Membrane attack complex of complement and neutrophils mediate the injury of acid aspiration

CONSTANTINOS KYRIAKIDES, WILLIAM AUSTEN, JR., YONG WANG, JOANNE FAVUZZA, LESTER KOBKIZ, FRANCIS D. MOORE, JR., AND HERBERT B. HECHTMAN
Departments of Surgery and Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

Kyriakides, Constantinos, William Austen, J r., Yong Wang, Joanne Favuzza, Lester Kobzik, Francis D. Moore, J r., and Herbert B. Hechtman. Membrane attack complex of complement and neutrophils mediate the injury of acid aspiration. J. Appl. Physiol. 87(6): 2357-2361, 1999.—A significant role for the alternative complement pathway in acid aspiration has been demonstrated by the observation that C3 genetic knockout mice are protected from injury. Utilizing C5-deficient mice, we now test the role of the terminal complement components in mediating injury. Lung permeability in C5-deficient mice was 64% less than in wild-type animals and was similar to wild-type mice treated with soluble complement receptor type 1, which gave a 67% protection. Injury was fully restored in C5-deficient mice reconstituted with wild-type serum. The role of neutrophils was established in immunodepleted wild-type animals that showed a 58% protection. Injury was further reduced (90%) with the addition of soluble complement receptor type 1, indicating an additive effect of neutrophils and complement. Similarly, an additional protection was noted in C5-deficient pneumonic mice, indicating that neutrophil-mediated injury does not require C5a. Thus acid aspiration injury is mediated by the membrane attack complex and neutrophils. Neutrophil activity is independent of C5a.

inflammation; complement activation; polymorphonuclear leukocytes; pneumonia; murine

ACID ASPIRATION leads to acute lung injury and is a potentially serious complication in trauma and surgical patients (11, 20). Furthermore, it is the leading cause of the acute respiratory distress syndrome after elective surgery (3). The reported mortality after major aspiration ranges between 30 and 94% (1, 3, 21). The pulmonary injury after acid aspiration has been described as biphasic (13). There is an early and direct chemical airway injury characterized by copious, protein-rich bronchial secretions resulting in neutralization of the acid (1). This is followed by a delayed humoral (complement, cytokine, eicosanoid) and cellular (neutrophil, macrophage) response leading to distal alveolar injury and increased capillary leak (4, 5, 8, 13, 16, 19).

Complement inhibition with the C3b and C4b antagonist soluble complement receptor type 1 (sCR1) or depletion with cobra venom factor have reduced both local and systemic organ injuries (18, 22, 24). More specifically, it is activation of the alternative complement pathway that leads to pulmonary injury, demon-

strated by protection of C3, but not C4, genetic knockout mice against injury (24). However, the mechanism by which complement activation leads to or aggravates injury remains unclear. In general, complement promotes inflammation via soluble and cell-bound activation fragments. The cleavage of C3 and C5 and generation of their respective anaphylatoxins C3a and C5a, may be a mechanism of polymorphonuclear leukocyte (PMN) recruitment, adhesion, and activation. The final membrane-inserted attack complex C5b-9 may lead to cellular activation as well as cause cell injury or osmotic lysis. It can also act as a neutrophil-signaling molecule (23). In addition, experimental neutrophil depletion or elastase inhibition have been shown to moderate pulmonary injury after acid aspiration (5, 7, 12, 24).

In this study of acid aspiration, it is hypothesized that complement-mediated injury is C5b-9 dependent. Utilizing C5-deficient mice, we examined the relative inflammatory contributions of the C5b-9 and C5a terminal complement components in mediating acid aspiration injury.

METHODS

Mice. Male congenic C5-deficient (B10.D2/oSnJ) and wild-type (B10.D2/nSnJ) control mice, purchased from Jackson Laboratories (Bar Harbor, ME), were used in all experiments. The animals were maintained in accord with the guidelines of the Committee on Animals of Harvard Medical School and those by the Guide for the Care and Use of Laboratory Animals (DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20892).

