Published in final edited form as:

J Trauma. 2009 December ; 67(6): 1182–1190. doi:10.1097/TA.0b013e318187a2bf.

Predictive Modeling and Inflammatory Biomarkers in Rats with Lung Contusion and Gastric Aspiration

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Abstract

This study uses statistical predictive modeling and hierarchical cluster analyses to examine inflammatory mediators and cells in bronchoalveolar lavage (BAL) as putative biomarkers in rats with blunt trauma lung contusion (LC), gastric aspiration (combined acid and small gastric food particles, CASP), or a combination of the two. Specific parameters assessed in the innate pulmonary inflammatory response were leukocytes, macrophages and polymorphonuclear neutrophils (PMNs) in BAL, whole lung myeloperoxidase (MPO) activity, and a series of cytokines/chemokines present in BAL at 5 or 24 hr post-injury: tumor necrosis factor- α (TNF α), interleukin (IL)-1 β , IL-6, interferon- γ (IFN- γ), IL-10, macrophage inflammatory protein-2 (MIP-2), cytokine-induced neutrophil chemoattractant-1 (CINC-1), and monocyte chemoattractant protein-1 (MCP-1). Rats with LC, CASP, LC+CASP all had severe lung injury compared to uninjured controls based on decreased arterial oxygenation and/or increased BAL albumin at 5 or 24 hr post-insult. However, the injury groups had distinct overall patterns of inflammation that allowed them to be discriminated accurately by hierarchical cluster analysis (29/30 and 35/37 rats were correctly classified in hierarchical clusters at 5 and 24 hr, respectively). Moreover, predictive analyses based on an extension of standard receiver operator characteristic (ROC) methodology discriminated individual animals and groups with similar high accuracy based on a maximum of two inflammatory parameters per group (29/30 and 36/37 rats were correctly classified at 5 hr and 24 hr, respectively). These results support the possibility that inflammatory biomarker profiles could be developed in the future to improve the diagnosis and management of trauma patients with unwitnessed (occult) gastric aspiration who have an increased risk of clinical acute lung injury (ALI) or the acute respiratory distress syndrome (ARDS).

Keywords

gastric aspiration; lung contusion; lung injury; inflammation; biomarkers; ALI/ARDS

INTRODUCTION

Lung contusion (LC) is an important and common problem in the care of trauma patients. Thoracic injury is involved in nearly one third of all acute trauma admissions to the hospital^{1–3}, and many of these patients have associated pulmonary tissue contusion. In addition to causing acute respiratory deficits, LC frequently leads to pneumonia and is an independent risk factor for the development of acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS)^{1–3}. Clinically, LC generates symptoms ranging from a limited, transient bout of hypoxia to progressive lung injury and respiratory failure and ALI/ARDS. On chest X-ray, LC injury may not be apparent as an infiltrate for a few days post-insult. CT scans are frequently obtained in routine management of thoracic trauma patients, but lower lobe pulmonary contusion in particular is difficult to differentiate from gastric aspiration.

Gastric aspiration is a major direct pulmonary cause of ALI/ARDS, and occurs frequently in trauma patients who have a brief loss of consciousness or risk factors such as recent food or alcohol intake^{4, 5}. In one prospective study⁴, 34% of trauma patients had evidence of aspiration at the time of endotracheal intubation by first responders at the accident site. However, many cases of gastric aspiration are unwitnessed or unreported, and gastric aspiration pneumonitis in a clinical setting is often a diagnosis of exclusion in patients without other known direct causes of respiratory compromise^{4, 5}. The scenario of concomitant LC injury plus occult gastric aspiration in chest trauma patients may be significantly under-appreciated, since affected individuals have a readily apparent presumptive cause of lung injury from trauma alone. Occult gastric aspiration may significantly increase the risk of progression to ALI/ARDS in blunt trauma patients. An ability to accurately identify patients with contusion plus aspiration early in their course of hospitalization has the potential to facilitate the future development and testing of better targeted therapeutic interventions in addition to improving general clinical management and prognostication.

