Tumor Necrosis Factor-α Blockade Prevents Neutrophil CD18 Receptor Upregulation and Attenuates Acute Lung Injury in Porcine Sepsis without Inhibition of Neutrophil Oxygen Radical Generation

Alastair C. J. Windsor,[‡] Ciaran J. Walsh,[‡] Patrick G. Mullen,[‡] Daniel J. Cook,[‡] Bernard J. Fisher,[*] Charles R. Blocher,[‡] Sandra K. Leeper-Woodford,[*] Harvey J. Sugerman,[‡] and Alpha A. Fowler III[*§]

Departments of [*]Medicine, [‡]Surgery, and [§]Pathology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0519

Abstract

Tumor necrosis factor (TNFα), both by direct action and by trafficking cells of the immune system, is implicated in cardiopulmonary derangements and PMN-mediated microvascular injury associated with gram-negative sepsis. We examined the effects of pretreatment with a monoclonal antibody to TNFα on PMN function, hemodynamic derangements, and alveolar capillary membrane damage in a septic porcine model. Anti-TNFα profoundly improved hemodynamic consequences in this model. Reduction in PMN CD11/18 receptor expression, lung myeloperoxidase activity, and attenuation of peripheral neutropenia (all $P < 0.05$) indicate that pretreatment significantly reduced lung sequestration of PMNs seen in septic controls. In contrast, PMN oxygen radical (O_2^-) generation was not significantly different from unprotected septic animals. Despite the presence of circulating PMNs primed for O_2^- burst, alveolar capillary membrane damage, assessed by bronchoalveolar lavage protein content and arterial Po_2 was markedly attenuated in the treatment group ($P < 0.05$). We conclude that anti-TNFα suppresses systemic hemodynamic actions of TNFα. Further, it prevents upregulation of PMN adhesion receptors inhibiting PMN/endothelial cell interaction. This prevents formation of a "microenvironment," protected from circulating oxidant scavengers, into which sepsis-activated PMNs release their toxic products. Pretreatment with anti-TNFα monoclonal antibody thus affords global protection in porcine Gram-negative sepsis. (*J. Clin. Invest.* 1993. 91:1459–1468.) Key words: cytokines • neutrophil • β_2 integrins • septic lung injury

Introduction

Gram-negative septicemia in hospitalized patients is invariably associated with high morbidity and mortality (1). Case fatality rates of 20–60% reflect the frequent development of acute lung injury (2) and multiple nonpulmonary organ failure (3), and the lack of effective therapeutic agents needed to prevent the high frequency of patient deterioration in sepsis syndrome.

Many pathophysiological derangements associated with gram-negative sepsis result from the release of endotoxin (4, 5), the LPS component of bacterial cell walls, into the circulation. One key role of LPS appears to be the initiation of a cascade of "communication proteins" elaborated and released by the reticuloendothelial system (5). These proteins or cytokines play an important role in inflammation, both by direct action on cells at sites of infection and by trafficking other cells of the immune system, such as polymorphonuclear leukocytes (PMNs) (6). Of the numerous cytokines now recognized, tumor necrosis factor-α (TNFα)[1] has emerged in recent years as a critical chemical mediator of sepsis syndrome (7–9). TNFα is a 17-kD peptide produced predominantly by members of the mononuclear phagocyte system in response to particulate and soluble inflammatory stimuli (10). Transcription of the gene for TNFα proceeds rapidly after exposure to inflammatory stimuli, with resultant extracellular release occurring within 15 min (11). The TNFα gene codes for a 233–amino acid protein, which undergoes proteolytic cleavage of a 76-residue signal peptide leaving a 157–amino acid active cytokine (12). This secreted protein contains one intrachain disulfide bridge and exists as a dimer or trimer in circulation (13).

Significant clinical and experimental evidence implicates TNFα as central to the pathogenesis of septic shock. Elevated TNFα plasma levels are detected with greater frequency in septic patients (14) and plasma levels of TNFα correlate in some series with severity of illness and mortality rates (15). Several animal and human studies show prompt surges of TNFα in circulation after intravenous injection of LPS (16, 17), with metabolic and pathophysiological consequences that mimic Gram-negative septicemia (18). Animals (e.g., C3HEJ mice) resistent to the effects of endotoxin appear to have protection conferred by a genetic inability to manufacture native TNFα (19, 20). These findings strongly suggest a pivotal role for TNFα in the evolution of septic shock from Gram-negative aerobic organisms.

A growing body of evidence implicates blood PMNs as primary mediators of end organ damage associated with multisystem organ failure in sepsis, particularly the alveolar capillary membrane damage characteristic of sepsis-associated acute lung injury (21, 22). Using a porcine model of septic acute lung injury, our laboratory has previously correlated the appearance of TNFα in the circulation with priming of PMNs for toxic oxygen metabolite generation, increased expression of

Address correspondence to Alpha A. Fowler III, M.D., Associate Professor of Medicine, Director of Research Activities, Division of Pulmonary/Critical Care Medicine, Medical College of Virginia, P.O. Box 50 MCV Station, Richmond, VA 23298-0519.

Received for publication 11 June 1992 and in revised form 20 October 1992.

J. Clin. Invest.

0021-9738/93/04/1459/10 $2.00
Volume 91, April 1993, 1459–1468

1. *Abbreviations used in this paper:* ARDS, acute respiratory distress syndrome; BAL, bronchoaveolar lavage; ELAM-1, endothelial leukocyte adhesion molecule-1; GMP-140, granule membrane protein-140; ICAM-1, intercellular adhesion molecule-1; r, recombinant, TNFα, tumor necrosis factor-α; WBC white blood cell.

PMN β_2 integrins, and consequent loss of PMNs from the circulation (23). In vitro studies indicate that TNFα activates PMNs for oxidant generation, phagocytosis, degranulation, and adherence (24–26). Although augmentation of these critical functions primarily prepares PMNs for first line immune defense, these functions are equally capable of precipitating host tissue damage.

In light of this evidence, we hypothesized that TNFα provides a link between bacteremia and PMN activation and that activated PMNs interact with the pulmonary endothelium, giving rise to alveolar capillary membrane damage by the action of toxic metabolites. To verify this we used an mAb directed at biologically active circulating TNFα. We sought to sever this TNFα link, thereby preventing PMN activation and thus attenuating the lung injury associated with the porcine model of experimental sepsis. In light of systemic TNFα effects (18), we also predicted a modification of the hemodynamic derangements characteristic of experimental Gram-negative septicemia (27). Our studies readily demonstrate significant protection against lung injury and altered hemodynamic performance in this model. Of striking interest was the discovery of conflicting effects on PMN function, with near complete inhibition of CD11/18 adhesion receptor expression but persistence of enhanced oxygen radical generation. We suggest that inhibition of adhesion receptor expression prevents the interaction of the activated PMN with pulmonary capillary endothelium and therefore prevents toxic PMN metabolites from mediating alveolar capillary membrane injury. Further, by preventing both direct systemic actions of TNFα and PMN–endothelial interaction, mAb to TNFα also inhibits the evolution of the cardiopulmonary derangements typical of this experimental model.

Methods

mAb to TNFα. The IgG1 antibody used for these studies was kindly provided by Miles Inc. (Berkeley, CA). The antibody was purified from murine hybridoma culture harvests via cell separation, polyethylene glycol precipitation, anion exchange, and size-exclusion chromatography. Purified antibody was 99% pure with fully functional binding to human TNFα. Endotoxin levels in all lots were < 2 pg/mg protein (Limulus assay). Stabilization was performed with glycine and maltose before lyophilization. Lyophilized mAb was stored at 4°C and reconstituted in sterile H_2O immediately before infusion.

Animal preparation and conditioning. Yorkshire pigs were obtained from a commercial vendor and housed in the Virginia Commonwealth University vivarium for 3–5 d before study. All animals received benzethine and procaine penicillin G (300,000 U each) intramuscularly 48 h before study as a part of preoperative conditioning. 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. Young swine weighing 15–20 kg were preanesthetized with intramuscular ketamine hydrochloride (25 mg/kg) and placed supine. Sodium pentobarbital (10 mg/kg) was then administered intravenously to induce anesthesia. Tracheostomy was performed and the trachea intubated with a cuffed endotracheal tube (Argyle, Tullamore, Ireland). Mechanical ventilation was commenced using a large animal ventilator (Harvard Apparatus, Boston, MA) using 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. Throughout the period of study, anesthesia was maintained by continuous-infusion pentobarbital (5 $mg \cdot kg^{-1} \cdot h^{-1}$). Indwelling catheters were placed in the left common carotid artery for systemic arterial pressure monitoring and arterial blood gas determination as well as the left external jugular vein for infusion of saline, *Pseudomonas* organisms, and mAb. An indwelling balloon-tipped pulmonary arterial catheter was inserted via the right external jugular vein and positioned in the pulmonary artery via pressure monitoring for measurement of pulmonary arterial pressure, pulmonary arterial occlusion pressure, central venous pressure, and thermodilution cardiac output (28) (COM1; American Edwards, Santa Ana, CA).

