

The Role of Tumor Necrosis Factor- α in the Pathogenesis of Aspiration Pneumonitis in Rats

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Background: Aspiration pneumonitis is characterized by proteinaceous pulmonary edema and acute infiltration of neutrophils into the alveolar space. This study examined the role of the proinflammatory cytokine, tumor necrosis factor- α (TNF- α), on the pathogenesis of the injury produced by the different components that may be present in the aspirate, acid, or gastric particles.

Methods: Rats were injured by intratracheal instillation of a vehicle containing acid or gastric particles. TNF- α concentration of bronchoalveolar lavage fluid was determined using a bioassay. upregulation of lung TNF- α mRNA was also measured. The effect of intratracheal anti-rat TNF- α treatment was assessed by lung protein permeability, blood gases, and lung myeloperoxidase activity.

Results: Injury vehicle alone and acid injury resulted in a small TNF- α peak 1–2 h after injury in the lavage fluid. Both particulate and acidic particulate groups produced a much more robust TNF- α signal that reached a plateau at 2–4 h after injury and declined at 8 h. Upregulation of TNF- α mRNA was only detected in the particulate-containing groups. Acidic par-

ticulate exposure yielded a synergistic increase in protein permeability and decrease in blood oxygenation. Anti-TNF- α treatment reduced protein permeability and myeloperoxidase activity and increased blood oxygenation in the groups exposed to only acid. Such treatment had no effect on either of the particulate containing injuries.

Conclusions: TNF- α is differentially manifested according to the components that make up the aspirate but the levels of TNF- α expression do not correlate with the severity of the resultant injury. However, the reduction in acid-induced lung injury by anti-TNF- α treatment indicates that TNF- α plays a role in the pathogenesis of aspiration pneumonitis. (Key words: Acid aspiration; ARDS; particulate aspiration; pulmonary injury.)

ASPIRATION pneumonitis is an acute inflammatory process that occurs following intrapulmonary deposition of gastric contents. This lung injury is facilitated by the obtundation of protective airway reflexes, such as occurs during general anesthesia. A 1986 study found that aspiration of gastric contents, a potentially lethal complication of the perioperative period, occurred in approximately 1 in 2,131 patients receiving anesthesia.¹ A more recent study (anesthetic procedures between 1985 and 1991) revealed that, with the use of prophylactic measures, the incidence has been reduced to 1 in 3,216.² However, the pathogenesis of aspiration pneumonitis has yet to be elucidated, and the indicated treatment of the condition is still merely supportive. A third of patients with acute aspiration pneumonitis develop adult respiratory distress syndrome (ARDS), with its high associated mortality rate, particularly when particulate matter is present in the aspirate.^{3–5} Furthermore, the occurrence of undetected aspiration can be indicted in a substantial number of cases of postoperative pulmonary dysfunction. Therefore, the development of strategies to decrease the severity of this form of lung injury, once it has occurred, is important in lessening the morbidity and mortality rates associated with this perioperative complication.

The nature of the inflammatory response to gastric aspiration is characterized by the acute infiltration of

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neutrophils into the alveolar space and is similar to the histologic picture of other neutrophil-mediated experimental models of lung injury.⁶⁻¹¹ Tumor necrosis factor- α (TNF- α) is a potent early-response proinflammatory cytokine that has been shown to be integral in the pathogenesis of other lung-injury models that are neutrophil-mediated.¹²⁻¹⁵ TNF- α leads to activation of leukocytes and induces the expression of endothelial adhesion molecules that play a major role in the leukocyte-endothelial interactions that lead to neutrophil migration into the alveoli. Other laboratories have also produced evidence that TNF- α is involved in acid-aspiration pneumonitis.¹⁶⁻¹⁸ However, the role of this cytokine in the pathogenesis of this lung injury has not been fully established. Furthermore, the TNF- α response to the particulate component that may be present in the aspirate has not been investigated. We hypothesize that the inflammatory response to aspiration of gastric contents differs according to the contents of the aspirate and that TNF- α plays a significant role in that difference. Thus, the purpose of this study has been to examine the role of TNF- α in the pathogenesis of aspiration pneumonitis and examine the response of this cytokine to the specific gastric components (acid, particulate) of the aspirate.

Materials and Methods

All procedures performed on rats in this study were approved by the State University of New York at Buffalo's Institutional Animal Care and Use Committee and complied with all state, Federal, and National Institutes of Health regulations.

Preparation of Gastric-aspirant Injury Vehicles

Four different injury vehicles were prepared for this study: (1) normal saline, pH = 5.3; (2) normal saline adjusted to pH = 1.25 with reagent HCl (acid); (3) 40 mg/ml gastric particles in normal saline, pH = 5.3 (particulate); and (4) 40 mg/ml gastric particles in normal saline adjusted to pH = 1.25 with reagent HCl (acidic particulate).¹¹ All four of these injury vehicles were instilled into the lungs of the test animals at a dose of 1.2 ml/kg. In addition, a fifth injury group was included, normal saline adjusted to pH = 1.25 with reagent HCl and delivered at a dose of 2.4 ml/kg (high-volume acid). The gastric particles were prepared by collecting the stomach contents from necropsied rats, washing three times in normal saline, pH = 5.3 and filtering through sterile, gauze sponges (to remove large "chunks").¹¹ Af-

ter autoclaving for 25 min (20 psi, 121°C), the filtrate was centrifuged at 3000 rpm for 2 min at room temperature (2,000g, GS-6R centrifuge with GH-3.8 rotor, Beckman Instruments, Palo Alto, CA), the supernatant discarded, and the pellet weighed (particle concentrations reported are calculated based on this wet weight). A stock solution of gastric particles was prepared by suspending the pellet in normal saline, pH = 5.3 to 100 mg/ml, and was used to prepare the particulate-containing injury vehicles.

Rat Gastric-Aspiration Model

Anesthesia was induced in male, 250-300-g, Long-Evans rats (Harlan Sprague-Dawley, Indianapolis, IN) in a glass chamber with 3-4% halothane in 50% O₂/air and maintained with 1.5% halothane, delivered by nose cone with spontaneous breathing. Gas concentrations were monitored continuously using a RASCAL II Ramon light-scattering spectrophotometer (Ohmeda, Salt Lake City, UT). Animals were placed on a 60-degree upright dissection board and suspended by the front teeth, exposing the ventral side of the neck.⁹ A 2-cm midline incision was made to expose the trachea into which an 18-gauge, 1.25-inch Teflon catheter (Becton Dickinson Infusion Therapy Systems, Sandy, UT) was inserted to just above the carina and secured with 1-0 silk.⁷ A 1-ml syringe was used to instill the appropriate injury vehicle into the lungs through the catheter. One minute later, the catheter was removed and the trachea and neck incisions were closed with 6-0 and 4-0 silk, respectively. The animal was removed from the inclined board and allowed to awaken in a cage maintained at 37°C with a servocontrolled heat lamp while spontaneously breathing room air. Food and water were provided *ad libitum* until harvest.

