lavage protein content and neutrophil count and arterial oxygen tension are significantly improved by administration of inhaled nitric oxide in septic swine. We have also shown the neutrophil priming for oxidant burst is attenuated by inhaled nitric oxide. Finally, we have observed that transendothelial migration of activated neutrophils is inhibited by administration of inhaled nitric oxide, even though neutrophil sequestration in the lung is not different from septic animals. These data suggests that inhaled nitric oxide attenuates acute lung injury associated with gram-negative sepsis by disrupting events associated with neutrophil migration and/or neutrophil respiratory burst. Due to its design, this study is incapable of addressing either the long term survival of nitric oxide treated animals or the potential deleterious effects of altering neutrophil function.

V. PATTERNS OF ENDOTHELIAL CELL INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1) AND E-SELECTIN (ENDOTHELIAL CELL ADHESION MOLECULE-1, ELAM-1) EXPRESSION

Introduction. Leukocyte adhesion molecules are expressed on the surfaces of endothelial cells previously activated by cytokines released at sites of inflammation. E-selectin, also known as Endothelial Leukocyte Adhesion Molecule-1 (ELAM-1), and Intercellular Adhesion Molecule-1 (ICAM-1) are structurally unrelated adhesion molecules critical for the capture and extravasation, respectively, of circulating granulocytes, monocytes, and T-lymphocytes. Soluble variants of these molecules, devoid of their transmembrane and cytoplasmic domains, are found in the serum of patients with bacterial sepsis and other inflammatory diseases and are thought to be shed from the surface of endothelial cells. The physiological and/or clinical significance of these soluble adhesion molecules is not clear. Recently, Sessler et al found soluble ICAM-1 and E-Selectin levels to be predictive of in hospital mortality and the development of multiple organ failure (8).

Using quantitative sandwich ELISAs for soluble E-selectin and soluble ICAM-1, we measured the amounts of these molecules present in culture media and cell lysates of cultured endothelial cells activated with tumor necrosis factor (TNFα) or interleukin-1 (IL-1ß) over a wide concentration range. The purposes of the study were to determine whether shedding is related to endothelial cell cytotoxicity and how increased cytokine concentrations alter distributions of shed versus cell-associated molecules. Finally we questioned whether actual quantities of E-selectin and ICAM-1 shed were different following exposure to these two cytokines critical to the septic process.

Methods. Recombinant human TNFα and IL-1ß were purchased from R&D Systems. During cytokine treatment, EC were maintained in M-199, 1% FBS, 9% Nu-Serum, and 1% antibiotic/antimycotic. At termination, conditioned media were collected, the cultures were washed twice with Hanks' Balanced Salt Solution containing 0.02% bovine serum albumin (HBSS/BSA), and cells were harvested by scraping in a third change of HBSS/BSA. Cell lysates were produced by sonication. Aliquots of media and lysates were stored at -20°C until analysis of adhesion molecules and at 4°C until analysis of lactate dehydrogenase (LDH) activity.

Cytotoxicity was determined by release of LDH into culture medium. LDH activity was measured in a microplate assay (OD_{490nm}) based on conversion of lactate to pyruvate. Total LDH in media and lysates was determined, cytotoxicity was expressed as percentage of the total released into the media, and survival was expressed as percentage of the total remaining in the cells. Cellular morphology was monitored by phase contrast microscopy.

E-selectin in media and lysates was measured in a quantitative sandwich ELISA sensitive to 0.1 ng/ml. This assay was developed and performed at the Maryland Research Laboratories of Otsuka America Pharmaceutical, Inc. ICAM-1 in the same media and lysates was measured in a quantitative sandwich ELISA sensitive to 0.3 ng/ml (T Cell Diagnostics, Inc.). Amounts of adhesion molecules in media and lysates

were expressed as ng/cm² of culture growth surface. The cell density was approximately 5 x 10^4 cells/ cm². Results are reported as mean ± SD.

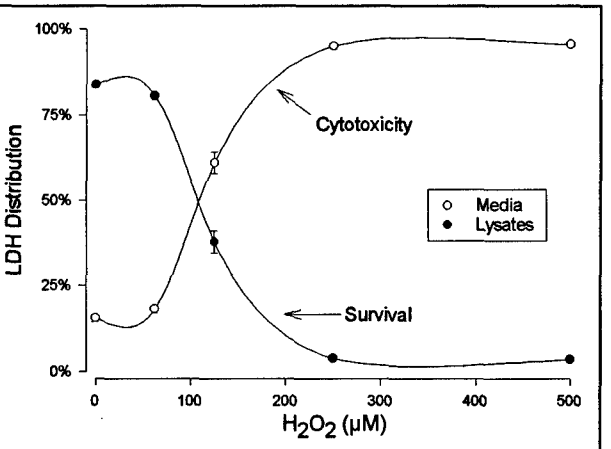

Figure 34 H_2O_2 Dose Response.

