Mediators of Lung Injury in Mice Following Systemic Activation of Complement

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Acute lung injury has been produced in mice by the intravenous injection of cobra venom factor. The marked attenuation of lung injury in C5-deficient mice indicates an absolute requirement for C5 in the development of lung injury. Similar studies carried out in beige mice suggest that leukocytic proteinases play, at best, a limited role in the injury. Neutrophil or platelet depletion resulted in a marked reduction in the extent of lung injury, suggesting that both platelets as well as neutrophils contribute to the injury. Treatment of mice with catalase provided a marked degree of protection from the lung injury, while treatment with superoxide dismutase produced limited protection, which suggests that H_2O_2 or its derivatives are involved in the induction of acute lung injury. By the use of transmission electron microscopy, areas of lung vascular injury, as manifested by extensive blebbing of endothelial cells, were associated with intravascular aggregates of platelets, neutrophils, and fibrin. Finally, lipoxygenase and thromboxane synthetase inhibitors afforded some protection against cobra venom factor-induced acute lung injury, while cyclooxygenase inhibitors gave variable results. These data suggest that acute lung injury in mice following systemic activation of complement has an absolute requirement for C5, is dependent on a role of both neutrophils as well as platelets, and can be linked to the generation of toxic oxygen products by neutrophils. (Am J Pathol 1985, 119:92–100)

ACUTE INJURY of lung vascular endothelial cells has been documented to occur in rats undergoing systemic complement activation.[1] Neutrophils have been shown to play a critical role, apparently through generation of H_2O_2 and its conversion in the presence of ionic iron to hydroxyl radical ($^{\cdot}OH$).[1,2] In this model an intact complement system has been shown to be required for the development of acute lung injury, the injury being preceded by the appearance in serum of C5-related chemotactic activity. However, although the complement chemotactic peptide C5a has been postulated to be a key mediator implicated in the development of lung injury, the precise complement activation product(s) involved is not known. The vascular infusion of complement-activated serum into rabbits and sheep yields variable evidence of acute lung injury,[3–5] although stimulation of human neutrophils with zymosan-activated human serum causes the release *in vitro* of ^{51}Cr from labeled human endothelial cells.[6]

Using congenic strains of mice, we have now demonstrated that C5 is required for the induction of acute lung injury produced by systemic complement activation. In this model evidence is also presented suggesting that neutral proteinases of neutrophils may not be required for the development of the initial stages of vascular injury, that oxygen radicals are involved in the ensuing lung injury, that neutrophils as well as platelets contribute to the injury, and that lipoxygenase products and certain cyclooxygenase products may be involved in the full expression of the tissue injury.

Materials and Methods

Animals

The following strains of mice were used: C57 Bl/J6, beige mice (C57 Bl/J6, bg+/+) and their heterozygous controls, (C57 Bl/J6, bg+/−), and $B_{10}D_2$/Osn and

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$B_{10}D_2$/Nsn mice, which are C5-deficient and C5-sufficient, respectively (Jackson Laboratory, Bar Harbor, Maine). Except where indicated, 9-week-old, male C57 Bl/J6 mice were routinely used in the experiments.

Lung Injury

Lung injury was induced by intravenously injected cobra venom factor (CVF) in a manner similar to that reported for the rat[1]: 1.0 unit CVF was used per mouse. The mice were anesthetized with 5 mg ketamine hydrochloride (Parke-Davis Co., Ann Arbor, Mich) and exsanguinated 30 minutes after the CVF injection. Purification and characterization of the CVF preparation are reported elsewhere.[1] Lung injury was measured by permeability of ^{125}I-bovine serum albumin (BSA) which had been injected intravenously with the CVF. Lung injury was determined by the ratio of the radioactivity remaining in the lungs after vascular perfusion with 2.0 ml saline to the amount of radioactivity present in 100 μl blood obtained from the animal at the time of sacrifice. Blood was collected from the posterior vena cava in a heparinized syringe.

Treatments

Catalase (Sigma Chemical Co., St. Louis, Mo) was derived from bovine liver and contained 11,000 units/mg protein and was mixed with saline and injected intraperitoneally as well as intravenously with the CVF. Superoxide dismutase (Sigma Chemical Co., St. Louis, Mo) was isolated from bovine blood and had an activity of 2800 U/mg protein. Injection of superoxide dismutase (SOD) into mice was similar to the protocol used for catalase.

Antiplatelet serum was produced in young adult, female New Zealand white rabbits by primary and booster immunizations with the use of platelets isolated from heparinized mouse blood. Material used for the immunizations consisted of 0.5 ml incomplete Freund's adjuvant containing 5×10^7 platelets obtained from C57Bl/J6 mice. Multiple intradermal sites were used for injection, with a 2-week interval between the initial and the boosting immunizations. Blood was collected 10 days later from the immunized rabbits and tested for platelet-depleting activity in mice by the intraperitoneal injection of 0.5 ml rabbit serum followed by monitoring of hematologic parameters (hematocrits, platelet counts, total leukocyte and absolute neutrophil counts) 18 hours later. Antiserum to neutrophils from C57Bl/J6 mice was obtained by similar immunization of white New Zealand rabbits using neutrophils from glycogen-elicited peritoneal exudates.