This study examines the feasibility and accuracy of using hierarchical cluster analysis based on parameters measured in the innate pulmonary inflammatory response to characterize specific patterns of mediator production and differentiate isolated LC injury from the combination of LC + gastric aspiration in rats. In addition, complementary statistical modeling approaches recently detailed by Hutson et al⁶ based on modified receiver-operator characteristic (ROC) methods are used to document the predictive accuracy of ‘best fit’ subsets of inflammatory mediators in differentiating these lung injuries. The gastric aspirate studied is a clinically-relevant combination of acid plus small non-acidified gastric food particles (CASP) utilized in our prior studies on mechanisms of aspiration-induced lung injury in rodents^{7–11}. Bilateral closed-chest LC injury in rats is induced by blunt thoracic impact from a falling weight as defined in our recent publications^{12, 13}. A primary hypothesis tested is that predictive models based on a maximum of 2–3 inflammatory mediators will be sufficient to distinguish with at least 90% accuracy between rats given LC, CASP, LC+CASP, and uninjured controls at a given time point of injury (5 or 24 hr post-insult).

MATERIALS AND METHODS

Hierarchical cluster and predictive modeling here used data on measurements of arterial oxygenation and albumin, cells, and cytokines/chemokines in bronchoalveolar lavage (BAL) from male, 250–300 g Long-Evans rats (Harlan Sprague-Dawley, Indianapolis, IN) divided randomly into four injury groups: 1) uninjured controls, 2) lung contusion (LC), 3) gastric aspiration (CASP), and 4) LC+CASP. Subgroups of animals were sacrificed at 5 and 24 hr post-injury to allow assessments of lung injury and inflammation at times relevant for patients presenting with acute respiratory pathology following blunt trauma-induced lung contusion and/or gastric aspiration. A total of 8–12 animals were examined per group at each time point.

All animal studies were approved by the Institutional Animal Care and Use Committee at the University at Buffalo-SUNY, and complied with New York State, Federal, and National Institutes of Health regulations. A summary of animal methods is given below, followed by details of statistical modeling methods.

Induction of isolated lung contusion (LC) injury

Following induction of halothane anesthesia, a 300 g cylindrical weight was dropped from a height of 68 cm (impact energy = 2.00 Joules) through a vertical guiding tube onto a Lexon plate with an attached precordial shield placed on the chest of a supine rat^{12, 13}. The precordial shield directed the impact force bilaterally to the lungs so as to prevent cardiac trauma^{12, 13}. The external chest impact energy of 2.00 Joules was chosen to minimize rat mortality in experiments using the combined insults of contusion and gastric aspiration, and is similar to impact levels used in other closed-chest experimental models of LC¹⁴. Direct traumatic forces to the lungs were not measured, but open-chest animal studies by Gayzik et al¹⁵ indicate that significant contusion can be caused by direct pulmonary impact forces two orders of magnitude smaller than the 2 J energy applied to the closed chest here. Our prior work has documented that severe LC is present at the histological and gross levels in this rat model^{12, 13}.

Gastric acid/food particle aspiration (CASP) lung injury

Gastric aspiration was simulated in halothane-anesthetized animals by tracheal instillation of CASP (1.2 ml/kg containing 40 mg/ml of small gastric food particles adjusted to a pH of 1.25 with hydrochloric acid)⁷⁻¹¹. Acid and particulate concentrations in CASP were the maximum non-lethal levels identified in our prior work⁸⁻¹⁰. Gastric particles were prepared from the stomach contents of healthy Long-Evans rats washed in normal saline, and coarse filtered through gauze⁷. The distribution of particle diameters in equivalent gastric preparations has previously been reported by Knight et al⁹ to be bimodal, with peaks at ~4.5 and 13 μm and a mean diameter of <10 μm . The CASP aspirate was instilled through a 14 gauge endotracheal tube, with anatomical placement verified by continuous end-tidal CO₂ tracing obtained with a RASCAL II Raman light scattering spectrophotometer (Ohmeda, Salt Lake City, UT). In experiments where CASP was superimposed on LC, contused rats were first allowed to recover in 100% O₂ until regular, spontaneous breathing was obtained (usually 8–10 minutes) prior to being re-anesthetized with halothane and given CASP. In all animals, the chest wall was compressed by hand and rapidly released as CASP was instilled into the endotracheal tube with a 1 ml syringe followed by a 0.5 ml air bolus chaser to ensure complete delivery of the aspirate and facilitate its rapid distal alveoli dissemination. Following induction of LC, CASP, or LC +CASP injury, rats were maintained in room air until being assessed for oxygenation and BAL levels of albumin, cells, and inflammatory mediators at 5 and 24 hr.

