Oleic-Acid–Induced Lung Injury in the Rat

Failure of Indomethacin Treatment or Complement Depletion to Ablate Lung Injury

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The purpose of this study was to establish a rat animal model of acute respiratory distress syndrome using the intravenous injection of oleic acid. Further, we attempted to inhibit the development of lung injury by pretreatment of the rats with indomethacin or cobra venom factor. Histologic evidence of lung injury was apparent within hours after the administration of a single intravenous injection of oleic acid. By 24 hours, interstitial and intraalveolar edema and hemorrhage were noted with vascular congestion and an extensive interstitial infiltrate. The lungs appeared virtually normal by 12 days, with no evidence of chronic lung injury. Multiple injections of oleic acid also did not progress into chronic pulmonary inflammation. Treatment of the rats with indomethacin or cobra venom factor had no effect on ablating acute lung injury. An animal model of adult respiratory distress syndrome is presented which does not progress to chronic lung injury. (Am J Pathol 1981, 103:376–383)

THE OLEIC ACID MODEL of pulmonary edema has been well studied both physiologically and histologically. Intravenous injection of pure oleic acid into several different animal species has been observed to cause respiratory distress. Physiologically the reported changes include increased pulmonary artery and right atrial pressures, decreased systemic pressures, diminished lung compliance and severe hypoxemia.[1–9] Histologically the model is characterized by an acute hemorrhagic alveolitis that has been shown in the dog to progress to a fibrosing alveolitis.[9–12]

Despite the extensive work that has been done in examining oleic-acid–induced pulmonary changes, the precise mechanism of injury remains unclear. Elucidation of this mechanism may be important in advancing our understanding and our approach to the management of the adult respiratory distress syndrome (ARDS). Platelets and granulocytes have been implicated by some workers in the pathogenesis of the fat embolism syndrome.[6,13–16] Other workers have proposed that fatty acids cause a local tissue reaction in the lung, leading to inflammation.[6,12] The ensuing sequence of alveolar edema and atelectasis result in progressive ventilatory compromise.

Virtually all of the experimental work has been done in larger laboratory animals such as dogs, sheep, and rabbits. Establishment of this model of acute lung injury in a smaller animal, such as the rat, in which immune mechanisms have been well studied would facilitate controlled studies of relevant mediator systems. We present here a convenient protocol for the induction of respiratory distress in the rat with intravenous oleic acid. A brief description of the acute and chronic changes induced by oleic acid are presented. Data are also presented demonstrating that treatment of the rats with indomethacin or cobra venom factor had no effect on ablating acute lung injury.

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Materials and Methods

Animals

Adult male inbred Fischer 344 rats, weighing 175–200 g, were obtained from Charles River, Inc. (Wilmington, Mass). The animals were free of respiratory disease and housed in isolation from all other laboratory animals.

Reagents

Oleic acid (approximately 99% pure) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, Mo). Cobra venom factor (CVF) was purchased from Cordis Laboratories (Miami, Fla) and indomethacin, from E. Lilly and Co. (Indianapolis, Ind).

Administration of Oleic Acid

Various amounts of pure oleic acid, injected intravenously in a manner similar to that of experiments with large animal models[1-12] resulted in a high mortality. In order to reduce the dose of oleic acid, a suspension of oleic acid in a saline–albumin solution was made as follows. Oleic acid was shaken vigorously in a 0.1 mg/dl (wt/vol) solution of albumin in sterile normal saline in a volume ratio of 1:5. Of this oleic acid suspension, 250 μl was injected intravenously via the penile vein. Prior to oleic acid administration, rats were anesthetized with 25–30 mg of ketamine hydrochloride (Bristol Laboratories, Syracuse, NY) injected intraperitoneally.

Single Dose Experimental Protocols

Histologic Evaluation at Several Time Points

A single intravenous injection of 250 μl of the oleic acid suspension was given to rats. They were then sacrificed by exsanguination under ketamine anesthesia at 10 minutes, 30 minutes, 1 hour, 6 hours, 24 hours, 4 days, 12 days, and 4 weeks after injection. For light-microscopic study, portions of the lungs were fixed in 10% buffered formalin (pH 7.4), embedded in paraffin, and stained with hematoxylin and eosin. For electron microscopy, lungs were fixed in 4% cacodylate-buffered glutaraldehyde and postfixed in 2% cacodylate-buffered osmium tetroxide.