Nitrogen mustard (Mustargen, Merck Sharp and Dohme, West Point, Pa) was injected intravenously in a volume of 0.2 ml and in an amount of 5.5 mg/kg 3 days prior to use of mice in the experimental protocols. The mice were evaluated for neutropenia 3 days later and considered neutropenic if they had less than 200 neutrophils/cu mm blood.

Mice were treated 6 hours before intravenous injection of CVF with a variety of inhibitors according to the following protocols: 1) indomethacin (Sigma Co., St. Louis, Mo), a cyclooxygenase inhibitor, 5 mg/kg body weight intraperitoneally; 2) BW775C (Burroughs Wellcome Co., Research Triangle, NC), a combined cyclooxygenase and lipoxygenase inhibitor, 50 mg/kg intraperitoneally; 3) U63557 (gift of Dr. Robert Gorman, Upjohn Co., Kalamazoo, Mich), a thromboxane synthetase inhibitor, 10 mg/kg intraperitoneally; 4) diphenhydramine (Parke-Davis Co., Ann Arbor, Mich), a H_1 histamine receptor antagonist, 1 mg intraperitoneally; 5) cimetidine (SmithKline Beckman, Philadelphia, Pa), a H_2 histamine receptor antagonist, intraperitoneally; 6) ibuprofen (Upjohn Co., Kalamazoo, Mich), a cyclooxygenase inhibitor, 10 mg/kg intraperitoneally; 7) piroxicam (Pfizer, New London, Conn), a cyclooxygenase inhibitor, 10 mg/kg intraperitoneally; 8) nordihydroguaiaretic acid, NDGA (Sigma Chemical Co., St. Louis, Mo), a combined lipoxygenase and cyclooxygenase inhibitor, 25 mg/kg intraperitoneally; 9) nafazatrom (Miles Laboratories, Elkhart, Ind), a lipoxygenase inhibitor, 25 mg/kg intraperitoneally; 10) methysergide nucleate (Dorsey Laboratories, Lincoln, Neb), 1 mg intraperitoneally. The specificity of these inhibitors has recently been described in detail, with the use of ^{3}H-arachidonic-acid-labeled neutrophils, a variety of agonists, and analysis of cyclooxygenase and lipoxygenase products by immunochemical analysis and by high-pressure liquid chromatography.[7]

Morphologic Analysis of Lung Tissues

Lungs from animals were routinely examined 30 minutes after injection of CVF. The lungs of selected mice were fixed by airway inflation with glutaraldehyde, embedded in plastic, and sectioned for examination by light and transmission electron microscopy.

Statistical Analysis

Data were expressed as the mean ± standard error of the mean. The Student t test was used for analysis of statistical significance (with calculation of P values) of the data.

Table 1—Requirement for C5 in Acute Lung Injury

Experimental group*	Mouse strains†	Treatment with CVF	Number of mice	Lung vascular permeability	
				Individual values	mean ± SEM
A	C57 BL/J6	–	10	0.086, 0.137, 0.163, 0.106, 0.177, 0.175, 0.278, 0.180, 0.115, 0.116	0.153 ± 0.017
B	C57 BL/J6	+	10	0.402, 0.708, 0.638, 0.440, 0.912, 0.849, 0.820, 0.947, 0.711, 0.955	0.738 ± 0.063
C	$B_{10}D_2$, old line	–	8	0.084, 0.126, 0.138, 0.250, 0.135, 0.189, 0.131, 0.111	0.143 ± 0.019
D	$B_{10}D_2$, old line	+	12	0.124, 0.282, 0.107, 0.117, 0.219, 0.113, 0.327, 0.177, 0.282, 0.287, 0.137,0.235	0.201 ± 0.023
E	$B_{10}D_2$, new line	–	5	0.295, 0.298, 0.310, 0.258, 0.293	0.291 ± 0.007
F	$B_{10}D_2$, new line	+	5	0.512, 0.459, 0.666, 0.861, 0.811	0.662 ± 0.080

* Calculated *P* values: A versus B, <0.001; C versus D, <0.05; E versus F, <0.005; D versus F, <0.001.
† Old line is C5-deficient; new line is C5-sufficient.