Cytotoxicity and H_2O_2 Dose Response. One goal of these experiments was to determine if shedding of E-selectin and ICAM-1 into the media of cytokine-treated HUVEC is related to cytotoxicity and consequent solubilization of adhesion molecules from the membranes of lysed cells. To document the effectiveness of the LDH assay in measuring cytotoxicity in cultures of HUVEC, cells were damaged with H_2O_2 and cytotoxicity and survival were assessed using the LDH assay (Figure 34). Cells were exposed to H_2O_2 in HBSS/0.02% BSA for one hour, then washed and re-fed with M-199 containing 4% FBS. After 18 hours the media were collected and the washed cells were scraped and lysed. LDH activity in the medium and lysate was determined for each culture. The percentage of the total LDH activity in the medium was an index of cytotoxicity and the percentage in the lysate was an index of survival. Cytotoxicity increased linearly at concentrations of H_2O_2 between 50 and 200 μM, reaching a plateau at higher doses. Survival followed an inverse linear curve between the same doses. Spontaneous release of LDH in control cultures was usually 15-20%. This study demonstrated that the LDH assay was sensitive in detecting cellular lysis above that occurring spontaneously in confluent cultures maintained in lowered-serum media.

E-selectin TNF Time Course. Figure 35 documents shedding of E-selectin into the culture media after 6 and 24 hours of continuous activation with TNFα. At 6 hours, shedding was not significantly increased at any concentration of TNFα. At 24 hours, shedding increased significantly at the lowest concentration and then remained level. Cytotoxicity, measured by release of LDH into the same media, was consistent with spontaneous levels of LDH release across the dose range. Thus, TNFα significantly increased shedding of E-selectin into the media between 6 and 24 hours but did not induce concomitant lysis of HUVEC. Phase contrast microscopy of the same cultures at 6 and 24 hours confirmed that the monolayers were intact.

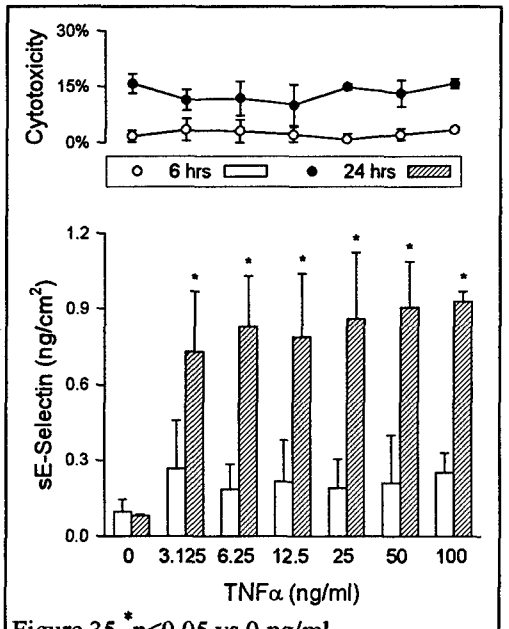

Figure 35 *p<0.05 vs 0 ng/ml.

TNF Stimulation of HUVEC. The effect of TNFα on the levels of cellular versus shed E-

selectin at 24 hours was examined in another experiment. The percentages of LDH activity in the media (cytotoxicity) and lysates (survival) of cultures incubated with TNFα for 24 hours was not significantly different than control (0 ng/ml) at any concentration of TNFα. The same media and lysates were also analyzed for soluble E-selectin (Figure 36) and ICAM-1 (Figure 37). After incubation with TNFα for 24 hours, the amount of E-selectin shed into the media increased incrementally with dose at concentrations up to 2.5 ng/ml. The amounts of shed E-selectin were 3-8 fold higher than those of cell bound E-selectin. Cell bound E-selectin was significantly higher than its control only at the 2.5 ng/ml concentration.

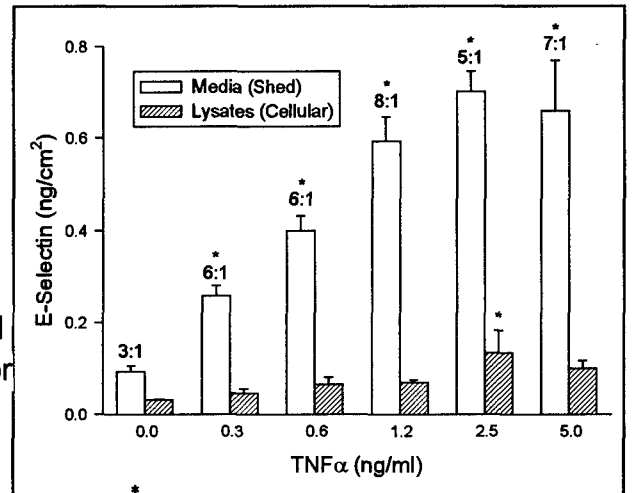

Figure 36 *p<0.05 vs 0 ng/ml.