Bronchoalveolar lavage (BAL). BAL was performed through the indwelling endotracheal tube using a fiberoptic bronchoscope (Model BF 4; Olympus Corp., New Hyde Park, NY) at 0 and 300 min in the right and left lungs, respectively. The distal end of the bronchoscope was gently wedged into third- or fourth-order bronchi of the middle and lower lobes. Each lobe was lavaged with two aliquots of 25 ml sterile saline. BAL fluid was centrifuged at 400 *g* and 4°C for 10 min, and the supernatant was stored at 4°C. Cell pellets were resuspended in Dulbecco's PBS containing 0.01% BSA. Cell counts were determined using a hemacytometer, and slide-directed cytocentrifugation was performed (Shandon Southern Instruments, Inc., Sewickley, PA). Differential counts were performed on 200 cells stained using a modified Wright-Giemsa (Diff-Quik®; Baxter Scientific, McGaw Park, IL). BAL protein was measured in the noncellular fraction by the bicinchoninic acid method (29).

Total white blood cell counts. Arterial blood samples were drawn into sterile glass tubes containing 0.15% EDTA and kept at 4°C (Vacutainer). Small aliquots of blood were set aside for white blood cell counts and blood smear differentials, which were performed as described above. The remainder was centrifuged at 500 *g* and 4°C for 20 min and the resulting plasma was stored at −20°C.

PMN isolation. At 0 and 300 min 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 (30). Cells counts and viability were confirmed using hemacytometer and trypan blue exclusion, respectively.

TNF assay. The L929 murine fibroblast bioassay was used to measure plasma TNFα levels (31). L929 cells were seeded into 96-well flat-bottomed microtiter plates (Costar Corp., Cambridge, MA) at a density of 4×10^4 cells per well in 100 μl DME with 5% FCS (DME–FCS; GIBCO/Bethesda Research Laboratories, Gaithersburg, MD) and incubated overnight at 37°C in a 5% CO_2, 95% air atmosphere. Spent medium was removed and replaced with 100 μl of DME–FCS containing 2 μg/ml actinomycin D (Merck Sharp & Dohme, West Point, PA). 100 μl of serial $\log_2$ dilutions of test samples was added in duplicate. Recombinant human TNFα (Cetus Corporation, Emeryville, CA) was used as a positive control. Plates were incubated 18 h (37°C, 5% CO_2). Medium was decanted and the remaining viable cells were stained for 10 min with 0.5% crystal violet in 20% methanol, rinsed in water, and air dried. Absorbance at 550 nm was determined using a microplate reader (Model EL309; Bio-Tek Instruments, Inc., Burlington, VT) blanked to noncellular reagent wells (100% cytotoxicity). Infinite dilutions of rTNFα were considered 100% survival. The percent cytotoxicity was calculated using the formula % cytotoxicity = $1 - ({}^{OD}\text{sample}/{}^{OD}\text{control})$. Units of TNF$\alpha$ were defined as the reciprocal of the dilution resulting in 50% cytotoxicity.

Immunophenotyping. Direct immunophenotyping was performed using a mAb (60.3; Oncogen, Seattle, WA) that recognizes a functional epitope on the CD18 adhesion receptor, and an isotypic IgG2a control (Mouse IgG2a, UPC 10; Sigma Chemical Co., St. Louis, MO). Both antibodies were previously conjugated with FITC (32). Arterial blood samples from study animals were drawn into polypropylene tubes containing 0.15% EDTA and 0.1% NaN_3 and immediately placed on ice. 100-μl aliquots of blood were then incubated with an equal volume of mAb 60.3 or IgG2a control for 20 min at 4°C such that the final concentrations were at antibody excess (previously established by antibody titration curves). Samples were washed twice with PBS containing

0.1% EDTA, 0.1% NaN_3, and 0.2% BSA at 4°C. Erythrocytes were lysed with NH_4Cl buffer and cells were resuspended in PBS. Cells were shielded from light at 4°C before analysis. Analysis was performed on a flow cytometer with a four-decade, 1,024-channel, logarithmic amplifier (FACScan® Becton Dickinson & Co., Mountain View, CA). PMNs were gated according to forward angle and 90° light scatter characteristics. A minimum of 5,000 events were analyzed for each sample, and the mean channel fluorescence of gated PMNs was calculated. Mean channel fluorescence, a logarithmic function, was converted to a linear scale using fluorescent microbead standards and Quickcal software (Flow Cytometry Standards Corp., Research Triangle Park, NC). Fluorescence due to the nonspecific IgG2a antibody was subtracted from mAb 60.3 fluorescence to yield specific binding that directly reflects the expression of CD11/18. Results are expressed as molecules of equivalent soluble fluorochrome.

Superoxide anion assay. Superoxide anion production was determined by measuring the SOD inhibitable reduction of cytochrome *c* using a dual-beam spectrophotometer (Shidmadzu Scientific Instruments Inc., Columbia, MD). PMNs (10^6/ml) and cytochrome *c* (100 μM) were combined in a thermostat-controlled stirred cuvette (37°C). An identically prepared reference cell contained reaction products plus SOD (300 U/ml). The reaction was started by adding phorbol myristate acetate (PMA) (200 ng/ml) to each cuvette. The change in absorbance at 550 nm was continuously recorded for 10 min. Results are expressed as the rate of O_2^- production ($nmol \cdot min^{-1} \cdot 10^{-6}$ PMN) based on an extinction coefficient of: $\Delta\epsilon_{550\,nm} = 2.10 \times 10^4$ M/cm.

Hypochlorous acid HOCl production. Generation of the long-lived PMN oxidant, HOCl, was measured in freshly isolated blood PMNs by the taurine-trapping method (33). Briefly, quintuplicate 100-μl aliquots of freshly isolated PMN (4×10^6/ml PBS) were added to the wells of a flat-bottomed microplate (Costar Corp.). 100 μl of PBS containing 30 mM taurine, 1.5 mM previously reduced 5-5′-dithio-bis-2-nitrobenzoic acid, and 400 ng/ml PMA was added to initiate the reaction. The decrease in absorbance at 410 nm was recorded (vs. noncellular reagent blanks) in a microplate reader (Bio-Tek Instruments, Inc.) over 30 min. The concentration of TNB was determined using the extinction coefficient: $\Delta\epsilon_{410\,nm} = 1.36 \times 10^4$ M/cm.

Lung myeloperoxidase content. At 300 min animals were killed by infusion of pentobarbital (100 mg/kg) and the right lung was excised immediately. Multiple random samples from all lobes were obtained, weighed, and homogenized (s-45 homogenizer; Virtis Co. Inc., Gardiner, NY) in 4 ml of 20 mM potassium phosphate buffer (pH 7.4). The homogenate was then centrifuged (40,000 *g*, 4°C, 30 min; L5-65 Ultracentrifuge; Beckman Instruments, Inc., Fullerton, CA). The pelleted material was resuspended in 4 ml 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB) (Sigma Chemical Co.) and frozen at −70°C. Before assay batched samples were thawed, sonicated for 90 s, incubated for 2 h (60°C), and centrifuged (1,000 *g*, 30 min, 4°C). Myeloperoxidase content was assessed by adding 50 μl of each sample to quadruplicate wells of a 96-well microplate. 50 μl of 0.025% dimethoxybenzidine (Sigma Chemical Co.) in 50 mM potassium phosphate buffer containing HTAB was then added. The reaction was started by the addition of 50 μl of 0.01% H_2O_2 and the OD at 460 nm was measured at 0, 1, 2, and 3 min, previously established as the linear interval of the reaction kinetics. The average change in OD over the period of observation was compared with OD of 10^6 freshly isolated porcine PMNs prepared in an identical fashion as noted for lung parenchymal samples. Results are expressed as units of equivalent myeloperoxidase activity per gram of lung tissue.

Experimental design. Three groups of animals were studied. Group I (control, $n = 10$) received a 60-min intravenous infusion of sterile saline. Group II (sepsis, $n = 10$) received a 60-min intravenous infusion of live *Pseudomonas aeruginosa,* PAO strain (5×10^8 CFU/ml at 0.3 $ml \cdot 20\ kg^{-1} \cdot min^{-1}$). Group III (anti-TNFα, $n = 8$) were pretreated with mAb to TNFα (5 mg/kg i.v.) 15 min before an infusion of live bacteria similar to group II animals.

Statistical analysis. Data are presented as means±SEM. Differences between and within groups were analyzed using analysis of variance with Tukey's studentized range test. Statistical significance was assumed for a P value < 0.05.