Results

Requirements of C5 for Full Expression of Lung Injury

Systemic activation of complement was carried out in complement-sufficient (C57 Bl/J6) mice by the intravenous injection (via a tail vein) of 1.0 unit CVF. As shown in Table 1, there was nearly a fivefold increase in the extent of lung injury as defined by increased lung vascular permeability using ^{125}I-bovine serum albumin as a marker of vascular permeability (from a value of 0.153 ± 0.017 to 0.738 ± 0.063; Table 1). We next employed congenic strains of C5-deficient ($B_{10}D_2$, old line) and C5-sufficient ($B_{10}D_2$, new line) mice in order to determine whether C5 was a critical requirement for the induction of acute lung injury. As shown in Table 1, in C5-deficient (old line) mice there was less than a doubling in lung vascular permeability (from 0.143 ± 0.019 to 0.201 ± 0.023) when mice given the CVF were compared with those given intravenous saline. In rather striking contrast, the congenic C5-sufficient (new line) strain had a higher "background value" (0.291 ± 0.007), which increased significantly (to 0.662 ± 0.080) following the injection of CVF. If the "background" values for lung permeability changes are subtracted from the lung permeability values caused by the injection of CVF, it can be calculated that the lung permeability changes in the C5-deficient strain were, on average, only 15.6% of the values obtained in the C5-sufficient mice. These studies suggest that C5 is a critical requirement for the induction of acute lung vascular injury in mice following the systemic injection of CVF.

Catalase-Induced Protection From Acute Lung Injury

Our earlier studies in rats have demonstrated that pulmonary vascular damage following systemic activation of complement is chiefly related to interstitial vascular endothelial cell injury, which can be ascribed to production by neutrophils of H_2O_2 (and its conversion to $^{\cdot}OH$) and that systemic treatment of rats with catalase affords significant protection against lung injury.[1,2] Protection is calculated by subtracting the mean lung permeability value of saline-treated animals (0.125) from the mean of the CVF-injected group, dividing by 0.780 (derived by 0.905–0.125), and then subtracting this value from 1.000 and multiplying the value by 100 to derive a percentage number. Similar studies were performed in mice (C57 Bl/J6) given intravenous injections of 1.0 unit CVF. As shown in Table 2, there was more than a sevenfold increase in lung vascular permeability (to 0.905 ± 0.070) in mice treated with CVF. In a dose-dependent manner, the intravenous injection of 6, 12, and 24 mg catalase (the first dose containing 66,000 units enzyme activity) achieved significant degrees of protection (52.6%, 56.7%, and 73.5%, respectively), while the intravenous injection of SOD at a dose of 24 mg (containing 67,200 enzyme activity) produced a much lesser degree (19.9%) of protection. These data are similar to our prior findings in the rat[1] and are consistent with the concept that H_2O_2 or some conversion products are the key factors responsible for lung vascular injury.

Comparing the number of neutrophils and platelets in the blood of 4 mice treated with 24 mg catalase or with injections of saline, catalase treatment resulted in a mean increase of 40% in the number of neutrophils and no change in the number of platelets. Thus, the protective effects of catalase are not due to induction of a leukopenia or a thrombocytopenia.

Role of Cellular Elements of Blood in Lung Vascular Injury

A recent report has indicated that neutrophils play a key role in the development of acute lung injury in

Table 2—Catalase-Induced Protection Against Acute Lung Injury

Treatment with CVF	Amount of enzyme injected	Number of mice	Lung vascular permeability		Protection (%)*	Significance (*P* value)
			Individual values	Mean ± SEM		
–	0	6	0.168, 0.232, 0.100, 0.044, 0.108., 0.098	0.125 ± 0.026		
+	0	7	0.799, 0.745, 0.923, 1.03, 0.808, 0.769, 1.26†	0.905 ± 0.070		<0.001
+	6 mg catalase (66,000 units)	2	0.477, 0.510	0.494 ± 0.0 ± 17	52.6	<0.001
+	12 mg catalase	7	0.388, 0.498, 0.752, 0.420, 0.441, 0.477, 0.510	0.463 ± 0.0 ± 8	56.7	<0.001
+	24 mg catalase	2	0.395, 0.269	0.332 ± 0.064	73.5	<0.01
+	24 mg SOD (67,000 units)	3	0.822, 0.827, 0.625	0.756 ± 0.087	19.9	<0.001

* See text for method of deriving these values.
† Reference values for calculation of *P* values were obtained from this group of 7 data points.

rats following systemic complement activation.[1] Experiments were designed in mice to determine whether neutrophils and platelets might be involved in the pathogenesis of lung vascular injury following injection of CVF. Five manipulations, as described in Table 3, were employed. Treatment of C57 Bl/J6 mice with the intraperitoneal injection of normal rabbit serum did not appreciably change the hematologic parameters as defined by hematocrits, total leukocyte counts, absolute neutrophil counts, and platelet counts. When mice were given intraperitoneal injections (18 hours earlier) of rabbit serum obtained from rabbits hyperimmunized to murine platelets, there was a profound depletion (>98%) in the number of blood platelets in the treated mice, with a fall from a mean count of 1.338×10^6/cu mm to 15,000/cu mm blood. Platelet-depleted mice had a slight reduction (15.9%) in the blood neutrophil levels when compared with mice given saline. There was no significant change in the hematocrit levels of platelet-depleted mice.

