Immunopathology of a two-hit murine model of acid aspiration lung injury

J. A. Nemzek, D. R. Call, S. J. Ebong, D. E. Newcomb, G. L. Bolgos, and D. G. Remick. Immunopathology of a two-hit murine model of acid aspiration lung injury. Am. J. Physiol. Lung Cell. Mol. Physiol. 278: L512–L520, 2000.—In a two-hit model of acid aspiration lung injury, mice were subjected to nonlethal cecal ligation and puncture (CLP). After 48 h, intratracheal (IT) acid was administered, and mice were killed at several time points. Recruitment of neutrophils in response to acid was documented by myeloperoxidase assay and neutrophil counts in bronchoalveolar lavage (BAL) fluid and peaked at 8 h post-IT injection. Albumin in BAL fluid, an indicator of lung injury, also peaked at 8 h. When the contributions of the two hits were compared, neutrophil recruitment and lung injury occurred in response to acid but were not greatly influenced by addition of another hit. Neutrophil sequestration was preceded by elevations in KC and macrophage inflammatory protein-2α levels. When KC was blocked with specific antiserum, neutrophil recruitment was significantly reduced, whereas albumin in BAL fluid was not affected. In conclusion, murine KC mediated neutrophil recruitment but not lung injury in a two-hit model of aspiration lung injury.

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MATERIALS AND METHODS

All studies were reviewed and approved by the University Committee on Use and Care of Animals at the University of Michigan (Ann Arbor).

CLP. Female BALB/c mice (19–20 g) were obtained from Harlan (Indianapolis, IN) and maintained under standard laboratory conditions. The mice were anesthetized with an intraperitoneal injection of 87 µg ketamine (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA) and 13 µg/g mouse of xylazine (Rompun, Bayer, Shawnee Mission, KS), and the abdomen was prepared for surgery with chlorhexidine (Nolvasan Surgical Scrub, Fort Dodge Laboratories). Heat-sterilized instruments were used to open the abdomen and exteriorize the cecum. The distal portion of the cecum was ligated with 3-0 silk suture material (Ethicon, Somerville, NJ) and was perforated twice with a 25-gauge needle. A small amount of fecal material was extruded to ensure wound patency. The cecum was then returned to the abdomen, and the abdominal cavity was closed with 3-0 silk in a simple continuous pattern. The skin incision was closed with stainless steel wound clips (Becton Dickinson, Sparks, MD). Immediately after surgery, each mouse received a 1-ml subcutaneous injection of warm (37°C) normal saline and was placed in an incubator (37°C) until recovery from anesthesia. Beginning 2 h after surgery, each animal received a 1-ml subcutaneous injection of imipenem-clindamycin (0.5 mg/mouse of Primaxin, Merck, West Point, PA) reconstituted in 5% dextrose every 12 h.

Intratracheal injection. Mice receiving intratracheal (IT) injections were anesthetized as described in CLP. The ventral aspect of the neck was prepped with chlorhexidine, and a 0.5-cm incision was made on the midline with the use of heat-sterilized instruments. By directly visualizing the trachea, an IT injection of 80 µl was made in 20-µl increments with a 0.5-ml insulin syringe with a 28-gauge needle. The skin incision was closed with a stainless steel wound clip. The mice received an IT injection of either normal saline or an acidic solution (HCl in one-third strength normal saline, pH 1.5). The acidic solution replicates the pH and osmolality of gastric fluid (16). IT injections were administered 48 h after surgery in animals that were subjected to CLP.

Study design. In an initial kinetics study, groups of animals (n = 9–18/group) underwent CLP and, 48 h later, IT injection of acid. The animals were then killed 3, 5, 8, 15, and 24 h after IT injection. A zero-time-point control 48 h after a 25-gauge puncture; IT, intratracheal.

