

Pulmonary Histopathology in an Experimental Model of Chronic Aspiration Is Independent of Acidity

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Gastroesophageal reflux has become a major health concern in industrialized countries, with drugs aimed at blocking acid production being more frequently prescribed than any other drug. Damage to lung tissue as a result of chronic aspiration of gastric fluid is a primary health risk associated with gastroesophageal reflux, with such aspiration being suspected in the induction or exacerbation of asthma and other lung diseases. In this study, a rodent model of chronic aspiration was used to characterize the pulmonary histopathology produced by repetitive aspiration events and to investigate the pathologic roles of individual gastric fluid components such as acid and particulate food matter. Rats exposed to chronic aspiration of whole gastric fluid developed a pathology distinct from that of acute lung injury, characterized by granulomatous interstitial pneumonitis with prominent formation of multinucleated giant cells. This pattern of injury could be reproduced with chronic aspiration of particulate food matter and with chronic aspiration of pH-neutralized gastric fluid, but not with chronic aspiration of hydrochloric acid. Thus, since acid-neutralizing therapy is currently the mainstay of treatment for patients with reflux-associated respiratory symptoms, these results strongly suggest that alternative therapeutic approaches aimed at preventing chronic-aspiration induced lung injury may be warranted. *Exp Biol Med* 233:1202–1212, 2008

Key words: chronic aspiration; gastroesophageal reflux disease, GERD; lung injury; pulmonary

Introduction

Aspiration of gastric contents has long been recognized as a cause of acute lung injury and subsequent pulmonary failure (1). Beginning with the description of “Mendelson Syndrome” in 1946, the phenomenon of lung damage following a single, large-volume aspiration event has been repeatedly observed in the clinical arena as well as demonstrated experimentally in animal models (2, 3). What remains to be thoroughly investigated is the role that more chronic forms of aspiration play in pulmonary injury. Gastroesophageal reflux disease (GERD) is particularly interesting in this regard, as it is a known source of repetitive aspiration events and therefore a potential contributor to many chronic lung diseases.

The rationale for investigating GERD as a possible mechanism of chronic lung injury stems from clinical observations linking GERD to various forms of pulmonary disease. GERD is extremely common in Western countries, with a reported prevalence of up to 45% in the adult population (4, 5). Importantly, a higher prevalence of GERD has been associated with a number of chronic lung diseases including cystic fibrosis, asthma, idiopathic pulmonary fibrosis, and chronic obstructive pulmonary disease (6–8). Other epidemiologic data suggest that GERD is the most common cause of chronic aspiration. One study demonstrated a 28% incidence of aspiration events in patients with GERD, with 60% of patients affected by aspiration demonstrating significant respiratory symptoms (9). Furthermore, a substantial percentage of cases of chronic lung disease have no identifiable cause (10), and the majority of patients suffering from unexplained chronic respiratory symptoms have occult GERD (11).

Because GERD is highly prevalent in patients with severe chronic lung disease, it is commonly observed in those who undergo lung transplantation. The presence of GERD in transplant recipients is correlated with a faster decline in allograft function (12), which can in many cases be at least partially reversed by anti-reflux surgery (13). These obser-

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variations have led some authors to speculate that aspiration injury may contribute to development of the bronchiolitis obliterans syndrome and subsequent allograft rejection (14). Ongoing experimental work in our lab supports this hypothesis, as we have seen accelerated graft dysfunction in lung-transplanted rats receiving gastric fluid aspirations.

Although these clinical and experimental data are compelling, there is little information available regarding the cellular and molecular mechanisms behind GERD-associated chronic lung injury. In particular, the precise etiologic agent(s) of tissue damage are unknown. Gastric fluid may contain acid, food particles, proteases and other enzymes, and, in some circumstances, bile. Many of these components have already been demonstrated to cause acute lung injury in the setting of a single experimental aspiration event (15, 16), but the exact magnitude of their contribution to chronic lung injury has not yet been elucidated. Because the acidity of aspirated fluid has been shown to be a key pathologic factor in acute lung injury models (17), it might be reasonable to hypothesize a similar role for acid in chronic aspiration models.

If we hypothesize that the acidity of aspirated gastric fluid is largely responsible for pulmonary tissue damage, the advent of acid-inhibiting drugs might have lessened the association between GERD and chronic lung diseases. In fact, the use of proton pump inhibitors (PPIs) in patients with GERD and unexplained pulmonary disease is now widespread, but the epidemiology remains unchanged, and response to therapy is often less than desirable (18, 19). Trials of PPI therapy in patients with GERD and asthma have thus far produced mixed results, providing no clear evidence that reducing gastric acidity improves lung function (6, 20). Limited studies of anti-reflux surgery have produced better functional outcomes, indicating that eliminating aspiration completely may be more advantageous than simply neutralizing the aspirated fluid (21). Currently available clinical data therefore suggest that gastric acid may not be the sole agent responsible for chronic aspiration-associated lung damage.

In this experiment, we hypothesized that components of gastric fluid other than acid contribute significantly to the long-term pulmonary damage seen with chronic aspiration. We sought first to characterize the histologic tissue response to repeated aspiration of whole gastric contents, and then to identify the individual constituents most responsible for the observed pathology. A rat model was used to simulate chronic aspiration of whole gastric fluid or its individual components. Lung histopathology as well as local and systemic markers of inflammation were then evaluated to assess the contribution of each component to the global pattern of chronic injury.

Materials and Methods

All procedures involving animals were approved by the Duke University Institutional Animal Care and Use Committee prior to the investigation.

Preparation of Gastric Fluid and Components. Whole gastric fluid was obtained from male F344 rats (Harlan Sprague Dawley, Indianapolis IA) as described previously (22). The fluid used in these experiments was a pooled sample from multiple rats, first strained through a 70-micron mesh to eliminate large particles and then stored at -80°C until needed. The pH of the gastric fluid was measured at 2.2 immediately prior to use. The particulate content of the gastric fluid was estimated to be 0.5% based on the mass of insoluble material in the gastric fluid that could be recovered by centrifugation for 10 minutes at $14,000 \times g$, and the bile concentration was calculated to be approximately 1% based on the visible-light spectrum of the gastric fluid.