Two approaches were employed to achieve neutrophil depletion: nitrogen mustard and antineutrophil serum. Nitrogen mustard treatment of mice caused a 17% drop in the hematocrit levels (to 38.3 ± 1.04), a leukopenia (93% fall in total circulating leukocytes), an 84.4% fall in levels of circulating neutrophils (to 226 ± 51/cu mm) and a 12.5% drop (to $1.17 \pm 0.056 \times 10^6$/cu mm) in platelet levels of blood (Table 3). In the group of mice treated with antineutrophil serum, there was no drop in the hematocrit, a mild leukocytosis (to 9.58×10^3 cells/cu mm), a profound neutropenia (falling from 1452 ± 164 in controls to 203 ± 50 cells/cu mm in treated mice) and a mild (9.5%) fall in levels of platelets in the blood.

C57 Bl/J6 mice treated with the various reagents described above were studied for the development of acute lung injury following intravenous infusion of 1.0 unit CVF. As shown in Table 4, there was approximately a sevenfold increase in lung vascular permeability in mice treated with CVF when compared with mice given normal rabbit serum or saline. Prior treatment of mice with normal rabbit serum had a relatively small depressing effect (7.4%) on the degree of lung vascular permeability (mean value of 0.908 ± 0.142). On the other hand,

Table 3—Specificity of Cellular Depletion

Treatment*	Number of animals	Parameter measured			
		Hematocrit (%)	Total leukocytes† $\times 10^{-3}$	Neutrophils $\times 10^{-3}$	Platelets† $\times 10^{-6}$
Saline	8	46.2 ± 0.95	7.14 ± 0.57	1.452 ± 0.164	1.338 ± 0.053
Normal rabbit serum	7	44.3 ± 0.75	7.25 ± 0.57	1.785 ± 0.265	1.24 ± 0.041
Anti-platelet serum	15	46.7 ± 1.04	6.34 ± 0.50	1.22 ± 0.258	0.015 ± 0.002
Nitrogen mustard	10	38.3 ± 1.04	0.50 ± 0.097	0.226 ± 0.051	1.17 ± 0.056
Anti-neutrophil serum	8	49.9 ± 0.55	9.58 ± 0.661	0.203 ± 0.050	1.21 ± 0.066

* Animals were injected with 0.5 ml saline or serum 18 hours before intravenous injection of 1.0 unit CVF. Nitrogen mustard was administered intravenously 3 days prior to injection of CVF.
† Cells per cubic millimeter.

Table 4—Effect of Cellular Depletion on Acute Lung Damage

Material injected into mice*	Treatment with CVF	Number of mice	Lung vascular permeability		Protection (%)†	Significance (*P* value)
			Individual values	Mean ± SEM		
Saline		5	0.086, 0.137, 0.163, 0.106 0.177	0.124 ± 0.036		
Normal rabbit serum	–	4	0.115, 0.222, 0.175, 0.149	0.165 ± 0.026		
Saline	+	3	0.921, 0.771, 1.22‡	0.971 ± 0.132		
Normal rabbit serum	+	3	1.16, .667, 0.897	0.908 ± 0.142	7.4	>0.05
Anti-platelet serum	–	4	0.267, 0.080, 0.176, 0.131	0.163 ± 0.040		<0.005
Anti-platelet serum	+	8	0.419, 0.471, 0.281, 0.464 0.418, 0.430, 0.454, 0.351	0.411 ± 0.023	66.1	<0.001
Anti-neutrophil serum	–	4	0.109, 0.128, 0.108, 0.096	0.110 ± 0.007		
Anti-neutrophil serum	+	8	0.109, 0.125, 0.350, 0.296, 0.570, 0.375, 0.477, 0.306	0.341 ± 0.050	74.3	<0.001
Nitrogen mustard	+	9	0.135, 0.546, 0.381, 0.247, 0.321, 0.148, 0.405, 0.164, 0.242	0.288 ± 0.046	80.6	<0.001

* See Table 3 and text for details.
† Calculated as described in the text. Reference data are from mice given saline and CVF.
‡ Reference group for calculation of *P* values.

severe platelet depletion (described in Table 3) resulted in 66.1% protection against lung injury as defined by diminished lung vascular permeability. As had occurred in CVF-treated rats,[1] neutrophil depletion in mice produced by pretreatment either with antiserum or with nitrogen mustard had significant protective effects (74.3% and 80.6%, respectively) in the development of lung vascular injury. In the absence of cobra venom factor, neither platelet depletion nor neutrophil depletion alone had a significant effect on lung vascular permeability values following the infusion of saline and ^{125}I-bovine serum albumin (Table 4). These data suggest that both neutrophils as well as platelets may contribute to the development of acute lung injury in mice following systemic complement activation.