Table 1. Treatment groups for comparison of hits

<table>
<thead>
<tr>
<th>Number of Hits</th>
<th>First Hit</th>
<th>Second Hit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0</td>
<td>IT-saline</td>
</tr>
<tr>
<td>Add</td>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>CLP-saline</td>
<td>1</td>
<td>CLP</td>
</tr>
<tr>
<td>CLP-acid</td>
<td>2</td>
<td>CLP</td>
</tr>
</tbody>
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None, no previous intervention or surgery; CLP, cecal ligation and puncture; IT, intratracheal.
immunoglobulin was biotinylated with NHS-LC-biotin (Pierce). The specificity of the antibodies was evaluated by Western blot of mouse plasma with the use of the biotinylated antibodies. A direct ELISA employing the nonbiotinylated antibodies was then developed. A borate buffer (120 mM NaCl, 50 mM H3BO4, and 16 N NaOH) was used to create a standard curve and to dilute lung lavage samples (1:1,000). The wells of a 96-well microtiter plate (Nunc Immunoplate, Neptune, N J ) were coated with 50 µl of sample or standards and were allowed to incubate overnight at 4°C. The plate was washed (5 washes, 250 µl/well, 15 s/wash) with an automatic plate washer (Bio-Tek Instruments). The remaining steps were conducted at room temperature on an orbital shaker unless otherwise indicated. To block nonspecific binding sites, Blocker Casein in PBS (Pierce) was added to each well (150 µl/well) and incubated for 1 h. Polyconal, rabbit, and anti-mouse albumin were diluted 1:8,000 (6.9 µg/ml) in a dilution buffer consisting of PBS with 10% Blocker Casein in PBS. After the plate was washed, the diluted polyconal antibody was added (50 µl/well) and allowed to incubate for 1 h. The plate was washed, and then goat anti-rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) was diluted 1:8,000 and incubated for 1 h (50 µl/well). The plate was washed, and 3,3',5,5'-tetramethylbenzidine (TMB; mixed 3 parts hydrogen peroxide in acetate buffer to 1 part TMB; Genzyme Diagnostics, San Carlos, CA) was added (100 µl/well). The plate was incubated in the dark for 20 min, and then the reaction was stopped with 1.5 N sulfuric acid (100 µl/well). The absorbance was read immediately on a biokinetix plate reader (Bio-Tek Instruments) at 456 and 590 nm.

Cytokine measurements. Cytokines were measured in plasma and lung lavage fluid. TNF was measured with the WEHI 164 subclone 13 fibrosarcoma cell line bioassay (7). In a flat-bottomed, 96-well microtiter plate (Costar, Cambridge, MA), a standard curve of recombinant human TNF (Cetus, Emeryville, CA) and samples were serially diluted in RPMI 1640 medium with 1% FCS and 1 mM L-glutamine. WEHI cells were suspended at a concentration of 5 × 10^6 cells/ml in RPMI 1640 medium containing 10% FCS, 1 mM L-glutamine, 30 µg/ml gentamicin, and 0.5 µg/ml actinomycin D (Calbiochem, La Jolla, CA). The suspension was added to the plate (100 µl/well) and incubated overnight in a humidified chamber (37°C and 5% CO2). At least 18 h later, cell proliferation reagent WST-1 (Boehringer Mannheim, Mannheim, Germany) was diluted 1:3 in RPMI 1640 medium and then added to the plates (10 µl/well). The absorbance was read 24 h later on a biokinetix plate reader at 465 nm, with a reference wavelength at 630 nm.

IL-6. IL-6 was measured with a B-9 cell line bioassay following previously described methods (4). In a 96-well microtiter plate (Costar), a standard curve of human recombinant IL-6 (PeproTech, Rocky Hill, NJ) and samples were serially diluted in Iscove’s modified Dulbecco’s medium ( Gibco BRL) containing 5% FCS, 1 mM L-glutamin, 100 U/ml penicillin, and 100 U/ml streptomycin (BioWhittaker). B-9 cells were washed twice and then suspended at a concentration of 5 × 10^5 cells/ml in the same medium supplemented with 0.05 mM 2-mercaptoethanol (Sigma). The cell suspension was added to the plate (100 µl/well), and the plates were incubated for 3 days in a humidified chamber (37°C and 5% CO2). Cell proliferation reagent WST-1 was then diluted 1:3 in RPMI 1640 medium and then added to the plates (10 µl/well). The absorbance was read 7 and 24 h later on a biokinetix plate reader at 465 nm with a reference wavelength at 630 nm.

ELISAs. KC and MIP-2a were assayed in plasma and lung lavage fluid with sandwich ELISAs that were developed in this laboratory using previously described procedures (3, 23). Rabbit polyclonal antibodies were raised against murine recombinant KC and MIP-2a and were used as the capture antibodies for the ELISAs (50 µl/well, incubation overnight). BAL fluid samples were diluted 1:5 and plasma samples were diluted 1:10 for testing (50 µl/well, incubation 1 h). A standard curve was prepared with recombinant KC or MIP-2a (50 µl/well, incubation 1 h). Normal mouse plasma (final concentration 10%) was added to the standards in ELISAs measuring plasma cytokines to correct for the effects of plasma interference. The same polyclonal antibodies used for capture were biotinylated for use as detection antibodies (50 µl/well, incubation 1 h). The color reagent used was TMB. The reaction was stopped with 1.5 N sulfuric acid, and the absorbance was read with a biokinetix plate reader at 456 and 590 nm.