A suspension of food particles was prepared by grinding standard laboratory rodent chow (Lab Diet 5015, PMI Nutrition) in water to a fine slurry, and filtering the slurry through a 70-micron mesh to eliminate large particles. The food powder was then lyophilized, resuspended in normal saline at 5% weight-by-volume, and stored at -80°C . The pH of the suspension was measured at 6.8 immediately after preparation. As gastric fluid contents *in vivo* are highly variable, this 5% concentration was designed to be approximately 10-fold greater than the particulate content of our collected gastric fluid. Technical aspects of the gastric fluid collection procedure required the rats to consume no food for approximately 12 hours prior to collection, so the fluid we obtained likely under-represented the average particulate concentration of gastric contents. In our component studies we chose to aim for a higher (although still presumably physiologic) concentration of each constituent to ensure that any pathologic contributions from that constituent would be adequately identified, if present.

Bile was collected from F344 rats by cannulation of the bile duct and collection of the bile using a modification of the method described by Kamada and Calne (23). For this procedure, the bile duct was dissected free from the portal vein and an incision made in its anterior wall. The drainage catheter was inserted into the bile duct via the choledochotomy and secured with a circumferential 6-0 silk suture. The 1.2 mm outside diameter polyethylene tube used to cannulate the bile duct was left long and brought through a small stab wound in the right upper quadrant abdominal wall. With the rat suspended in a restrainer cage, all bile was collected in a test tube connected to the distal end of the tube.

A solution of 10% whole bile in normal saline was administered in these aspiration experiments, again targeting approximately a 10-fold greater concentration than that observed in the gastric fluid we collected for the experiment. The pH of the bile solution was 7.4. A solution of hydrochloric acid was titrated to pH 2.2 to match the acidity of the whole gastric fluid. Neutralized gastric fluid was prepared by titrating 12.5 mL whole gastric fluid to pH 7.0 with 1.1 mL 0.67 M sodium hydroxide solution prior to

storing at -80°C . Control rats received instillations of sterile normal saline.

Instillation of Gastric Fluid or Components.

Male F344 rats (10–12 weeks, 250–300 g) were briefly anesthetized with inhaled isoflurane and then sedated with 40 mg/kg intraperitoneal methohexital (Monarch). They were orotracheally intubated with the sheath of a 14-gauge intravenous catheter, and tube placement confirmed by successful ventilation with a small-animal mechanical ventilator (SAR-830, IITC Inc., Woodland Hills, CA). The animals were then placed in the left lateral decubitus position with their heads inclined to 40 degrees. A curved silastic catheter was passed through the endotracheal tube such that its tip extended 3–4 mm beyond the end of the tube, with the curvature directed toward the left mainstem bronchus. The desired fluid aspirates were introduced through the catheter and the rat maintained in the left lateral decubitus position for 15 minutes. This technique has been previously employed by our lab (22) to introduce fluid selectively into the left lung with a high degree of reproducibility.

Rats received aspirations of whole gastric fluid ($n = 6$), neutralized gastric fluid ($n = 7$), hydrochloric acid ($n = 6$), bile solution ($n = 5$), food suspension ($n = 6$), or normal saline ($n = 7$) at doses of 0.5 mL/kg body weight. In order to avoid acute lung injury and subsequent morbidity, this dose was selected to be significantly less than the maximal non-lethal dose reported in the literature for single aspirations of both hydrochloric acid and particulate matter (24). In all groups, instillations were conducted once weekly for a total of nine weeks, a time course which our preliminary data has shown to be sufficient to induce chronic pathology.

Sample Collection and Evaluation. One week after the final aspiration, rats were again anesthetized as described above. Whole blood was withdrawn from the inferior vena cava, and the serum separated and frozen at -80°C for cytokine analysis. A tracheotomy was created, and the heart, lungs, and trachea were explanted en bloc. Bronchoalveolar lavage (BAL) was then performed separately on the right and left lungs using 1% bovine serum albumin (BSA) in PBS, 1.5 mL for each lung. The BAL fluid was centrifuged and frozen at -80°C for cytokine analysis. Portions of all three lobes (right lung, left upper lobe, and left lower lobe) were fixed in 10% neutral formalin and then cut in 5 μm thick cross-sections through the middle of the lobe so that each section included hilum and periphery. Sections were placed on positively charged slides (Superfrost® Plus slides, Port City Diagnostics, Inc., Wilmington, NC), deparaffinated, and stained with hematoxylin and eosin using standard procedures.

Stained slides were then evaluated by a pulmonary pathologist, and the degree of lung injury scored using a semi-quantitative grading system. This novel grading scale assigned scores from 0 to 3 to lung tissue samples ranging from normal to severely damaged (See Table 1). Intra-alveolar white blood cell (WBC) infiltrates were also

Table 1. Semi-Quantitative Grading System for Chronic Aspiration-Induced Pneumonitis

Grade	Histologic findings
0	Normal lung. No significant difference from controls.
1	Septal congestion with increase in interstitial edema and cellularity. Focal atelectasis. Findings involve at least 25% of tissue sample. No granulomas.
2	Mild interstitial pneumonitis, nodular intra-alveolar histiocyte accumulations. Rare granulomas.
3	Granulomatous pneumonitis with prominent giant cells. Septal collapse with areas of atelectasis. Accumulations of mucin and debris in airways.

quantified. From each lung lobe, two microscopic high-power fields were randomly selected. The number of WBCs per high-power field were counted and then averaged across the two sampled fields.