Lack of Role of Neutrophil Proteinases in Acute Lung Injury Following Systemic Complement Activation

In earlier studies it was shown that in mouse neutrophils the bulk (>80%) of lysosomal protease activity has a pH optimum at neutrality, that most of this enzyme activity has characteristics similar to human leukocyte elastase, and that in neutrophils of beige mice the enzyme activity is virtually absent.[8,9] We employed beige mice with the homozygous trait (bg +/+) and congenic mice with the heterozygous defect (bg +/−) for these studies. The latter group has neutrophils that almost fully express neutral protease activity.[9] These two congenic strains (bg +/+ and bg +/−) were given injections of CVF, and the degree of resulting acute lung damage (as measured by lung vascular permeability changes) was assessed. Although the number of mice available for the study was limited, the data in Table 5 reveal that the degree of lung damage in the homozygous strain of beige mice was slightly diminished (1.37 ± 0.099) after infusion of the CVF, as compared with lung injury in the heterozygous strain (1.52 ± 0.247). These data suggest that the virtual absence of neutral protease in neutrophils of beige mice confers little protection against acute lung injury following systemic complement activation. Accordingly, it appears that lysosomal neutral proteases of neutrophils are not essential during the first 30 minutes for the full expression of acute lung vascular injury in mice following systemic activation of the complement system.

Morphologic Factors in Lung Damage

Transmission electron microscopic examination of lungs from mice given venous injections of CVF 30 minutes earlier revealed prominent aggregates of neutrophils and platelets within the pulmonary interstitial capillaries (Figure 1). Many platelets showed evidence of degranulation. Many neutrophils were in direct contact with vascular endothelial cells, which showed evidence of extensive blebbing (Figure 2). In addition, edema involving subendothelial regions was apparent (Figures 1, 2). Evidence of cell damage (cytoplasmic blebbing) appeared to be limited to lung vascular endothelial cells, because alveolar lining cells were intact and no intraalveolar hemorrhage was present.

Table 5—Acute Lung Injury in Beige Mice

Genotype	Treatment with CVF	Number of mice	Lung vascular permeability	
			Individual values	Mean ± SEM
bg +/−	−	2	0.281, 0.108	0.195 ± 0.087
bg +/−	+	4	0.788, 1.89, 1.66, 1.73	1.52 ± 0.247
bg +/+	−	2	0.280, 0.112	0.197 ± 0.085
bg +/+	+	4	1.12, 1.44, 1.31, 1.59	1.37 ± 0.099

Inhibitor-Induced Protection Against Acute Lung Injury

Mice were treated with a series of inhibitors for determination of whether mediators responsible for the acute lung injury could be tentatively identified. The doses of inhibitors employed were based on a recent report by Kunkel et al[10] in which mice were treated with doses that were nontoxic. The biochemical specificity of the inhibitors was shown in the same report. As the data in Table 6 (Experiment A) indicate, the cyclooxygenase inhibitor indomethacin had mild protective effects (34.6%), while treatment of mice with BW755C (having combined cyclooxygenase and lypoxygenase inhibitory activity,[7]) and with the thromboxane synthetase inhibitor,[11] U63557, produced 58.7% and 36.4% protection, respectively. Neither treatment with the serotonin antagonist methysergide nor treatment with the H_1 histamine receptor antagonist diphenhydramine produced significant protection from acute lung injury, although the H_2 histamine receptor antagonist cimetidine caused 44.7% protection from lung injury.

In another series of experiments (Table 6, Experiment B), the cyclooxygenase inhibitors ibuprofen and piroxicam failed to protect the animals. In fact, there was an increase in CVF-induced acute lung injury in mice pretreated with these drugs. NDGA, which has inhibitory activity for both cyclooxygenase and lipoxygenase,[7] afforded 21.2% protection from acute lung injury, while the lipoxygenase inhibitor nafazatrom gave 42.7% protection from acute lung injury. These data suggest that lipoxygenase pathways may be required for the full expression of lung injury, that serotonin does not play a significant role, and that histamine (via a H_2 receptor) and thromboxane may play some role in the development of lung injury.