Bronchoalveolar Lavage

In experiments to determine TNF- α activity in the lungs following aspiration injury, bronchoalveolar lavage was performed at various times following instillation of the aspirant (each time point represents one animal because this was a terminal procedure). The animal was anesthetized by intraperitoneal injection of 260 mg of sodium pentobarbital, and the neck incision was reopened to expose the trachea. A 16-gauge, 1.25-inch Teflon catheter was inserted into the trachea, distal to the inoculation incision, and secured in position with a suture. A longitudinal incision was made through the diaphragm and sternum and the rib cage was spread open, carefully, to avoid puncturing the lungs. A three-

way stopcock with two 20-ml syringes attached, one empty and the other containing 14 ml sterile, phosphate-buffered saline (PBS) (137 mM NaCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, 0.9 mM CaCl₂, 0.5 mM MgCl₂, pH = 7.4), was attached to the catheter and 2 × 7-ml PBS instilled into the lungs and recovered. The collected lavage fluid was pooled, centrifuged at 2100 rpm for 5 min at 4°C (700g, GS-6R centrifuge with GH-3.8 rotor, Beckman Instruments), and the supernatant aliquoted and stored at -20°C.

TNF-α Cytotoxicity Bioassay

WEHI 164, subclone 13, cells, a TNF-α-sensitive cell line derived from a mouse fibrosarcoma (a generous gift from Dr. Steven L. Kunkel, Department of Pathology, University of Michigan, Ann Arbor, Michigan), were cultured to approximately 90% confluency in culture medium containing RPMI-1640, 2 mM L-glutamine, 10% fetal calf serum (GIBCO-BRL, Grand Island, NY), and 3 μg/ml gentamicin (Sigma Chemical, St. Louis, MO) in T75 flasks at 37°C in 5% CO₂. Cells were prepared for the assay by detaching with 0.25% trypsin and 0.02% EDTA (Sigma Chemical, St. Louis, MO), adding 10 ml culture medium per flask, centrifuging at 800g for 5 min at room temperature (GS-6R centrifuge with GH-3.8 rotor, Beckman Instruments), and resuspending in culture medium supplemented with 1 μg/ml actinomycin D (Calbiochem, La Jolla, CA) to a concentration of 500,000 cells/ml. One hundred microliters of cell suspension was added to each well of a flat-bottom 96-well plate (Sarstedt, Newton, NC) containing 100 μl of two-fold serial dilutions of unknown samples, in triplicate, or known concentrations of human rTNF-α standards (R & D Systems, Minneapolis, MN) in diluting medium: RPMI-1640, 2 mM L-glutamine, 1% fetal calf serum, and 15 mM HEPES (Sigma Chemical). Following 20 h of incubation at 37°C in 5% CO₂, 20 μl of filter sterilized 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical) in PBS (5 mg/ml) was added to each well. After an additional 4-h incubation, 150 μl was removed from each well and replaced with 100 μl of 0.04-N HCl in isopropanol. The plates were incubated for 20 h in the dark at room temperature, and the absorbance at 570 nm (OD₅₇₀) was measured using a 3550-UV microplate reader (Bio-Rad, Hercules, CA). A standard curve (reverse sigmoid in shape) of the OD₅₇₀ versus log TNF-α concentration was plotted and the TNF-α concentration of each sample was determined from the dilution closest to the inflection point of the standard curve. This assay has a detection limit of approximately 1 pg/ml.¹⁹

The assay is based on the specific cytotoxicity of the WEHI 164, subclone 13, cells to TNF-α. MTT is used as a cell viability indicator, being taken up and cleaved to a dark-blue formazan product by active mitochondria, which is quantitated spectrophotometrically. Increasing TNF-α concentration results in increased cell death and, therefore, a reduced absorbance at 570 nm.²⁰

Northern Blot Analysis of TNF-α mRNA Levels in Injured Lungs

All reagents and equipment were maintained RNase-free during experiments involving RNA extraction. Whole lungs were dissected at 0, 1, 2, and 4 h after instillation of the injury vehicle, following flushing of the pulmonary circulation with 10 ml saline, *via* right-ventricle injection. The harvested lungs were snap-frozen in liquid nitrogen. Total RNA was extracted from whole-lung homogenates using a guanidinium isothiocyanate/chloroform-based technique (TRIZOL Reagent, GIBCO-BRL), followed by isopropanol precipitation. Purified rat-lung total RNA, 12 μg, was fractionated on a 1% agarose formaldehyde gel and transferred to a nylon membrane (Zetabind, CUNO, Meriden, CT) by capillary action. A full-length cDNA probe for the rat TNF-α gene (708 base pairs) was PCR-cloned from an IgG immune complex-injured rat lung using the following primers: 5'-ATGAGCACGGAAAGCATGATCCGA (sense); 5'-tcacagagcaatgactccaaagta (antisense), with the following cycle conditions: 94°C × 30 s, 52°C × 1 min, 72°C × 1 min 30 s, for 35 cycles. The amplified product was sequenced and confirmed to be rat TNF-α.²¹ This cDNA probe was radiolabeled with [³²P]dCTP (Pharmacia, Piscataway, NJ) by random priming. Radioactivity of the probe was determined by an LS6500 scintillation counter (Beckman Instruments) and 1.5 × 10⁷ cpm was applied to the blot with hybridization occurring at 65°C for 16 h. Autoradiography of the blots was performed at -70°C on X-OMAT-AR film (Eastman Kodak, Rochester, NY). The resultant autoradiograph was digitized and analyzed with a microprocessor-controlled GS-700 imaging densitometer and Molecular Analyst version 1.5 software (Bio-Rad). The TNF-α mRNA and corresponding 28S ribosomal RNA bands were integrated from the acquired image and the TNF-α mRNA bands normalized to a constant 28S ribosomal RNA level.²²

In Vivo Anti-TNF-α Treatment

The affinity-purified IgG fraction of goat anti-rat TNF-α antisera was administered intratracheally to assess whether "locally expressed" TNF-α was required for full

expression of gastric aspiration injury.^{12,23} The production, purification, and characterization of this polyclonal antibody has been fully described.²⁴ Anti-TNF- α , or control goat IgG (Lampire Biological Laboratories, Pipersville, PA), was administered intratracheally (1.5 or 3.0 mg/kg) with the injury vehicle, in the case of normal saline and particulate injuries, or 1 min following injury vehicle instillation at 0.6 ml/kg for acid, high-volume acid, and acidic particulate injuries *via* the same intratracheal catheter. Treatment with anti-TNF- α was not administered with the acid-containing injury vehicles because of the potential for inactivation of the antibody. Another group of animals was included in these experiments in which no injury or treatment solution was instilled into the lungs to provide basal levels of the indices measured.