Results

Plasma TNF activity

Plasma TNFα levels surged in group II (sepsis) animals within 60 min, reaching a peak of 4.54±0.47 U/ml at 120 min, and remained significantly elevated over baseline and control values at 300 min. Group I (control) and group III (anti-TNFα) animals showed no significant increase in plasma TNFα activity throughout the study period (Fig. 1).

Physiology of porcine sepsis and effects of anti-TNFα antibody

Group II (sepsis) animals exhibited significant cardiopulmonary derangements after onset of sepsis (Table I). These derangements included early-phase pulmonary arterial hypertension and rapidly developing systemic arterial hypotension associated with significant deterioration of cardiac output. In the latter phases of sepsis, group II (sepsis) animals failed to recover cardiac function and exhibited sustained pulmonary arterial hypertension and systemic arterial hypotension, which was associated with an evolving metabolic acidosis and the development of significant arterial hypoxemia over the period of observation (Fig. 2). In contrast, group III (anti-TNFα) animals showed significant improvement in many cardiopulmonary derangements after the onset of *Pseudomonas* sepsis. After an initial decline in cardiac output during the 60-min *Pseudomonas* infusion, group III (anti-TNFα) animals exhibited rapid recovery to baseline levels (Table I). The progressive systemic arterial hypotension and metabolic acidosis observed in group II (sepsis) animals were not observed in group III animals (anti-TNFα). Further, anti-TNFα treatment abolished the development of significant systemic arterial hypoxemia (Fig. 2). However anti-TNFα failed to improve the early septic pulmonary arterial hypertension.

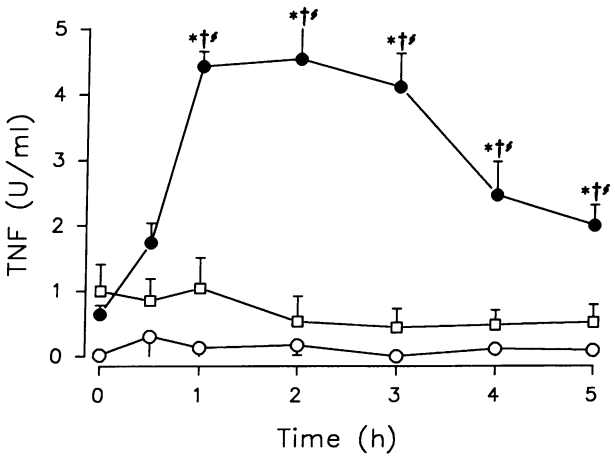

Figure 1. Plasma TNF bioactivity. Plasma levels of TNF in septic (●) animals surged during the first 60 min of study, reaching peak levels by 120 min, and failed to return to baseline by 300 min. Animals pretreated with anti-TNFα (□) (5 mg/kg, i.v.) 15 min before the onset of *Pseudomonas* sepsis exhibited no biologically active TNFα in circulation for the duration of study. (○) control animals. $^*P < 0.05$ vs. control; $^\dagger P < 0.05$ vs. anti-TNFα; $^\S P < 0.05$ vs. baseline.

Table I. Hemodynamics

		Time (h)						
		0	0.5	1	2	3	4	5
CI (liter · min^{-1} · m^2)	Control	3.3±0.2	3.5±0.2	3.3±0.2	3.2±0.2	3.1±0.2	2.9±0.1	2.9±0.2
	Septic	3.1±0.2	2.1±0.2*§	2.5±0.3§	2.5±0.2§	2.1±0.1*‡§	1.7±0.1*‡§	1.5±0.2*‡§
	Anti-TNF	3.9±0.4	2.4±0.2*§	2.6±0.3§	3.1±0.3	2.9±0.3	2.3±0.2*§	2.6±0.3§
PAP (mm Hg)	Control	12.9±1.0	13.9±1.1	15.4±1.0	15.4±0.9	16.5±1.4§	16.1±1.2	15.9±0.9
	Septic	14.9±1.2	46.3±1.6*§	36.6±1.5*§	29.0±1.4*§	32.0±1.7*‡§	32.6±2.2*‡§	30.7±2.9*§
	Anti-TNF	11.3±0.8	41.7±2.4*§	35.7±2.8*§	24.7±1.6*§	23.0±1.1*§	22.8±2.2§	23.5±1.6§
SAP (mm Hg)	Control	94.6±2.1	99.4±4.9	105.0±3.5	110.0±3.1§	110.0±3.0§	105.0±6.4	110.4±4.8§
	Septic	98.7±5.4	133.0±5.5*‡§	98.0±8.1	74.5±7.3*§	79.0±7.4*	84.0±6.7	88.0±9.8
	Anti-TNF	91.2±2.2	103.7±6.4	102.5±5.3	88.3±4.9	90.0±3.7	90.0±3.4	99.2±6.5

Despite the early pulmonary hypertension and fall in cardiac output seen in both septic protected and unprotected animals, anti-TNFα pretreatment significantly improved late hemodynamic derangements of this model. After 60 min of sepsis significant recovery of cardiac output and systemic hypotension in addition to reduction in the pulmonary hypertension were observed in the treatment group. * $P < 0.05$ vs. control; ‡ $P < 0.05$ vs. anti-TNFα; § $P < 0.05$ vs. 0 h. Data represent mean±SEM.

BAL protein analysis

The recovery of instilled BAL fluid at 0 and 300 min was consistently high (≈ 70% return) and did not differ across groups.

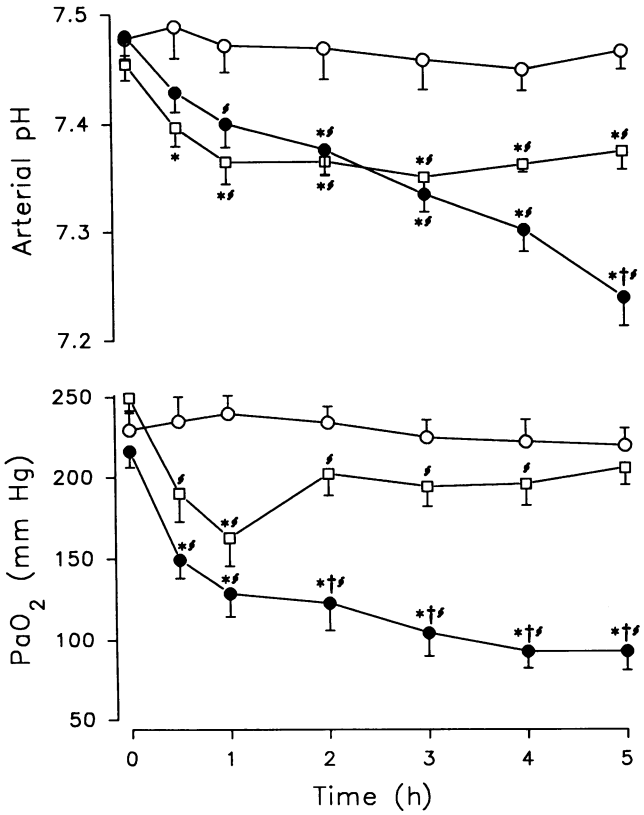

Figure 2. Arterial pH and PaO_2. Septic (●) unprotected animals developed progressive arterial acidosis and a significant decrease in arterial oxygen tension over the course of study. Despite an early decrease in both variables in anti-TNFα– (□) (5 mg/kg, i.v.) treated animals, significant stabilization and return of values to near control levels occurred beyond 60 min. (○) control animals. * $P < 0.05$ vs. control; † $P < 0.05$ vs. anti-TNFα; § $P < 0.05$ vs. baseline.

Baseline BAL protein content was similar in all three groups. In group I (control) animals BAL protein content at 300 min did not differ from baseline (140±18 vs. 132±21 μg/ml). In contrast, BAL protein content at 300 min in group II (sepsis) animals was more than fivefold higher than baseline (770±158 vs. 137±15 μg/ml, $P < 0.05$). Although group III (anti-TNFα) animals also showed an increase in BAL protein content at 300 min (313±48 vs. 141±19 μg/ml), this was significantly less than that observed in group II (sepsis) ($P < 0.05$) (Fig. 3).

Neutrophil trafficking

Peripheral white cell counts. Group II (sepsis) animals became significantly neutropenic within 30 min, reaching a nadir at 120–180 min (Fig. 4). Circulating white blood cell (WBC) counts fell by > 80% and remained depressed throughout the study period. Pretreatment with anti-TNFα antibody altered the WBC profiles, producing a biphasic response. Group III (anti-TNFα) animals initially showed a significant drop in circulating WBC within 30 min, reaching a 50% reduction by 120

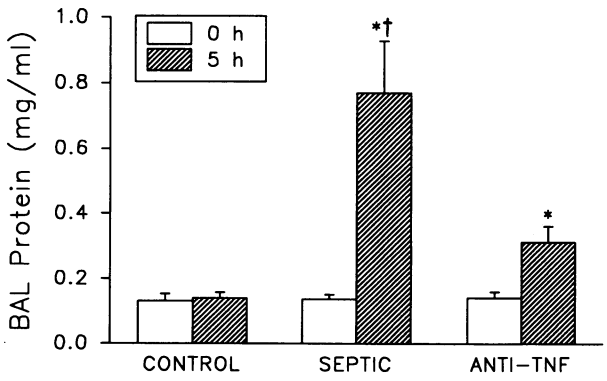

Figure 3. BAL protein content. Septic animals (●) exhibited a significant increase in protein content of the distal airspaces of the lung over 300 min of observation. Anti-TNFα (□) (5 mg/kg, i.v.) pretreatment resulted in significant attenuation of the protein leak. (○) control animals. * $P < 0.015$ vs. 0 h; † $P < 0.05$ vs. control or anti-TNFα.