Quantitative Assessment of Lung Injury (Lung Permeability Ratio)

Changes in lung permeability were assessed by the extravascular accumulation of radiolabeled albumin in lung as described by Johnson and Ward.[17] Twelve hours prior to oleic acid injection, the rats were intraperitoneally injected with 0.5 μg of radiolabeled albumin in 1.0 ml NS. At the time of sacrifice a 1.0 ml sample of blood was collected from each animal and radioactivity measured in the deep-well scintillation counter. In addition, the pulmonary vasculature was perfused with 10 ml saline by injection into the right ventricle of the heart under direct visualization. Total radioactivity was measured for each lung in the scintillation counter. Data are calculated as the ratio of total radioactivity in an individual lung to the radioactivity in 1 ml of blood from the corresponding rat. Lungs, with the upper airways intact, were dissected en bloc, inflated, and fixed with formalin for histologic examination.

Indomethacin Treatment

Treated animals were given 5 mg of indomethacin intraperitoneally 4 hours before the administration of 250 μl of the intravenous oleic acid suspension. Control animals for this group received indomethacin, but no oleic acid. Positive and negative control animals received the oleic acid suspension alone or a 0.1 mg/dl albumin solution alone, respectively. All animals were sacrificed by exsanguination 1 hour after oleic acid or albumin injection. Damage was assayed histologically and by permeability changes, as described above.

C3 Depletion

Treated animals were given intravenous injections of 40 units Cobra venom factor (CVF) 4 hours prior to the administration of the oleic acid suspension. Serum complement levels were determined prior to the injection of CVF and at the time of sacrifice using the standard CH_{50} assay as described by Kabat and Mayer.[18] Control animals received CVF but no oleic acid.

Complement Levels After Oleic Acid Infusion

Serum was collected from a group of 5 rats for CH_{50} assay by serial tail bleeds prior to the injection of the oleic acid suspension, and 10 minutes, 1 hour, and 6 hours after injection. Data are expressed as the ratio of hemolytic complement at each time point to the hemolytic complement for each rat prior to the injection of oleic acid.

Statistical Analysis

Student's unpaired *t* test was used for analysis of the data.[19]

Multiple Dose Experimental Protocols

One group of 4 animals was injected intravenously with oleic acid suspension on 4 successive days and

then sacrificed at 4 weeks. Controls were injected with 1 mg/dl albumin solution.

A second group of 7 animals received weekly injections of oleic acid suspension for 7 weeks and were sacrificed 3 weeks after the last injection. Control animals again received similar injections of albumin solution. Lung injury was assessed histologically. The lungs from each animal were inflated and fixed with 4% buffered glutaraldehyde and submitted for light- and electron-microscopic study. Hematoxylin and eosin and Gomori trichrome stains were made for light-microscopic examination and standard uranyl acetate and lead citrate stains for electron-microscopic examination.

Results

Administration of Oleic Acid

Intravenous injections of pure oleic acid in doses as small as 80 μl were associated with a greater than 50% mortality, and this approach was abandoned because of the impracticality of injecting smaller amounts. The LD_{50} when oleic acid was suspended in a 1:5 volume ratio with the albumin solution was approximately 60 μl of oleic acid. This provided a convenient volume for the injection of 300 μl. When only 250 μl of the suspension was used, few animals died before sacrifice. The concentration of albumin used was the minimal concentration to the nearest order of magnitude that kept the oleic acid suspended long enough for us to comfortably inoculate the animals.

Histologic Evaluation of Lung Injury

Within minutes after the injection of oleic acid there were notable histologic changes, such as marked perivascular interstitial and intraalveolar edema, vascular congestion, intravascular coagulation, and intraalveolar hemorrhage; by 24 hours there was an extensive interstitial infiltrate with prominence of the alveolar lining cells and of alveolar macrophages (Figures 1–4). The lesion progressed in a manner characteristic of respiratory distress syndrome, which has been extensively described both in man and other animal models.[1-12] The lesion did not progress to pulmonary fibrosis or any form of chronic pulmonary inflammation in any of the animals studied, neither in animals which received a single injection of oleic acid nor in those animals which received multiple injections.

Effects of Indomethacin Treatment and C3 Depletion

Pretreatment with CVF was shown by CH_{50} assay to deplete serum complement greater than 95%. Grossly, the lungs of animals treated with indomethacin and oleic acid or CVF and oleic acid had the same hemorrhagic appearance as those of animals treated with oleic acid alone. Histologically, all three groups presented a similar picture of hemorrhagic alveolitis.