For cytokine analysis, serum and BAL samples were thawed, and levels of IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN- γ , and TNF- α were assayed using a BioSource bead-based multiplex immunoassay (Invitrogen-BioSource, Carlsbad, CA). Readings were taken on a BioPlex/Luminex-100 dual laser array reader and data analyzed using Bio-Plex Manager software (BioRad Laboratories, Hercules, CA). Analysis of the data reveals that the baseline fluorescence in the presence of the BAL fluid was shifting compared to the blanks and other controls without BAL fluid. This shift was likely due to surfactant or some other factor in the BAL fluid that is apparently not present in the other reagents used in the study. However, the fluorescence intensity was linear over the range of interest in samples with BAL fluid, and thus relative but not absolute measures of cytokine levels in the BAL fluid could be obtained.

Statistical Analyses. Statistical analyses were performed using GraphPad Prism software (v. 3.00). All comparisons of quantitative data across three or more groups utilized the one-way ANOVA, with a Bonferroni correction for multiple analyses when post-tests were required. Comparisons between two groups utilized the Student's t test, which was paired when comparing right and left lungs. Because the histologic grading data are semi-quantitative and non-parametric, the Kruskal-Wallis rank sum and Dunn's multiple comparison tests were used for these analyses. Right and left lungs were compared using the Wilcoxon matched pairs test. All P values reported are two-sided.

Results

Histology. Histologic specimens were first examined for quality and degree of inflammation. Lungs treated with each component of gastric fluid were compared with those receiving normal saline and whole gastric fluid. Treated left

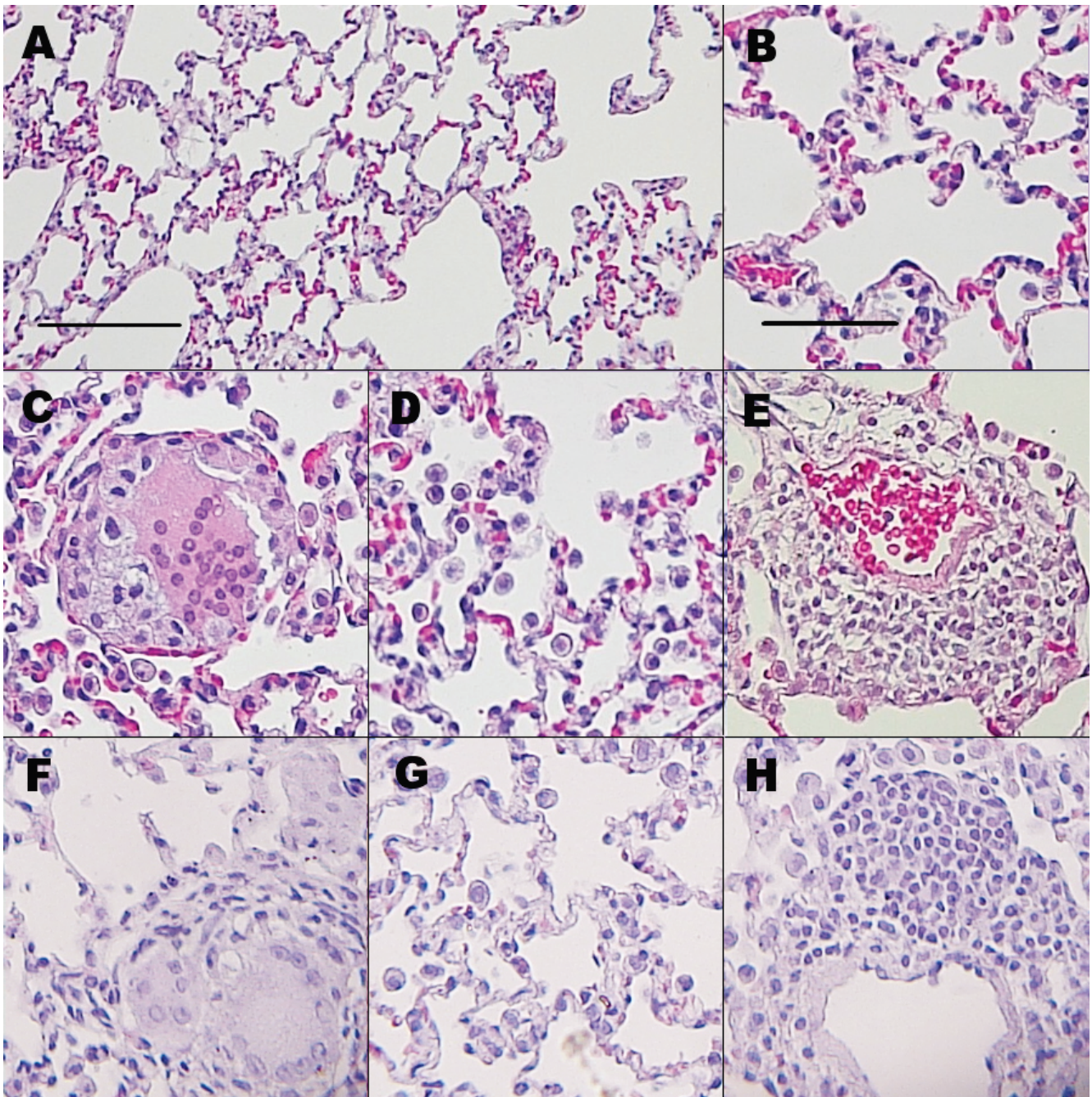


Figure 1. Histopathology of lungs receiving chronic aspirations of normal saline, gastric fluid, or neutralized gastric fluid. Sections taken after nine weeks. A–B. Left lungs treated with normal saline showing no pathology. C–E. Left lungs treated with whole gastric fluid showing multi-nucleated giant cells, chronic inflammatory infiltrates, and perivascular infiltrates. F–H. Left lungs treated with neutralized gastric fluid showing multi-nucleated giant cells, chronic inflammatory infiltrates, and perivascular infiltrates. Bar = 100 μ m (A) and bar = 50 μ m (B–H).

lungs were also compared to untreated right lungs in each rat. Rats receiving aspirations of normal saline showed no evidence of acute or chronic cellular infiltrates in either lung and no observable difference in histology between left and right lungs (Fig. 1A–B). In contrast, left lungs of rats treated with whole gastric fluid demonstrated an interstitial and perivascular pneumonitis, with prominent formation of multinucleated giant cells and scattered granulomas

(Fig. 1C–E). Untreated lungs from gastric fluid-aspirated rats lacked this pathology, closely resembling lungs treated with saline.

