The Effects of Two Antiinflammatory Pretreatments on Bacterial-induced Lung Injury

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Background: Two antiinflammatory therapies that have been effective in preventing acid-induced lung injury were evaluated. Specifically, their effects on a subsequent bacterial-airspace challenge were compared. Bacteria were instilled 24 h after acid-induced lung injury. Pseudomonas aeruginosa PAO-1 was used as the bacteria, because its effects in healthy lungs was documented previously.

Methods: New Zealand white rabbits were anesthetized and three pretreatments were administered: (1) pentoxifylline pretreatment (a 20 mg/kg bolus dose and then 6 mg·kg⁻¹·h⁻¹ given intravenously), (2) 1 ml anti-tumor necrosis factor α antiserum given intravenously, or (3) normal saline given intravenously. The pretreatments were shown previously to prevent acid-induced lung injury. Then 1.2 ml/kg hydrochloric acid (HCl), pH 1.25, was instilled into the rabbits' right lungs. All the animals then underwent mechanical ventilation for 8 h. Twenty-four hours after the acid instillation, the rabbits were anesthetized again and 2 ml/kg (10⁷ colony forming units/ml) PAO-1 was instilled into their left lungs. The rabbits’ breathing was aided by mechanical ventilation for another 8 h, and then were killed and exsanguinated.

Results: Both pretreatments attenuated the acid-induced lung injury of the noninstilled left lungs. Arterial oxygen tension and the lung edema of pretreated, acid-exposed animals were significantly and almost equally improved (compared with no pretreatments) by either of the pretreatments. However, when the bacteria were instilled into the left lungs 24 h after the acid injury, the pentoxifylline pretreatment but not the anti-tumor necrosis factor α pretreatment prevented much of the bacteria-induced lung injury. Pentoxifylline pretreatment significantly improved the measurements of left lung edema and epithelial and endothelial permeability. There was also a trend for improved oxygenation in the pentoxifylline-pretreated and infected animals. In contrast, the anti-tumor necrosis factor α pretreatment did not prevent the bacteria-induced lung injury and increased some of the measurements of lung injury.

Conclusions: Two antiinflammatory therapies that prevented acid-induced lung injury to the noninstilled left lungs had significantly different effects on a subsequent bacteria-induced lung injury to the left lungs. The therapies differed in their mechanism of tumor necrosis factor α blockade, and this may have affected the bacteria-induced injury to the lungs. (Key words: Cytokines; inflammation; lung.)

BACTERIAL lung infections commonly occur several days after acid aspiration.¹,² The mechanisms for the
immediate and direct acid-induced lung injury and the later indirect injury to the nonexposed lung regions by the inflammatory response to acid have been investigated; however, the effect of both of these lung injuries on subsequent bacterial infections have not. These issues are of particular interest given the clinical trials using antiinflammatory therapies and the high risk for bacterial pulmonary infections to develop in patients with acid injuries.1,2

Several antiinflammatory therapies decrease indirect, acid-induced lung injury, including neutrophil depletion, modification of neutrophil activation or neutrophil adhesion,3-8 complement depletion, and the blockade of cytokines, including tumor necrosis factor α (TNF-α) and interleukin 8 (IL-8).9-11 The blockade of TNF-α has been evaluated extensively in animal investigations and in human clinical trials of sepsis1-4; the blockade of TNF-α in patients with sepsis has been associated with an increase in the mortality rate.15 The clinical usefulness of TNF-α blockade, therefore, is still unclear. This investigation compared the effects of two pretreatments that block TNF-α activity by different mechanisms: pentoxifylline and polyclonal antiserum to TNF-α. Although the mechanism is not clear, pentoxifylline has been documented to inhibit the production of TNF-α mRNA and protein in vitro.12 After pentoxifylline is stopped, a rebound in TNF-α production occurs.13 Antiserum blocks activity of TNF-α protein with no known rebound. Therefore, the two treatments differ in their mechanisms of TNF-α blockade and the potential for post-treatment rebound. Given these different effects of pentoxifylline and anti–TNF-α antiserum, we set out to determine whether these two therapies would differ in their effects on an airspace bacterial challenge given to animals 24 h after they had sustained acid-induced lung injuries.

Materials and Methods

Surgical Preparation and Ventilation

The protocol for these studies was approved by the University of California San Francisco Animal Research Committee.

Male New Zealand white rabbits (weight, 2-4.2 kg) initially were anesthetized with 25 mg/kg pentobarbital given intravenously via 20-gauge Angiocaths (Deseret Medical, Becton Dickinson & Co., Sandy, UT) that were inserted into their carotid veins. The rabbits’ necks were shaved, and after repeated povidone–iodine applications to the neck, tracheostomies were performed; endotracheal tubes with a 5-mm ID were inserted. The animals’ breathing was aided by mechanical ventilation using constant-volume pumps (Harvard Apparatus, Millis, MA), and anesthesia was maintained with halothane. The tidal volumes were 10 ml/kg with 3 cm H2O positive end-expiratory pressure and an inspired oxygen fraction of 1.0 to maintain oxygenation after lung injury. The respiratory rates were adjusted to maintain the arterial carbon dioxide tension (Paco2) between 35 and 45 mmHg. The rabbits were placed in the supine position, and 0.3 mg/kg pancuronium was administered intravenously for neuromuscular blockade. Right carotid arterial catheters were placed to measure blood pressure and to sample arterial blood gases.