Lung injury severity variables

To assess the severity of lung injury, oxygenation was measured as the arterial partial pressure of oxygen (PaO₂) divided by the fraction of inspired oxygen (FiO₂) following a five minute period of breathing 98% oxygen and 2% halothane just prior to sacrifice. Albumin concentration in BAL as a reflection of permeability injury was measured by ELISA using a polyclonal rabbit anti-mouse albumin antibody (generously provided by Dr. Daniel Remick, Boston University) and a HRP-labeled goat anti-rabbit IgG (BD Biosciences Pharmingen, San Diego, CA). The assay yielded similar results when either rat or mouse albumin (Sigma, St. Louis, MO) was used to generate the standard curves, indicating significant cross-reactivity of the anti-mouse albumin antibody with rat albumin. The lower detection limit of the assay was 5.8 ± 1.6 ng albumin/ml, and the upper limit of the linear portion was 500 ± 110 ng albumin/ml ($n = 14$ assays). BAL samples were typically diluted 1:1000 – 1:10,000 so the resulting absorbance would fall on the linear portion of the standard curve.

Inflammatory response variables

Cluster analyses and predictive statistical modeling studies focused on parameters in the innate pulmonary inflammatory response as putative biomarkers. Detailed methodologies for measuring inflammatory variables (cells and cytokines/chemokines) in BAL are given in our prior work ^{7, 8, 10, 13}. In brief, numbers of total leukocytes, macrophages, and polymorphonuclear neutrophils (PMNs) in BAL were measured by centrifugation, hemocytometer counting, and differential cell analysis. Concentrations (pg/ml) of tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , IL-6, interferon- γ (IFN γ), and IL-10, plus the rodent CXC chemokines macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant-1 (CINC-1), and the CC chemokine monocyte chemoattractant protein-1 (MCP-1), were measured in cell-free BAL by ELISA methods using specific antibodies. In addition, biologically-active TNF α was also quantitated by a cytotoxicity bioassay ⁷. These representative mediators were chosen because they reflect different facets of the innate pulmonary inflammatory response to injury. IL-1 β and TNF- α are early response pro-inflammatory cytokines, while IL-6 and IL-10 are important immuno-modulating cytokines. CINC-1 and MIP-2 are neutrophilic chemokines, and MCP-1 is a CC chemokine implicated in the transformation of acute inflammatory lung injury to a sub-acute stage in rodents ^{7, 8, 10, 13}. In addition to inflammatory mediators in BAL, whole lung myeloperoxidase (MPO) activity was also measured and examined in cluster analyses and statistical models. Although less accessible as a future clinical biomarker due to its source from lung tissue as opposed to lavage, MPO activity was a valuable parameter in these initial animal studies because it reflected the potential relevance of variables relating to neutrophil activation in the lungs. MPO levels were quantitated following tissue protease inhibition, tissue homogenation, extraction, centrifugation, and spectrophotometric assay using KH₂PO₄, H₂O₂, and o-dianisidine dihydrochloride. Details of all reagents, commercial sources, and methodological protocols used are in fully discussed in our prior work ^{7, 8, 10, 13}.

Hierarchical cluster analyses

Graphical hierarchical cluster analyses were performed using JMP5.1 software (SAS Institute, Cary, NC) to analyze data on inflammatory mediators and cells in BAL at 5 and 24 hr post-injury. Initial cluster analyses focused on overall patterns of inflammation in the various injury groups (LC, CASP, LC+CASP, and uninjured controls). Additional hierarchical cluster analyses were then done focusing on specific inflammatory variables identified as being most predictive of injury group status (see below for predictive modeling methods). In all cluster analyses, a single linkage approach was used to define the distance between rank profiles of mediators and/or cells to display their degree of correlation. Results from analysis are presented as dendrograms (tree-like diagrams), with more closely correlated cytokines/cells clustered together and less correlated combination of variables located farther apart in terms of their respective branches. The hierarchical cluster analysis software used here required that all mediators analyzed be present at levels above the limit of assay detection. This led to some variation in animal numbers depending on the injury time point and group considered, as well as on whether cluster analysis involved only subsets of mediators (i.e., as in the case of final predictive hierarchical clusters compared to initial overall clusters). Analyses based on ROC predictive modeling (see below) included all surviving animals at all time points without exclusion.