Histological analysis. Formalin-fixed lung tissues from the 8-h groups were embedded in paraffin and sectioned. The cut sections were stained with hematoxylin and eosin. The prepared slides were evaluated by a pathologist (D. G. Remick) blinded to the sample treatment groups.

Results. Peripheral blood counts. As determined with the automated counter in this laboratory, normal mice have a total white blood cell (WBC) count of 17.70 ± 1.03 × 10^3 cells/µl (neutrophils = 6.01 ± 0.17 × 10^3 cells/µl and lymphocytes = 10.69 ± 0.17 × 10^3 cells/µl). In our two-hit acid aspiration model, the peripheral WBC counts were low after CLP and 3 h after injection of IT acid (Fig. 1). The total WBC counts reflected changes in both neutrophil and lymphocyte counts. In an earlier study, this laboratory demonstrated that WBC cell counts decrease to 4.0–5.0 × 10^3 cells/µl (2.00–2.50 × 10^3 cells/µl for both lymphocytes and neutrophils) within 48 h of a 25-gauge CLP and gradually increase over 4–5 days to reach total counts of 10.00–12.00 × 10^3 cells/µl (6). In contrast to that study, the sepsis-associated low WBC counts seen for 3 h after IT injection of acid increased significantly by 5 h. This demonstrates a significant systemic response to the local instillation of acid and suggests that a WBC reserve was mobilized, probably from bone marrow. Further evaluation at 8 h demonstrated that both CLP groups (CLP-saline and CLP-acid) had lower WBC counts than groups that were not subjected to CLP (saline and acid; Fig. 2).

BAL cell counts. In a previous study (6), our laboratory demonstrated that 48 h after 25-gauge CLP, BAL cell counts are 100,000/mouse or less and the cells are exclusively alveolar macrophages. In our two-hit model of acid aspiration, the total cell counts increased after IT injection of acid and peaked at 8 h (Fig. 3). This increase in cell numbers appears to be almost exclusively due to an increase in neutrophil counts that also
peaked at 8 h post-IT injection of acid. Further evaluation of the 8-h time point demonstrated that the neutrophil counts were greater in the acid versus the saline group and in the CLP-saline group versus the CLP-acid group (Fig. 4). The increases appear to be related to the IT administration of acid and not to the CLP. Although two hits caused an increase in neutrophil influx (7.73 ± 2.05 × 10^4/mouse in the CLP-acid group) compared with one hit (4.95 ± 1.88 × 10^4/mouse in the acid group), the difference was not significant.

Whole lung MPO assay. The MPO assay was performed after lung lavage and vascular perfusion; therefore, it represents neutrophil activity within the lung tissue or adhered to the capillary endothelium. MPO levels increased quickly in the two-hit model, with a significant peak 5 h after IT injection of acid. These levels increased over 1st 8 h postacid. This increase was due to a rise in neutrophil numbers from 3 to 8 h. *P < 0.05 compared with 3-h time point.

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data document the movement of neutrophils into the lung shortly before the influx of neutrophils into the alveolar space (Fig. 5A). Evaluation at 8 h showed less MPO activity in the CLP-saline and CLP-acid groups (Fig. 5B). There was no difference between the acid and CLP-acid groups, again suggesting that the inflammatory response to two hits was not synergistic. However, these values may simply reflect changes in peripheral blood values. The septic animals had lower peripheral WBC counts, and, therefore, fewer neutrophils were available to adhere to the capillary endothelium.

Histology. Evaluation of the 8-h time point revealed similar findings in the no-hit, one-hit, and two-hit groups. There were small numbers of neutrophils present in the alveolar capillaries and septa without significant disruption of the normal architecture of the lung at the light-microscopic level.