Aspiration protocol. Mice aged 8–12 wk and weighing 25–30 g were anesthetized with intraperitoneal pentobarbital sodium (60 mg/kg), weighed, and their necks were washed with ethanol. Through a midline neck incision, a 0.75-cm-long, 16-gauge catheter (Angiocath, Becton-Dickinson, Sandy, UT) was placed in the upper trachea under direct vision and secured with 4/0 silk sutures. The tracheostomy was cannulated with a 22-gauge, 1-cm-long angiocath, and 2 ml/kg of 0.1 N HCl (Sigma Chemicals, St. Louis, MO) or 0.9% saline (sham) was instilled into the trachea proximal to the carina by using a 1-ml syringe. Previous studies performed in our laboratory have shown that this injury in mice is at maximum severity when 2 ml/kg of 0.1 N HCl are instilled (24). At that dose, experimental mortality is <10%. Five minutes before aspiration, 1 µCi of 125I-albumin (ICN, Irvine, CA) in 0.3 ml of 0.9% saline was infused intravenously via a tail vein. Mice were allowed to breathe spontaneously. They were maintained in a supine position and kept anesthetized by intermittent intraperitoneal pentobarbital sodium injection. They were covered throughout the experiment to maintain body temperature.

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Four hours after aspiration, the animals were euthanized by an intraperitoneal pentobarbital sodium overdose (90 mg/kg). Through a midline sternotomy, blood was aspirated from the right ventricle, and its gamma radioactivity was measured (Packard, Downes Grove, IL). Through the tracheostomy, the lungs were instilled with 0.5 ml of 0.9% saline by using a 1-ml syringe. After 1 min, the bronchoalveolar (BAL) fluid was aspirated. This process was performed three times over a 3-min period by using a total of 1.5 ml of 0.9% saline. A total of 0.9 ± 0.1 ml of BAL fluid was retrieved. Lung permeability index (PI) is reported as the ratio of radioactivity per gram of BAL fluid retrieved to radioactivity per gram of blood. An identical mouse preparation, save for the omission of administration of 125I-albumin, was used to quantitate neutrophils harvested from lung lavage. Another set of mice that underwent no lavage was used for immunohistochemical analysis of complement deposition. In this group of animals, the trachea and lungs were inflated with optimal cutting-temperature compound (Miles) and immediately frozen.

Complement inhibition with sCR1. Both classic and alternative pathways of complement activation were inhibited by an intravenous bolus of sCR1 (AVANT Immunotherapeutics, Needham, MA) administered 5 min before acid aspiration. Each animal received 1 mg/kg, a dose previously determined to effectively inhibit the complement (10, 25).

Wild-type serum reconstitution of C5-deficient mice. Blood was aspirated from the right ventricle of wild-type, complement-sufficient mice after euthanasia. The blood was collected in a glass tube and allowed to clot for 5 min. After centrifugation at 5,000 g for 5 min at 0°C, the serum was aspirated and kept on ice. C5-deficient mice were infused with 0.5 ml of wild-type serum 16 h before acid aspiration.

PMN depletion. Mice underwent uncomplicated neutropenia by a tail vein injection of rabbit anti-mouse PMN antibody (20 mg/kg; Accurate, Westbury, NY) 16 h before acid aspiration. Venous blood samples were taken just before aspiration and at the time of euthanasia. The blood was used for leukocyte counting, and blood smears were Wright’s stained for differential counts.

Complement inhibition in neutrophil-depleted mice. Animals were given a tail vein injection of anti-PMN antibody (20 mg/kg) 16 h before acid aspiration. Complement was inhibited by the intravenous administration of sCR1 (20 mg/kg) 5 min before aspiration. After 4 h, blood samples for total leukocyte counts and differentials were taken.

Neutrophil harvest from BAL fluid. Both lungs were lavaged five times over a period of 3 min by using a total of 2.5 ml of saline with 0.6 mM EDTA. The lavage fluid return of 2.3 ± 0.1 ml was centrifuged at 1,500 rpm for 15 min. The cellular pellet was resuspended in 1 ml of cold saline. Two hundred microliters of the fluid were injected in the cytospin apparatus (Shandon Lipshaw Cytospin 3) and run at 200 rpm for 4 min. The glass slides were stained with Diff-Quick (Dade, CH-3186 Düdingen, Switzerland) to identify macrophages. PMNs were counted in a blinded fashion. The results are expressed as total PMN count in BAL fluid (means ± SE).

C3 immunostaining. Immunoperoxidase labeling of C3 was performed on paraformaldehyde-fixed cryostat sections of lung by using goat anti-mouse C3 (5 mg/ml; Organon Teknia, Durham, NC) and a standard avidin-biotin protocol (15). Immunostaining of lung samples from sham animals was used as control. Injury was scored semiquantitatively by evaluating the presence of interstitial edema and intra-alveolar exudates by a respiratory pathologist (L. Kobzik) in a blinded fashion.