Lungs receiving individual components of gastric fluid were examined for an inflammatory pattern similar to that seen with whole gastric fluid. Those treated with neutralized gastric fluid were indistinguishable from those receiving whole gastric fluid, demonstrating the same pneumonitis

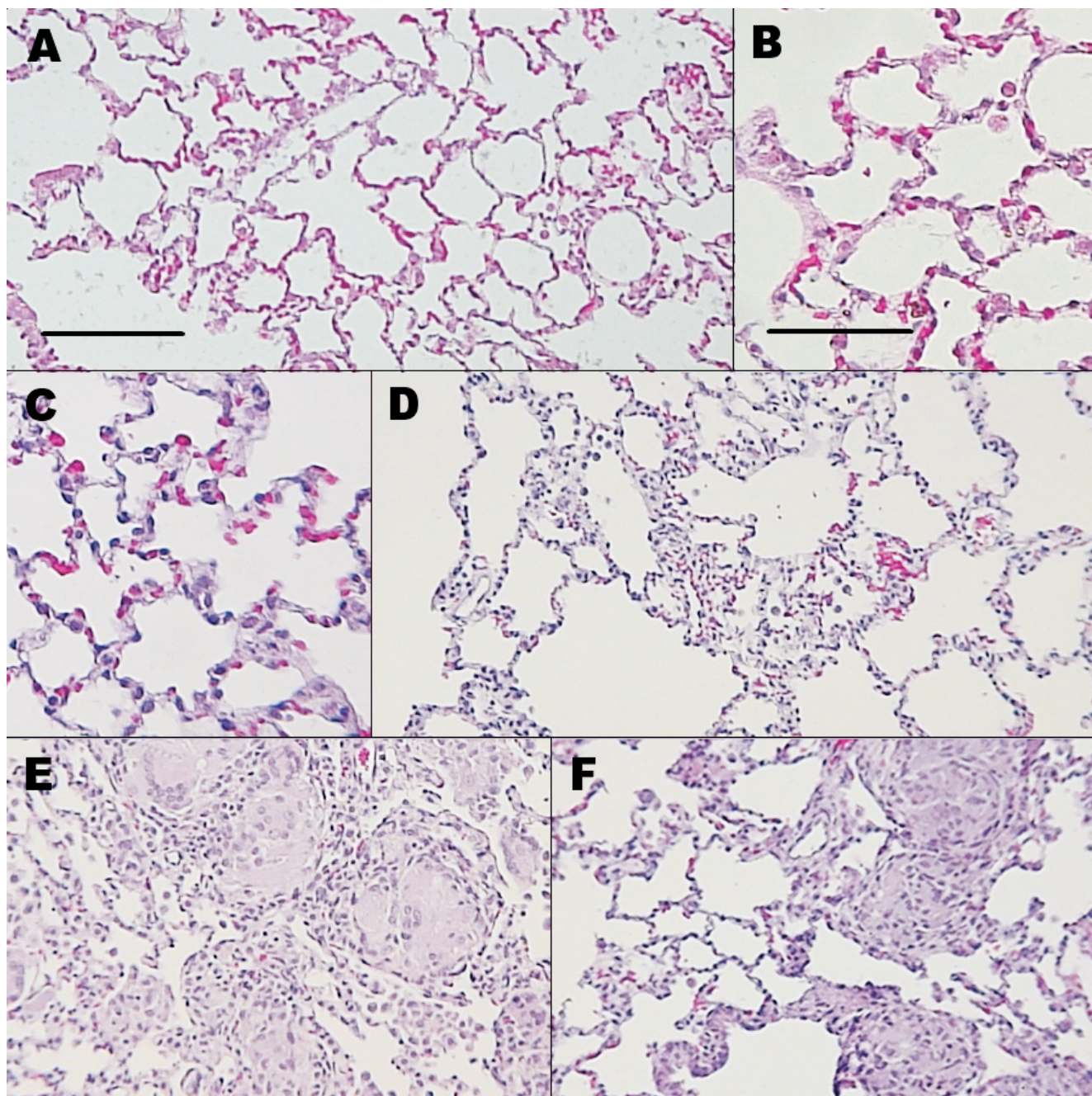


Figure 2. Histopathology of lungs receiving chronic aspirations of hydrochloric acid, bile, or food suspension. Sections taken after nine weeks. A–B. Left lungs treated with hydrochloric acid showing no pathology. C–D. Left lungs treated with bile showing no pathology. E–F. Left lungs treated with food suspension showing multi-nucleated giant cells, chronic inflammatory infiltrates, and effacement of lung architecture. Bar = 100 μ m (A, D–F) and bar = 50 μ m (B–C).

with giant cell and granuloma formation (Fig. 1F–H). In rats treated with hydrochloric acid or bile, the lung specimens were indistinguishable from controls (Fig. 2A–D). Lungs treated with food suspension showed the most severe pathology, with large, well-formed granulomas causing effacement of the lung architecture (Fig. 2E–F).