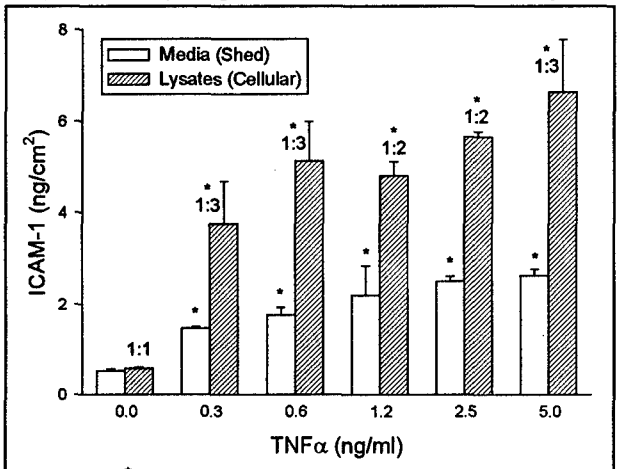

Figure 37 *p<0.05 vs 0 ng/ml.

In the same experiment, shedding of ICAM-1 increased significantly at the lowest concentration of TNFα and continued to increase up to 5.0 ng/ml. Cell bound ICAM-1 was markedly increased at the lowest concentration and then gradually increased. The ratios of shed:cell bound ICAM-1 were reversed and lower than those for E-selectin; most ICAM-1 was cell bound while most E-selectin was shed.

IL-1 Stimulation of HUVEC. The effect of IL-1ß on the levels of cellular versus shed E-selectin at 24 hours was examined in another experiment. The percentages of LDH activity in the media (cytotoxicity) and lysates (survival) of cultures incubated with IL-1ß for 24 hours was not significantly different than control (0 ng/ml) at any concentration of IL-1ß. The same media and lysates were also analyzed for soluble E-selectin (Figure 38) and ICAM-1 (Figure 39). Shedding of E-selectin increased significantly at the lowest concentration of IL-1ß and then remained level. Cell bound E-selectin increased significantly and incrementally up to 1.2 ng/ml with a plateau at higher concentrations. The ratios of shed:cell bound E-selectin were only 2:1 or 1:1 after

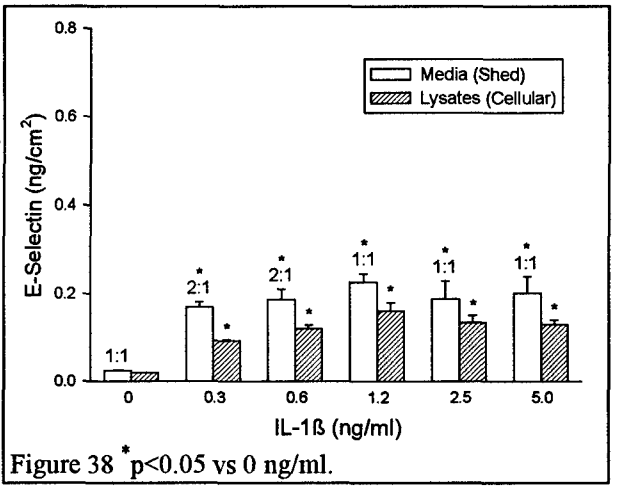

Figure 38 *p<0.05 vs 0 ng/ml.

activation with IL-1ß, in contrast to ratios of 5:1 to 8:1 after activation with TNFα.

In the same experiment, shedding of ICAM-1 increased significantly at the lowest concentration of IL-1ß and then remained level (Figure 39). Cellular ICAM-1 increased significantly and precipitously at the lowest concentration of IL-1ß and also leveled off at higher doses. Again, the ratios of shed:cellular ICAM-1 were reversed with regard to E-selectin, but the differences in the ratios were smaller.

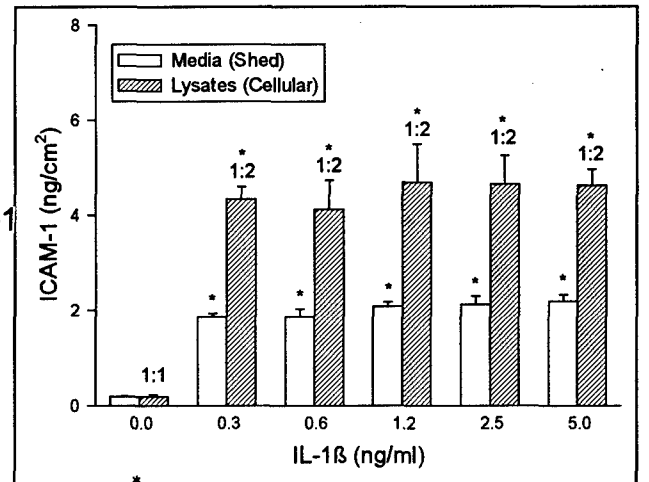

Figure 39 *p<0.05 vs 0 ng/ml.