Animals treated with indomethacin and albumin solution, CVF and albumin solution, or albumin solution alone, had grossly and histologically normal lungs at necropsy. There was no statistically significant difference in permeability among these groups, and all control data were grouped together for comparison with treatment groups.

There was an approximately 7-fold increase in pulmonary vascular permeability in animals given oleic acid over control animals, but there was no significant difference in permeability among the groups treated with oleic acid and indomethacin, oleic acid and CVF, or oleic acid alone (Figure 5).

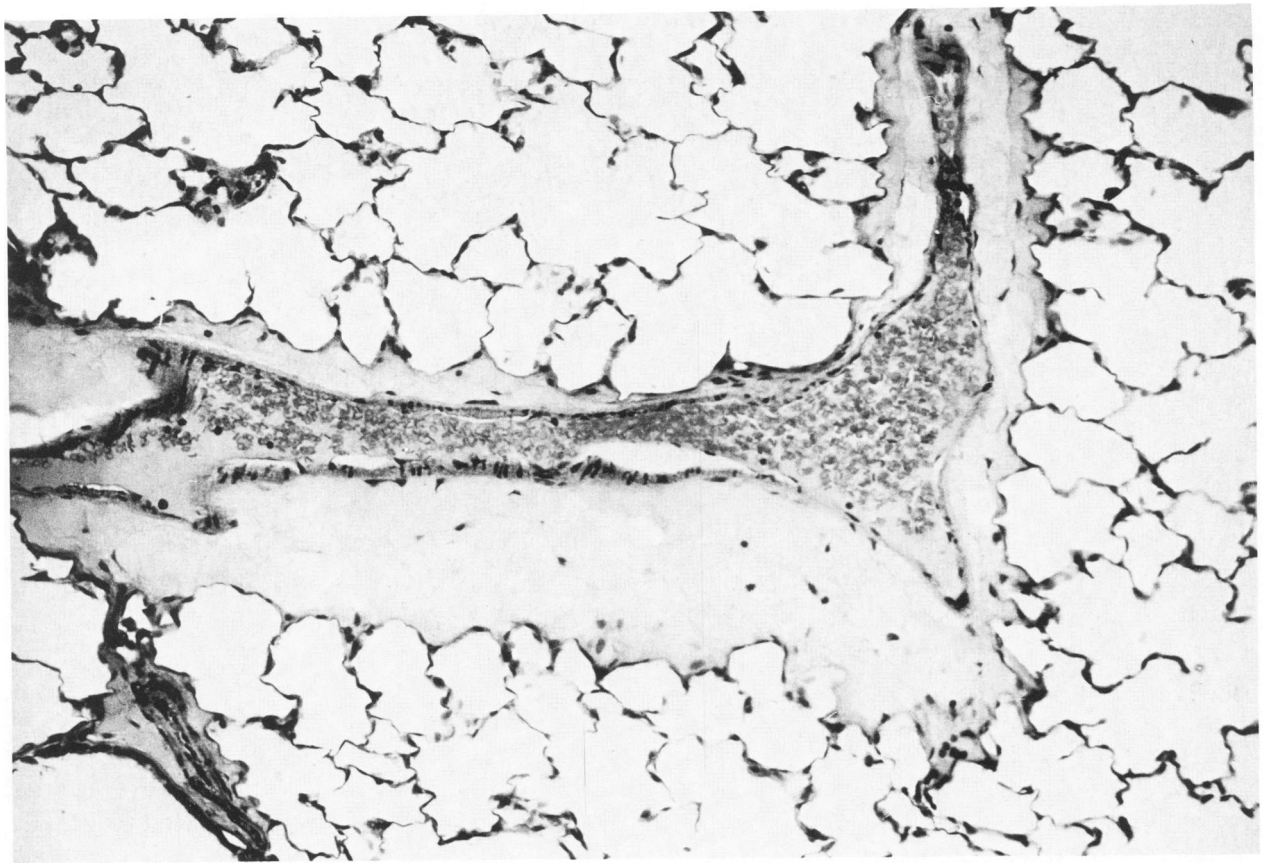
Complement Levels After Oleic Acid Infusion

The serum hemolytic complement levels of animals treated with intravenous oleic acid showed no significant changes over control values at 10 minutes, 1 hour, or 6 hours after oleic acid injection.