Statistical analysis. Results are presented as means ± SE. Groups were subjected to analysis of variance followed by Student’s t-test with the Bonferroni correction for multiple comparisons. Percentage reduction in PI was calculated after subtraction of the background value determined in sham animals.

RESULTS

In C5-deficient mice (n = 11) after saline aspiration, PI of 0.0048 ± 0.0004 was similar to PI of 0.0053 ± 0.0004 in wild-type sham mice (n = 14). Acid aspiration in wild-type mice led to a marked rise in PI (0.074 ± 0.004; n = 28, P < 0.05, Fig. 1).

Role of complement and neutrophils. Complement antagonism with sCR1 (n = 8) reduced injury by 67% (P < 0.05, Fig. 1). Treatment with the anti-neutrophil antibody achieved an 87% neutropenia (mean absolute PMN count of 112 ± 36 vs. 847 ± 138 cells/ml in neutrophil-replete mice, P < 0.05). After acid aspiration, PMN-depleted mice demonstrated a 58% reduction in PI (n = 9, P < 0.05, Fig. 1). Combined sCR1 treatment and neutrophil depletion (mean absolute PMN count of 123 ± 41 cells/ml) yielded an additive 90% reduction in PI (n = 11, P < 0.05, Fig. 1). These data are similar to those reported by Weiser et al. (24) in the mouse.

Role of the terminal complement components. PI in C5-deficient mice (n = 25) was 64% lower than that of wild-type animals (P < 0.05). This was similar to
sCR1-treated animals, indicating that injury was mediated by the terminal complement components as well (Fig. 2). In addition, injury was completely restored in C5-deficient mice reconstituted with wild-type serum (n = 9, Fig. 2).

Role of C5a as a potential PMN chemoactivator. To test the neutrophil participation in mediating this injury via the C5a anaphylatoxin, a group of C5-deficient and wild-type animals had their lungs lavaged and the cells counted. Acid aspiration produced an accumulation of 484 ± 86 PMN in C5-deficient mice (n = 5), similar to 530 ± 128 PMN in injured wild-types (n = 5). The counts for saline-aspirated C5-deficient (n = 5, PMN 4 ± 2) and wild-types (n = 5, PMN 6 ± 2) were significantly lower (P < 0.05). To test for neutrophil activity in the absence of C5a, a group of C5-deficient animals (n = 8) was neutrophil depleted (mean circulating absolute PMN count of 119 ± 39 cells/µl) 16 h before acid aspiration. The PI in this group of animals was similar to than in combined treatment with sCR1 and neutrophil depletion. Furthermore, this represents a 64% reduction in PI compared with the C5-deficient acid-aspirated mice (n = 25, P < 0.05), indicating neutrophil activity without C5a in the latter group (Fig. 2).

C3 immunostaining. Lungs snap-frozen in optimal cutting-temperature compound 4 h after acid aspiration were stained for C3 (Fig. 3). The injury was assigned a score from 0 to 3, corresponding to normal, mild (<25% of sample area showing injury), moderate (25–50% of sample showing injury), and severe (>50% of sample area injured), respectively (Table 1). Intense C3 alveolar staining associated with fibrinous exudates was observed in the acid-aspirated wild-type and C5-deficient animals reconstituted with wild-type serum. This contrasted with significantly less immunoreactive exudative alveolar injury in acid-aspirated C5-deficient and wild-type animals treated with sCR1.

C3 immunoreactivity was restricted to intravascular spaces in sham saline-aspirated lungs of C5-deficient and wild-type groups.

DISCUSSION

Complement inhibition as well as depletion have been shown to moderate acid-induced pulmonary injury in various animal models (18, 22, 24). Furthermore, the involvement of the alternative complement pathway as an initiating mechanism has been demonstrated by protection against injury observed in C3, but not C4, genetic knockout mice (24). In the present study, C5-deficient animals unable to form the C5b-9 membrane attack complex were protected from injury (64%) to the same degree as were wild-type animals treated with the complement antagonist sCR1 (67%). In addition, the injury was restored (100%) in C5-deficient mice reconstituted with wild-type serum. These data indicate that, with regard to the complement segment of the inflammatory reaction, the terminal complement components are the key mechanism by which the alternative pathway exerts its effect.