Both TNFα and IL-1ß induced about 10-fold greater ICAM-1 than E-selectin over 24 hours (disregarding possible degradation). For E-selectin, all concentrations of TNFα and IL-1ß stimulated more expression than their respective controls, and at each concentration TNFα stimulated significantly more E-selectin than did IL-1ß. For ICAM-1, all concentrations of TNFα and IL-1ß also stimulated more expression than their controls, but TNFα stimulated significantly more expression than IL-1ß only at the two highest concentrations.

Summary

Cytokines released during acute inflammation exert multiple effects on endothelial cells, including activation which results in increased synthesis of various adhesion molecules for circulating leukocytes. During the pulmonary inflammation which occurs during sepsis-induced lung injury, leukocyte (particularly neutrophil) adhesion to and extravasation through the vascular endothelium is integral to the damage produced by activated leukocytes to the vascular endothelium and later to the alveolar epithelium. Without leukocyte adhesion, the pulmonary vascular-leak syndrome could be significantly reduced. Therefore, studies of the mechanisms of cytokine-induced adhesion molecule expression and cellular processing of these molecules by endothelial cells is important for logical design of strategies to inhibit leukocyte adhesion during sepsis induced lung injury. A question that remains unanswered is *"What is the significance of soluble adhesion molecules in the blood in patients with severe inflammatory diseases such as ARDS and sepsis?"* Soluble E-selectin in plasma is derived from endothelial cells, the only cell type that synthesizes this molecule. Most soluble ICAM-1 is likely derived from endothelial cells, although ICAM-1 is synthesized by a number of cell types. The present cell culture studies focused on several aspects of expression and shedding of E-selectin and ICAM-1 in the HUVEC culture system, which is a widely accepted in vitro model for studying vascular pathophysiology.

The experiments reported earlier in this section have generated the following novel information concerning activation of E-selectin and ICAM-1 in EC by TNFα and IL-1ß:

1) Shedding of E-selectin and ICAM-1 following cytokine-activation of EC is not a result of cell death;
2) Shedding of E-selectin and ICAM-1 increases with increasing concentrations of TNF α up to approximately 5 ng/ml. Shedding of E-selectin and ICAM-1 also increases following activation with IL-1ß, but increasing concentrations (0.3 to 5 ng/ml) produces less intense effects;
3) Greater quantities of E-selectin (3-8 fold) is shed than remains cell-associated at 24 hours after activation with TNFα. The amount of shed E-selectin is only 1-2 times the cell bound amount at 24 hours after activation with IL-1ß;
4) Greater amounts of ICAM-1 (2-3 fold) remain cell-associated than is shed at 24 hours after activation with either TNFα or IL-1ß;
5) TNFα induces significantly greater total E-selectin throughout the concentration range than does IL-1ß. TNFα also induces greater total ICAM-1 than does IL-1ß, but the difference is significant only at higher concentrations; and
6) Following activation with either cytokine, the total amounts (ng/cm^2) of ICAM-1 are about 10 fold greater than the total amounts of E-selectin.

In conclusion, these studies have revealed that soluble E-selectin and ICAM-1 are not derived by spontaneous solubilization of these molecules from the membranes of lysed endothelial cells. These molecules are found both in cell-bound and soluble forms in viable EC cultures. TNFα appears to induce greater expression and later shedding of these molecules than does IL-1ß. The significance of the soluble adhesion molecules free in circulation during episodes of sepsis remain unknown. One potentially important use for quantification of soluble adhesion molecule during septic events would be as a marker of vascular injury or activation. Experiments to evaluate this possibility are planned.

VI. EFFECTS OF INTRAABDOMINAL PRESSURE ON SYSTEMIC HEMODYNAMICS AND COMPARTMENT SPACE PHYSIOLOGY

Introduction. A final area of research which has direct implications and impact upon traumatized patients is the effect of elevated intra-abdominal pressure (IAP) on systemic and pulmonary hemodynamics and intracranial pressure (ICP). Elevated IAP is a common complication of major abdominal trauma which has significant adverse effects upon multiple organ systems. We have investigated this area recently attempting to more clearly define the expected adverse effects upon the cardiovascular and central nervous systems in the controlled setting of our laboratory. To enable accurate study we have sought to establish proper methods of monitoring such effects. Once the physiology is carefully defined and measured we will focus on development of effective treatment strategies. In order to study the relationship between IAP and hemodynamics and ICP a large animal model was developed.