Discussion

The acute lung damage following intravenous infusion of oleic acid has been studied as a model for the so-called chemical phase of posttraumatic pulmonary fat embolism.[12] Two major mechanisms have been proposed for the genesis of fat embolism. The first is the mechanical theory, proposed by Gauss,[20] in which fat is forced from traumatized adipose tissue into the bloodstream via a damaged blood vessel. This theory limits the relevance of the oleic acid model largely to posttraumatic fat embolism. Alternatively, the physicochemical theory of Lehman-Moore[21] proposes that some biologic event, initiated by various types of trauma, alters the natural emulsion of fat in the bloodstream, leading to the formation of large droplets of fat that act like emboli.[6,13,22] The syndrome of fat embolism has in fact been reported in many situations, including severe burns, poisoning, use of the pump oxygenator, acute infection, hemorrhagic shock, and others.[13,22-24] The physicochemical theory points to a wider application of the oleic acid model, including the many nonspecific causes of ARDS. In fact, the oleic acid model has been widely used as an animal model of ARDS, because it consistently reproduces the tachypnea, hypoxemia, loss of lung compliance, and microscopic atelectasis of ARDS.[1,2,4,8,9,25]

Despite its potential applications as a model, oleic acid pneumopathy is far from fully understood. The



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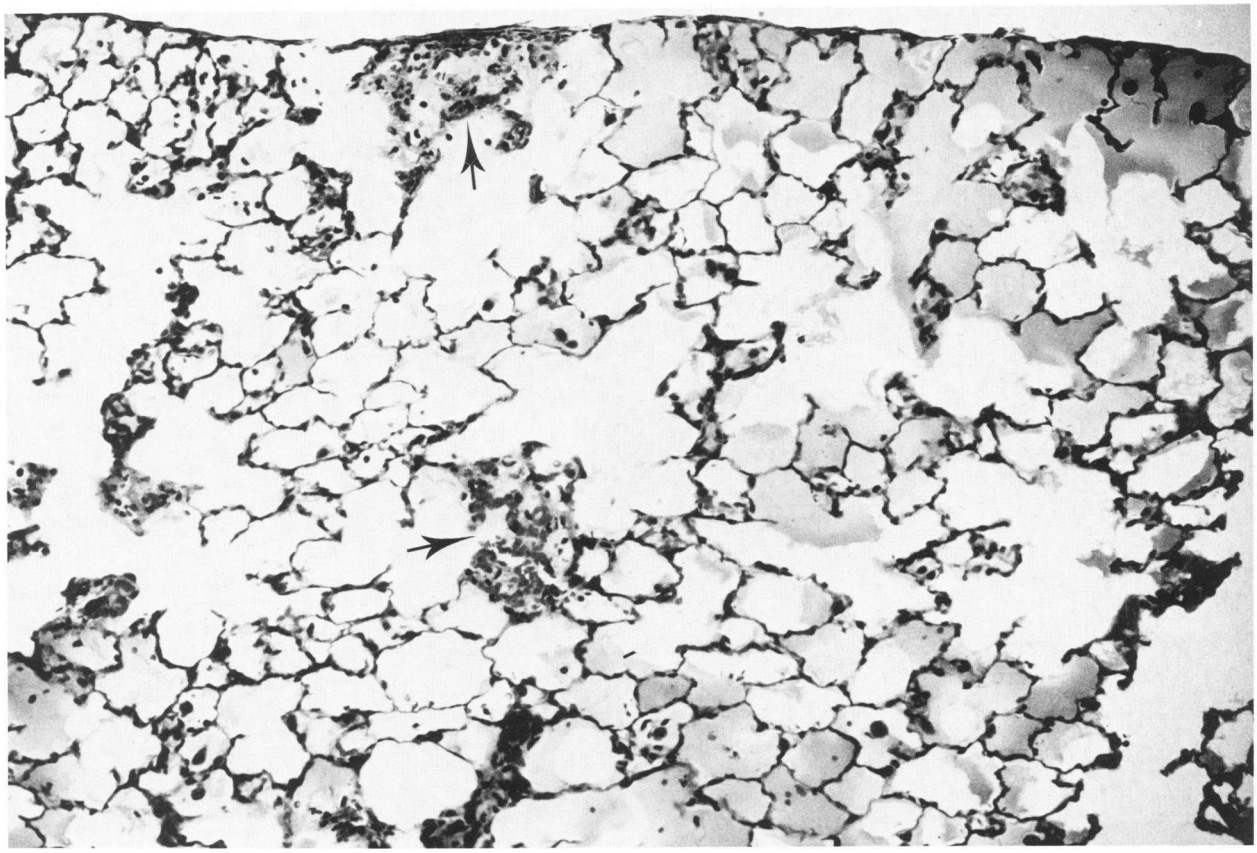