Discussion

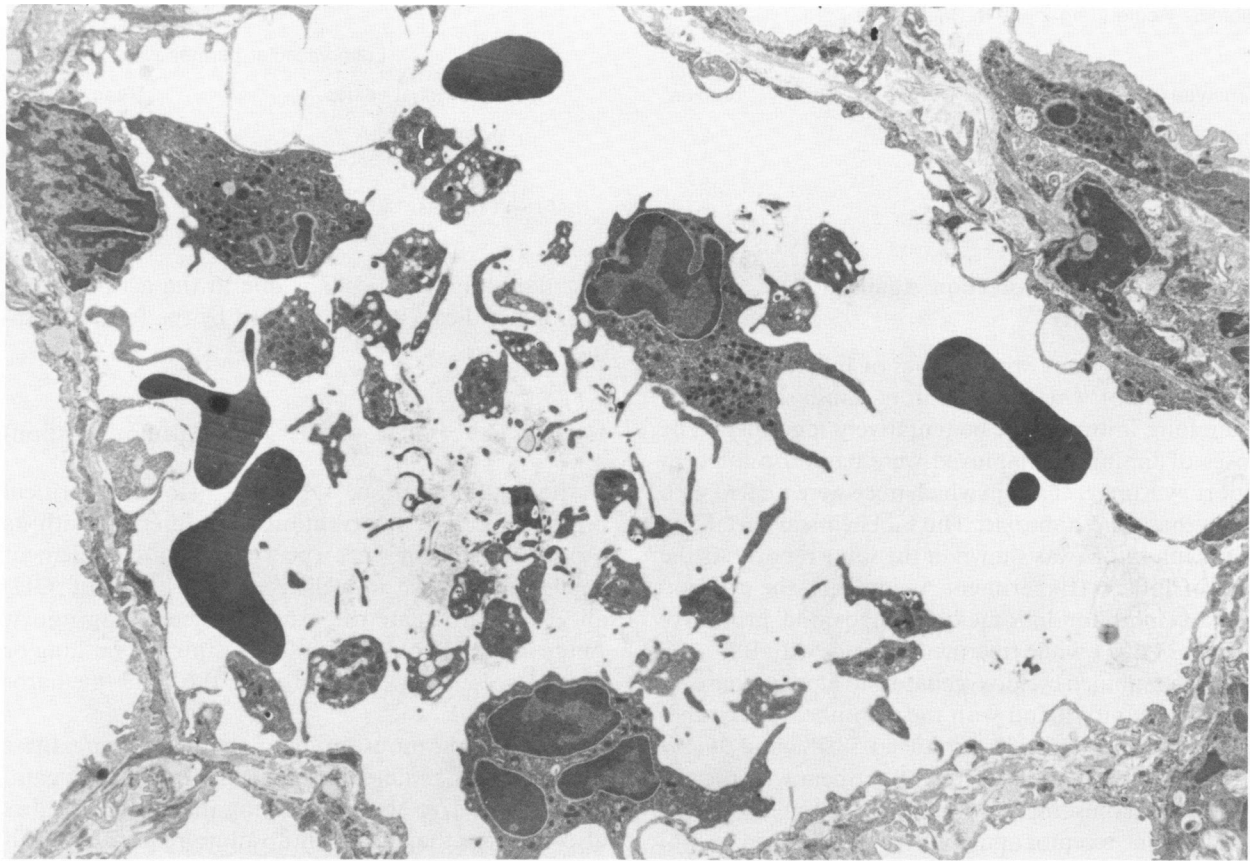
The studies outlined in this report take advantage of the availability of congenic strains of mice. In several respects, the data are similar to our recent reports of CVF-induced acute lung injury in rats.[1,2] In the rat model, there were absolute requirements for an intact complement system, neutrophils, Fe^{3+}, and H_2O_2. Accordingly, it has been postulated that damage of lung vascular endothelial cells is due to the action of the hydroxyl radical ($\cdot OH$) produced by the following sequence.

$$O_2^{\cdot} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$
$$H_2O_2 + Fe^{2+} \rightarrow OH^- + \cdot OH + Fe^{3+} \text{ (Fenton reaction)}$$

In the Fenton reaction, $\cdot OH$ appears to be the critical product, because interventions that interfere with its production or presence (neutrophil or complement depletion, catalase, iron chelators, scavengers of $\cdot OH$) all greatly attenuate the extent of acute lung injury. Acute immune-complex–induced injury of rat lung or dermal vessels appears to follow the same mediator pathways.[12]

Studies in the mouse point to a similar picture: there is an absolute requirement for neutrophils, and catalase is protective against the lung injury. The studies also indicate that C5 is an absolute requirement, although whether an activation product such as C5a is a critical mediator cannot be determined. Perhaps the C5-9 membrane attack complex is instrumental either in activating the neutrophil to generate oxygen radicals or directly causes damage to lung vascular endothelial cells or otherwise renders these cells susceptible to neutrophil-induced injury. It should be noted that Henson and his colleagues have suggested that exposure of the lung to prostaglandin E_2[3] somehow causes the tissue to become susceptible to injury produced by the systemic infusion of complement-activated serum. It is possible that an *in vivo* generated complement activation product may cause the lung to become susceptible to the effects of oxygen radicals produced by contact of blood neutrophils with C5a. Our studies in the mouse imply that generation of C3b is not sufficient to bring about ultimate injury of endothelial cells.