The Porcine Model. The procedure for animal preparation and instrumentation was identical to that described for the acute lung injury model in the introduction of this document. A low midline abdominal incision was made and a 14 french balloon catheter inserted into the bladder to measure bladder pressure. A 20 french pigtail catheter was placed into the left upper quadrant of the abdomen via a stab incision for measurement of intra-abdominal pressure (IAP) and infusion of fluid. The abdominal incision was closed in three layers and the exit points of the catheters sealed using purse-string sutures. A pleural catheter (rubber balloon containing 3 ml of saline sealed over polyethylene tubing) was inserted into the right pleural space between the 6^{th} and 7^{th} ribs and the incision closed at peak expiration to prevent pneumothorax. All catheters were connected to a pressure transducer (Honeywell, NY). Following baseline measurements, IAP was increased by infusion of an isosmotic solution of ethylene glycol ("Co-Lyte", Block Inc, NJ) via the abdominal catheter. IAP was initially increased to 10 mm Hg above baseline and then further elevated in 5 mm Hg increments. Following each pressure increase, all parameters were measured after a 30 minute stabilization period. At IAP 25 mm Hg above baseline intravascular volume was increased with an intravenous infusion of 0.9% saline in order to return the cardiac output to baseline levels. Following a further 30 minutes stabilization, all parameters were re-measured.

The identical model and protocol was used to study the effects of elevated IAP upon ICP. The differences in the models were the use of a 6 liter balloon placed into the peritoneal cavity and inflated with sterile water to increase the IAP, the placement of a catheter into the cisterna magna for measurement of ICP and variation of the animals' respiratory rate to maintain arterial carbon dioxide 35 – 40 mm Hg.

Results

These studies have led to a number of important findings of the effects of elevated IAP upon hemodynamics. First we observed that measurement of urinary bladder pressure by installation of 50 ml of saline into an empty bladder is an extremely accurate method of determining IAP (Figure 40).Second we observed that elevated IAP exhibits exerts multifaceted effects on cardiac function (Figure 41).We observed that cardiac output is decreased in direct proportion to incremental increases in IAP.

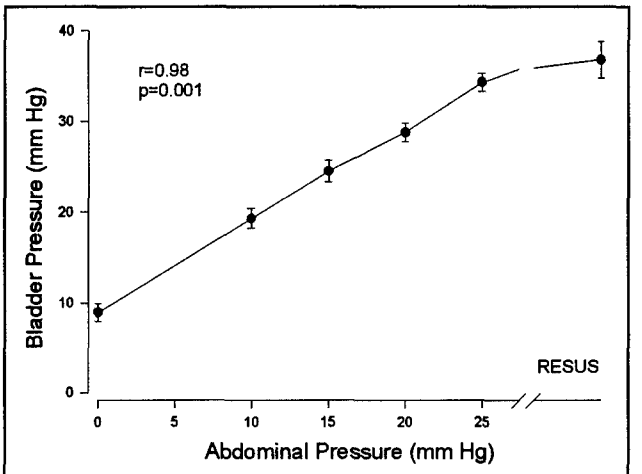

Figure 40

We further observed secondary dramatic increases in systemic vascular resistances during abdominal loading and postulate that diminished cardiac performance results from increased afterload. Elevated IAP was also found to exert significant effects upon central venous pressure, pulmonary artery pressure and pulmonary wedge pressures. These measurements were increased in direct proportion to elevations in IAP. Others have interpreted this as arising from increased cardiac end diastolic pressures with secondary left ventricular failure and redistribution of splanchnic blood volume to the central vasculature. However we devised a new parameter (the transarterial wedge pressure [wedge pressure - pleural pressure]) which provides greater accuracy for measure of left ventricular/left atrial end diastolic pressures (Figure 42).This index repeatedly showed that true estimates of left ventricular/left atrial end diastolic pressures actually diminished with increasing IAP and that the decreased cardiac outputs produced by elevated IAPs arises secondary to both an increased afterload and decreased cardiac filling.

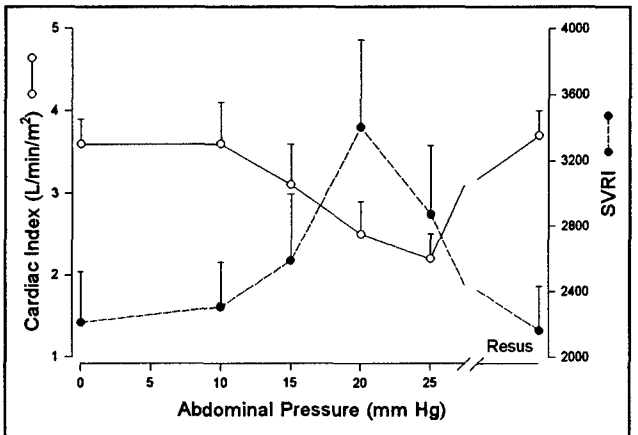

Figure 41

We believe our conclusions to be valid since clinically measured wedge pressures are actually the sum of the left atrial end diastolic pressure and pleural pressure. The low "transarterial wedge pressure" measured in our studies