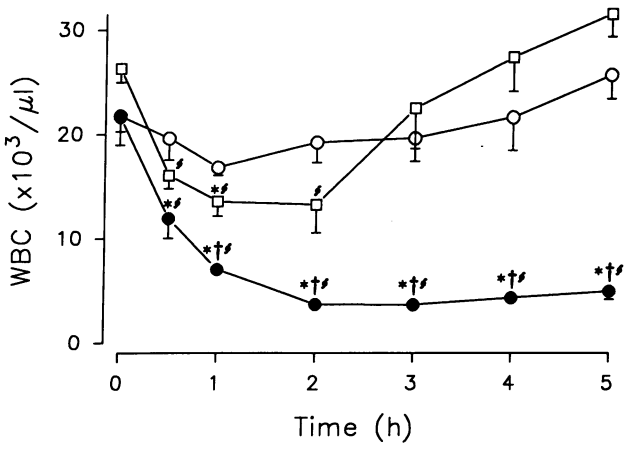

Figure 4. Peripheral blood white cell count. Septic animals (●) exhibited an acute and sustained fall in peripheral blood white cell counts over the 300 min of study. Anti-TNFα (□) (5 mg/kg, i.v.) pretreatment was associated with an early significant fall in white cell counts, which recovered to baseline levels beyond 120 min. (○) Control animals. $^{*}P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. anti-TNFα; $^{\S}P < 0.05$ vs. anti-TNFα.

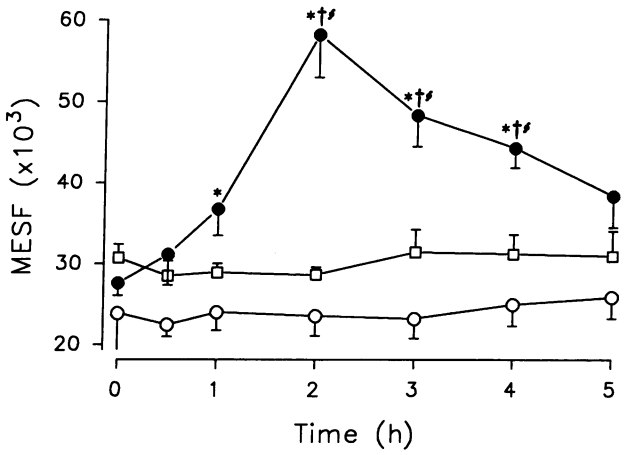

Figure 5. Neutrophil immunophenotyping. Septic animals (●) exhibited a significant increase in PMN CD11/18 receptor expression, which was maximal at 120 min and declined thereafter. Anti-TNFα (□) (5 mg/kg, i.v.) pretreatment abolished the PMN receptor upregulation for the duration of study. (○) control animals. $^{*}P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. anti-TNFα; $^{\S}P < 0.05$ vs. anti-TNFα.

min of observation. However, from 120 min until completion of the study, WBC counts rebounded to above baseline values (31.46±2.15 vs. 26.32±1.35 × $10^3/\mu l$). Relative to other cells present in circulation, neutrophilic populations accounted for the greatest decreases or increases in cell numbers in groups II and III, respectively (data not shown).

Circulating PMN morphology was monitored by examining cytocentrifuge preparations in animals from all groups during the 300-min period. Although total WBC counts differed significantly between group II (sepsis) and group III (anti-TNFα) animals at 300 min (Fig. 4), the maturity of the PMN forms found in circulation did not. Equal numbers of immature PMNs (i.e., band forms, myelocytes) were observed between septic and antibody-treated animals (data not shown).

Neutrophil CD11/CD18 expression. PMNs obtained from group II (sepsis) animals exhibited significant upregulation of CD11/18 expression compared with baseline and control values (Fig. 5). Peak values were observed from 120 to 240 min. In contrast, PMNs from group I (control) and group III (anti-TNFα) animals showed no significant upregulation of CD11/18 expression over the course of study (Fig. 5).

Lung neutrophil load. Myeloperoxidase content of lung tissue from animals in each study group was analyzed to assess lung PMN burden (Fig. 6). Group II (sepsis) animals exhibited significantly higher myeloperoxidase content in lung tissue when compared with group I (control) animals (51.6±9.9 vs. 11.3±2.8 U/g, $P < 0.001$). In group III (anti-TNFα) animals, pretreatment with anti-TNFα antibody greatly reduced lung PMN burden when compared with group II (sepsis) animals (25.4±3.3 vs. 51.6±9.9 U/g, $P < 0.05$). Thus, antibody treatment reduced lung PMN content despite an ongoing septic process.

Neutrophil transendothelial migration. PMN counts in recovered BAL lavage fluid, expressed as a percentage of the total recovered white cell count, were not significantly different between groups at time 0 (Fig. 7). In group II (sepsis) lavage recovered significantly ($P < 0.05$) more PMNs at 300 min (24.5±6.7, $P < 0.05$) than at 0 min (1.8±0.4) and group I (control) at 300 min (3.9±1.4). There was no significant increase in PMNs recovered from BAL at time 300 (13.6±6.5) compared with time 0 (4.7±1.4) in group III (anti-TNFα) animals.

Neutrophil oxidant production. PMNs obtained from group II (sepsis) animals at 300 min demonstrated a marked priming response for PMA-stimulated O_2^- production when compared with baseline PMNs, as noted by an increase in both rate of production and peak production of O_2^- (Fig. 8). In contrast, PMNs from group I (control) animals showed no priming over the course of study. Pretreatment with anti-TNFα antibody failed to attenuate enhanced PMN short-lived oxidant generation. We found that PMNs obtained at 300 min from group III (anti-TNFα) animals showed a similar degree of priming as that observed in group II (sepsis) animals.

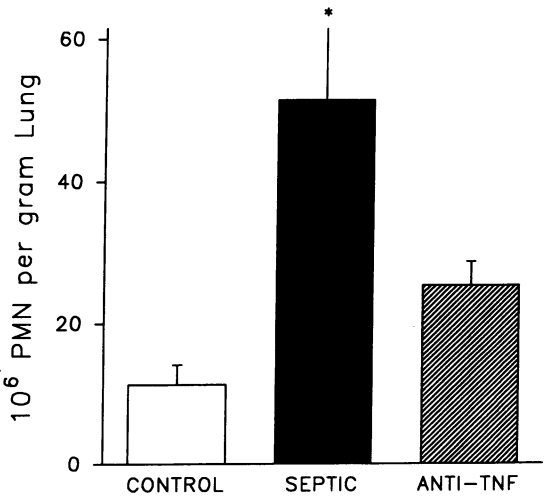

Figure 6. Lung neutrophil burden. Anti-TNFα pretreatment significantly reduced lung PMN burden as estimated by myeloperoxidase activity of lung homogenate compared with septic untreated animals. $^{*}P < 0.05$ vs. control or anti-TNFα.

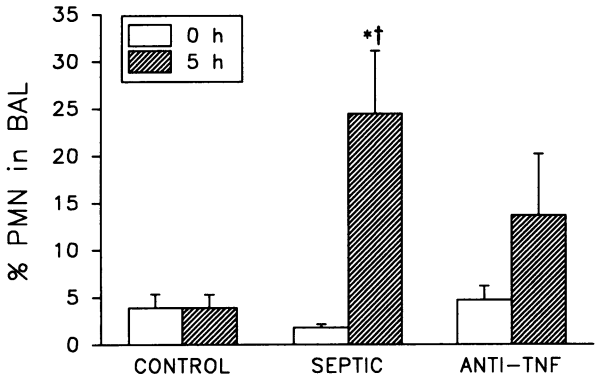

Figure 7. BAL neutrophil content. Septic animals exhibited significant increases in lung BAL PMN content at 300 min when compared with time 0 (baseline). However, anti-TNFα pretreatment attenuated PMN migration, BAL PMN counts at 300 min were not significantly different from baseline. $*P < 0.01$ vs. 0 h, $^{\dagger}P < 0.05$ vs. control.