Statistical predictive modeling

Statistical predictive models considered the four possibilities of: no injury (NI), LC, CASP, or LC+CASP. Specific modeling strategies utilized approaches recently defined by Hutson ^{6, 16}, which extend standard ROC methodology ¹⁷. In brief, lung injury type was modeled as a two-by-two outcome with injury groups discriminated by two levels (0 or 1) for the two injury

factors of LC and CASP, i.e., NI = (LC=0, CASP=0), LC = (LC=1, CASP=0), CASP = (LC=0, CASP=1), or LC+CASP = (LC=1, CASP=1). The basic algorithm considered population probability parameters given by Π_{00} = Probability (NI) = $\Pr(\text{LC=no, CASP=no})$; Π_{10} = $\Pr(\text{LC=yes, CASP=no})$; Π_{01} = $\Pr(\text{LC=no, CASP=yes})$; and Π_{11} = $\Pr(\text{LC=yes, CASP=yes})$. These population probability parameters were modeled as a function of a linear combination of inflammatory parameters (levels of specific cytokines/chemokines or inflammatory cells in BAL or levels of whole lung MPO) under the constraint that the probabilities sum to 1, i.e. $\Pi_{00} + \Pi_{10} + \Pi_{01} + \Pi_{11} = 1$. Related estimated (statistical cell) probabilities were defined during modeling as p_{00} , p_{10} , p_{01} and p_{11} , and the final predicted group assignment corresponded to the maximum of these estimated probabilities. For example, if the maximum statistical cell probability from among p_{00} , p_{10} , p_{01} and p_{11} was found to be p_{00} , then the predicted injury was NI. Modeling was initiated by considering the marginal probabilities $\Pi_{1\cdot}$ and $\Pi_{\cdot 1}$ that corresponded to $\Pr(\text{LC=yes})$ and $\Pr(\text{CASP=yes})$ as determined from standard logistic regression with covariate cytokine levels (marginal probability estimates for $\Pi_{1\cdot}$ and $\Pi_{\cdot 1}$ were denoted as $p_{1\cdot}$ and $p_{\cdot 1}$). The probability of the combined injury, $\Pr(\text{LC=yes, CASP=yes})$, was then assessed as a function of cytokine levels under the constraint $p_{00} + p_{10} + p_{01} + p_{11} = 1$ conditional on the marginal probabilities held fixed. Evaluations assuming that no synergistic injury was present used independent standard ROC curve methodology considering LC alone and CASP alone with the probability of the combined injury set equal to the product of the marginal probabilities ($\Pi_{11} = \Pi_{1\cdot} \Pi_{\cdot 1}$). Hypothesis-testing analyses examined the predictive accuracy of models incorporating a maximum of two inflammatory mediators at a fixed time of injury and for a fixed type of aspiration. Initially, individual inflammatory parameters were assessed for predictive accuracy, followed by additional modeling testing the ability of relevant parameter pairs. Calculations were limited to a maximum of two inflammatory parameters per margin and two parameters for LC+CASP to minimize over-fitting the data. For purposes of analysis, cytokine/chemokine concentrations below the limit of assay detection were defined as being at the known detection limit (an easy option for adaptation in handling low cytokine/chemokine levels in future clinical applications).

RESULTS

Lung injury severity

All animal groups exhibited significant lung injury based on decreases in $\text{PaO}_2/\text{FiO}_2$ and/or increases in albumin in BAL compared to uninjured controls. At 5 hr post-insult, all three injury groups (LC, CASP, LC+CASP) met oxygenation criteria for the clinical definition of ARDS by having $\text{PaO}_2/\text{FiO}_2 \leq 200$ mmHg¹⁸. At 24 hr post-insult, arterial oxygenation improved in all injury groups, but rats given LC+CASP or CASP had decreased $\text{PaO}_2/\text{FiO}_2$ ratios compared to rats with LC or uninjured controls (Figure 1A). Albumin levels in cell-free BAL were higher in all three injury groups compared to uninjured controls at both 5 and 24 hr post-injury, indicating the presence of permeability lung injury at both time points (Figure 1B). In terms of the injury groups, average albumin levels in BAL were ordered as LC+CASP > CASP ~ LC at 5 hr, and as LC+CASP > CASP > LC at 24 hr. A comparison of BAL albumin concentrations also showed that the mean value of this marker of permeability injury was increased in rats with LC+CASP at 24 hr compared to 5 hr post-insult, whereas mean BAL albumin levels were decreased at 24 hr compared to 5 hr in rats given LC or CASP alone (Figure 1B). However, although these group-specific differences in $\text{PaO}_2/\text{FiO}_2$ and albumin were present, values of both lung injury parameters for individual animals in different groups overlapped in many cases (Figure 1A, B). In contrast, intergroup response patterns of inflammatory parameters were fully distinct, including comparisons involving individual animals between groups as documented by hierarchical cluster analyses and ROC predictive modeling below.