Numeric grading of pulmonary histopathology using a semi-quantitative scoring system (See Table 1) supported the qualitative observations reported above. All left lungs

from rats treated with normal saline were scored at 0, representing no change from normal lung tissue (Fig. 3). All left lungs treated with whole gastric fluid demonstrated a median pneumonitis score of 2, which was significantly increased over untreated lungs from the same rats (right lung median 1, $P < 0.02$) as well as control lungs receiving normal saline (median difference 2, $P < 0.01$). Rats treated with neutralized gastric fluid showed a similar pattern of pathology, with all left lungs scoring 2. This represented a

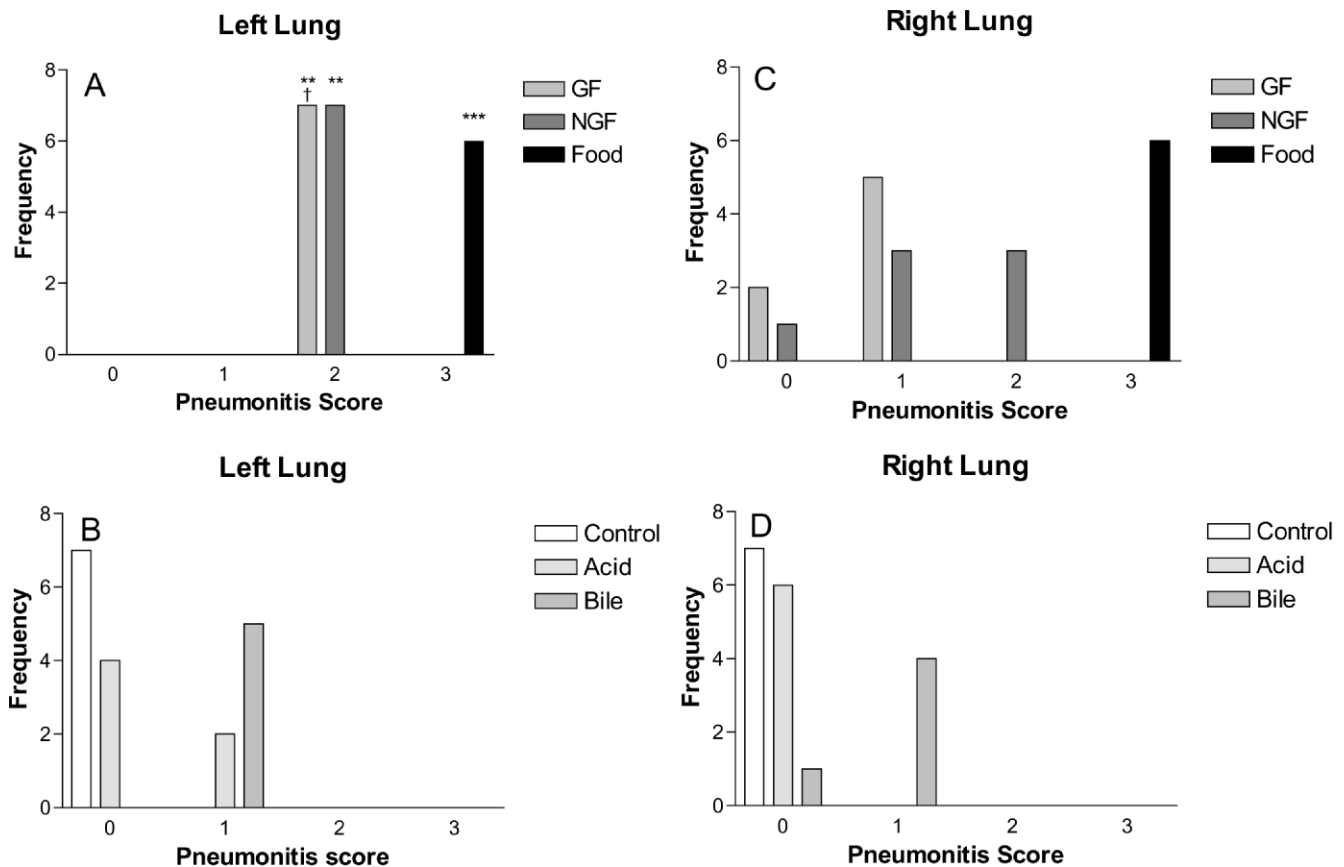


Figure 3. Frequency distributions of pneumonitis scores at nine weeks of lungs receiving chronic aspirations of whole gastric fluid or components. GF = gastric fluid, NGF = neutralized gastric fluid. Significance levels are reported over median score for each group, and correspond to comparison of right lungs to left lungs ($\dagger$) and treated left lungs to control left lungs (*). *, $\dagger$ $P < 0.05$, **, $\dagger\dagger$ $P < 0.01$, ***, $\dagger\dagger\dagger$ $P < 0.001$.

significant increase in normal saline controls (median difference 2, $P < 0.01$). Left lungs treated with neutralized gastric fluid showed a trend toward increased damage as compared to untreated right lungs of the same rats (right lung median score 1).

Examination of lungs treated with gastric fluid components demonstrated that little damage was produced by instillation of acid alone. Acid-treated lungs had a median score of 0 (range 0 to 1), which was not significantly different from untreated right lungs or saline controls. Lungs receiving food particles, by contrast, were severely damaged. In these animals, all left and right lungs received scores of 3, which was a marked increase over saline controls (median difference 3, $P < 0.001$). Left lungs treated with bile showed minor injury with a mean score of 1 (range 0 to 1); however, this was similar to the untreated right lungs from these animals, which were all graded at 1 and not significantly different from saline controls.

Finally, intra-alveolar cellular infiltrates were quantified as an additional measure of inflammatory severity (Fig. 4). Saline controls demonstrated few white blood cells (WBCs) per high power field in either treated left lungs (8.0 ± 0.4) or untreated right lungs (8.1 ± 1.4). Rats treated with gastric

fluid developed significant infiltrates on the left (40.5 ± 3.7) but not on the right (8.0 ± 0.9). These infiltrates were reproduced in left lungs receiving neutralized gastric fluid (35.4 ± 3.3) and food (24.5 ± 2.8), but not in their corresponding right lungs. Left lungs treated with acid showed no increase in alveolar white cells over controls (9.6 ± 0.9) while those receiving bile showed mild inflammation (14.5 ± 1.8).

The number of white blood cells (WBCs) per high-power field was increased in treated left lungs as compared to untreated right lungs in rats receiving whole gastric fluid (mean difference 32.5, $P < .001$), food (mean difference 15.5, $P < .05$), neutralized gastric fluid (mean difference 29.5, $P < .001$), and bile (mean difference 8.1, $P < .05$), but not in those rats treated with hydrochloric acid (mean difference 1.6, $P = .24$) or control (mean difference 0.1, $P = 0.94$).