Based on the use of beige mice, it would appear that complement-induced injury of lung vascular endothelial cells does not have an absolute requirement for the presence of neutral proteases derived from neutrophils, although in the rabbit chemotactic-factor–induced alveolar damage following airway instillation of synthetic chemotactic peptide is associated with the appearance of substantial amounts of neutrophil derived protease activity and the presence of oxidatively inactivated α_1-antiprotease.[13] It should also be stressed that while lung



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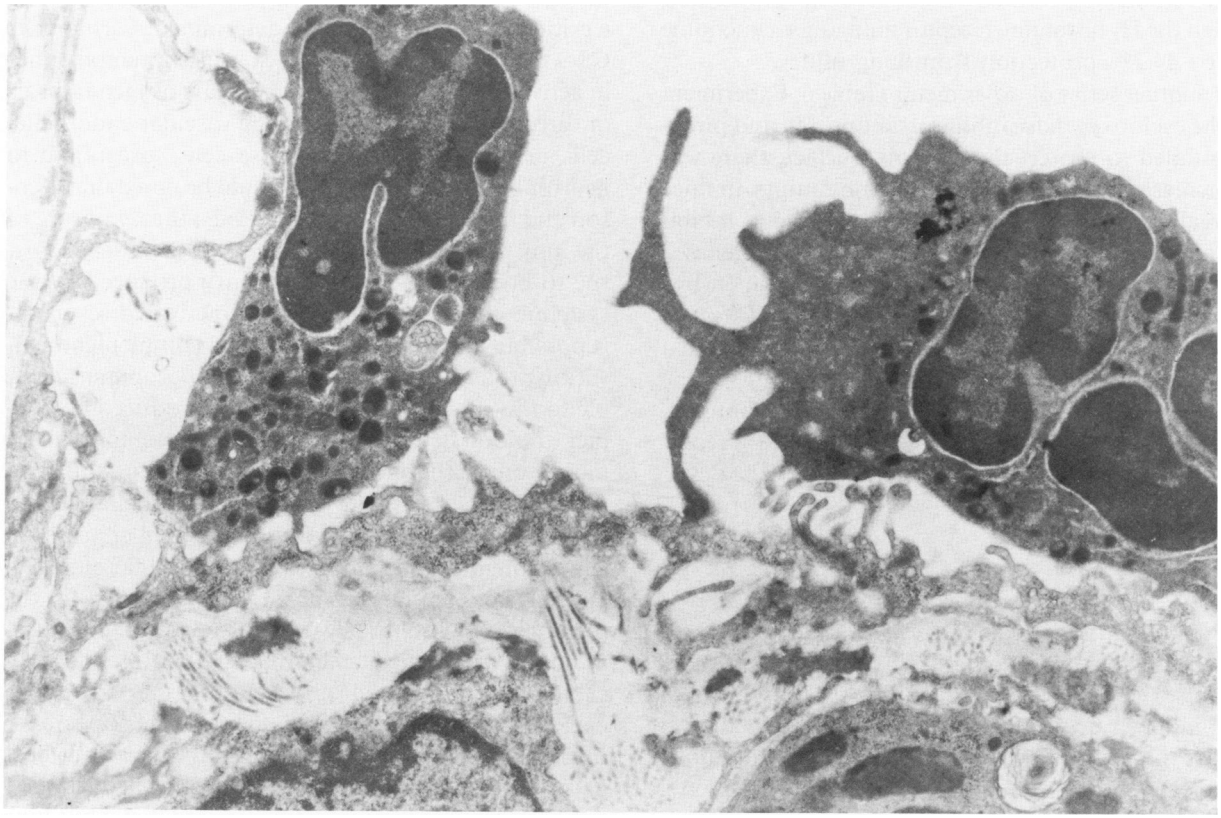

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Table 6—Protection Against Lung Injury in CVF-Treated Mice

Experimental treatment	Number of mice	Lung vascular permeability		Protection (%)	Significance (*P* value)
		Individual values	Mean ± SEM		
A					
None	8	1.44, 1.82, 1.85, 1.51, 1.33, 1.04, 1.56, 1.48*	1.50 ± 0.092		
Indomethacin	6	0.77, 1.09, 1.05, 0.89, 1.16, 0.95	0.98 ± 0.058	34.6	<0.001
BW775C†	4	0.44, 0.79, 0.61, 0.65	0.64 ± 0.072	58.7	<0.001
U-63557‡	5	0.769, 1.00, 1.00, 0.88, 1.12	0.95 ± 0.060	36.4	<0.005
Methysergide	5	1.60, 1.78, 1.52, 1.37, 2.04	1.66 ± 0.115	0	>0.05
Diphenhydramine	6	1.14, 1.24, 1.64, 0.95, 2.02, 1.26	1.37 ± 0.159	8.7	>0.05
Cimetidine	5	0.59, 1.04, 0.71, 0.83, 0.98	0.83 ± 0.026	44.7	<0.001
B					
None	4	1.98, 1.42, 1.82, 1.75*	1.74 ± 0.118		
Ibuprofen	6	2.56, 2.31, 3.76, 3.65, 2.86, 4.05	3.19 ± 0.292	0	<0.005
Piroxicam	4	1.16, 2.22, 2.05, 2.63	2.01 ± 0.309	0	>0.05
NDGA	4	1.45, 1.52, 1.01, 1.53	1.37 ± 0.123	21.2	>0.05
Nafazatrom	5	0.945, .753, 1.16, 0.784, 1.34	0.996 ± 0.112	42.7	<0.005