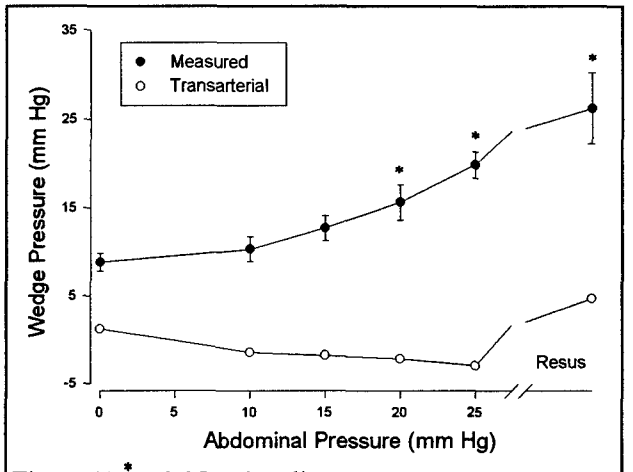

Figure 42 *p<0.05 vs baseline.

supports the hypothesis that the measured wedge pressure measured in the conventional intraluminal fashion was spuriously elevated due to the transmitted effects of a high IAP on pleural pressure. We further demonstrated that volume expansion could be used to return cardiac outputs to baseline levels, lending further support to the conclusion that *elevated IAP leads to a relatively hypovolemic state* which is not detected by standard pulmonary arterial catheter measurements, and which is improved with volume expansion rather than diuresis.

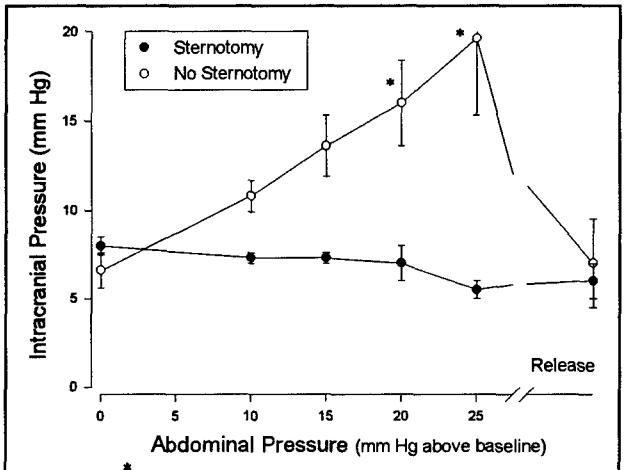

Figure 43 *p<0.05 vs baseline.

Several studies were performed evaluating the effects of elevated IAP upon the ICP. These studies yielded several interesting new findings. First, we demonstrated that rising IAP produces increased ICP (Figure 43). Second, the mechanism producing increased ICP is an increased resistance to cerebral venous outflow secondary an IAP-induced elevation in central venous pressure (Figure 44).Third, elevated central venous and other central vascular pressures observed in animals with increased IAP are mediated by the elevated pleural pressure which results from increased IAP. This conclusion was confirmed by performing midline sternotomies and pleurotomy. We found that opened pleural spaces prevented any increase in pleural pressure. Finally, it was shown that volume expansion to return cardiac output to baseline, as in the previous study, led to a further increase in ICP in animals with elevated IAP (Figure 45).

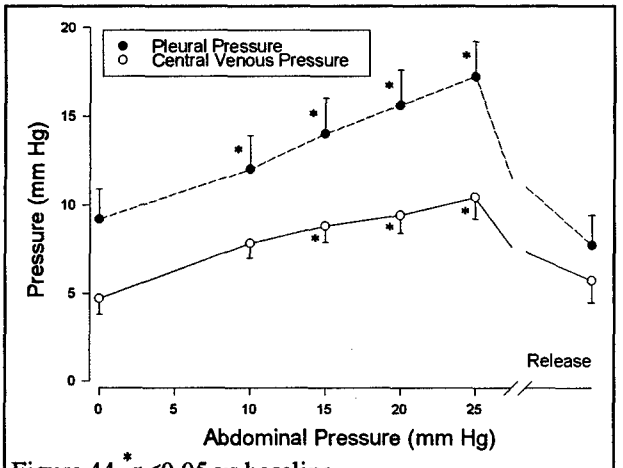

Figure 44 *p<0.05 vs baseline.

During the coming months we will focus our studies upon the effects of elevated IAP on renal function and renally-produced mediators of arterial pressure such as renin and aldosterone.