Lung Injury Assessment

Alveolar-capillary wall integrity was quantified by measuring the translocation of radiolabeled albumin from the blood into the lung. Blood gases were measured to assess gas-exchange efficiency. Immediately prior to injury, 100,000 cpm of ¹²⁵I-BSA (Dupont NEN Research Products, Boston, MA) in 1 ml 2% BSA was injected into the penile vein. At 5 h after injury, the animals were exposed to 80% oxygen, breathing spontaneously, for 10 min then anesthetized with halothane (as described previously except with 80% oxygen in air as the carrier gas). A midline peritoneal and thoracic incision was made and approximately 500 μ l of blood collected from the abdominal aorta with a heparinized syringe and 22-gauge needle. One milliliter of venous blood was collected from the inferior vena cava. The vena cava was cut to exsanguinate the animal, and the pulmonary vasculature was cleared of residual blood by injecting 10 ml of sterile PBS into the beating heart's right ventricle. The lung was excised and its ¹²⁵I activity determined using a 1282 CompuGamma CS gamma counter (Wallac, Gaithersburg, MD). The activity in the 1-ml blood sample was also measured and the lung albumin permeability index (PI) calculated as a simple ratio of the activity in the lung (minus background) to that in 1 ml blood (minus background).⁷

The arterial blood sample was injected into an ABL4 blood gas analyzer (Radiometer America, Westlake, OH) and the gas-exchange efficiency calculated as Pa_{O_2}/Fi_{O_2} , where Pa_{O_2} is the arterial oxygen partial pressure in millimeters of mercury and Fi_{O_2} is the inspired oxygen concentration (80%).²⁵

Myeloperoxidase Assay

Myeloperoxidase (MPO) activity in lung homogenates was measured as a surrogate marker of lung neutrophil infiltration. After counting on the gamma counter, harvested lungs were homogenized on ice using a Polytron TP-2000 tissue homogenizer (Brinkman Instruments, Westbury, NY) in 3 ml buffer containing 50 mM KH_2PO_4 , 13.7 mM hexadecyltrimethylammonium bromide, and 5 mM EDTA, pH = 6.0. The homogenate was then sonicated on ice for 4 \times 10-s pulses using a Sonifier Cell Disruptor 350 with microtip probe (Branson Ultrasonics, Danbury, CT) and centrifuged at 3200 rpm for 30 min at 4°C (2300g, GS-6R centrifuge with GH-3.8 rotor, Beckman Instruments). The resultant supernatant was assayed for MPO activity by combining 50 μ l of sample with 1.5 ml assay buffer containing 50 mM KH_2PO_4 , 176 μ M H_2O_2 , and 525 μ M o-dianisidine dihydrochloride, pH = 6.0, in a cuvette and continually recording the absorbance at 460 nm for 2 min using a DU-650 spectrophotometer (Beckman Instruments). The spectrophotometer was blanked with 50 mM phosphate buffer before reading the sample. MPO activity was expressed as the absorbance change (at 460 nm) per minute (ABS/min) over the linear portion of the curve.²⁶

Statistical Analysis

Intergroup comparisons (*i.e.*, TNF- α bioactivity from particulate-injured *vs.* acidic particulate-injured at 1 h after injury, or PI from acid-injured IgG-treated *vs.* anti-TNF- α antisera-treated) were made using the Student two-sample *t* test after F tests to determine if equal or unequal variance assumptions were appropriate. To guard against the possibility of the data not being normally distributed, Mann-Whitney tests were also conducted. All comparisons were performed using two-tailed tests. To test for interaction between acid and particulate injury, with respect to the injury (PI, Pa_{O_2}/Fi_{O_2}) or inflammation (MPO) parameters, two-factor analyses of variance were performed among the groups receiving similar treatments (*i.e.*, IgG or anti-TNF- α). The effect of normal saline, acid, or high-volume acid aspiration on TNF- α bioactivity in bronchoalveolar lavage fluid was analyzed at each harvest time point for significant differences from no injury (0 h) by only the nonparametric Mann-Whitney test because the data were decidedly non-normal. Values are expressed as mean \pm SD, with *n* = total number of animals in each group. Differences were accepted as significant for *P* < 0.05.

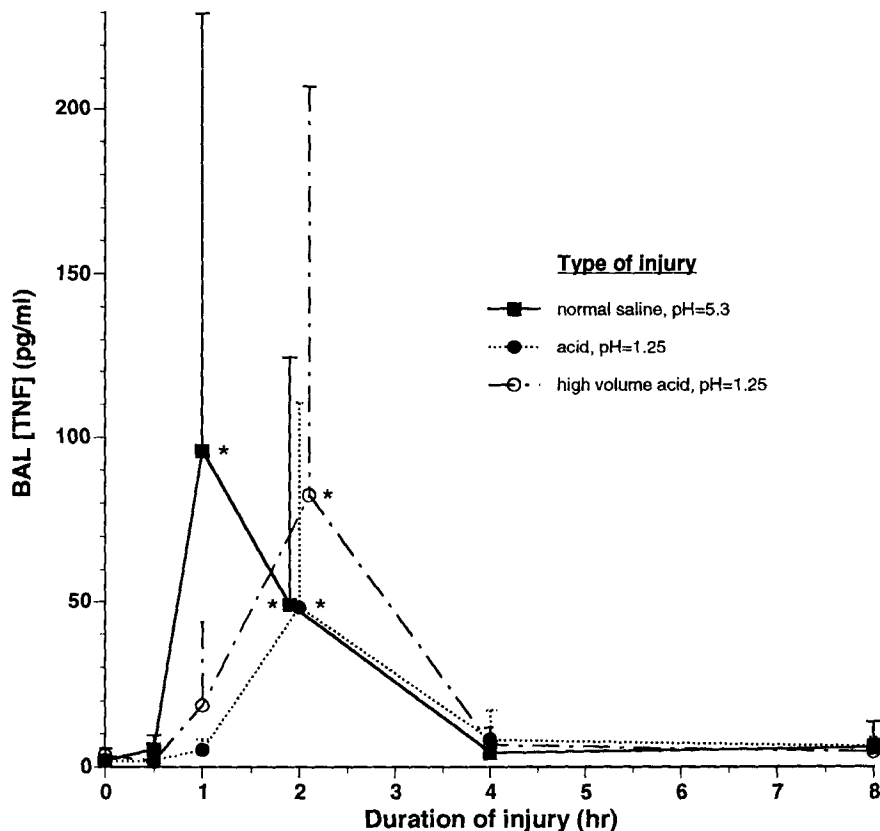


Fig. 1. Rats had 1.2 ml/kg (2.4 ml/kg for the high-volume acid group) of the indicated injury vehicle instilled into their lungs *via* a tracheal catheter. At the indicated time, the animal was harvested and bronchoalveolar lavage (BAL) was performed with 2 x 7 ml phosphate-buffered saline. The clarified, recovered BAL fluid was assayed for TNF- α activity by a cytotoxicity bioassay. Data at 2 h are offset for clarity. $n = 6$ for each point. * $P < 0.05$ compared with 0 h (no injury).