To standardize the volume of fluids given to the animals, all animals received the same quantity of normal saline and other intravenous fluids throughout the study.

Experimental Protocols Using Pentoxifylline or Tumor Necrosis Factor α Antiserum as Pretreatments for Acid-induced Lung Injury

The comparable effects of the two pretreatments on acid-induced lung injury were documented in 59 rabbits in 8-h experiments (fig. 1).

In group 1 (intravenous saline plus phosphate-buffered saline [PBS] given intratracheally), nine animals received saline intravenously for 1 h (the vehicle used in the pretreatments) and then 1.2 ml/kg PBS, pH 7.4, was instilled into their right lower lobes as described.1 Less than 5% of the instillate was found in the opposite lung, as confirmed by radioactive tracer measurements at the discontinuation of the experiments. If spillover into the opposite lung was detected, the experimental results were discarded.

In group 2 (intravenous saline plus HCl given intratracheally), nine animals received saline intravenously for 1 h and then HCl (1.2 ml/kg, pH 1.25) was instilled into their right lower lobes.3

In group 3 (nonimmune serum given intravenously plus HCl given intratracheally), three animals received 1 ml nonimmune goat serum that was given intravenously 15 min before the acid was instilled into their right lower lobes.

In group 4 (pentoxifylline given intravenously plus HCl given intratracheally), nine animals received 20 mg/kg pentoxifylline given intravenously 1 h before the acid was instilled. During the 8-h experimental interval, these animals received 6 ml·kg⁻¹·h⁻¹ pentoxifylline as a continuous infusion.
In group 5 (anti–TNF-α given intravenously plus HCl given intratracheally), nine animals received 1 ml goat anti-rabbit TNF-α antiserum 15 min before the acid was instilled into their right lower lobes. The timing and dose used to administer these agents was based on previous experiments that provided TNF-α blockade for at least 8 h (J. Mathison, personal communication, June 1997).

During the 8-h interval, all the rabbits were kept in the right lateral decubitus position; blood samples were obtained each hour, beginning 1 h before the acid instillation. Blood pressure, arterial blood gases, and peak airway pressures were recorded hourly.

After 8 h, the rabbits were anesthetized deeply with pentobarbital and killed by transection of their abdominal aortas. The lungs were removed through a sternotomy and the right and left bronchi were clamped. After the opposite lung was clamped, each lung was lavaged by instilling and withdrawing 15 ml sterile saline three times. All liquid obtained from the lavages was placed in tubes containing 0.15% EDTA; the lavage was centrifuged at 1,500g for 20 min at 4°C and the supernatant was frozen at −70°C. Cells were stained with gentian violet and Wright-Giemsa, and a hemocytometer was used to count them. The total protein concentration of the lavage fluid was measured using the Lowry method. Myeloperoxidase activities were assayed in the lungs and small intestines of the rabbits. We measured the plasma levels of TNF-α and bronchoalveolar lavage samples of TNF-α and IL-8, because both cytokines have been documented as the major inflammatory mediators involved in causing acid-induced lung injury.18

Preparation of Pretreatments with Pentoxifylline
Pentoxifylline (supplied by Hoechst Japan, Tokyo, Japan) was dissolved in sterile normal saline to a concentration of 10 mg/ml and filtered through a 0.45-mm filter (Millipore, Bedford, MA) before injection. For these experiments, 20 mg/kg pentoxifylline was administered intravenously 1 h before acid instillation, and 6 mg·kg⁻¹·h⁻¹ pentoxifylline was infused continuously for the 8-h interval. This dose is one we and others have found to effectively prevent acid-induced lung injury.

Goat Anti-rabbit Tumor Necrosis Factor α Antiserum
The goat anti-rabbit TNF-α antiserum was prepared by Drs. Mathison and Kravchenko at the Scripps Research Institute and shipped to the University of California at San Francisco. All blood was collected using sterile pyrogen-free devices, and serum was processed in glassware that had been heated overnight at 200°C to inactivate endotoxin. The goat anti-rabbit TNF-α antiserum was found to have more than 10⁶ neutralizing units/ml, which was determined by incubating equal volumes of rabbit TNF-α (4 × 10⁶ U/ml) and diluted antiserum at 37°C for 30 min, followed by measurement of residual TNF-α cytolytic activity using an L929 cell assay. Nonimmune goat serum was used in control studies. The dose of antiserum given (1 ml) leads to a lack of detectable TNF-α activity for 8 h (personal communication, J. Mathison).