Initial hierarchical cluster analysis involving all inflammatory mediators and cells

Results for individual animals were analyzed in the context of patterns of inflammatory mediators rather than individual cytokines. Initial two-way hierarchical cluster analyses covered the full range of measured inflammatory variables (cells and mediators) to assess overall patterns of inflammation in the injury groups (Figures 2A, 2B). Rats injured with LC, CASP, or LC+CASP had very different overall patterns of inflammation. Intergroup differences for individual animals based on overall hierarchical cluster analysis were particularly prominent at 5 hr post-injury (Figure 2A). Differences in overall hierarchical cluster profiles were also present between rats given LC, CASP, and LC+CASP at 24 hr post-injury, but were less pronounced than at the 5 hr post-injury time point (Figure 2B). Because total lung MPO activity is not a readily obtainable future biomarker for human patients, hierarchical clusters at 5 and 24 hrs were also constructed without this variable (data not shown). Without the inclusion of MPO, animals with LC and LC+CASP all remained clustered within the appropriate injury group. However, without the inclusion of total lung MPO, 2 rats with CASP were misplaced within the LC+CASP cluster at 5 hr post-insult. In addition, at 24 hr post-insult, 1 rat with CASP was misplaced in the LC cluster and 1 rat with CASP was misplaced in the LC+CASP cluster when whole lung MPO was not included.

Predictive modeling based on inflammatory mediators for rats at 5 and 24 hrs

ROC modeling based on the levels of one or two inflammation-related parameters per group in BAL provided accurate predictions of the type of injury (LC, CASP, or LC+CASP) (Table 1). The most accurate predictive mediators varied as a function of injury group and time. At 5 hr, parameters with the greatest predictive utility were IL-10 levels and macrophage numbers for rats with LC, MPO activity for rats with CASP, and IL-10 for rats with LC+CASP. Overall, 29/30 rats were correctly classified for the type of injury based on modeling using these inflammatory parameters at the 5 hr post-injury time point (one rat with CASP was misclassified as LC+CASP) (Table 1). A similar high degree of predictive accuracy was found for lung injury type at 24 hr post-injury. Based on measured levels of IL-10, TNF- α or PMNs in BAL at 24 hr, 36/37 rats were correctly classified (one rat in the LC group was misclassified as LC+CASP) (Table 1). These ROC predictive modeling results document that distinct inflammatory signature profiles accompany injury with LC, CASP, and LC+CASP.

Hierarchical clusters based on specific biomarkers identified in prediction model

Additional two-way cluster analyses were performed based on the most predictive biomarkers identified in modified ROC modeling. At 5 hr post-injury, BAL IL-10, BAL macrophage numbers, and whole lung MPO activity were examined. Twenty-nine of thirty rats with LC, CASP or LC+CASP were clustered into the correct 3 separate groups based on these variables (one rat with LC+CASP was misclassified as having LC) (Figure 3A). At 24 hr post-injury, analysis based on BAL levels of TNF- α , IL-10, and PMNs allowed all but two of thirty-seven rats to be clustered into the proper separate injury groups (one rat with CASP was misclassified as having LC, and one rat with CASP was misclassified as having LC+CASP) (Figure 3B).