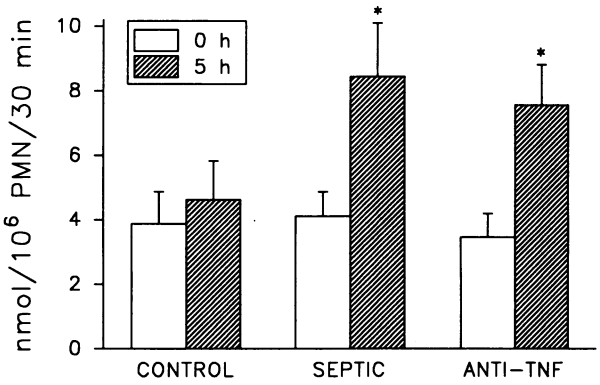

Figure 9. HOCl production. PMNs isolated from septic animals at the conclusion of the study were primed for long-lived oxidant generation in response to PMA stimulation compared with PMNs isolated at time 0. Anti-TNFα failed to attenuate this priming. $*P < 0.01$ vs. 0 h.

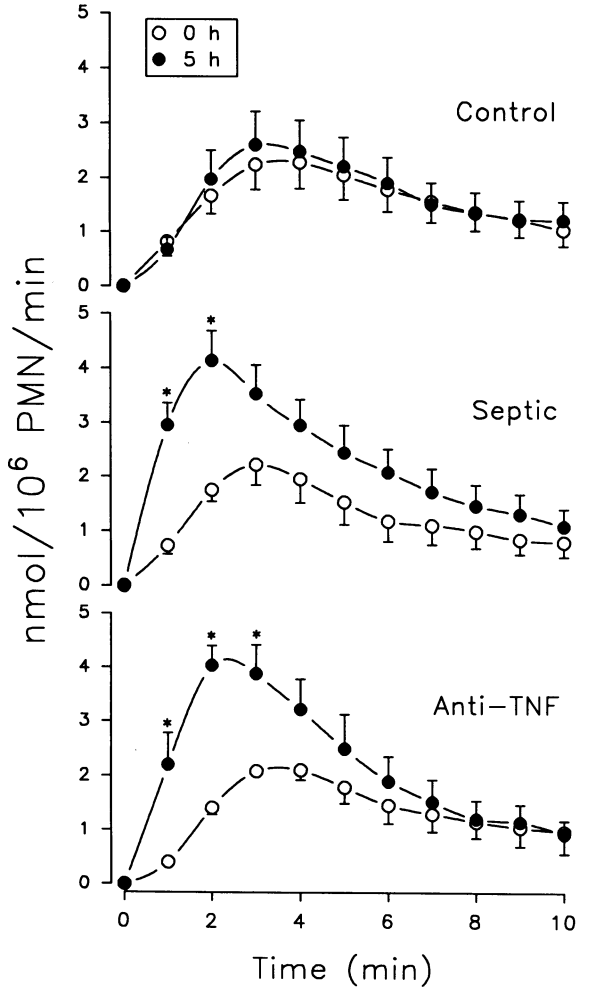

Figure 8. Superoxide anion production. PMNs isolated from septic animals at the conclusion of the study were primed for superoxide generation, as shown by a shorter lag time and greater respiratory burst in response to PMA stimulation compared with control PMNs isolated at time 0. Anti-TNFα pretreatment failed to attenuate this priming. $*P < 0.05$ vs. h.

Comparable findings were observed in PMN production of the long-lived oxidant, hypochlorous acid (HOCl). *Pseudomonas* sepsis (group II sepsis) resulted in significant priming of PMA-stimulated PMN HOCl production. This was not attenuated by pretreatment with anti-TNFα antibody (group III) (Fig. 9).

Elimination of staining artifact

To control for potential functional interference of CD18 immunophenotyping by anti-TNFα antibody, three in vitro studies were performed. In experiment 1, porcine PMN were isolated from control animals and incubated (30 min at 37°C) with either recombinant (r)TNFα (1,000 U/ml) or PMA (200 ng/ml) in the presence or absence of anti-TNFα antibody. After incubation PMN were stained with mAb 60.3 antibody to assess measurable CD18 expression. Results are expressed as percent of control fluorescence. Anti-TNFα antibody did not prevent CD18 upregulation in cells exposed to PMA (303±2.3 control, 284±12 anti-TNFα, $P < 0.05$). However, anti-TNFα prevented upregulation of rTNFα-treated PMN (207±2.7 control vs. 95±15 anti-TNFα antibody, $P < 0.05$). Thus anti-TNFα antibody binds to TNFα in vitro, preventing upregulation of CD18, but fails to interfere with measurement of PMA-stimulated CD18 expression.

To determine whether the lack of PMN CD18 upregulation in antibody-treated septic animals was a mAb 60.3 epitope-specific phenomenon, an additional CD18 mAb (IB4) that crossreacts with porcine PMNs was used (34). We found that IB4 fluorescence patterns on PMNs from both septic and septic anti-TNFα–treated animals were identical to fluorescence patterns presented for mAb 60.3 (Fig. 5), suggesting that the results are not epitope specific.

Finally, we examined whether failed upregulation of PMN CD18 was a permanent cell surface defect, perhaps resulting from interference by anti-TNFα/TNFα complexes or anti-TNFα nonspecific Fc interactions with PMNs. In this study, PMNs were isolated as described above in four animals at baseline (before antibody and *Pseudomonas* infusion) and at 5 h after antibody and organism infusion. Isolated PMNs were incubated with rTNFα (1,000 U/ml) or PMA (200 ng/ml) for 30 min at 37°C. CD18 expression was then measured using mAb 60.3. PMNs from antibody-treated animals isolated after

a 5-h septic insult remained capable of significantly upregulating CD18 receptors when exposed to both rTNFα and PMA (229±8 and 338±25% of control fluorescence, respectively, at baseline; 168±12 and 315±56%, respectively, at 5 h, $P < 0.05$). Thus, failed upregulation of CD18 observed in vivo was not a "permanent" PMN defect. PMN from antibody-treated septic animals obtained at 5 h upregulated CD18 significantly less after rTNFα exposure than did rTNFα-stimulated baseline PMN (168±12 vs. 229±8%, $P < 0.05$). These findings may result from nonspecific Fc binding of anti-TNFα antibody to PMN, subsequently reducing rTNFα activity in vitro. In all cases, however, rTNFα and PMA produced CD18 fluorescence that was significantly greater than control unstimulated PMN.

Discussion

In this as in other studies, the key humoral response to unprotected sepsis is the early phasic surge of TNFα in the circulation. Abolition of this TNFα response by passive immunization with a mAb to TNFα profoundly alters the evolution of both the hemodynamic and alveolar capillary membrane derangements associated with this model of Gram-negative septicemia.

Of particular interest is the finding that isolation and inhibition of only one of many humoral components of the complex chemical network associated with sepsis affords such global protection. With respect to hemodynamic effects, we observed maximal protection at 60 min of sepsis and beyond. This is in keeping with previously reported hemodynamic protection afforded by a higher dose of anti-TNFα (15 mg/kg) (35). In both of these studies, early-phase pulmonary arterial hypertension and decrements in cardiac index persist, despite anti-TNFα antibody pretreatment. An early fall in arterial oxygen tension (PaO_2) and an arterial acidosis was observed in antibody-treated animals, however, both variables recovered to near baseline levels beyond 60 min. These findings suggest that early changes in cardiopulmonary variables are mediated by other humoral components of sepsis, (e.g., thromboxane), an observation confirmed by previous reports using cyclooxygenase inhibition (36) and the detection of high thromboxane levels at 30 min in animals treated with high dose (15 mg/kg) anti-TNFα (35).

Significant evidence has accumulated from in vitro and in vivo studies that suggests that PMNs play a significant role in many inflammatory states. Of particular importance is evidence that suggests that PMNs are primary cellular mediators of acute lung injury associated with sepsis. Histological examination of postmortem lungs of PMN-sufficient patients who died from acute respiratory distress syndrome (ARDS) invariably shows large numbers of sequestered PMNs in pulmonary microvasculature (37–40). Further evidence is provided by studies that show large numbers of PMNs recovered from BAL fluid of patients suffering from ARDS (41, 42). High levels of PMN elastase–α_1 protease inhibitor complexes are found in the same lavage fluids, suggesting that PMNs rapidly migrated from the vascular space and were actively degranulating (43–45). Weiland and others correlated the appearance of PMNs in the lavage fluid of ARDS patients with clinically deteriorating oxygenation (42) and with the development of an acute peripheral neutropenia reported in numerous animal models of sepsis-induced acute lung injury (46–48). Preinjury depletion of circulating PMNs in these models attenuates the resulting lung injury (49–53). More recently, functional manipulation of the PMN has provided striking evidence of the importance of PMNs to the development of lung injury. Inhibiting PMN oxygen radical generation using cyclooxygenase inhibitors (e.g., ibuprofen) leads to significant attenuation of lung injury in animal models when used in pre- or postinjury treatment protocols (36, 54). Application of mAbs directed against the CD11/18 components of the PMN adhesion receptor complex significantly attenuates lung injury and prevents the neutropenia associated with tissue sequestration during sepsis (55, 56). The early morphological evidence suggested an intimate association between PMNs and lung injury whereas attenuation of lung injury by manipulating PMN function may more definitively indicate a cause and effect relationship.