Results

Bronchoalveolar Lavage Fluid (TNF- α)

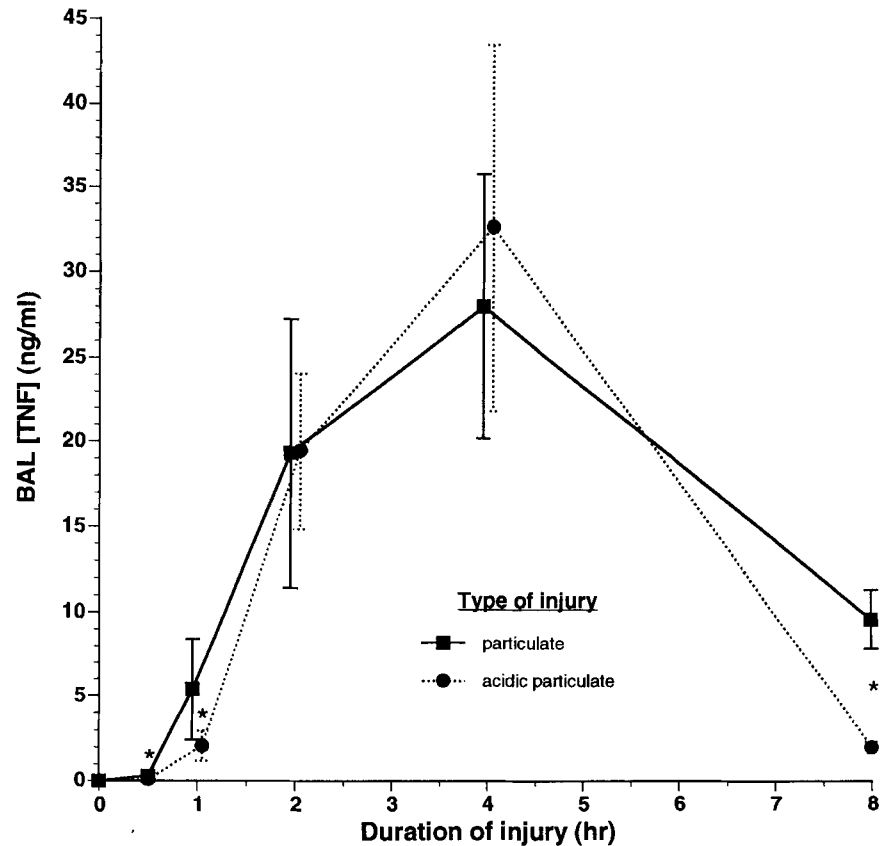
All study groups demonstrated a TNF- α response that was detectable in the lavage fluid by the WEHI assay, albeit with varying kinetics and amplitude. Figure 1 displays the time course of experiments with normal saline, acid, and high-volume acid as the injury vehicles. All three injury regimes produced TNF- α peaks that resolved to baseline by 4 h after injury. The response from normal saline peaked at 1 h after injury, 95.8 ± 133.8 pg/ml ($P < 0.01$ compared with no-injury levels), whereas the peak response from acid and high-volume acid did not occur until 2 h after injury, 48.3 ± 62.4 and 82.6 ± 124.8 pg/ml, respectively ($P < 0.025$ compared with no-injury levels). Groups receiving gastric particles produced a much more robust and sustained TNF- α response (fig. 2). Particulate and acidic-particulate groups produced greater amounts of lavage fluid TNF- α at all time points after injury compared with those injuries that did not contain gastric particles ($P < 0.0001$). At 2 h after injury, lavage fluid TNF- α levels in the particulate group were 390-fold greater than those in the nor-

mal saline group (19.3 ± 7.9 ng/ml *vs.* 0.05 ± 0.08 ng/ml), 400-fold greater than the acid group, and 235-fold greater than the high-volume acid group. The elevated TNF- α response was sustained through 4 h after injury in the particulate group (28.0 ± 7.8 ng/ml) and the acidic-particulate group (32.7 ± 10.8 ng/ml), a time at which the normal saline, acid, and high-volume acid lavage fluid TNF- α levels had returned to baseline (4.3 ± 3.4 , 8.2 ± 8.9 , and 6.7 ± 5.1 pg/ml, respectively). Interestingly, adding an acidic component to the gastric particulate-treated lungs initially delayed the onset of the TNF- α response (at 0.5 h, particulate = 300.8 ± 185.0 pg/ml *vs.* acidic particulate = 87.6 ± 115.5 pg/ml, $P < 0.05$; and at 1 h, particulate = 5.4 ± 3.0 ng/ml *vs.* acidic particulate = 2.1 ± 0.9 ng/ml, $P < 0.05$). It did not affect the peak plateau from 2 to 4 h after injury, but it did hasten the TNF- α response's decline (at 8 h, particulate = 9.6 ± 1.7 ng/ml *vs.* acidic particulate = 2.0 ± 0.3 ng/ml, $P < 0.0002$).

TNF- α Northern Blot Analysis

To determine the transcriptional state of TNF- α production in injured lung tissue, experiments were per-

Fig. 2. Same experiment as described for figure 1, except with the indicated injury vehicles. Data at 1, 2, and 4 h are offset for clarity. $n = 6$ for each point. * $P < 0.05$ between the two types of injury at the indicated time point. BAL = bronchoalveolar lavage.



formed with each of the injury groups and analyzed for TNF- α mRNA using Northern blots. Only the lungs from animals injured with aspirant vehicles that contained gastric particles (particulate and acidic particulate) demonstrated robust upregulation of TNF- α mRNA (fig. 3). This upregulation peaked between 1 and 2 h after injury and began to diminish by 4 h after injury. Expression of TNF- α mRNA could not be consistently detected in homogenized lung samples that had been injured with normal saline, acid, or high-volume acid using this technique (data not shown).

Effects of Anti-TNF- α Antibody Treatment on Lung Protein Accumulation

To assess the role that TNF- α plays in the pathogenesis of gastric aspiration lung injury, experiments with anti-rat TNF- α antibody treatment were conducted on animals with acid, high-volume acid, particulate, or acidic-particulate solutions instilled into the lungs. Normal saline injury was not included as an experimental group because the lung injury that results, as assessed by lung protein permeability and blood-gas analysis, is not suffi-

ciently different from untreated animals to detect effects of anti-TNF- α antisera treatment (PI = 0.40 ± 0.13 vs. 0.28 ± 0.09 , and $\text{PaO}_2/\text{FiO}_2 = 503.9 \pm 22.1$ mmHg vs. 550.6 ± 56.1 mmHg). Instillation of nonspecific goat IgG in the same manner as the anti-TNF- α antibody treatment was used as the control in all injury groups.

Treatment with anti-TNF- α antibody produced a significant reduction in albumin permeability of the alveolar-capillary wall produced by acid and high-volume acid, but not particulate or acidic-particulate lung injury (fig. 4). The PI was reduced 21.8% in the acid (0.86 ± 0.28 vs. 1.10 ± 0.36 , $P < 0.05$) and 27.5% in the high-volume acid injury group (1.32 ± 0.22 vs. 1.82 ± 0.29 , $P < 0.0005$). No reduction in this index of lung injury was demonstrable by anti-TNF- α antibody treatment of particulate pulmonary injury (0.95 ± 0.29 vs. 1.00 ± 0.23). Also, doubling of the anti-TNF- α dose, from 1.5 mg/kg to 3.0 mg/kg, still did not produce a significant reduction in particulate lung injury, as assessed by PI. The IgG-treated acidic particulate injury group had a PI of 5.07 ± 1.19 , which represented a 4.6-fold, synergistic increase ($P < 0.0001$) in PI over the similarly treated acid-injured animals, and a 5.0-fold increase in