DISCUSSION

The major goal of this study was to document that different patterns existed in the innate inflammatory response following injury to rats with LC, CASP, or LC+CASP, and determine if these differences allowed accurate diagnostic predictions of individual animal status based on a small number of inflammatory variables as putative biomarkers. Initial hierarchical cluster analyses indicated substantial differences in the overall innate inflammatory responses accompanying the three types of lung injury (Figure 2A, 2B). Modified ROC predictive modeling indicated that animals in the three injury groups could accurately be identified based on one to two parameters per group at 5 or 24 hr post-injury (Table 1). Only 2 out of 67 total

rats at 5 and 24 hr post-injury were misdiagnosed in these predictive ROC analyses (29/30 and 36/37 rats were correctly diagnosed at 5 and 24 hr post-injury, respectively). Hierarchical cluster analyses using the 3 most predictive biomarkers were also consistent in showing a high accuracy of injury discrimination (Figures 3A, B). With the exception of one rat at 5 hr (Figure 3A) and two rats at 24 hr (Figure 3B), all animals were clustered appropriately by this analysis. The cluster analysis and ROC statistical modeling results here lend support to the feasibility of eventually developing related inflammatory biomarker profile testing to identify patients with pulmonary contusion who have concomitant, unwitnessed (occult) gastric aspiration and may be of increased risk for progression to ALI/ARDS.

Given the high mortality of ALI/ARDS, it is increasingly evident that the presence of risk factors such as gastric aspiration should be identified as early as possible in the clinical course to facilitate the testing of new therapies and also to allow intervention before lung damage becomes progressive. A recent National heart, lung and blood institute (NHLBI) working group report has emphasized the importance of developing improved cellular and molecular methods in combination with animal and human studies to better understand the pathogenesis of ALI/ARDS and associated conditions including gastric aspiration¹⁹. It is becoming increasingly evident that individual etiologies responsible for ARDS need to be identified before specific therapeutic agents can be employed. The severe systemic pathology in indirect forms of ALI/ARDS has the potential to significantly limit the effectiveness of pharmacological and other therapies that target pulmonary pathology compared to the case of ALI/ARDS caused by direct lung injury. As one example, post-hoc analyses in two recent clinical trials of surfactant therapy in ALI/ARDS suggest greater efficacy in direct as opposed to indirect forms of these syndromes^{20, 21}.

The modified ROC statistical modeling approach used here has been applied in our prior work to discriminate between different sub types of gastric aspiration pneumonitis in rats and mice as reported by Hutson et al¹⁶. This earlier study examined rats, wild type mice, and MCP-1 (-/-) mice given one of three gastric aspirates: hydrochloric acid, small non-acidified gastric food particles, or a combination of the two in the form of CASP¹⁶. Gastric aspiration in patients with blunt thoracic trauma is most likely to involve both gastric acid and food particles as present in CASP. The study of Hutson et al¹⁶ showed that modified ROC statistical modeling based on BAL levels of a small number of inflammatory mediators in BAL could accurately identify the different gastric aspirates, consistent with findings in the present paper on the ability of related inflammatory parameters to identify the presence of CASP aspiration in rodents. However, analyses here focused on an even more clinically-relevant predictive question in documenting that biomarker-based comparisons could discriminate LC and LC + CASP injury, either of which might occur during blunt thoracic trauma. As noted above, unwitnessed gastric aspiration pneumonitis is often a clinical diagnosis of exclusion in patients without known causes of respiratory dysfunction, and an improved ability to define its presence in a setting of blunt trauma-induced lung contusion has the potential to improve patient management.

Studies here used hierarchical cluster analysis to further test the predictive applicability of mediators studied in modified ROC modeling. Previous studies by Knight et al⁸ have reported distinct patterns of cytokine/chemokine responses by cluster analysis in rats given acid, gastric particles, or CASP. This approach was extended here in cluster analyses showing that rats injured with LC, CASP, or LC + CASP had very distinct overall patterns of inflammation at both 5 and 24 hr post-insult based on a range of inflammatory parameters (numbers of total leukocytes, macrophages and PMNs in BAL, whole lung MPO activity, TNF- α , IL-1 β , IL-6, IFN- γ , IL-10, MIP-2, CINC-1, and MCP-1 (Fig. 2A, B). The finding that rats with LC, CASP, and LC+CASP could be discriminated with a high degree of accuracy based on hierarchical cluster analyses in addition to ROC-based statistical predictions provides further support for

the concept of developing useful diagnostic biomarker strategies in future clinical applications. Hierarchical cluster analyses based only on the three most predictive of the inflammatory parameters studied across injury groups at a given time were shown to discriminate between LC, CASP, and LC + CASP with a similar level of accuracy to that found in ROC analysis (three rats total were misclassified via cluster analysis in Fig. 3 compared to two rats total being misclassified by ROC analysis in Table 1 for combined data at 5 and 24 hr).