BAL fluid albumin. In the two-hit model, large quantities of albumin were present in lung lavage fluid from 3 to 24 h. Levels peaked at 8 h and then gradually declined (Fig. 6A). Evaluation at 8 h revealed a significant increase in BAL albumin in the acid group versus the saline group. Although not significant, the mean albumin levels in BAL fluid from the CLP-acid group were three times higher than those in the CLP-saline group (Fig. 6B). A 12% SDS-PAGE separating gel was used for electrophoresis of BAL samples from the CLP-saline and CLP-acid groups. The Coomassie-stained gels confirmed the presence of larger amounts of albumin in the CLP-acid group (Fig. 7). Because albumin in the BAL fluid is an indicator of loss of vascular integrity with leakage into the alveolar space (28), these results suggest that substantial lung injury occurred in response to the IT acid. It is interesting that the two-hit group (CLP-acid) did not show an increase in BAL fluid albumin over the one-hit group (acid). The addition of a second hit did not affect neutrophil influx or lung injury in a synergistic manner.

Using the CLP-acid two-hit model, we were able to demonstrate neutrophil recruitment into the lung and substantial lung injury. The mechanisms behind these findings were examined by evaluating cytokine responses in plasma and lung lavage fluid.
Cytokine analysis. TNF was not detected at appreciable levels in any of the lung lavage fluid or plasma samples (data not shown). The lower limit of detection of the assays was 20 pg/ml or less.

IL-6 in plasma reached the highest levels detected within 3 h of IT injection of acid and declined significantly by 5 h post-IT injection. Local IL-6 concentrations measured in BAL fluid were lower and showed a significant peak slightly later than the plasma IL-6 (Fig. 8). At the 8-h time point, plasma IL-6 was higher in the CLP-saline and CLP-acid groups (161 ± 101 and 142 ± 41 pg/ml, respectively) than in the saline and acid groups (14.6 ± 8.9 and 46.7 ± 16.3 pg/ml, respectively). Local levels of IL-6 were higher in the CLP-acid and acid groups (196.5 ± 43.0 and 34.9 ± 9.5 pg/ml, respectively) than in the saline and CLP-saline groups (17.7 ± 4.9 and 4.9 ± 2.9 pg/ml, respectively). IL-6, a marker of inflammation, appeared to compartmentalize with the insult delivered, showing greater increases in plasma with the septic insult and in the BAL fluid with the local lung injury.

Plasma concentrations of KC and MIP-2α were measured at their highest levels 3 h after IT injection of acid. These values declined quickly and remained low throughout the rest of the study (Fig. 9). Local levels of KC in BAL fluid peaked at 3 h and decreased significantly by 5 h. This occurred before the maximum increase in BAL fluid neutrophil counts (Fig. 10A). MIP-2α concentration in BAL fluid peaked at 5 h and declined gradually by 15 h (Fig. 10B). Overall, KC peaked earlier and at a higher concentration than MIP-2α. These findings prompted further evaluation of the roles of both KC and MIP-2α in neutrophil recruitment to the lung.

Antibody treatment. Three groups were treated with antiserum (control, anti-MIP, and anti-KC) after CLP but before acid injection. There were no significant differences in peripheral total WBC counts, whole lung MPO, or plasma or lung IL-6 levels (data not shown). The total cell counts in BAL fluid from both of the treated groups were not significantly different from those in the control group (data not shown). Likewise, the BAL fluid neutrophil counts in the anti-MIP-treated group were not significantly different from those in the control group (Fig. 11A). In contrast, the BAL fluid neutrophil counts in the anti-KC group were significantly lower than those in the control group (Fig. 11A). The neutrophil count in the anti-KC group was reduced by 69% compared with that in the control antiserum group. To determine whether capillary permeability was also affected when the chemokines were blocked, BAL fluid albumin levels were measured. It is interesting that the BAL fluid albumin concentrations
were not significantly different between the groups, suggesting that the degree of lung injury was not affected by blocking neutrophil influx (Fig. 11B).

**DISCUSSION**

Neutrophil recruitment and increased vascular permeability are hallmarks of acute lung injury. These changes have been reported in clinical cases of acid aspiration-induced lung injury in humans (20, 34); therefore, appropriate animal models of acid aspiration should produce these pathological findings. We were able to readily document both neutrophil influx and lung injury during the evaluation of our two-hit murine model of acid aspiration lung injury. After CLP and an IT injection of acid, animals in this study developed a systemic response, increased WBC counts and plasma IL-6, within hours. Concurrently, neutrophils moved into the lung as shown by early increases of whole lung MPO activity, and a short time later, influx of neutrophils into the alveolar space occurred, documented by cell counts of BAL fluid. Albumin also accumulated in the BAL fluid. Because albumin leakage is associated with the loss of vascular integrity, this indicates that actual lung injury occurred in response to the IT injection of acid. The time course of our model presented with a reliable pattern and appears similar to those seen in other studies. In a single-hit rat model, neutrophil influx was documented within 2–3 h of acid aspiration and numbers within the lung tissue returned to normal by 15 h (16). Another study (17) reported that maximum lung injury occurred as early as 4–6 h after IT acid. In addition to the necessary pathological findings, the two-hit model evaluated in our study allowed the benefit of clinical relevance by preceding the acid aspiration with another inflammatory event.