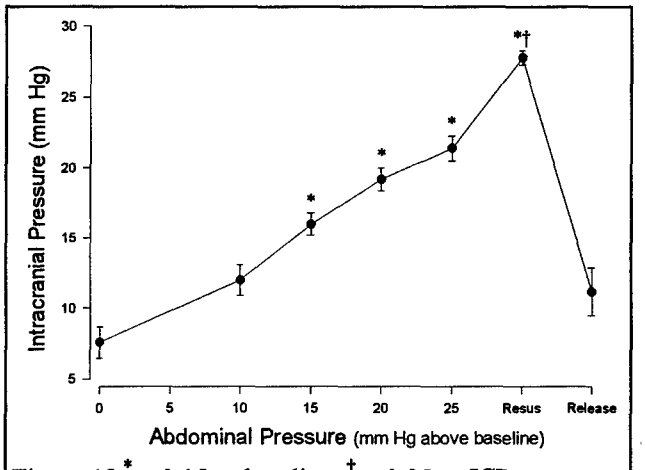

Figure 45 *p<0.05 vs baseline, †p<0.05 vs ICP at maximum IAP

VII. CONCLUSIONS

During period of support, we have performed studies focused upon attenuation of sepsis-associated lung injury using pharmacological and immunopharmacological approaches. Our work has examined unique and novel approaches to the treatment of sepsis induced lung injury demonstrating significant efficacy in most regimens examined. The results presented here underscore the extraordinary complexity and redundancy of acute inflammatory responses which leads to lung injury following the onset of sepsis. In multiple protocols we found that deletion of certain proinflammatory mediators or disruption of critical neutrophil or endothelial cell adhesion molecules effectively attenuated lung injury. However, a critical feature common to all studies performed to date is the *increasing loss of efficacy as treatments are delayed.* In virtually all treatment strategies examined the degree of protection achieved diminished with delayed administration. Thus, each pathway or biochemical system examined (e.g., plasma kallikrein-kinin system, cytokine system, lipopolysaccharide, etc.) exerts important biological effects at unpredictable points following onset of the systemic inflammation. This reality has repeatedly plagued human sepsis trials. Within the past 5 years separate human studies using agents as diverse as: anti-lipopolysaccharide antibody, anti-TNF-α antibody, interleukin-1 receptor antagonist, soluble TNF-α receptor, and cyclo-oxygenase inhibition have all either failed to alter overall outcome (i.e., severity of lung injury or mortality) altogether or have proven efficacy only in small subsets of larger patient cohorts.

The clear effectiveness of many agents administered at the outset of sepsis contrasted markedly with diminished efficacy in human studies or delayed administration animal studies. These findings suggest that the *time* at which agents are administered may be as important as the agents utilized. Thus far no acceptable physiological or biochemical marker has been described which will pinpoint a *"window of efficacy"*. Recently collaborative clinical studies performed between members of this laboratory and Dr. Curtis Sessler, (Associate Professor of Medicine, Pulmonary Critical Care Division, Virginia Commonwealth University School of Medicine) have generated renewed enthusiasm for a serum marker of injury. In a recently published study, Dr. Sessler found that soluble ICAM-1 and E-Selectin levels in the serum of patients with sepsis not only correlated with severity of injury and mortality but also correlated with the eventual appearance of nonpulmonary organ injury (8). We are encouraged by these findings and feel they are directly relevant to the research performed here.

After careful study of our results using the acute porcine sepsis model of lung injury, we have determined that a **more clinically relevant model** is needed to move into the next phase of our studies. The features required of a new model to achieve greater human clinical relevance are: 1) A time course following onset of infection to evidence of clinical sepsis and lung injury similar to that observed in hospitalized patients ($\approx$ 24 hours); 2) Infection with a bacterial pathogen commonly associated with human infection (e.g., E. Coli) and; 3) A route of infection which commonly predisposes to acute lung injury in both medical and surgical patients (i.e., peritonitis).

Porcine Subacute Lung Injury Model. As outlined in the last quarterly report ending 31 March 1995 we have begun developing a model of subacute lung injury. We have chosen peritoneal sepsis because of its importance to the practice of surgery and internal medicine and because of its importance to acute lung injury. We have chosen *E. Coli* in the development of this model because it is common and because prior work has been performed with *E. Coli* in other model systems. Further, *E. Coli* is a highly genetically predictable organism with known pathogenicity.

At the outset of study, animals are instrumented in an identical fashion to that used in acute (5 hour) studies. Following preparation, animals are maintained under general anesthesia via continuous infusion pentobarbital for the duration of study. Sepsis is induced by administering organisms intraperitoneally at a concentration of 2.0×10^{10} - 3.0×10^{10} CFU/kg. Organisms are administered through a mini-laparatomy incision using a specially designed catheter to afford maximal mixing and spread of organisms over the surface of the bowel. Following superfusion of the organisms onto the bowel the incision is closed and animals undergo continuous mechanical ventilation maintained under continuous sedation for the next 24 hours. In our initial studies we targeted for the development of lung injury to occur within 24 hours. Physiologic variables are measured hourly with plasma and blood samples taken at 0, 2, 4, 8, 12, 16, 20, 24 hours. The focus of the studies to date is seeking the desirable concentration of E. coli organisms which will produce lung injury predictably within a 24 hour time frame.