Assay for Tumor Necrosis Factor α Activity
The mouse L929 fibroblast assay was used to measure biologic TNF-α activity. Blood samples were drawn into sterile glass tubes containing 0.15% EDTA and maintained at 4°C. Specimens were centrifuged at 500g for 20 min at 4°C, and the plasma was frozen at −70°C until use. L929 cells were seeded onto flat-bottom 96-well microtiter plates at a density of 4 × 10⁴ cells/well and
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Table 1. TNF-α Activity in Plasma and in Bronchoalveolar Lavage Fluids (BALF) in Pretreated Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Plasma (U/ml)</th>
<th>BALF (positive number)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>2 h</td>
</tr>
<tr>
<td>Control groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline iv + PBS it</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Saline iv + HCl it</td>
<td>6</td>
<td>0.52 ± 0.83*</td>
</tr>
<tr>
<td>Nonimmune serum iv + HCl it</td>
<td>3</td>
<td>0.48 ± 0.69*</td>
</tr>
<tr>
<td>Pretreated groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTX iv + HCl it</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Anti-TNF serum iv + HCl it</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

Data are mean ± SD in plasma data. Lavage samples measured for TNF-α activity, levels were barely detectable and so are documented as positive or negative in plasma.
* Significant at P < 0.01 when compared with saline iv + PBS it, PTX iv + HCl it, anti-TNFα serum iv + HCl it by ANOVA.
iv = intratracheally; iv = intravenously.

grown to confluence overnight in Dulbecco minimal essential medium (GIBCO, Grand Island, NY) containing 1% penicillin-streptomycin and 5% fetal calf serum. The medium was removed from the confluent monolayers, and 100 ml Dulbecco minimal essential medium containing actinomycin D (final concentration, 5 mg/ml) was added to each well. One hundred microliters of each of the following was added to selected duplicate wells containing 1929 cells: (1) Dulbecco minimal essential medium (0% cytotoxicity), (2) serial dilutions of recombinant TNF-α (5 × 10⁻⁵ to 6 × 10⁻⁴ U/ml), (3) serial dilutions of plasma samples from each group, and (4) Dulbecco minimal essential medium in blank wells without cells (100% cytotoxicity). Plates were incubated for 20 h at 37°C in a 5% carbon dioxide incubator. After incubation, the medium was removed and the 1929 cells were stained for 10 min with 0.5% crystal violet in 20% methanol, rinsed in water, and air dried. The optical density of each well was determined by a microplate reader and calibrated to noncellular reagent blanks at a wavelength of 550 nm. The percentage of cytotoxicity of 1929 cells was calculated according to

\[
\text{Cytotoxicity} = \left( \frac{\text{OD well with 0% cytotoxicity} - \text{OD experimental sample well}}{\text{OD wells with 0% cytotoxicity} - \text{OD wells with 100% cytotoxicity}} \right) \times 100
\]

Myeloperoxidase Assay

The activity of this neutrophil enzyme was used as a measure of neutrophil sequestration in tissues; this assay is more sensitive for neutrophils in tissues than quantitative histologic analysis.¹⁷ One gram of tissue was blotted dry and homogenized in 10 ml potassium phosphate buffer, 0.01 M, (pH 7.4) containing 1 mM EDTA. Two milliliters homogenate and 5 ml potassium phosphate buffer, 0.01 M, containing 1 mM EDTA were mixed gently and then centrifuged at 10,000 g for 20 min at 4°C. The pellet was rehomogenized in 5 ml potassium phosphate buffer, 0.05 M, (pH 6) containing 0.5% hexadecyltrimethylammonium bromide. This suspension was freeze-thawed and sonicated using a Branson cell disrupter at 65 W for 1 min. A 0.1 ml aliquot was mixed with 0.79 ml potassium phosphate buffer, 0.08 M, (pH 5.4) and 0.1 ml tetramethylbenzidine 16 mM, dissolved in N,N-dimethylformamide at 37°C. After 2 min, 0.01 ml hydrogen peroxide, 30 mM, was added. After incubating for 3 min at 37°C, 0.05 ml catalase solution (300 mg/ml) was added. The mixture was diluted with 4 ml sodium acetate, 0.2 M, (pH 5) and then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was read in a spectrophotometer (UV-2200; Shimadzu, Kyoto, Japan). One unit of myeloperoxidase activity was defined as the amount of enzyme necessary to catalyze an increase in absorbance of 1.0/ min at 655 nm at 37°C.

Rabbit Interleukin-8 Assay

Rabbit IL-8 was measured, as described in detail,¹⁸ by enzyme-linked immunosorbent assay using two monoclonal antibodies directed against recombinant rabbit IL-8. Recombinant rabbit IL-8 and lavage and plasma samples were assayed over dilutions (1:10 to 1:80). The assay did not recognize human IL-8 or other closely

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related human cytokines (gro/MGSA, platelet factor 4, β-thromboglobulin), other human cytokines (TNF, IL-1), or chemotaxins (C5a, FMLP). The assay was sensitive to a lower limit of 12 pg/ml and was linear over the range from 12 pg/ml to 1,000 pg/ml. The intraassay coefficient of variation was approximately 5%.

Extravascular Lung-water Measurement

We determined the extravascular lung-water measurement (EVLW) according to the method we used before and it was expressed as grams/gram dry lung using a modification of the method reported by Selinger.19 In this assay, the blood content of lungs is measured so the water in the blood is excluded from the lung water.