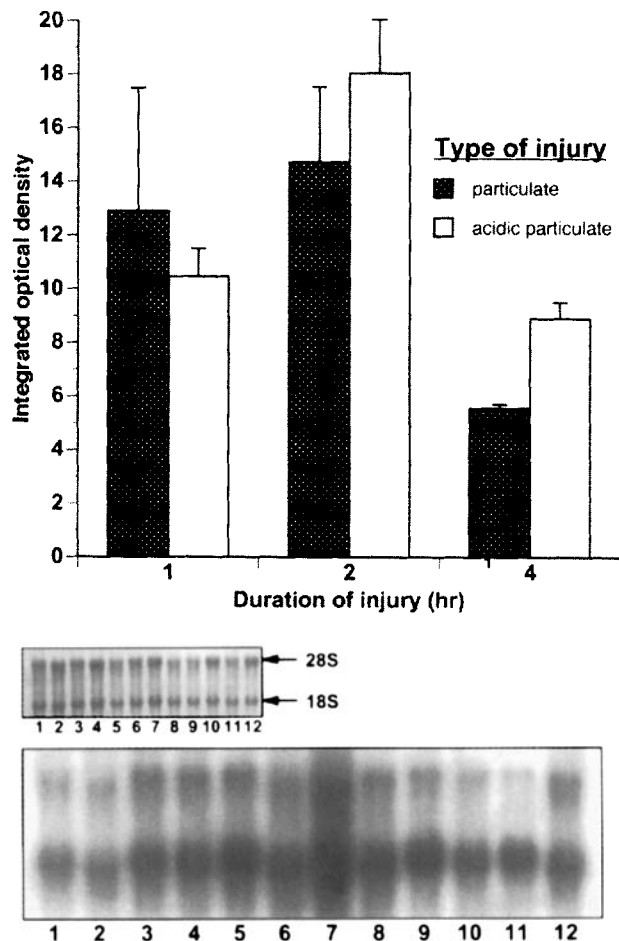


Fig. 3. Northern blot analysis of RNA isolated from whole lungs of rats injured with particulate or acidic particulate at the time points indicated. Probes were full-length cDNA for rat TNF- α , as described in Methods. Lanes 1 and 2: particulate, 4 h; lanes 3 and 4: acidic particulate, 4 h; lanes 5 and 6: particulate, 2 h; lanes 7 and 8: acidic particulate, 2 h; lanes 9 and 10: particulate, 1 h; lanes 11 and 12: acidic particulate, 1 h. Blot is representative of three separate experiments. Equal loading was confirmed by methylene-blue staining of 18S and 28S ribosomal RNA bands shown in inset. The bar graph represents densitometric analysis of the Northern blot. TNF- α mRNA bands were integrated and normalized to a constant 28S ribosomal RNA level. $n = 2$ for each point.

PI over the particulate-injured group. Anti-TNF- α antibody treatment had no significant effect on this synergistic increase ($P < 0.0001$) in lung injury from the combined gastric components.

Effects of Anti-TNF- α Antibody Treatment on Lung Function

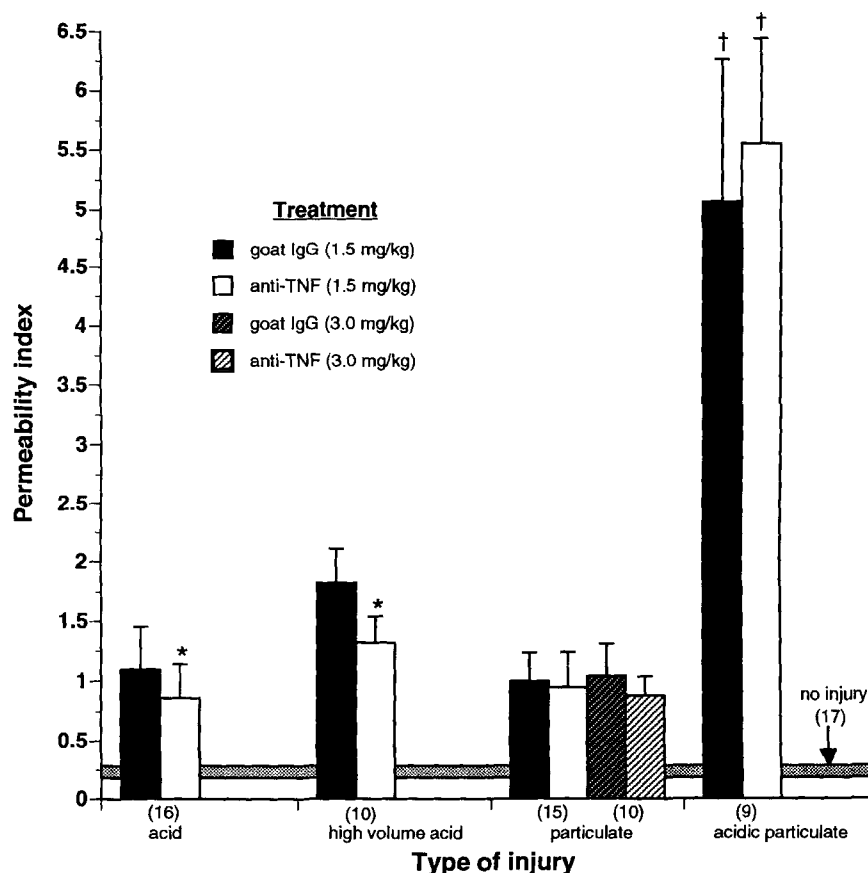
Impairment of lung function (*i.e.*, blood oxygenation) by the different injury models was also assessed. Acid

injury alone resulted in a 57% decrease in pulmonary efficiency, as measured by $\text{Pa}_{\text{O}_2}/\text{F}_{\text{I}_{\text{O}_2}}$ (fig. 5), compared with uninjured animals (238.7 ± 99.9 mmHg *vs.* 550.6 ± 56.1 mmHg, $P < 0.0001$). Increasing the volume of the acid instillate yielded a 73% reduction (149.8 ± 49.9 mmHg, $P < 0.0001$). However, particulate injury only caused an 11% reduction in $\text{Pa}_{\text{O}_2}/\text{F}_{\text{I}_{\text{O}_2}}$ (490.9 ± 78.1 mmHg, $P < 0.05$), even though pulmonary albumin permeability was increased to the same magnitude as the acid injury. The acidic-particulate group demonstrated a synergistic increase ($P < 0.02$) in lung injury, as measured by this parameter, yielding an 86% decrease (75.5 ± 15.6 mmHg, $P < 0.0001$). The effect of anti-TNF- α treatment on the pulmonary efficiency for each of the injury regimes mirrored the pattern for PI. Both the acid and the high-volume acid groups responded with increased gas exchange (by 77% in the acid group, to 423.2 ± 104.4 mmHg, $P < 0.0001$, and by 90% in the high-volume acid group, to 284.1 ± 126.9 mmHg, $P < 0.01$), whereas none of the groups containing gastric particles experienced a significant change when compared to their associated nonspecific IgG control groups. As with the lung protein permeability parameter, anti-TNF- α treatment did not effect the synergistic interaction ($P < 0.0001$) resulting from the combination of the acid and particulate injury modalities in regards to pulmonary gas-exchange efficiency.