When left lungs from the treatment groups were compared with controls, WBC counts were increased in lungs receiving whole gastric fluid (mean difference 32.5, $P < .001$), food (mean difference 16.6, $P < .001$), and neutralized gastric fluid (mean difference 27.6, $P < .001$) over those given normal saline. Counts were similar to

controls in lungs treated with hydrochloric acid or bile. Infiltrates seen in lungs treated with gastric fluid were generally the most severe, being significantly increased over infiltrates in lungs treated with food, with a mean difference between the two groups of 15.9 ($P < .001$). There was no significant difference in counts between rats receiving whole versus neutralized gastric fluid. WBC counts in untreated right lungs were similar across all groups and not significantly different than the baseline values observed in control lungs.

Cytokine Analysis of BAL Fluid. Because cytokine concentrations in BAL fluid are typically low, we expected that not all cytokines tested would be measurable given the sensitivity of our bead-based assay. Levels of IL-1 α , IL-1 β , IL-6, and IL-10, were detectable with our assay, whereas GM-CSF, IFN- γ , IL-2, IL-4, IL-12, and TNF- α were not. Given the clear separation of our lung specimens into those with significant granulomatous inflammation and those lacking this pathology, we wished to investigate whether changes in cytokine levels were correlated with this histopathology.

When treated lungs demonstrating granulomatous pathology (pneumonitis score > 2.0), were compared with those without (pneumonitis score < 2.0), several significant differences were revealed (Fig. 5). Treated lungs showing high-grade pathology had depressed levels of IL-1 α when compared to rats with minimal to no histologic findings. Absolute concentrations in the BAL fluid could not be calculated due to a shift in baseline between samples and blanks as described in the Materials and Methods section; however, the relationship between fluorescent intensity and concentration remained linear within the range of interest. Given this linear relationship between fluorescent intensity and concentration, it was calculated that there was an approximate decrease in IL-1 α levels of 7.4 pg/mL in the BAL fluid from lungs with a pneumonitis score > 2.0 ($P < 0.05$). These same lungs also demonstrated decreased levels of IL-1 β (mean difference 6.5 pg/mL, $P < 0.05$) and IL-6 (mean difference 0.33 pg/mL, $P < 0.05$) in their BAL.

Interestingly, levels of IL-1 α and IL-1 β tended to be similarly depressed in the BAL fluid of both right and left lungs of affected rats. Untreated right lungs of rats whose left lungs showed granulomatous inflammation had significantly lower levels of IL-1 α (mean difference 10.3 pg/mL, $P < 0.05$) and IL-1 β (mean difference 6.3 pg/mL, $P < .05$) in their BAL when compared to the BAL of right lungs of rats with lower histologic scores. This was not the case for IL-6, however, which appeared to be depressed only in the BAL of lungs subjected to chronic aspiration. Levels of IL-10 were significantly decreased (mean difference 6.0 pg/mL, $P < 0.01$) in the BAL of untreated right lungs (but not the BAL of treated left lungs) of rats whose left lungs had granulomatous inflammation.

We then attempted to further characterize the contribution of each gastric fluid component by comparing cytokine levels across specific treatment groups. A trend toward