Experimental Protocols Using Pretreatments for Acid-instilled Rabbits before Bacterial Challenge

The rationale for these experiments was to determine the effect of the two antiinflammatory pretreatments described on animals with acid-induced lung injuries when they were exposed to a subsequent intrapulmonary bacterial challenge. Because bacterial infections tend to occur more than 2 h after acid aspiration in patients,1 the animals were allowed to recover for 24 h after the acid-induced lung injury.

Sixteen rabbits were pretreated with pentoxifylline, anti-TNF-α antiserum, or saline before acid or PBS was instilled into their right lower lungs, as described in the first group of experiments. After a recovery period of 24 h, a bacterial inoculum was instilled into their left lower lungs, and the rabbits were monitored for an additional 8 h (fig. 1).

We included four groups of rabbits in these experiments, but the following general protocol was used for each.

On day 1, 8 h after acid or PBS was instilled into the right lower lobes, the anesthetic was discontinued, the endotracheal tubes were shortened and left in place, and the rabbits were allowed to resume spontaneous ventilation. After the rabbits were fully awake and mobile, they were returned to their cages. The rabbits were monitored closely throughout the night for respiratory distress, and their lungs were suctioned every 2 h through their endotracheal tubes. On day 2, 24 h after the intrapulmonary acid or PBS, the animals were anesthetized again with 25 mg/kg pentobarbital given intravenously, and mechanical ventilation was resumed. The rabbits’ breathing was aided by mechanical ventilation, as on the previous day, and they were maintained with halothane anesthesia, as in the first group of experiments. Angiocaths (22 g) were placed in their ear veins and 0.5 mCi 151I-albumin (Merck-Frosst, Kirkland, Quebec, Canada) was administered as a vascular protein tracer (described in the following paragraph). The rabbits were placed in the left lateral decubitus position and 5-French feeding tubes were placed into their left lower lungs via the endotracheal tubes.

An instillate (2 ml/kg) containing 10⁶ colony forming units (cfu)/ml PAO-1, a well-characterized strain of Pseudomonas aeruginosa,20-22 and 0.5 mCi 125I-albumin (Merck-Frosst) as an airspace protein tracer was instilled into the left lungs over 30 min. We used PAO-1 as the bacterial strain because previous experiments reported the quantity of lung injury induced by this strain at this dose in uninjured lungs.20 Blood pressure, arterial gas exchange, and peak airway pressures were monitored every 30 min. Eight hours after the bacteria was instilled (32 h after the intrapulmonary acid or PBS), the animals were anesthetized deeply and killed by transection of their abdominal aortas. The lungs were removed via median sternotomies. PE-50 catheters were placed into the left lower lobes to retrieve all of the remaining instillates. The right and left lungs were homogenized separately and samples were taken using a sterile technique for bacterial cultures and to measure radioactivity and extravascular lung water. Samples of the homogenized lungs were diluted with sterile water and quantitatively cultured so the colony forming units in each lung could be determined.

The four animals in group 1 (saline given intravenously plus PBS given intratracheally plus bacteria) received intravenous saline as pretreatment, 1 h before the instillation of PBS (1.2 ml/kg) into their right lower lungs, before the bacterial challenge.

The four animals in group 2 (saline given intravenously plus HCl given intratracheally plus bacteria) received intravenous saline as pretreatment and then HCl (1.2 ml/kg, pH 1.25) into their right lower lungs before the bacterial challenge.

The four animals in group 3 (pentoxifylline given intravenously plus HCl given intratracheally plus bacteria) received intravenous pentoxifylline (20 mg/kg given intravenously) 1 h before the acid instillation, and then a 6 mg · kg⁻¹ · h⁻¹ infusion of pentoxifylline for 8 h. The pentoxifylline infusion was discontinued; 16 h later, the animals were anesthetized again and received their intrapulmonary bacterial challenge.

The four animals in group 4 (anti-TNF-α given intravenously plus HCl given intratracheally plus bacteria) received anti-TNF-α antiserum (1 ml given intrave-
nously) as pretreatment 15 min before acid was instilled into their right lower lungs. They then received bacterial challenges in the same manner as the other three groups.

Calculations of Endothelial and Alveolar-Epithelial Barrier Permeability to Protein

To evaluate endothelial permeability, we measured the accumulation of the vascular protein tracer in the extravascular spaces of the lung, as we have before. Accumulation of the vascular protein tracer $^{131}I$-albumin was calculated by the total extravascular counts of $^{131}I$-albumin in the lung divided by the average counts in the plasma after 8 h, and it was expressed as plasma equivalents in milliliters.