Mediators found here to have particular predictive utility included IL-10 and whole lung MPO activity in addition to the pro-inflammatory cytokine TNF- α and the levels of inflammatory cells (neutrophils or macrophages) in BAL (Table 1). The identification of IL-10 as a sensitive biomarker of LC injury at 5 hr suggests the importance of a compensatory anti-inflammatory response generated by the lung at this time. Elevated levels of IL-10 in peripheral blood monocytes and decreased levels of IL-10 in splenic macrophages have previously been reported in LC injury, suggesting that T-helper-2 responses may be exaggerated in this condition²². TNF α , which was found here to be a sensitive biomarker of gastric aspiration at the 24 hr time point (Table 1), has previously been shown to be produced by alveolar macrophages in response to the particulate component of CASP²³. Also identified as being especially important in predicting CASP lung injury were increases in lung MPO activity (Table 1), a finding consistent with the significant component of neutrophil-induced inflammation known to exist in gastric aspiration⁸. Since total lung MPO is not obtainable from BAL samples, related markers that reflect the degree of inflammatory neutrophil activity such as BAL myeloperoxidase or neutrophil elastase^{24–26} may be alternative candidates for study in further biomarker research.

In terms of eventual clinical applications, biomarker analyses in patients based on mediators or parameters from the innate inflammatory response have potential advantages over physiological parameters related to respiratory function or injury severity in discriminating pulmonary contusion from the combination of contusion plus aspiration. Both of these conditions have the ability to cause significant acute respiratory deficits in patients, in analogy with the decreased oxygenation and increased permeability injury in rats with LC and LC + CASP shown here and reported previously by Raghavendran et al²⁷. The combination injury of LC + CASP did lead to more severe permeability lung injury than present in CASP or LC based on increases in mean BAL albumin levels at 24 hr compared to 5 hr in the combination injury group (Figure 1B). However, in a practical sense, the magnitude of respiratory deficits in a clinical setting will span an overlapping range so that individuals affected with either condition will exhibit significant respiratory pathology. Our rat studies support the ability of focused inflammatory biomarker analyses to discriminate LC, CASP, and LC + CASP with a high degree of accuracy, although significant additional research will be needed to document if this potential translates readily to human patients.

Another important practical consideration for future clinical applications involves the physical site of inflammatory mediator sampling. The present study examined inflammatory signatures obtained from the pulmonary air spaces by lavage, but it would be far more ideal if diagnostic and prognostic algorithms for patients could be developed based on inflammatory biomarkers in blood samples. Recent studies in humans with ARDS have attempted to identify unique serum biomarkers associated with poor outcomes^{28, 29}. Decreased plasma levels of protein-C and increased levels of plasminogen activator inhibitor-1 were associated with increases in mortality and adverse outcomes in a group of patients with ALI/ARDS²⁹. Similar conclusions were derived based on higher circulating levels of angiopoietin-2 obtained from a surgical population with ALI/ARDS²⁸. However the etiologies of ARDS are many and varied, and unique disease subgroups within the overall population of affected patients may be more likely to yield diagnostic biomarkers. Recently we have attempted to identify serum biomarkers in animals (rats and mice) following lung contusion and gastric aspiration (data not shown). Unfortunately, rodent blood samples present unique difficulties due to the presence of

inhibitory factors, making it more difficult to assess alterations in the normal responses of blood leukocytes using ligands like lipopolysaccharide (LPS). However, the promising BAL cluster analysis and predictive modeling findings presented here and in our earlier work^{8, 16} certainly support at least the conceptual feasibility of eventually developing meaningful biomarker tests of this kind in a clinical setting.

Acknowledgments

The authors gratefully acknowledge the support of grants K08 GM-73826 (KR), DC-14685 (KR), HL-48889 (BAD, PRK) from the National Institutes of Health, plus the support of the Buswell foundation at the University at Buffalo (KR).