To examine the interaction of complement and neutrophils, particularly with regard to the anaphylatoxin C5a, a group of C5-deficient animals were immunodepleted of neutrophils before undergoing aspiration. This led to a 64% reduction in injury compared with neutrophil-replete C5-deficient mice, indicating a role for the neutrophil in this injury without C5, C5a, or other terminal complement products. Further evidence that C5a is not operative in this setting as a chemoattractant is the observation that neutrophil accumulation in the BAL was similar in both injured wild-type and C5-deficient animals. These data strongly suggest that the complement action in mediating injury is via the formation of the membrane attack complex. This porelike structure inserts itself into the plasma membrane of target cells at the site of injury, resulting in unchecked influx of water and ions such as calcium. The membrane perturbation leads to second-messenger signaling, enzyme activation, and possible osmotic lysis (9).

Additional support for the role of the membrane attack complex in mediating injury comes from the results of histological injury score of lung tissue sections of C5-deficient and wild-type mice. A positive relationship exists between PI and the severity of alveolar injury in acid-aspirated wild-type animals, C5-deficient mice reconstituted with wild-type serum, and C5-deficient mice without reconstitution. Acid-aspirated C5-sufficient animals had significantly more
immunoreactive alveolar tissue damage, edema, and intra-alveolar exudates than did C5-deficient mice. The present understanding of complement activation is that it proceeds in a cascade, whereby cleavage of C3 and binding of C3b covalently to the activator surface precedes the activation of C5. In concert with this thesis, there should be as much C3 deposition in acid-aspirated wild-type mice as in acid-aspirated C5-deficient mice. Our observation was of increased C3 deposition in the C5-sufficient mice. A possible explanation for this observation is that the initial C3 activation and deposition is at a low level and is thus faintly registered by immunohistochemistry. Subsequent alveolar injury due to C5b-9 then allows for further cellular activation of C3, becoming more pronounced by immunostaining and reflecting a greater overall injury in the complement-sufficient groups.

Neutropenic wild-type mice had a 58% reduction in lung injury after acid aspiration. This is similar to previous reports including other animal species (5, 6, 8, 14, 24). Prior work from this laboratory has examined other potent mediators of PMN chemotaxis in addition to C5a. Experimental lavage with leukotriene B4 into the airways induced the synthesis of local tumor necrosis factor-α that, in turn, leads to PMN diapedesis (6). The importance of eicosanoids after aspiration was documented by the observation that acid aspiration induced preliminary synthesis of leukotriene B4 and thromboxane A2, coincident with the recruitment and activation of neutrophils (8). When eicosanoid synthesis was inhibited by lavaging antagonists, the influx of neutrophils was abrogated. Another chemoattractant is interleukin-8. Levels of this cytokine rise in BAL after acid aspiration (2, 17). Treatment with anti-interleukin-8 monoclonal antibody has attenuated PMN sequestration and lung injury. A similar effect was observed with the intravenous administration of lidocaine, which is postulated to suppress cytokine production by direct action on alveolar endothelium, macrophages, and pneumocytes.

Table 1. Semiquantitative evaluation of bronchoalveolar injury

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Injury Score</th>
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<tbody>
<tr>
<td>Wild-type sham</td>
<td>7</td>
<td>0.21 ± 0.15</td>
</tr>
<tr>
<td>Wild-type injured + sCR1</td>
<td>7</td>
<td>0.93 ± 0.07*</td>
</tr>
<tr>
<td>Wild-type injured</td>
<td>5</td>
<td>2.20 ± 0.12</td>
</tr>
<tr>
<td>C5D sham</td>
<td>7</td>
<td>0.14 ± 0.14</td>
</tr>
<tr>
<td>C5D injured</td>
<td>5</td>
<td>0.90 ± 0.10*</td>
</tr>
<tr>
<td>C5D injured + wild-type serum</td>
<td>5</td>
<td>1.80 ± 0.20</td>
</tr>
</tbody>
</table>

Values are means ± SE. n = no. of mice per group. sCR1, soluble complement receptor type 1; C5D, C5-deficient mice. *P < 0.05 compared with wild-type injured mice.
mocytes (17). Thus acid aspiration appears to stimulate eicosanoid and chemokine release, likely from activated alveolar endothelial and epithelial cells as well as mast cells and alveolar macrophages, which, in turn, are the primary recruitment mechanisms for neutrophils.

In conclusion, acid aspiration results in an inflammatory injury mediated in an additive fashion by the membrane attack complex and neutrophils. Neutrophil sequestration and activation is independent of C5a. The therapeutic implication is that the treatment should inhibit the activity of both terminal complement components as well as neutrophils.

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Address for reprint requests and other correspondence: H. B. Hechtman, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115 (E-mail: hhechtman@bwh.harvard.edu).

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