Evidence supporting a linkage between PMNs and vascular injury has prompted a close examination of the effect of anti-TNFα on PMN kinetics and PMN function. We found that characteristic acute increases in surface expression of CD11/18 adhesion receptors, observed in septic-unprotected animals, were abolished by anti-TNFα pretreatment. A series of in vivo and in vitro experiments confirmed that observations of suppression of CD11/CD18 upregulation by anti-TNFα antibody were not artifactual.

In light of evidence suggesting that increasing numbers of receptors may not correlate with adhesive function (57, 58), we examined indicators of receptor activity. Inhibition of PMN receptor expression in this study was accompanied by a reduction in lung PMN sequestration, measured as a reduction in myeloperoxidase activity of postmortem lung samples and attenuation of the peripheral neutropenia, both consistent with reduced PMN adhesive function. Significant in vitro data suggest that the CD11/18 receptor complex and its interaction with a complementary adhesion receptor, intercellular adhesion molecule-1 (ICAM-1), expressed on inflamed endothelium, is necessary for transendothelial migration (59). Altered CD11/18–ICAM-1 interaction in the current study brought about by altered CD11/18 function was again confirmed by the reduction in the number of PMNs recovered from the alveolar spaces at the conclusion of the study, indicating reduced PMN migration. Despite an early decrease in peripheral WBC, the profound neutropenia associated with unprotected sepsis was abolished by anti-TNFα treatment. The similarity in maturity of PMNs at 300 min suggests that recovery of circulating numbers of PMN is not solely the result of an enhanced marrow response. Thus, PMNs in anti-TNFα–treated animals appear to return to the circulating pool from tissue capillary beds in greater numbers than they are being removed or sequestered.

Transient early-phase peripheral neutropenia (Fig. 4) is a phenomenon noted in previous reports from our laboratory (56). Recent evidence suggests a two-stage process involving separate adhesion receptor families (i.e., selectins and β_2 integrins) may promote early PMN sequestration after onset of sepsis (60–63). After exposure to proinflammatory peptides (e.g., cytokines and complement), endothelium initiates adhesion by expression of the selectin's (64) granule membrane protein-140 (GMP-140) and later endothelial leukocyte adhesion molecule-1 (ELAM-1). Unstimulated PMNs loosely bind to these molecules via another selectin, designated lectin adhesion molecule-1 or L-selectin. L-selectin/GMP-140, ELAM-1

binding initiates a rolling process and arrests PMNs at sites of inflamed endothelium. Local exposure to inflammatory mediators (platelet-activating factor, interleukin 8, etc.) at sites of arrested movement activates PMNs resulting in upregulation of CD11/18 receptors. Upregulated CD11/18 complexes bind to endothelial ICAM-1, whose expression is also increased on inflamed endothelium (59). This binding provides strong adhesive forces necessary for migration of PMNs across vascular endothelium (59). Coincident with PMN activation and upregulation of CD11/18 receptors, there is equally rapid shedding of PMN L-selectin receptors, resulting in release of the initial arresting receptor complexes. Early loss of PMNs from the circulation in our studies is unlikely to be due to L-selectin/ELAM-1 binding, since ELAM-1 expression is dependent upon protein synthesis and is maximal at 4 h. However, GMP-140 stored in cytosolic Wiebel-Palade granules within endothelial cells can be mobilized to the cell surface within minutes of cellular activation and is therefore upregulated within the time frame of this neutropenia (65). We speculate that early-phase peripheral neutropenia observed in our studies results from L-selectin/GMP-140 interaction, which produces temporary lung sequestration. PMNs removed from circulation in this fashion but prevented from upregulating CD11/18 receptors because of TNFα blockade reenter the circulation upon shedding L-selectin receptors. Thus, no CD11/18-dependent adhesion or migration occurs as PMNs are released back into the circulating pool, returning the WBC to near control levels.

Administration of anti-TNFα therefore exerts significant effects on kinetics and upon CD11/18 adhesion receptor expression in septic PMNs. Bainton et al. (66) suggest that upregulation of β_2 integrins is due to surfacing of membrane-associated vacuoles, constitutively expressing the MAC1, CD11b/18 heterodimer. Thus, inhibiting the biological activity of TNFα by antibody exposure is likely to interfere with receptor surfacing. TNFα acts through two specific surface receptors (67), with binding resulting in changes in membrane fluidity and chemical composition of target cells (68). Prevention of these membrane events by blocking circulating TNFα activity in sepsis may inhibit vital steps required for emergence of preformed intracellular receptor glycoproteins necessary for PMN adhesion. The possibility that this finding in our model resulted in permanent inhibition of receptor expression was also investigated. Results indicated that the PMN obtained from antibody-treated septic animals retained the ability to upregulate their CD18 receptors in the absence of anti-TNFα antibody.

Numerous reports point to a role for TNFα in both priming PMNs for oxidant burst activity in response to secondary stimuli and for inducing generation and release of reactive oxygen species (69, 70). Korchak and Weissman (71) reported that receptor–ligand interaction results in measurable changes in target cell membrane electropotentials before activation of the membrane-associated NADPH oxidase enzyme system. Seeds et al. (72) later postulated that changes in membrane potentials could regulate potential-dependent conformational changes of membrane proteins (i.e., "electromorphostasis") and thus control early membrane-associated cellular functions. One might therefore predict that a proposed anti-TNFα–mediated, alteration in membrane configuration responsible for inhibiting CD11/18 receptor expression would also affect oxidant metabolism. This was not the case in our studies. Generation of both short- and long-lived oxidant species in the treatment group was not different from that observed in unprotected sepsis. Nathan (73) and Laurent et al. (74) correlated CD11/18-dependent adhesion with enhanced oxygen radical generation in vitro. They suggested a role for integrin interactions in priming of PMNs for subsequent oxidant burst in response to secondary humoral mediators such as TNFα. The current study indicates that this interaction may not be as critical under in vivo conditions. These findings confirm a study by Whitin and Cohen (75) that a dissociation exists between oxygen radical generation and PMN aggregation under a variety of conditions in vitro.

Thus, we have demonstrated that isolation of a single chemical mediator, TNFα, leads to paradoxical effects on PMN function in this model of experimental sepsis. It seems likely that the persistence of enhanced oxidant generation by PMNs obtained from anti-TNFα–treated animals results from the presence in circulation of humoral mediators other than TNFα. Endotoxin, eicosanoids, and complement degradation products prime PMNs for enhanced oxidant burst (76). The continued presence of these and other proinflammatory mediators in the circulation of these animals thus activate the PMN in TNFα-deficient sepsis. Despite the presence of other humoral mediators, we observed no upregulation of CD11/18 receptors in the anti-TNFα–treated animals. These results contrast sharply with in vitro studies that suggest mediators such as complement degradation products and LPS are capable of upregulating CD11/18 receptors in the absence of TNFα (57). These findings further suggest that, in this model, biologically active TNFα is required by the PMN to promote surface expression of β_2 integrins during sepsis (vide supra).

Finally, our results show excellent protection against alveolar capillary membrane injury with preserved gas exchange. BAL protein concentration, an indicator of protein leak from the pulmonary microvasculature, was significantly reduced when compared with unprotected animals at 300 min. Despite the early drop in arterial oxygenation, we observed complete recovery to approximately control levels beyond 60 min. This observed protection is likely due in part to blocking the direct effects of TNFα on endothelial surfaces. It is of further interest that, although the PMNs remain capable of oxygen radical generation, they fail to mediate significant endothelial injury typical of septic-unprotected animals. This protection would appear to support the "microenvironment theory" (77) of PMN-mediated tissue injury. This theory suggests that adherence of activated PMNs to endothelium creates an intercellular microenvironment into which PMNs secrete toxic products such as reactive oxygen metabolites and proteinases and that this microenvironment is protected from the action of circulating oxidant scavengers and proteinase inhibitors permitting unchecked endothelial injury. Our work suggests that preventing upregulation of CD11/18 receptors after onset of sepsis inhibits PMN adhesion and thus prevents the formation of sufficient microenvironmental surface area in the pulmonary microvasculature to produce the extent of endothelial damage observed in unprotected animals. The postulate that there is reduced PMN adhesion and hence sequestration in lung microvasculature is supported by the observed reduction in lung myeloperoxidase activity in the antibody-treated compared with untreated septic animals.