Two measurements were used to monitor the bidirectional flux of albumin across the alveolar-epithelial barrier. In the first method, the accumulation of $^{125}I$-albumin (the airspace protein tracer) in the plasma was measured. The $^{125}I$-albumin in the circulating plasma was determined by multiplying the radioactivity in a sample of plasma obtained at the end of the experiment by the estimated plasma volume [body weight in grams $\times 0.07(1 - \text{hematocrit} \times 100^{-1})$]. The $^{125}I$-albumin in the plasma is expressed as a percentage of the $^{125}I$-albumin that had been instilled in the lungs. The total quantity of $^{125}I$-albumin instilled into the lung was determined by multiplying the radioactivity (counts $\cdot$ min$^{-1} \cdot$ gram$^{-1}$) of the alveolar instillate, in duplicate samples, by the total volume instilled into the lung. The residual $^{125}I$-albumin remaining in the lung at the end of the experiment was calculated by multiplying the average radioactivity of two 0.5-g samples obtained from the lung homogenate by the total volume of the lung homogenate. The total counts in the lung homogenate were added to the counts recovered in the aspirated alveolar fluid to equal the residual $^{125}I$-albumin in the lung. According to these calculations, we can account for 95% or more of the instilled radioactivity.

With the second method, we measured the vascular protein tracer $^{131}I$-albumin entering the airspace compartment of the lung. The $^{131}I$-albumin radioactivity in the plasma during the course of the experiment was averaged, and the $^{131}I$-albumin radioactivity in the airspaces was expressed as a ratio over the mean plasma counts.

Statistical Analyses

Data are presented as the mean ± 1 SD of the mean. Arterial blood gas data are shown as median and ranges. Differences between the baseline and 8-h blood gas data were analyzed using paired Student $t$ tests; differences between the 8-h data of the different groups were compared using analysis of variance and a Student–Newmann–Kuels test. We accepted a $P$ value < 0.05 as significant.

Results

Pretreatments for Acid-instilled Rabbits before Bacterial Challenge

Arterial Gas Tension. The pentoxifylline or anti-TNF-α antisera pretreatments inhibited the acid-induced decrease in oxygenation to the same degree; there was no significant difference between the arterial oxygen tensions ($P_{aO_2}$) in the two pretreatment groups (Table 2). Therefore, in terms of arterial oxygenation, the two agents were of similar benefit. The administration of nonimmune goat serum before the instillation of the acid did not inhibit the decrease in oxygenation (Table 2) or change any parameter measured after acid instillation compared with administration of saline before the instillation of acid. Blood pressures, airway pressures, and heart rates were not different among the groups (data not shown).

Myeloperoxidase Levels. The pentoxifylline and anti-TNF-α antisera pretreatments both prevented the acid-induced increases in myeloperoxidase levels in the acid-instilled lungs, in the noninstilled lungs, and in the small intestines (fig. 2).

<table>
<thead>
<tr>
<th>Control groups</th>
<th>N</th>
<th>Baseline</th>
<th>8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline iv + PBS it</td>
<td>6</td>
<td>519 (512–601)</td>
<td>567 (555–586)</td>
</tr>
<tr>
<td>Saline iv + HCl it</td>
<td>6</td>
<td>542 (498–587)</td>
<td>385† (63–433)</td>
</tr>
<tr>
<td>Nonimmune serum iv + HCl it</td>
<td>3</td>
<td>540 (511–560)</td>
<td>346‡ (290–377)</td>
</tr>
<tr>
<td>Pretreated groups</td>
<td>6</td>
<td>566 (537–601)</td>
<td>541 (361–619)</td>
</tr>
<tr>
<td>Anti-TNF serum iv + HCl it</td>
<td>6</td>
<td>594 (542–631)</td>
<td>574 (517–599)</td>
</tr>
</tbody>
</table>

Data are median (range).

† $P < 0.05$ versus baseline.
‡ $P < 0.01$ versus 8 h data of groups saline iv + PBS; PTX iv + HCl; and anti-TNF serum iv + HCl by ANOVA.

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Extravascular Lung-water Measurements. The pentoxifylline and anti-TNF-α antiserum given as pretreatments completely inhibited the increase in EVLW of the noninstilled lungs; neither of these pretreatments inhibited the increase in the EVLW of the acid-instilled lungs (fig. 3).

Tumor Necrosis Factor α Activity. Both pretreatments blocked TNF-α activity in the plasma and in the bronchoalveolar lavage fluids of acid-instilled animals (table 1). In contrast, acid-instilled rabbits without pretreatment had TNF-α activity in both plasma and bronchoalveolar lavage fluids (table 1). Therefore, both the pentoxifylline and anti-TNF-α antiserum were equally able to block TNF-α activity during the first 8 h.

Interleukin-8 Activity. Pretreatment with pentoxifylline, but not pretreatment with anti-TNF-α antiserum, inhibited increases in the IL-8 concentration in the lavage fluids obtained from the noninstilled lungs (fig. 4). Neither of the pretreatments inhibited the increases of IL-8 concentrations in the lavage fluids obtained from the acid-instilled (right) lungs (fig. 4) or in the plasma of the acid-instilled animals (data not shown).

Although the two pretreatments lead to significantly different IL-8 concentrations in the bronchoalveolar lavage fluid, the myeloperoxidase activity, an indicator of neutrophil number, was similar for both pretreatment groups in the noninstilled and in the acid-instilled lungs (fig. 2).