Effects of Anti-TNF- α Antibody Treatment on Neutrophil Recruitment

Previous work from our laboratory has demonstrated that alveolar neutrophil infiltration is involved in acid, gastric-particulate, and acidic-particulate aspiration injury.^{7,9} MPO activity in lung homogenates from injured animals treated with anti-TNF- α antibody or nonspecific IgG was determined to ascertain TNF- α 's involvement in neutrophil infiltration (fig. 6). The acid and high-volume acid groups were the only groups in which anti-TNF- α antibody reduced neutrophil infiltration (0.29 ± 0.14 *vs.* 0.58 ± 0.43 ABS/min for acid and 0.29 ± 0.07 *vs.* 0.44 ± 0.19 ABS/min for high-volume acid, $P < 0.05$). Anti-TNF- α antibody treatment did not change the MPO activity in the particulate or acidic-particulate groups. Comparing the IgG-treated groups, the relationship of the MPO activity of the acidic-particulate group (0.96 ± 0.35 ABS/min) to that of the acid alone (0.58 ± 0.43 ABS/min) or particulate alone (0.71 ± 0.23 ABS/min) groups demonstrated no interactive effects ($P < 0.30$) between the acid and particulate injuries with respect to neutrophil infiltration. This lack of an interactive effect

Fig. 4. Rats were injured by instillation of the indicated injury vehicle through an intratracheal catheter. Animals were treated *via* the same catheter as the injury vehicle with nonspecific goat immunoglobulin G (IgG) or goat anti-rat TNF- α (purified IgG fraction) at the indicated dose. At the time of injury, 1 ml of 125 I-BSA in 2% BSA (100,000 cpm) was injected into the penile vein. At 5 h postinjury, the albumin permeability index was determined. Horizontal bar indicates mean \pm SD of an uninjured rat group. Parenthetical numbers denote *n* for the groups above the numbers. * $P < 0.05$ between the different treatment groups. Permeability index increased for all experimental groups compared with uninjured animals, $P < 0.05$. † $P < 0.0001$ for a synergistic interaction between acid and particulate injury within similar treatment groups.



was also true when comparing the same injury groups that had been treated with anti-TNF- α antibody ($P < 0.19$).

Summary of TNF- α Response and Subsequent Injury Parameters

Table 1 offers a semiquantitative, "visual" presentation of how different injury-induced TNF- α responses (as determined in lavage fluid) compare with the resulting injury and inflammation parameters measured. Clearly, a direct correlation between the level of TNF- α and the lung injury resulting from acid, particulate, or acidic particulate instillation cannot be drawn.

Discussion

Aspiration of gastric components leads to an acute pulmonary inflammatory injury that is characterized by proteinaceous edema in the lung, a decrease in pulmonary gas-exchange efficiency, and neutrophil infiltration into the interstitial and alveolar space from the intravas-

cular compartment, as well as various systemic inflammatory responses.^{6-9,11,16-18,25,27-37} Patients that experience this condition are at risk for developing ARDS, with its associated high mortality rate.^{3,4,38} There is considerable evidence that the proinflammatory cytokine, TNF- α , plays an important role in the pathophysiology associated with acute pulmonary inflammation resulting from a variety of lung insults, including gastric aspiration.^{10,12-16,18,23,24,39,40} This study has examined the role of TNF- α in the pathogenesis of aspiration pneumonitis by characterizing the TNF- α response to different gastric components and determining if it is required for full expression of the resulting lung injury. This has been done with the goal of developing therapeutic strategies for this potentially lethal complication of anesthesia and surgery.

Previously, we have demonstrated that the rat aspiration pneumonitis model involves a biphasic injury pattern, with an "early phase" (0-1 h after aspiration) that is associated with the direct chemical effects of the acid, and an additional "late phase" (4-6 h after aspira-

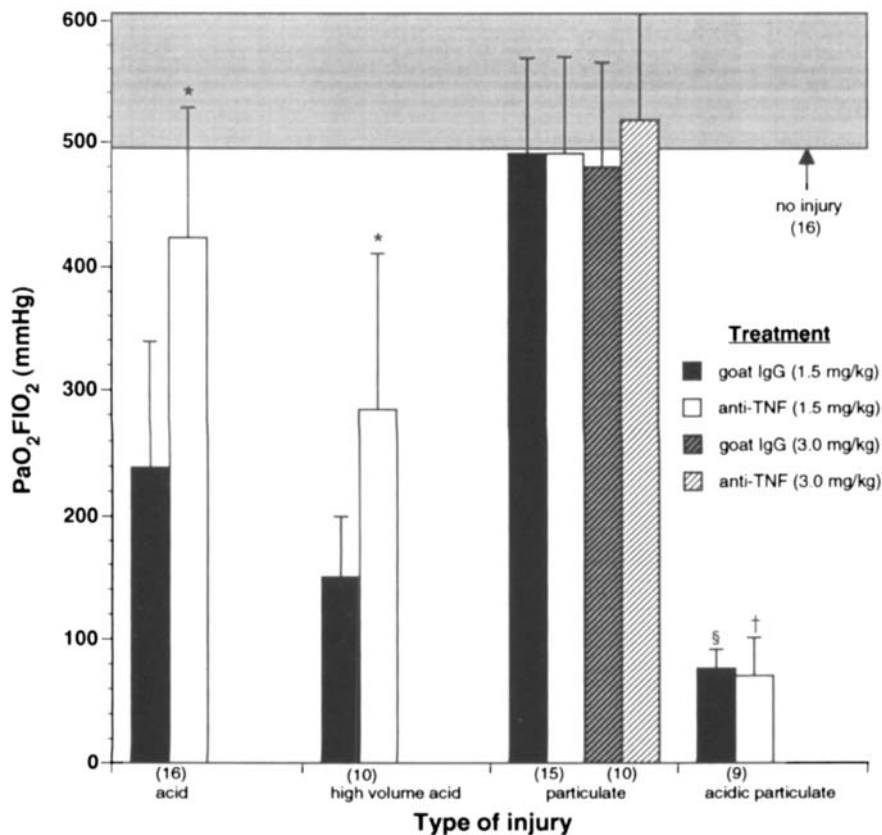


Fig. 5. Same experiment as described for figure 4, except blood gases were determined from samples taken from the abdominal aorta at 5 h after injury ($F_{iO_2} = 0.8$ for 10 min before harvest) and Pa_{O_2}/F_{iO_2} calculated. Horizontal bar indicates mean \pm SD of an uninjured rat group. Parenthetical numbers denote n for the groups above the numbers. * $P < 0.01$ between the different treatment groups. Except for the particulate-injured/3 mg/kg anti-TNF- α -treated group, Pa_{O_2}/F_{iO_2} decreased for all experimental groups compared to uninjured animals, $P < 0.05$. § $P < 0.02$ for a synergistic interaction between acid and particulate injury within the IgG-treatment group. † $P < 0.0001$ for a synergistic interaction between acid and particulate injury within the anti-TNF- α treatment group.

tion) inflammatory injury. Neutrophils have been clearly established, by morphometric analysis and depletion studies, as necessary for the additional inflammatory lung injury of the "late phase."^{7,9} These results have been confirmed by other laboratories.^{27,31,33} We have also reported that aspiration of washed and filtered gastric particulate matter causes an inflammatory lung injury similar to the "second phase" of the acid aspiration. Maximal leakage of protein occurs at 4–6 h after deposition of the food particles into the trachea and corresponds to infiltration of large numbers of neutrophils into the lung. There is not a significant "first phase" injury as with acid aspiration.¹¹ The data presented here support those previous results (figs. 4 and 6).