In conclusion, the application of a mAb against TNFα in

experimental sepsis confers both hemodynamic and alveolar capillary membrane protection. Further, antibody treatment alters PMN kinetics as a result of inhibiting CD11/18 receptor expression but has no effect on PMN oxygen radical generation. This study has isolated critical mechanisms through which TNFα acts in an in vivo situation, complicated by the complex network of other chemical and cellular components of the septic response. Such global protection in this pretreatment model begs further examination of this antibody in post-treatment protocols and future clinical trials.

Acknowledgments

The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

The authors acknowledge the assistance of Miles Incorporated and thank them for supplying the mAb to TNFα for these studies.

This work was supported in part by the United States Army Medical Research Acquisition Activity (DAMD 17-86-C-6168) and American Lung Association of Virginia. A. C. J. Windsor is supported in part by Ethicon Foundation, Wellcome Trust, and Fulbright Commission.

References

1. Bone, R. C. 1991. The pathogenesis of sepsis. *Ann. Intern. Med.* 115:457–469.

2. Byrne, K., P. D. Carey, and H. J. Sugerman. 1987. Adult respiratory distress syndrome. *Acute Care.* 13:206–234.

3. Abrams, J. H., and F. B. Cerra. 1989. Multisystem organ failure. *Surg. Rounds.* 12:44–56.

4. Brigham, K. L., and B. Meyrick. 1986. Endotoxin and lung injury. *Am. Rev. Respir. Dis.* 133:913–927.

5. Glausner, M. P., G. Zannetti, J. D. Blaumgartner, and J. Cohen. 1991. Septic shock pathogenesis. *Lancet.* 338:732–736.

6. Brown, J. M., M. A. Grosso, and A. H. Harken. 1989. Cytokines, sepsis and the surgeon. *Surg. Gynecol. Obstet.* 169:568–575.

7. Rock, C. S., and S. F. Lowry. 1991. Tumor necrosis factor. *J. Surg. Res.* 51:434–445.

8. Michie, H. R., P. J. Guillou, and D. W. Wilmore. 1989. Tumor necrosis factor and bacterial sepsis. *Br. J. Surg.* 76:670–671.

9. Michie, H. R., and D. W. Wilmore. 1990. Sepsis and tumor necrosis factor —bedfellows that cannot be ignored. *Ann. Surg.* 212:653–654.

10. Kelley, J. 1990. Cytokines of the lung. *Am. Rev. Respir. Dis.* 141:765–788.

11. Ulich, T. R., K. Guo, and J. del Castillo. 1989. Endotoxin induced cytokine gene expression in vivo. *Am. J. Pathol.* 134:11–14.

12. Beutler, B., and A. Cerami. 1987. Cachectin: more than a tumor necrosis factor. *N. Engl. J. Med.* 316:379–385.

13. Aggarwal, B. B., W. J. Kohr, P. E. Hass, B. Moffat, S. A. Spencer, W. J. Henzel, T. S. Bingman, G. E. Nedwin, D. V. Goeddel, and R. N. Harkins. 1985. Human tumor necrosis factor. Production, purification and characterization. *J. Biol. Chem.* 260:2345–2353.

14. Damas, P., A. Reuter, P. Gysen, J. Demonty, M. Lamy, and P. Franchimont. 1989. Tumor necrosis factor and interleukin-1 serum levels during severe sepsis in humans. *Crit. Care Med.* 17:975–978.

15. Marano, M. A., Y. Fong, L. L. Moldawer, H. Wei, S. E. Calvano, K. J. Tracey, P. S. Barie, K. Manogue, A. Cerami, G. T. Shires, et al. 1990. Serum cachectin/tumor necrosis factor in critically ill patients with burns correlates with infection and mortality. *Surg. Gynecol. Obstet.* 170:32–38.

16. Hesse, D. G., K. J. Tracey, Y. Fong, K. R. Manogue, M. A. Palladino, A. Cerami, G. T. Shires, and S. F. Lowry. 1988. Cytokine appearance in human endotoxemia and primate bacteremia. *Surg. Gynecol. Obstet.* 166:147–153.

17. Michie, H. R., K. R. Manogue, D. R. Spriggs, A. Revhaug, S. O'Dwyer, A. Cerami. 1988. Detection of circulating tumor necrosis factor after endotoxin administration. *N. Engl. J. Med.* 318:1481–1486.

18. Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey, A. Zentella, J. D. Albert, et al. 1986. Shock and tissue injury induced by recombinant human cachectin. *Science (Wash. DC).* 234:470–474.

19. Cross, A. S., J. C. Sadoff, K. E. Bernton, and P. Gemski. 1989. Pretreatment with recombinant murine tumor necrosis factor-α/cachectin and murine interleukin-1 protects mice from lethal bacterial infection. *J. Exp. Med.* 169:2021–2027.

20. Kawakami, M., and A. Cerami. 1981. Studies of endotoxin-induced lipoprotein lipase activity. *J. Exp. Med.* 154:631–639.

21. Weiss, S. J. 1989. Tissue destruction by neutrophils. *N. Engl. J. Med.* 320:365–375.

22. Rinaldo, J. E., and J. W. Christman. 1990. Mechanics and mediators of adult respiratory distress syndrome. *Clin. Chest Med.* 2:621–632.

23. Walsh, C. J., S. K. Leeper-Woodford, P. D. Carey, D. J. Cook, D. E. Bechard, A. A. Fowler, and H. J. Sugerman. 1991. CD18 adhesion receptors, tumor necrosis factor and neutropenia during septic lung injury. *Surg. Res.* 50:323–329.

24. Larrick, J. W., D. Graham, K. Toy, L. S. Lin, and B. M. Fendly. 1987. Recombinant human tumor necrosis factor causes activation of human granulocytes. *Blood.* 69:640–644.

25. Shalaby, M. R., M. A. Palladino, S. E. Hirabayashi, T. E. Eessalu, G. D. Lewis, H. M. Shepard, and B. B. Aggarwal. 1987. Receptor binding and activation of polymorphonuclear neutrophils by tumor necrosis factor-alpha. *J. Leukocyte Biol.* 41:196–204.

26. Livingston, D. H., S. H. Appel, G. Sonnenfield, and M. A. Malangoni. 1989. The effect of tumor necrosis factor-α and interferon-γ on neutrophil function. *J. Surg. Res.* 46:322–326.

27. Byrne, K., T. D. Sielaff, B. Michna, P. D. Carey, C. R. Blocher, A. Vasquez, and H. J. Sugerman. 1990. Increased survival time after delayed histamine and prostaglandin blockade in a porcine model of severe sepsis-induced lung injury. *Crit. Care Med.* 18:303–308.

28. Byrne, K., and H. J. Sugerman. 1988. Experimental and clinical assessment of lung injury by measurement of extravascular lung water and transcapillary protein flux in ARDS. A review of current techniques. *J. Surg. Res.* 44:185–203.

29. Brown, R. E., K. L. Jarvis, and K. J. Hyland. 1989. Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal. Biochem.* 180:136–139.

30. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 97(Suppl.):77–89.

31. Flick, D. A., and G. E. Gifford. 1984. Comparison of in vitro cell cytotoxic assays for tumor necrosis factor. *J. Immunol. Methods.* 68:167–175.

32. Goding, J. W. 1983. *Monoclonal Antibodies: Principles and Practice.* Academic Press Inc., Orlando, FL.

33. Weiss, S. J., R. Klein, A. Slivka, and H. Wei. 1982. Chlorination of taurine evidence for hypochlorous acid generation. *J. Clin. Invest.* 70:598–607.

34. Stahl, G. L., M. P. Fletcher, E. A. Amsterdam, and J. C. Longhurst. 1991. Role of granulocytes and C5a in myocardial response to zymosan activated serum. *Am. J. Physiol.* 261:H29–H37.

35. Walsh, C. J., H. J. Sugerman, P. G. Mullen, P. D. Carey, S. K. Leeper-Woodford, G. J. Jesmok, E. F. Ellis, and A. A. Fowler. 1992. Monoclonal antibody to tumor necrosis factor-α attenuates cardiopulmonary dysfunction in porcine gram negative sepsis. *Arch. Surg.* 127:138–145.

36. Carey, P. D., K. Byrne, J. K. Jenkins, T. D. Sielaff, C. J. Walsh, A. A. Fowler, and H. J. Sugerman. 1990. Ibuprofen attenuates hypochlorous acid production from neutrophils in porcine acute lung injury. *J. Surg. Res.* 49:262–270.

37. Metchnikoff, E. 1887. Sur la lutte des cellules de l'organisme contre l'invasion des microbes. *Ann. Inst. Pasteur (Paris).* 1:41–72.

38. Riede, U. N., H. Joachim, J. Hassenstein, U. Costabel, W. Sandritter, P. Augustin, and C. Mittermyer. 1978. The pulmonary air-blood flow barrier of human shock lungs. *Path. Res. Pract.* 162:41–72.