LIST OF ABBREVIATIONS

LC	lung contusion
CASP	combination of acid and small gastric particles
ARDS	acute respiratory distress syndrome
ALI	acute lung injury
BAL	bronchoalveolar lavage
TNF	tumor necrosis factor
IL-1	interleukin 1
IL-6	interleukin 6
IL-10	interleukin 10
MIP-2	macrophage inflammatory protein
CINC-1	cytokine-induced neutrophil chemoattractant-1
MCP-1	macrophage chemoattractive polypeptide
IFN- γ	interferon- γ
MPO	myeloperoxidase

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Figure 1. Severity of lung injury parameters in rats with lung contusion (LC), gastric aspiration (CASP) or both (LC+CASP)

Panel A: Arterial oxygenation (PaO₂/FiO₂). **Panel B:** BAL levels of albumin. Data are Mean ± SEM for n = 6–12 rats at 5 or 24 hr following injury with LC, CASP, or LC+CASP (mean values are shown within each graphical bar). Arterial blood was analyzed for the partial pressure of oxygen (PaO₂) after a 5 min period of breathing 98% oxygen (FiO₂ = 0.98). The concentration of albumin was determined by ELISA in cell-free BAL supernatant. Kruskal-Wallis statistical analysis was performed on data at each time point, and 6 inter-group comparisons were made with a Bonferroni correction for multiple comparisons such that

$p < 0.0083$ was considered significant (family-wise α error = 0.05). * $p < 0.001$ compared to uninjured controls; # compared to LC alone; & compared to CASP alone.

Figure 2A
5 hr

Figure 2B
24 Hr

Figure 2. Heat-map dendrograms showing hierarchical cluster analyses relating the degree of correlation between different inflammatory parameters in rats with lung contusion (LC) and/or aspiration lung injury (CASP)

Panel A: 5 hr post-injury. Panel B: 24 hr post-injury. Cluster dendrograms are based on statistical analyses of combined data from rats subjected to LC, CASP, LC+CASP, or uninjured controls. Smaller rank profile distances in the figure correspond to higher rank correlations between pairs of the variables and vice-versa. The two-way cluster indicates the measured parameters. All the parameters measured to assess innate inflammation were used in the clusters shown. All injured animals were clustered appropriately into their distinct group (LC, CASP, LC+CASP) at each of the two time points studied based on overall hierarchical cluster patterns that included the full panel of inflammatory parameters assessed (cytokines/chemokines and whole lung MPO activity). See text for details.

Figure 3A
5 hr

Figure 3B
24 hr

Figure 3. Heat map dendrograms showing hierarchical cluster analyses relating the degree of correlation between the three most predictive inflammatory parameters in rats with LC, CASP, and LC+CASP

Panel A: Cluster results using predictive inflammatory parameters from 5 hr ROC analysis; Panel B: Cluster results using predictive inflammatory parameters from 24 hr ROC analysis. Dendrograms showing hierarchical clusters were redrawn using BAL levels of IL-10 and macrophages and total lung MPO at 5 hr, and BAL levels of PMNs, IL-10 and TNF- α levels (measured by ELISA and bioassay) at 24 hr. All animals with the exception of one with LC +CASP at 5 hr, and two with CASP at 24 hr, are shown to cluster accurately within the appropriate groups (LC, CASP, LC+CASP, or uninjured control). See text for details.

Statistical modeling predictions of injury group based on measured inflammatory parameters at 5 and 24 hr.

Table 1

Mediators at 5 hr post-injury (total n=30)					
Actual Group					
Predicted Group	NONE	LC	CASP	LC+CASP	Best predictive mediators
NONE	6	0	0	0	---
LC	0	8	0	0	IL-10, BAL macrophages
CASP	0	0	6	0	Total lung MPO
LC+CASP	0	0	1	9	IL-10
Total	6	8	7	9	
Mediators at 24 hr post-injury (total n=37)					
Actual Group					
Predicted Group	NONE	LC	CASP	LC+CASP	Best predictive mediators
NONE	6	0	0	0	---
LC	0	11	0	0	IL-10, BAL PMN's
CASP	0	0	10	0	TNF- α
LC+CASP	0	1	0	9	TNF- α
Total	6	12	10	9	

* Details of statistical modeling approaches are given in Methods. None = no injury, LC = lung contusion, CASP = combination of acid and small gastric particles, MPO = myeloperoxidase, BAL = bronchoalveolar lavage, IL-10 = interleukin 10, PMNs = polymorphonuclear neutrophils, TNF- α = tumor necrosis factor alpha.