Effects of the Pretreatments and Acid Instillations on Subsequent Bacteria-induced Left Lung Injury Arterial Gas Tension. Eight hours after the bacteria was instilled, the PaO₂, was significantly less in the untreated acid-instilled animals and in the anti-TNF-α antiserum-pretreated acid-instilled animals compared with their baseline values (table 3). Furthermore, the 8-h PaO₂ values in these two groups were significantly less than those in the other two groups (table 3).

Endothelial Permeability. Pretreatment with pentoxifylline was associated with significantly less EVLW in the left lungs after bacterial challenge than was the
TWO ANTIMFLAMMATORY PRETREATMENTS

Table 3. Arterial Oxygen Tension in Pretreated versus Control Rabbits (8 h after Bacterial Challenge)

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>N</th>
<th>Baseline (24 h after Acid Instillation)</th>
<th>8 h after Bacterial Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline iv + PBS it + bacteria</td>
<td>4</td>
<td>447 (423–548)</td>
<td>404 (333–546)</td>
</tr>
<tr>
<td>Saline iv + HCI it + bacteria</td>
<td>4</td>
<td>523 (267–575)</td>
<td>289*§ (66–378)</td>
</tr>
<tr>
<td>Pretreated groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTX iv + HCI it + bacteria</td>
<td>4</td>
<td>466 (315–577)</td>
<td>481 (262–537)</td>
</tr>
<tr>
<td>Anti-TNF serum iv + HCI it + bacteria</td>
<td>4</td>
<td>385 (237–440)</td>
<td>70†† (32–86)</td>
</tr>
</tbody>
</table>

Data are median (range).
* P < 0.05 versus baseline.
† P < 0.01 versus baseline by paired t test.
‡ P < 0.01 versus 8 h data of all others groups by ANOVA.
§ P < 0.05 versus 8 h of saline iv + PBS it + bacteria, PTX iv + HCI it + bacteria by ANOVA.
iv = intravenously; it = intratracheally.

anti-TNF-α pretreatment (fig. 5). Pentoxifylline pretreatment also was associated with significantly less accumulation of the vascular protein tracer in the left lungs after bacterial challenge than was the anti-TNF-α pretreatment, and anti-TNF-α pretreatment was associated with significantly greater accumulation of the vascular protein tracer (fig. 6).

Epithelial Permeability. After the bacterial instillation, the efflux of the $^{125}$I-albumin airspace tracer from the left lungs into the circulation was greater in the animals that had acid instilled compared with the animals that had PBS instilled (fig. 7). Although not statistically different, these results suggest that the acid injury to the right lung affected the response of the left lungs to the bacterial challenge and accounted for approximately 5% of the observed $^{125}$I-albumin efflux from the airspaces of the left lungs. Pretreatment with pentoxifylline, but not with anti-TNF-α antiserum, prevented the increase in $^{125}$I-albumin efflux in the animals with acid instilled after the bacterial challenge (fig. 7). The epithelial permeability of the left lungs decreased significantly in the pentoxifylline-pretreated animals.

Bacterial Culture Data

There were no differences between the numbers of bacteria in the bacteria-instilled (left) lungs of animals, regardless of whether they were pretreated with pen-

Fig. 3. The extravascular lung water. The extravascular lung water measurement of the noninstilled lungs increased significantly in the animals that received acid instillations, an increase that was blocked equally by both pretreatments. Pretreatments did not block the increase in extravascular lung water measurement in the acid-instilled lungs. Significantly different from the animals that received phosphate-buffered saline (P < 0.05). Significantly different from the animals that received phosphate-buffered saline, pentoxifylline pretreatment, and anti-tumor necrosis factor α pretreatment (P < 0.01).
toxifylline (3.3 ± 5.2 × 10^{10} cfu/ml tissue homogenate) or with anti-TNF-α antiserum (2.7 ± 2.2 × 10^{10} cfu/ml); nor was there a difference between the numbers of bacteria in the noninstilled (right) lungs of animals pretreated with pentoxifylline (0.3 ± 0.7 × 10^{10} cfu/ml) or with anti-TNF-α antiserum (4.9 ± 9.6 × 10^{10} cfu/ml).

Discussion

To meet the first objective of our investigation, we showed that the two antiinflammatory pretreatments, pentoxifylline and anti-TNF-α antiserum, were similar in their ability to inhibit the initial lung injury induced by unilateral acid instillation. Although neither agent could prevent the increase in EVLW in the acid-instilled lung, both agents prevented the increase in EVLW seen 8 h after acid instillation in the noninstilled, left lungs. Both agents inhibited the decrease in PaO₂ caused by the acid instillation, and both agents inhibited the increase in myeloperoxidase activity of the acid-instilled lungs, of the noninstilled left lungs, and of the small intestines. The two agents were similar in other respects; pretreatment with either the anti-TNF-α antiserum or the pentoxifylline abolished TNF-α activity in the bronchoalveolar lavage fluids and in the plasma of animals that had acid instilled during the first 8 h. Therefore, the pentoxifylline and the anti-TNF-α antiserum pretreatments similarly inhibited the initial indirect lung injury, which depended on the acid-induced generation of cytokines, including TNF-α.