39. Bachofen, M., and E. R. Weibel. 1977. Alterations of the gas exchange apparatus in adult respiratory insufficiency associated with septicemia. *Am. Rev. Respir. Dis.* 116:589–615.

40. Orell, S. R. 1971. Lung pathology in respiratory distress following shock in adults. *Acta. Pathol. Microbiol. Scand.* 79:65–76.

41. Fowler, A. A., T. M. Hyers, J. Fischer, D. E. Bechard, R. M. Centor, and R. O. Webster. 1987. The adult respiratory distress syndrome cell populations and soluble mediators in the air spaces of patients at high risk. *Am. Rev. Respir. Dis.* 136:1225–1231.

42. Weiland, J. E., W. B. Davis, J. F. Holter, J. R. Mohammed, P. M. Dorinsky, and J. E. Gadek. 1986. Lung neutrophils in the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 133:218–225.

43. Lee, C. T., A. M. Fein, M. Lippman, H. Holtzman, P. Kimbel, and G. Wienbaum. 1981. Elastolytic activity in pulmonary lavage fluid from patients with adult respiratory distress syndrome. *N. Engl. J. Med.* 304:192–196.

44. McGuire, W. W., R. G. Spragg, A. M. Cohen, and C. G. Cochrane. 1992. Studies on the pathogenesis of the adult respiratory distress syndrome. *J. Clin. Invest.* 69:543–553.

45. Fowler, A. A., S. Walchak, P. C. Giclas, P. H. Heines, and T. M. Hyers. 1982. Characterization of antiproteinase activity in adult respiratory distress syndrome. *Chest.* 81(Suppl.):50s–51s.

46. Brigham, K. L., W. C. Woolverton, L. H. Blake, and N. C. Staub. 1974. Increased sheep lung vascular permeability caused by pseudomonas bacteremia. *J. Clin. Invest.* 54:792–804.

47. Meyrick, B., and K. L. Brigham. 1983. Acute effects of E. coli endotoxin on the pulmonary microcirculation of anesthetized sheep. *Lab. Invest.* 48:458–470.

48. Jenkins, J., P. D. Carey, K. Byrne, H. J. Sugerman, and A. A. Fowler. 1991. Sepsis induced lung injury and the effects of ibuprofen pretreatment. *Am. Rev. Respir. Dis.* 143:155–161.

49. Flick, M. R., A. Percel, and N. C. Staub. 1981. Leukocytes are required for increased lung microvascular permeability after microembolization in sheep. *Circ. Res.* 48:344–351.

50. Johnson, A., and A. B. Malik. 1980. Effect of granulocytopenia on extravascular lung water content after microembolization. *Am. Rev. Respir. Dis.* 122:561–566.

51. Helfin, A. C., and K. L. Brigham. 1981. Prevention by granulocyte depletion of increased vascular permeability of sheep lung following endotoxemia. *J. Clin. Invest.* 68:1253–1260.

52. Stephens, K. E., A. Ishizaka, Z. Wu, J. W. Larrick, and T. A. Raffin. 1988. Granulocyte depletion prevents TNF mediated acute lung injury in guinea pigs. *Am. Rev. Respir. Dis.* 138:1300–1307.

53. Hinson, J. M., A. A. Hutchinson, M. L. Ogletree, K. L. Brigham, and J. R. Snapper. 1983. Effect of granulocyte depletion on altered lung mechanics after endotoxemia in sheep. *J. Appl. Physiol.* 55:92–99.

54. Carey, P. D., S. K. Leeper-Woodford, C. J. Walsh, K. Byrne, A. A. Fowler, and H. J. Sugerman. 1991. Delayed cyclooxygenase blockade reduces the neutrophil respiratory burst and tumor necrosis factor levels in sepsis-induced acute lung injury. *J. Trauma.* 31:733–741.

55. Ismail, G., M. L. Morganroth, R. F. Todd, and L. A. Boxer. 1987. Prevention of pulmonary injury in isolated perfused rat lung by activated human neutrophils preincubated with anti-Mo1 monoclonal antibody. *Blood.* 69:1167–1174.

56. Walsh, C. J., P. D. Carey, D. J. Cook, D. E. Bechard, A. A. Fowler, and H. J. Sugerman. 1991. Anti-CD18 antibody attenuates neutropenia and alveolar capillary membrane injury during gram negative sepsis. *Surgery.* 110:205–212.

57. Larson, R. S., and T. A. Springer. 1990. Structure and function of leukocyte integrins. *Immunol. Rev.* 114:181–217.

58. Buyton, J. P., M. R. Phillips, S. B. Abramson, S. G. Slade, G. Weissman, and G. Winchester. 1990. Mechanisms regulating the recruitment of CD11/18 to the cell surface is distinct from that which induces adhesion in homotypic neutrophil aggregation. *In* Leukocyte Adhesion Molecules. Springer-Verlag, New York. 72–83.

59. Smith, C. W., S. D. Marlin, R. Rothlein, C. Toman, and D. C. Anderson. 1989. Cooperative interaction of LFA-1 and MAC-1 with ICAM in facilitating adherence and transendothelial migration. *J. Clin. Invest.* 83:2008–2017.

60. Kishimoto, T. K., R. A. Warnock, M. A. Jutila, E. C. Butcher, C. Lane, D. C. Anderson, and C. Wayne-Smith. 1991. Antibodies against human neutrophil LECAM-1 and endothelial cell ELAM-1 inhibit a common CD18 independent adhesion pathway in vitro. *Blood.* 78:805–811.

61. Picker, L. J., R. A. Warnock, A. R. Burns, C. M. Doerschuk, E. L. Berg, and E. C. Butcher. 1991. The neutrophil selectin LECAM-1 presents carbohydrate ligands to vascular selectins ELAM-1 and GMP-140. *Cell.* 66:921–933.

62. von Adrian, U. H., D. J. Chambers, L. M. McEvoy, R. F. Bargatze, K. E. Arfors, and E. C. Butcher. 1991. Two step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and leukocyte β2 integrins in vivo. *Proc. Natl. Acad. Sci. USA.* 88:7538–7542.

63. Kishimoto, T. K. 1991. A dynamic model for neutrophil localization to inflammatory sites. *J. NIH. Res.* 3:75–77.

64. Springer, T. A., and L. A. Lasky. 1991. Sticky sugars for selectins. *Nature (Lond.).* 349:196–197.

65. Lawrence, M. B., and T. A. Springer. 1991. Leukocytes roll on a selectin at physiological flow rates: distinction from and prerequisite for adhesion through integrins. *Cell.* 65:859–873.

66. Bainton, D. F., L. J. Miller, T. K. Kishimoto, and T. A. Springer. 1987. Leukocyte adhesion receptors are stored in peroxidase-negative granules of human granulocytes. *J. Exp. Med.* 166:1641–1653.

67. Shepherd, V. L. 1991. Cytokine receptors of the lung. *Am. J. Respir. Cell Mol. Biol.* 5:403–410.

68. Matsubara, N., S. Fuchimoto, and K. Orita. 1991. Tumor necrosis factor-alpha induces translocation of protein kinase C in tumor necrosis factor sensitive cell lines. *Immunology.* 73:457–459.

69. She, Z., M. D. Wewers, D. J. Herzyk, A. L. Sagone, and W. B. Davis. 1989. Tumor necrosis factor primes neutrophils for hypochlorous acid production. *Am. J. Physiol.* 257:I338–I345.

70. Berkow, R. I., D. Wang, J. H. Larrick, R. W. Dodson, and T. H. Howard. 1987. Enhancement of neutrophil superoxide production by preincubation with recombinant human tumor necrosis factor. *J. Immunol.* 139:3783–3791.

71. Korchak, H. M., and G. Weissman. 1978. Changes in membrane potential of human granulocytes antecede the metabolic responses to surface stimulation. *Proc. Natl. Acad. Sci. USA.* 75:3818–3822.

72. Seeds, M. C., J. W. Parce, P. Szejda, and D. A. Bass. 1985. Independent stimulation of membrane potential changes and the oxidative metabolic burst in polymorphonuclear leukocytes. *Blood.* 65:233–240.

73. Nathan, C. 1987. Neutrophil activation on biological surfaces. *J. Clin. Invest.* 80:1550–1560.

74. Laurent, F., A. M. Benoliel, C. Capo, and P. Bongrand. 1991. Oxidative metabolism of polymorphonuclear leukocytes. *J. Leukocyte Biol.* 49:217–226.

75. Whitin, J. C., and H. J. Cohen. 1985. Dissociation between aggregation and superoxide production in human granulocytes. *J. Immunol.* 134:1206–1211.

76. Anderson, B. O., J. M. Brown, and A. H. Harken. 1991. Mechanisms of neutrophil mediated tissue injury. *J. Surg. Res.* 51:170–179.

77. Harlan, J. M. 1987. Neutrophil mediated vascular injury. *Acta. Med. Scand. Suppl.* 715:123–129.