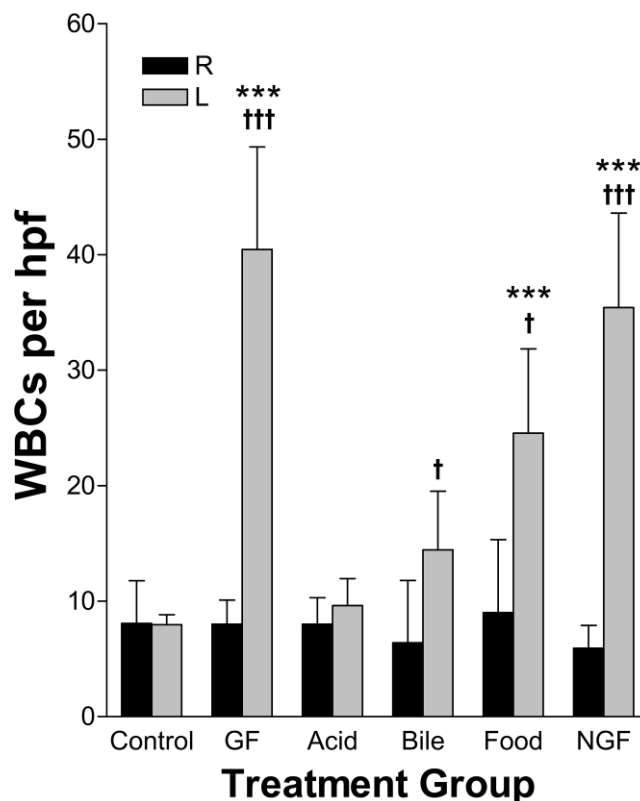


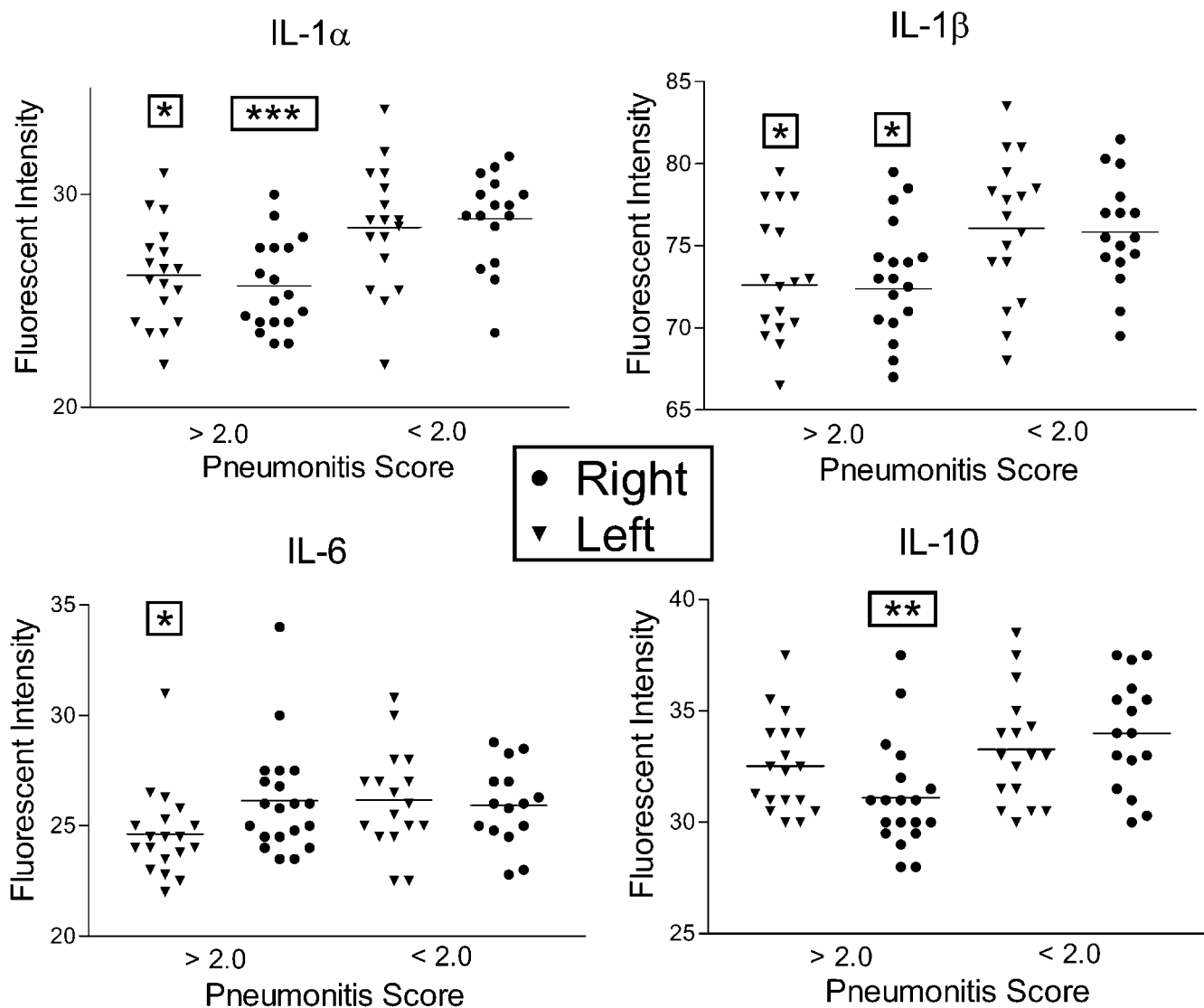
Figure 4. Mean intra-alveolar infiltrates at nine weeks in lungs receiving chronic aspirations of whole gastric fluid or components. GF = gastric fluid, NGF = neutralized gastric fluid. Error bars represent 95% confidence intervals. Significance levels are for comparison of right lungs to left lungs (†) and for comparison of treated left lungs to control left lungs (*). *, † $P < 0.05$, **, †† $P < 0.01$, ***, ††† $P < 0.001$.

decreased levels of IL-1 α was noted in the BAL from left and right lungs of rats receiving gastric fluid, whole gastric fluid and food, but it was not statistically significant. Given the small sample size and the relatively large biologic variability in cytokine production, this study was apparently underpowered to detect the potential effects of individual gastric fluid components on the cytokine environment.

Levels of IL-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN- γ , and TNF- α were also tested in serum samples from all rats. Cytokines were more easily detected in serum than in BAL fluid; however, intra-group variability was high and no significant differences in cytokine levels in BAL fluid were observed across groups. These data were consistent with the absence of a systemic immune response in response to aspiration. When combined with the observation that histopathology occurs primarily in the treated left lung, the cytokine results support the idea that the effects of aspiration in the rat model are highly localized.

Discussion

In light of the many clinical associations linking GERD with chronic lung disease, we designed this study to characterize the histopathologic and immunologic effects of



chronic gastric fluid aspiration on lung tissue. We also sought to identify the specific components of gastric fluid that contribute most to development of pulmonary pathology. Lung histology from these rats indicates that chronic aspiration of whole gastric contents results in a granulomatous pneumonitis with prominent chronic inflammatory infiltrates and giant cell formation. The lack of any pathology in left or right lungs of rats treated with normal saline implies that neither the process of intubation itself nor the instillation of an innocuous fluid is sufficient to account for the chronic histologic change seen in our model.

The interstitial pneumonitis and granuloma formation seen with whole gastric fluid aspiration were reproduced in rats receiving instillations of food particles and neutralized

gastric fluid, but not with instillations of hydrochloric acid. This suggests that particulate matter found in gastric contents is a major contributor to the histopathologic changes observed with repetitive aspiration, regardless of the pH of the aspirated fluid. The failure of gastric fluid neutralization to ameliorate the pulmonary damage and the lack of any pathology with hydrochloric acid administration alone further support a lesser role for acid in the observed histopathology.

These findings contrast with studies of acute aspiration in which the acidity of the inhaled fluid was critically important to the lung injury which followed (25). Animals in those experiments typically received a higher volume of aspirate (1.0 to 4.0 mL/kg per dose) and died within minutes

to 24 hours after the instillation, depending on dose. They developed a histologic pattern of lung injury similar to that seen in the Acute Respiratory Distress Syndrome (ARDS), with massive pulmonary edema, hemorrhage, and hyaline membrane formation. Our model produces a markedly different histology, does not cause acute mortality, and suggests that the underlying pathophysiologies of acute and chronic aspiration injury may be quite distinct, with the latter more dependent on the particulate content of inhaled fluid rather than its acidity.