After a reliable model had been established, another of the important goals of this study was to evaluate the relative contribution of the two hits, sepsis and IT acid, to the development of acid-induced lung injury. It is interesting that neither neutrophil counts nor albumin concentrations in the BAL fluid showed significant increases when two hits were delivered compared with one hit. Therefore, the effects of two hits could not be considered synergistic or even additive in regard to neutrophil recruitment or lung injury. However, it should be noted that the plasma albumin levels measured by ELISA were lower in the septic groups than in the nonseptic groups and this may confound comparison of these groups (data not shown). Our findings are contrary to hypotheses that multiple inflammatory insults may augment immune responses and lead to organ failure (5). Recent reports have demonstrated in animal models that neutrophil recruitment to the lung was enhanced when hemorrhagic shock was followed by LPS (8) and when sepsis was followed by direct lung injury with immune complexes or LPS (2). The lack of synergistic response seen in our study compared with other two-hit models could reflect different types and/or order of inflammatory insults. Alternatively, the absence of a significant increase in neutrophils within the
lung after two hits might be explained by a decrease in neutrophil stores caused by the initial septic insult.

The final goal of this study was to begin to establish the mechanisms behind neutrophil recruitment and lung injury in the two-hit model. To examine the mediators of neutrophil recruitment, the murine C-X-C chemokines KC and MIP-2α were chosen for study. These chemokines were chosen because of their similarities in sequence homology to IL-8 (31, 35), which has been implicated in neutrophil recruitment in human cases of ARDS (20) and in a rabbit model of acid aspiration lung injury (9). KC and MIP-2α were also examined because each has demonstrated their effects on neutrophil recruitment in other models of lung injury (10, 14, 15, 27, 30). In our two-hit model, KC appeared to play the major role in neutrophil recruitment. KC demonstrated the earliest and highest peak in concentration and preceded neutrophil influx. Most convincingly, blockade of KC with specific antiserum reduced neutrophil influx by 69%. These findings are similar to those found in studies examining neutrophil recruitment in response to CINC, a rat C-X-C chemokine with 90% sequence homology to KC (32). The studies in rats demonstrated increases in CINC (KC) mRNA levels and neutrophil recruitment after IT LPS (15, 30). In addition, treatment with antiserum (30) or purified polyclonal antibodies (10) against rat CINC (KC) decreased neutrophil influx after LPS by ~70%. In another two-hit study, the augmented recruitment of neutrophils seen in animals subjected to hemorrhage followed by LPS was also dependent on rat CINC and not MIP-2α (8). It has been suggested that neither of these chemokines works alone (10, 14), and this cannot be completely ruled out by our study because treatment with anti-MIP antiserum did lower neutrophil counts slightly. Nonetheless, in our model, KC appears to be a major chemoattractant for neutrophils responding to acid aspiration.

Also of interest in our two-hit model, the albumin levels detected in the animals treated with KC antiserum did not show a reduction in albumin leakage despite a decrease in BAL fluid neutrophil numbers. It has been reported that neutrophils and their proteases are both necessary and sufficient to produce the second phase of injury after acid aspiration (17). However, the results of our study imply that factors other than neutrophil recruitment also contribute to acid aspiration lung injury. These findings are supported by the incomplete reduction of lung injury in neutrophil-depleted rabbits subjected to acid aspiration (9). Several other mediators of acid aspiration lung injury have been investigated and include direct insult from the acid (16), complement activation (24, 26, 33), and free radical formation (21). It would appear that further investigations into the mechanisms of lung injury in acid aspiration lung injury are warranted.

In summary, this study documented systemic and local changes in a two-hit murine model of acid aspiration lung injury. Although the aspiration of acid produced dramatic neutrophil influx and lung injury, the combination of sepsis and acid aspiration did not create a heightened response. In this model, it appears that the murine chemokine KC plays a major role in neutrophil recruitment; however, the neutrophil is not solely responsible for the development of lung injury.

REFERENCES