Our second major objective was to learn whether the administration of either of the two antiinflammatory pretreatments affected the quantity of lung injuries induced by a subsequent bacterial airspace instillation. Previously, we showed that instillation of the same inoculum of the same bacteria, PAO-1, caused moderate lung injury. The parameters of lung injury measured previously included an efflux of the alveolar tracer into the circulation of approximately 10% and an increase of the EVLW to 6.3 g water/g dry weight. These values are similar to those we measured in the current experiments; the group of animals pretreated with saline, which received PBS into their right lungs and then had the PAO-1 instilled into their left lungs, had nearly identical increases in these measurements of lung injury.

In the current experiments, the bacteria were instilled after an initial acid-induced lung injury to determine the combined effects of the two injuries. The bacterial instillation was delayed for 2 h after the acid-induced lung injury to mimic the usual clinical scenario. Pentoxifylline as a pretreatment was clearly superior to the anti-TNF-α antiserum. Pentoxifylline pretreatment significantly blocked most of the lung injury in the bacteria-instilled lungs, as measured by the EVLW, the accumulation of the vascular protein tracer, and the efflux of the alveolar protein tracer. Although the arterial oxygenation measured in the pentoxifylline-pretreated acid-instilled animals was not statistically different from that of the anti-TNF-α animals, there was no overlap in the oxygen tensions measured in the two pretreatment groups. Therefore, in three of four parameters of lung injury that we measured, pentoxifylline pretreatment led to significant improvements in a delayed, bacteria-induced lung injury.

In contrast, the anti-TNF-α antiserum pretreatment did not protect against the bacterial-induced lung injury. All the parameters of lung injury measured in the animals pretreated with anti-TNF-α antiserum were similar to the measurements found in the animals that were pretreated with saline and acid instilled and that subsequently received bacterial instillations, or they were even more abnormal. These results suggest that, although the anti-TNF-α antiserum may have initially improved the acid-induced lung injury, this pretreatment was of no benefit to the animals when they were challenged subsequently with a bacterial infection. In fact, there was a trend for an increased mortality rate (data not shown) in the animals pretreated with anti-TNF-α antiserum compared with the animals pretreated with pentoxifylline.

How pentoxifylline pretreatment protects animals against lung injury is not known. Although both pretreatments decreased TNF-α activity for the first 8 h, there is reason to suppose that there was a different long-term effect. There may be, for example, a decreased duration, a decreased intensity of blockage of TNF-α after pentoxifylline than after polyclonal antiserum, or both. Evidence from clinical studies suggests that increasing doses of anti-TNF-α and more extensive neutralization of TNF-α may be accompanied by an increase in the mortality rate in patients with sepsis. Several studies have found that TNF-α is important to host defense against intracellular bacteria and fungi, and its blockade also may impair host defense against gram-negative bacteria, as used in this study. Pentoxifylline has known anti-TNF-α effects, although via a different, more proximal mechanism than TNF-α blockade. It protects cells from TNF-α-mediated injury and decreases the rate of TNF-α mRNA production in vitro in response to endotoxin stimulation.
Pentoxifylline also protects against endotoxin-induced shock in vivo by decreasing TNF-α levels. In vitro studies, after pentoxifylline treatment is discontinued, cells have enhanced TNF-α production, which is thought to be caused by an enhanced sensitivity of the endothelial components in the signaling pathway. Although TNF-α activity was absent from the plasma in the infected animals that were pretreated with pentoxifylline and with the anti-TNF-α antiserum, TNF-α activities may have been different locally in the lungs of the two pretreatment groups.

Pentoxifylline also has many antiinflammatory actions separate from its effects on TNF-α. It has been shown to decrease neutrophil-mediated lung injury in many different experimental paradigms. Pentoxifylline decreases leukocyte aggregation and inhibits superoxide anion generation. Pentoxifylline, however, does not prevent the CD18-dependent or independent phase of complement-mediated neutrophil sequestration within the pulmonary microvasculature, nor does it affect the release of neutrophils from the bone marrow. Because neutrophil emigration into the alveolar space is not known to be altered directly by pentoxifylline, the decrease in neutrophil influx seen with pentoxifylline is probably caused by the pentoxifylline-mediated effects on mediators of neutrophil influx.

Because IL-8 blockade can prevent acid-induced lung injury, IL-8 measurements were made in the bronchoalveolar lavage fluids. Compared with pretreatment with anti-TNF-α antiserum, pretreatment with pentoxifylline was associated with significantly lower IL-8 levels in the lavage fluids from the noninstilled lungs, despite similar IL-8 concentrations in the acid-instilled lungs. Because TNF-α blockade was similar with the two pretreatments, this result suggests that pentoxifylline may decrease IL-8 levels through mechanisms other than blocking of TNF-α. Because both pretreatments prevented the increase in myeloperoxidase activity of the noninstilled lungs, blockade of IL-8 alone does not appear to account for the decrease in the numbers of neutrophil influx seen in the noninstilled lungs, although the distribution of polymorphonuclear cells between the interstitial and the alveolar compartments may have been different. Blockade of IL-8 has been shown to inhibit acid-induced lung injury, and therefore the lower IL-8 levels may account for some or all of the inhibition of the subsequent bacteria-induced lung injury measured in the infected animals that were pretreated with pentoxifylline compared with those that were pretreated with anti-TNF-α. Although the mechanism of IL-8 induction and the role of IL-8 in these experiments is not known, the persistently low IL-8 levels in the animals pretreated with pentoxifylline suggests an effective antiinflammatory effect of the pentoxifylline.