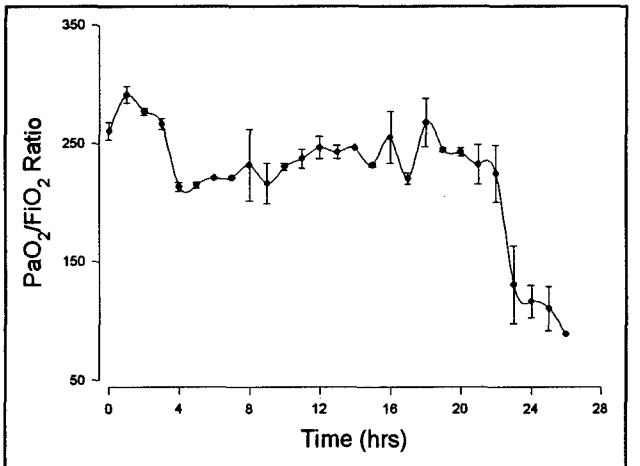

Figure 46 Effect of E.coli superfusion onto the bowel of anesthetized pigs.

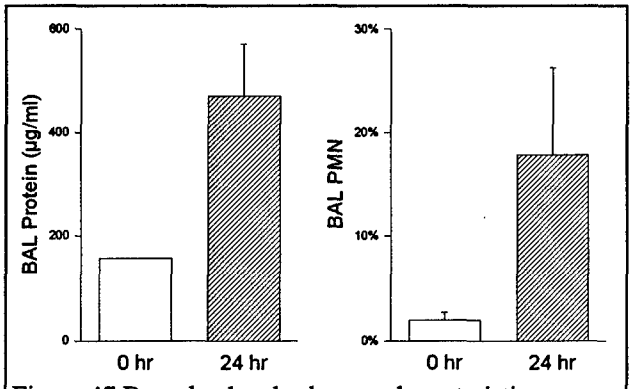

Figure 47 Bronchoalveolar lavage characteristics following E. Coli superfusion onto the bowel

Figure 46 shows the results of PaO_2/FiO_2 ratios in six animals studied to date. We have observed the development of significant lung injury between 15 and 20 hours as evidenced by descending PaO_2/FiO_2 ratios. Figure 47 shows the results of BAL protein and BAL neutrophil counts demonstrating clear evidence of both enhanced permeability and neutrophil migration, findings consistent with lung injury. We have found that concentrations above 2.75×10^{10} CFU/kg produce early mortality from shock without development of injury. Circulating blood neutrophils from the first six animals exhibit a state of enhanced oxidant burst. We view this as a strong beginning, however, the model will need additional refinement.

Using Soluble Serum Markers To Define The "Therapeutic Window". Following the results of recently completed human studies, we have chosen soluble E-Selectin serum levels as a marker of endothelial cell injury. We have begun work on the development of a sandwich ELISA for porcine soluble E-Selectin using reagents (polyclonal antibodies to porcine E-Selectin) obtained from Dr. Martyn Robinson of Celltech Corp, Berkshire, England. Following ELISA development, we will begin our studies by measuring soluble E-Selectin titers in serum samples obtained at 0, 2, 4, 8, 12, 16, 20, 24 hour time points. Serum E-Selectin titers will then be carefully correlated with physiological and bronchoalveolar lavage indicators of lung injury in the subacute model. We predict that subacute animals will exhibit surging E-Selectin serum titers and that a *threshold of injury* will be identified beyond which significant correlations will be found between soluble E-Selectin levels and the development of lung injury.

The second phase of this project will then focus on the use of soluble E-Selectin levels as a guide to therapeutic intervention. For these studies therapeutic intervention (similar to those outlined in this report) will be undertaken at varying times prior to the biochemical "threshold of injury". A biochemically targeted approach as outlined here will provide a greater chance of therapeutic success.

Additional Acute Studies. We plan to continue a careful examination of novel therapeutic modalities for treatment of sepsis-associated lung injury. As in the past, we will continue to employ the acute sepsis model to initially examine the efficacy of treatment modalities which appear promising. Recently we have begun work with two agents which are potent and appear effective. These are first inhaled nitric oxide and finally a new class of infusion agents: the lazeroids.

The Lazeroids. The lazeroids are a new class of synthetic compounds which exhibit potent antioxidant properties. Prior studies using lazeroid compounds have demonstrated potent capacity to down regulate neutrophil oxidant burst as well as to mediate complete protection in models of ischemia reperfusion. We are in the initial stages of using compound OPC 6535 provided to us by Otsuka America Pharmaceuticals. We have preliminary data in three acutely septic animals that the compound produces significant protection from sepsis induced lung injury. The results of these studies will be the subject of future reports.

LABORATORY PUBLICATIONS (5/1/93 - PRESENT)

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