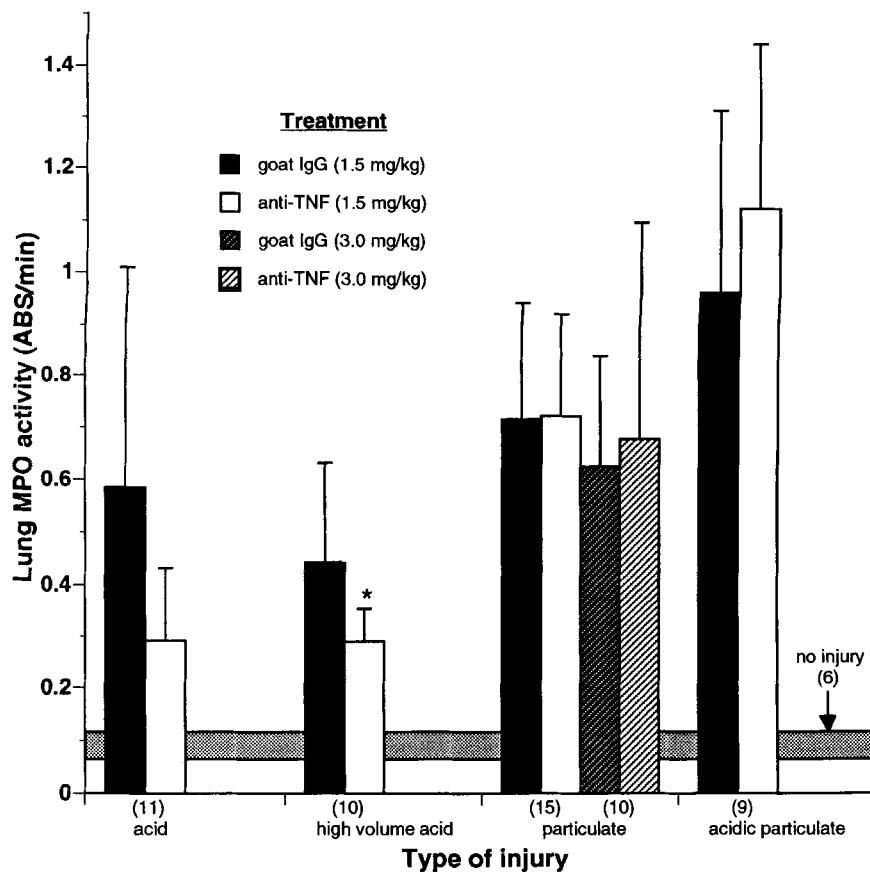
In this study a small increase in TNF- α levels in the bronchoalveolar lavage fluid of rats receiving an instillate of normal saline into the lung was detected using a cytotoxicity bioassay. The temporal relationship of TNF- α release differs based on the pH of the instillate (with a low pH, pH = 1.25, TNF- α levels peak later than normal saline, pH = 5.3; fig. 1). Others have implicated TNF- α involvement in acid-aspiration injury but do not find detectable levels of TNF- α in the

bronchoalveolar lavage fluid from acid-injured lungs.^{17,36,37} The discrepancy between these reports and ours may be caused by their use of the L929 mouse fibroblast bioassay, which is not as sensitive for TNF- α detection as the WEHI bioassay.¹⁹ However, detectable increases in serum TNF- α levels have been reported 1–2 h after injury.^{17,18,37} In the current study, upregulation of TNF- α mRNA was not consistently detected in the normal saline-instilled animals at either pH. This result is consistent with the report from Ohara *et al.*,³⁴ in which upregulation of TNF- α mRNA could not be detected in alveolar macrophages (most likely source of TNF- α in acute lung injury¹²) isolated from acid-injured mouse lungs.

When gastric particulate material is instilled into the lungs, a large (4-log increase over uninjured controls at 4 h after instillation) and sustained (>8 h) TNF- α response is detected in the bronchoalveolar lavage fluid (fig. 2). TNF- α mRNA is also upregulated as a result of this injury (fig. 3). Other studies have demonstrated a similar TNF- α response using other types of particulate insult.^{13,15,39} The acidic-particulate combination injury results in a TNF- α response, as assessed in the bronchoal-

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Fig. 6. Same experiment as described for figures 4 and 5, except the excised lungs were homogenized and the clarified homogenate assessed for myeloperoxidase (MPO) activity. Horizontal bar indicates mean \pm SD of an uninjured animal group. Parenthetical numbers denote n for the groups above the numbers. * $P < 0.05$ between the different treatment groups. Myeloperoxidase activity increased for all experimental groups compared with uninjured animals, $P < 0.05$.



veolar lavage fluid, as well as with mRNA upregulation, that closely mirrors that of particulate alone in terms of intensity. However, the addition of the acidic component to the particulate injury inhibits the initial TNF- α response and accelerates its decline (fig. 2).

Goldman *et al.*¹⁶ demonstrated in an earlier study that

the pulmonary protein permeability, neutrophil infiltration into the lung, and lung wet/dry weight of rats injured by acid aspiration can be reduced by intraperitoneal administration of anti-TNF- α antisera. Similar results have been obtained in this study (figs. 4 and 6). In addition, intratracheal administration of anti-TNF- α anti-

Table 1. Comparison of Aspiration-induced TNF- α Response to Inflammatory Injury Parameters*

Parameter	Aspiration Injury Modality				
	No Injury or Normal Saline	Acid	High-volume Acid	Particulate	Acidic Particulate
Lung TNF- α response†	—	—	—	+++++	+++++
Increased lung protein permeability‡	—	+	++	+	+++++#
Reduced gas exchange efficiency§	—	+++	+++++	+	+++++#
Increased lung neutrophil infiltration	—	+++	+++	+++++	+++++

* Relative "level" compared with maximum (+++++) and minimum (—) attained.

† Assessed by bronchoalveolar lavage fluid [TNF α] integrated from 0 to 5 h postinjury.

‡ Assessed by lung protein permeability index measured from 0 to 5 h postinjury.

§ Assessed by Pa_{O₂}/Fi_{O₂} at 5 h postinjury.

|| Assessed by lung homogenate myeloperoxidase levels harvested at 5 h postinjury.

Synergistic interaction between acid and particulate injury.

body improves blood oxygenation in the animals injured with acid alone (fig. 5).

This study clearly supports the hypothesis that TNF- α plays an important role in inflammatory processes involved in acid-aspiration pneumonitis. The paradigm that recognition cytokines (*i.e.*, TNF- α , interleukin [IL]-1 β) "prime" the inflammatory response and lead to the elaboration of other inflammatory regulators (*i.e.*, chemokines, eicosenoids) is consistent with the results of this injury model.^{10,41} Inhibition of TNF- α limits the recruitment of neutrophils and decreases lung injury associated with acid aspiration. Because TNF- α is not by itself directly chemotactic, the obvious conclusion is that the cytokine either leads to the generation of direct regulators of neutrophil infiltration and activation by paracrine or autocrine mechanisms or enhances the action of these chemotactic agents.¹⁰ Additionally, the observation that TNF- α levels in the bronchoalveolar lavage fluid peaks later in both the low pH models (normal saline and particulate) suggests that a low pH aspirate may initially directly inhibit TNF- α release. Thus, we postulate that indirect effects of the insult lead to the later transient increases in TNF- α levels. A possible mechanism may involve the stimulation of alveolar macrophages and release of preformed TNF- α by cellular products or debris caused by low-pH damage to the cells lining the airways or alveoli. This stimulation appears to be transient and does not result in appreciable upregulation of TNF- α mRNA. This is consistent with the limited lung injury demonstrated by the low-pH model if there are no other additional insults.