The bacterial challenge introduced a complex toxic and inflammatory stimulus into the noninstilled lungs. Indeed, the lung injury induced by the Pseudomonas bacteria has been shown to result from the exoproteins of the bacteria and from the inflammatory response of the host to the bacteria. Because the numbers of bacteria were no different in the pretreatment groups, we suspect that the difference in injury seen in these experiments resulted from differences in the inflammatory host response as modulated by the two pretreatments. In fact, the only difference between the pretreatment groups noted before the bacterial challenge was the higher alveolar concentration of IL-8 in the anti-TNF-α group compared with the pentoxifylline pretreatment group. The higher inflammatory cytokine concentration may have contributed to a different host response to the bacterial challenge that followed. At least at the

Fig. 5. The extravascular lung water of the bacteria-instilled left lungs of the pretreated and control animals. In the pentoxifylline-pretreated animals, the extravascular lung-water measurement of the bacteria-instilled left lungs was significantly less than that in the animals pretreated with anti-tumor necrosis factor α. *Significant at P < 0.05 when compared with animals pretreated with anti-tumor necrosis factor α.
Fig. 6. The total plasma equivalents in the bacteria-instilled left lungs of the pretreated and control animals. In the animals previously exposed to acid, the total plasma equivalents of the opposite, bacteria-instilled lungs were significantly increased after bacterial challenge. This increase was blocked by pentoxifylline but not by anti-tumor necrosis factor α (TNF-α) pretreatment. In the animals pretreated with anti-TNF-α antisera, the total plasma equivalents were significantly increased compared with the control animals that had not received acid but had bacteria instillations. *Significant at P < 0.01 compared with animals pretreated with anti-TNF-α. †Significant at P < 0.05 compared with animals given saline intravenously plus HCl given intrathecally plus bacteria. ‡Significant at P < 0.05 compared with animals given saline intravenously plus phosphate-buffered saline given intrathecally plus bacteria.

Intervals evaluated, the pretreatments did not interfere with local lung antibacterial defenses.

The limitations of the current studies include the small number of animals used in these difficult experiments; the small numbers may have precluded our finding statistical differences (i.e., differences between oxygenation between the groups). In addition, we administered 100% oxygen to the animals during the experiments. This was done to limit the hypoxemia during the acid and bacteria-induced lung injuries, but it may have caused some of the lung injury that was measured. Nonetheless, because all the animals received the same oxygen concentrations, all the animals should have been affected equally; therefore, the ultimate conclusions of the investigation should be valid.

Many therapies have been proposed for acid-induced lung injury. The benefit of these therapies appears to be their ability to decrease the inflammatory response affecting the non-acid-injured lung. Several of the proposed therapies affect neutrophil migration, complement generation, and TNF-α activity. This study shows that two antiinflammatory therapies that equally prevented acid-induced lung injury had significantly different effects on a subsequent bacteria-induced lung injury. The results suggest that the mechanism of TNF-α blockade may be an important issue for host defense and that increases in IL-8 may contribute to lung injury. Because bacterial infections occur frequently in patients with acid aspiration, as they do in other patients with lung injury, potential therapies for lung injury need to be assessed in terms of their effect on subsequent bacterial infections.

Fig. 7. The percentage efflux of the alveolar protein tracer, a measure of lung epithelial injury, from the bacteria-instilled left lungs of control and pretreated animals. In the animals pretreated with pentoxifylline, the efflux of the alveolar protein tracer (125I-albumin) was significantly less than in the animals pretreated with saline–HCl or anti-tumor necrosis factor α. In fact, the animals pretreated with pentoxifylline had significantly less epithelial injury than did the control animals, which received bacteria but not acid. *Significant at P < 0.01 when compared with the group that received anti-tumor necrosis factor α intravenously plus HCl intrathecally plus bacteria and the group that received saline intravenously plus HCl intrathecally plus bacteria. †Significant at P < 0.05 when compared with the group that received saline intravenously plus phosphate-buffered saline intrathecally plus bacteria.
The interleukin-8 (IL-8) concentrations in the bronchoalveolar lavage from the acid-instilled right lung and the bacteria-instilled left lung of pretreated and control animals. There was no difference in the IL-8 concentrations in the bronchoalveolar lavage from the acid-instilled right lungs in the three groups of animals. However, the IL-8 concentration in the bronchoalveolar lavage from the bacteria-instilled left lungs of the animals pretreated with anti-tumor necrosis factor α was increased significantly compared with that of the other two groups. Significant at \( P < 0.01 \) compared with the other two groups of left lungs (pentoxifylline and saline).

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