* Reference group for calculation of *P* values.
† Combined cyclooxygenase and lipoxygenese inhibitor as documented elsewhere.[7]
‡ Thromboxane synthetase inhibitor.[10]

injury related to oxygen radical generation by neutrophils can occur in the absence of participation of leukocytic neutral proteases, there is recent evidence that a synergism exists between oxygen radicals and proteases. The presence of nanomolar amounts of H_2O_2 will greatly enhance the ability of serine proteases present in preparations of trypsin and rat neutrophil extracts to hydrolize a variety of substrates.[14] In addition, leukocytic proteases have the ability to cause the lifting off of endothelial cells from monolayers *in vitro* in a noncytotoxic manner.[15] Thus, proteases may participate in a variety of ways to bring about injury to, or alterations of, vascular endothelial cells.

The data presented in this paper represent an important and novel new observation, namely, that platelets potentiate the development of the full extent of acute lung injury following vascular infusion of CVF. It is unknown in what manner platelets contribute to the injury. There is evidence that platelets can transfer their arachidonate intermediates to endothelial cells, resulting in increased amounts of PGI_2 being produced by endothelial cells.[16,17] How this would lead to intensified damage of endothelial cells is not clear. Alternatively, there is also a "shuttle" of arachidonate intermediates between the platelet and the neutrophil, resulting in increased production by the neutrophil of di-HETE compounds.[18] By this mechanism the response of the neutrophil to complement-activated products may be accentuated, with the generation of larger amounts of toxic oxygen radicals and the release of greater amounts of lysosomal enzyme. Alternatively, platelet release of its glycoproteins, proteases, TxA_2 or other factors may also result in some direct or indirect insult to pulmonary vascular endothelial cells, causing them to become especially susceptible to the toxic oxygen products of activated neutrophils in a manner similar to the ability of prostaglandin E_2 to render pulmonary vascular endothelial cells susceptible to injury.[3]

Finally, the use of a variety of inhibitors of arachidonate derivatives gives some suggestion but no clear picture of mediator systems involved in development of acute lung injury. Consistent with early data from the CVF-induced model of acute lung injury in the rat,[1] our current studies in the mouse suggest that although

Figure 1—Transmission electron micrograph of the lung of mouse injected 30 minutes earlier with 1.0 unit CVF. Neutrophils and platelets are present in clusters within an interstitial pulmonary capillary. At higher magnification (not shown), platelets showed evidence of degranulation. Blebbing changes in endothelial cells are prominent. (×5500) **Figure 2**—Transmission electron micrograph of an interstitial vessel similar to that described in Figure 1. Two neutrophils are in direct contact with vascular endothelial cells, one of which shows evidence of bleb formation. There is evidence of edema in subendothelial areas. (×11,000)

complement-derived anaphylatoxins would be expected to cause histamine release *in vivo*, the lung permeability changes cannot be related to histamine acting via H_1-histamine receptors.[1] Why the H_2 receptor antagonist, cimetidine, has protective effects is unclear at the present time. The lack of protective effects in mice treated with the serotonin antagonist methysergide suggests that the lung injury is probably not linked to the release of serotonin from platelets. The ability of a variety of lipoxygenase inhibitors (BW755C, NDGA, or nafazatrom) to protect against acute lung injury could be explained by recent studies with rat neutrophils in which these inhibitors block the functional responses (enzyme secretion, oxygen radical generation) of neutrophils to a variety of agonists.[7] The inconsistent behavior of the cyclooxygenase inhibitors (indomethacin, ibuprofen, piroxicam) to protect against lung injury could also be explained on the differing effects of these inhibitors with respect to depressing oxygen-radical generation by activated neutrophils.[7]

The ability of ibuprofen and piroxicam to potentiate acute lung injury following infusion of CVF (Table 6) may be explained by the recent studies demonstrating that treatment of neutrophils with these inhibitors causes a "substrate shuttle" of arachidonic acid into the lipoxygenase pathway, yielding larger amounts LTB_4 and monoHETEs and an accentuated functional response of the neutrophil.[7] The ability of a specific thromboxane synthetase inhibitor, U63557, to protect from acute lung injury would be consistent with a role for TxA_2 produced either by the neutrophil[19] or by the platelet. A fuller explanation of these data will require extensive biochemical analysis of both neutrophils and platelets in the presence and absence of each inhibitor.

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