Treatment of animals with anti-TNF- α antibody that had been injured by instillation of gastric particles into the lungs demonstrated no significant changes in lung protein permeability, gas-exchange efficiency, or neutrophil lung infiltration. These results are different than those obtained in a lung-injury study using a model of inhalation of quartz particles model.¹³ In that study, rats pretreated with anti-TNF- α antibody have diminished infiltration of neutrophils into the lungs compared with rats pretreated with nonimmune IgG. The different types and size of particle, as well as the timing of the measurements (24 h *vs.* 5 h after injury in our experiments), may be the reasons for the differing results.

Considering the robust nature of the TNF- α response in both gastric-particle models, it is surprising that inhibition of the bioactivity of the TNF- α did not decrease the lung injury in either group of animals. There are several possible explanations for these findings. First, the experimental conditions may not have been able to pre-

vent the biologic effects of a sustained release of TNF- α with the antibody strategy that we employed. To demonstrate the requirement for TNF- α in the pathogenesis of the gastric-particulate injury may require larger dosages or continuous administration of the antibody.

Second, proinflammatory cytokine cascades have a significant amount of redundancy. Because of the overlapping actions of the many inflammatory cytokines, interpretation of data derived from inhibition studies may not be straightforward. Clinically, this can lead to many of the failures associated with therapies based on experimental data associated with cytokine cascades in disease (*e.g.*, strategies to improve outcomes in sepsis).⁴² This may be particularly important when the stimulus is multifactorial (*e.g.*, low pH and particulate). The most obvious and direct example of this is the overlapping responses of IL-1 β and TNF- α . These initiation cytokines have very similar actions in inducing subsequent cytokines that lead to inflammatory effector cell infiltration.⁴³ Additionally, inhibiting the bioactivity of one proinflammatory cytokine may lead to the overexpression of another. Thus, concurrent use of several blocking agents (*e.g.*, anti-IL-1 β and TNF- α) may be required to demonstrate overlapping actions in the pathogenesis of the lung injuries.

Finally, TNF- α also has a number of potentially beneficial effects involving the injury associated with the inflammatory response. This may also be why therapeutic strategies to decrease the bioactivity of TNF- α clinically in systemic inflammatory injury syndromes has failed. For example, sustained TNF- α is an important stimulant of IL-10, a cytokine that can prevent some of the untoward effects of acute inflammation and shift the inflammatory response to a less acute, chronic injury or reparative mode.⁴⁴ Additionally, TNF- α has been demonstrated to increase levels of antioxidants in the lung that are predicted to protect the lung from injury associated with oxidant-mediated damage.⁴⁵ Interestingly, in several models of lung infection, increasing TNF- α levels has been shown to be protective locally.¹⁴ Thus, decreasing TNF- α activity may directly decrease lung inflammation but also directly or indirectly decrease the innate anti-inflammatory defenses from being upregulated, leading to no detectable differences in lung injury.

An interesting difference between the acidic injury and the particulate injury emerged in the blood-gas data. Gastric particulate pulmonary injury produced a minimal (11%) reduction in oxygen-exchange efficiency, as measured by PaO₂/FiO₂, whereas the effect of acid aspiration was quite substantial, a 57% reduction (fig. 5). This was

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despite the fact that the acid- and particulate-injury regimens used in this study produced the same increase in pulmonary protein permeability (fig. 4).

Clearly, the mechanism of lung-function impairment (with regard to blood oxygenation) is different between the two gastric components and cannot be explained merely in terms of alveolar edema impeding gas diffusion. If this were the case, the pulmonary protein permeability measurements would have correlated with the gas-exchange indexes for the two different aspirate modalities. Alterations in the normal hypoxic pulmonary vasoconstriction response may offer a putative mechanism. As a result of the injury insult, acid, or particulate, the integrity of the alveolar-capillary barrier is compromised, producing proteinaceous pulmonary edema at the site of insult exposure. The resulting decrease in oxygen exchange would normally lead to a decrease in blood flow to the pulmonary vasculature of the injured area. This response prevents venous admixture and is controlled by endothelial vasomediators.⁴⁶⁻⁴⁸ In gastric particulate aspiration-induced inflammation this mechanism appears to be intact, whereas in the acid-injured lung, it does not. Stimulation of vasodilating mediators (*i.e.*, NO) by the acid insult, or inhibition of vasoconstricting mediators (*i.e.*, thromboxanes), may result in venous admixture. Likely sources of these mediators would be the vascular endothelium, alveolar macrophages, and neutrophils marginated in the pulmonary vasculature.⁴¹

Previously, we have demonstrated that aspiration of gastric particles suspended in an acid solution leads to a resultant injury to the alveolar-capillary boundary, as measured by protein leakage into the lung, that is synergistic compared with the two injury modalities alone.¹¹ The results of this study corroborate these previous findings (fig. 4) and also demonstrate a synergistic decrease in gas-exchange efficiency when the two gastric-aspirate components are combined (fig. 5). These results suggest that the combined injury insult produces such an overwhelming pulmonary edema that either blood oxygenation cannot be improved by hypoxic pulmonary vasoconstriction or the normal response mechanism is inhibited by the acid exposure.

Clearly, aspiration of gastric components produces a sequela of pulmonary inflammation that is dependent on the constituents of the aspirant. Aspiration of stomach acid, gastric particles, or their combination produces inflammatory injuries that are uniquely manifested and have different underlying mechanisms driving their pathogenic and reparative courses. This is demonstrated in this study by the lack of correlation between lung

protein permeability and gas-exchange efficiency changes induced by particulate aspiration injury, whereas these lung-injury parameters mirror each other in response to acid alone or acidic particulate aspiration. The synergistic increase in lung injury produced by the combination of the acidic and particulate components of gastric contents may, in part, be responsible for the potential for development of ARDS following an aspiration event. Interestingly, although elaboration of the proinflammatory cytokine, TNF- α , has been demonstrated to occur with each of the tested aspiration modalities, these changes in TNF- α expression or presentation are not reflected in the resultant lung-injury parameters tested. Therefore, TNF- α does not appear to be the singular mitigating factor producing the synergistic increase in lung injury. However, the evidence from this study does not exclude the possibility that the interaction of TNF- α with other inflammatory cascade components may be responsible for that process.

Finally, there are other potential risk factors that can confound the course of events. Introduction of throat-colonizing bacteria into the lung either by the aspiration event itself or intubation presents an entirely different stimulus for the immune/inflammatory system to deal with. Hyperoxia exposure may pose a risk, as well. We previously demonstrated that exposure to as little as 50% oxygen for 5 h or 98% oxygen for 2 h produced an exacerbation of the lung injury in this rat model of acid aspiration. Exposing the injured lung to high concentrations of oxygen, as is often the case in the critical-care setting, can add an additional insult to the preexisting ones and aggravate the situation with hyperoxic modification of the body's defenses.²⁵

Elucidating the cytokine cascades involved in "directing" each inflammatory response will clearly help us understand why some gastric-aspiration victims successfully recover on their own and why others develop ARDS. This knowledge can help us develop therapies to prevent this potentially lethal downward spiral.

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