In the chronic setting, exposure to particulate food suspension was so damaging that the visualized pathology exceeded the descriptive sensitivity of our pneumonitis scale. Because small quantities of aspirate may spill over into the right lung despite our selective technique, untreated right lungs in most groups did show a minor degree of injury when compared to controls. It appears that in rats treated with food, even this small inoculation of fluid into the right lung was sufficient to create the largest degree of damage describable by our scale. This finding is not unexpected given the pathology we observed in gastric fluid-treated rats, since the particulate content of the food suspension utilized in our experiments was approximately 10-fold higher than that in the harvested gastric fluid.

Bile was also investigated for a potential contribution to gastric fluid-induced pulmonary pathology; however, its role remains ambiguous at the conclusion of this study. Although bile-treated lungs did demonstrate some degree of tissue damage, the findings were restricted to mild edema and increase in interstitial cellularity. No granulomatous changes were seen, and the histopathology that was observed was as likely to be seen in untreated right lungs as in treated left lungs. These observations were made despite the fact that we administered approximately 10-fold greater concentrations of bile in the study than were present in the whole gastric fluid. While aspiration of bile may in fact be harmful, we therefore cannot conclude whether it contributes to the injury produced by aspiration of whole gastric fluid, or instead represents a completely different spectrum of pathology.

The discovery of a granulomatous pneumonitis in lungs exposed to particulate solutions like gastric fluid or food is not entirely surprising, as granuloma formation is a recognized tissue reaction to the presence of a foreign body. We also know, however, that granulomas are seen as precursor lesions to fibrosis in many chronic interstitial lung diseases (26). Because minimal fibrosis occurred in our lungs over the time course of this experiment, the question then becomes whether these aspiration-induced granulomas have the potential to progress to fibrosis if allowed to develop further.

Current understanding of the immunologic cascade leading from granulomatous inflammation to pulmonary fibrosis is incomplete; however, several authors have suggested that a change in the local cytokine profile may

select the fibrotic pathway over recovery (27, 28). In this experiment, we observed a decrease in IL-1 α , IL-1 β , and IL-6 levels in lungs demonstrating visible granulomatous inflammation. We also noted a depression of IL-10 levels in the untreated right lungs of these same animals. While it is not possible to construct the entire inflammatory picture from these observations alone, it is interesting that IL-1, a substance with a controversial role in granulomatous inflammation and fibrosis, was the cytokine most correlated with histopathologic change in our experiment. This finding merits further investigation, perhaps by utilization of IL1 inhibition to potentially affect the development of pneumonitis.

Several studies have specifically investigated the role of IL-1 in pulmonary fibrosis. In rat lungs, transient overexpression of IL-1 β causes acute inflammation, but a subsequent decrease in IL- β concentrations to undetectable levels typically precedes pulmonary fibrosis (29). In human lungs, immunohistochemical studies have shown high levels of IL-1 β to be correlated with pre-fibrotic states, while low levels are seen in tissue with chronic fibrotic disease (30). IL-1 β therefore seems to participate in the inflammatory environment which precedes fibrosis, but typically decreases before TGF- β levels rise and histologic change begins. In this experiment we examined cytokine levels at one time point only. Our observation that IL-1 β levels were decreased in lungs showing visible granulomatous inflammation may indicate that these histopathologic lesions have fibrotic potential.

If aspiration-induced granulomatous disease does indeed provide a setup for pulmonary fibrosis, our findings regarding the relative contributions of individual gastric fluid components become therapeutically important. The current mainstay of treatment for GERD-associated pulmonary symptoms is medical therapy with proton-pump inhibitors (PPIs). In our study, we could not produce any significant lung pathology with chronic administration of acid alone. More importantly, when gastric fluid was neutralized (as it would be at least partially with PPI use), the development of granulomatous inflammation was not aborted. This is not to say that acid plays no role in lung injury. We did note that the cellular infiltrates were more severe with whole gastric fluid than with food alone, so perhaps acid can potentiate the effect of particulate aspiration, even if it is not sufficient to induce pathology in and of itself. In fact, a synergistic interaction of acidic and particulate insults has been recently demonstrated in the setting of experimental acute lung injury.

Our results therefore suggest that chronic aspiration of particulate matter is injurious to the lung, and that typical gastric fluid contains enough particulate matter to elicit this effect. In the rat, this pathology is independent of acidity and cannot be prevented by gastric fluid neutralization. Clinical data in humans are largely absent from the literature, but small observational studies support the

injurious potential of particulate matter. Histologic samples taken from four patients with chronic aspiration-associated lung disease found a predominance of multinucleated giant cells surrounding food particles (31). If these data are confirmed by larger studies, anti-reflux strategies in patients most at risk for pulmonary disease may need to consider more than control of gastric pH. In those patients, a surgical strategy such as fundoplication, which actively obstructs the reflux of all gastric contents, might prove more successful than medical therapy alone.

The results of these experiments clearly suggest areas for future study. The process of evolution from granuloma to fibrosis occurs over time, and so sampling at various time points both earlier and later than the nine weeks chosen in this protocol might shed additional light on the temporal progression of the aspiration-associated inflammation. It would also be desirable to clearly correlate the histologic presence of granulomatous inflammation with loss of pulmonary function via spirometric or compliance testing of aspiration-damaged lungs. Finally, further understanding of the regulation of the immunologic checkpoint between granuloma and fibrosis would be of immense value in understanding the natural history of many fibrotic lung diseases.

In conclusion, this study characterized the histopathologic response to chronic aspiration of gastric contents as a granulomatous pneumonitis, which is distinct from the pattern seen with acute aspiration injury. The granulomatous inflammation could be reproduced by administration of any solution that contained particulate food matter, but not with acid alone. Removing the acid from gastric fluid did not ameliorate the inflammation. We hypothesize that aspiration-induced granulomas have the potential to progress to pulmonary fibrosis, and we therefore conclude that acid is not the only component of gastric fluid with the potential to injure lung tissue. In fact, acidity may be substantially less harmful than particulate matter when aspiration is chronic. These results may suggest altering the clinical management of reflux-associated lung disease to focus less on controlling gastric fluid acidity and more on eliminating aspiration events in their entirety.

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