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Figure 1—Section of rat lung ten minutes after intravenous injection of oleic acid. Marked perivascular interstitial edema can be seen. (H&E, ×200) **Figure 2**—Six hours after injection of oleic acid there is widespread intraalveolar edema and some hemorrhage. Necrosis of septal walls is also present (*arrows*), and there is some infiltration of septal walls with neutrophils. (H&E, ×200)

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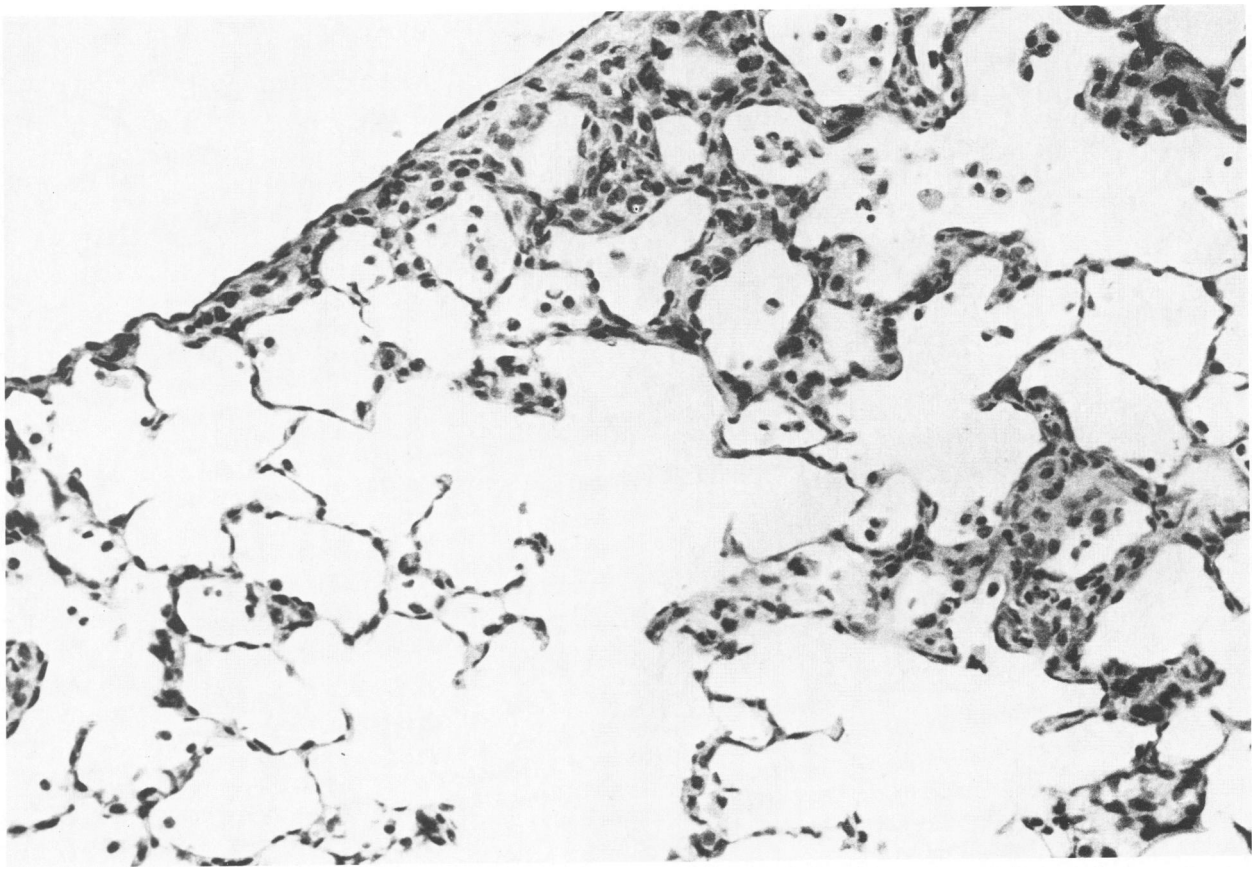

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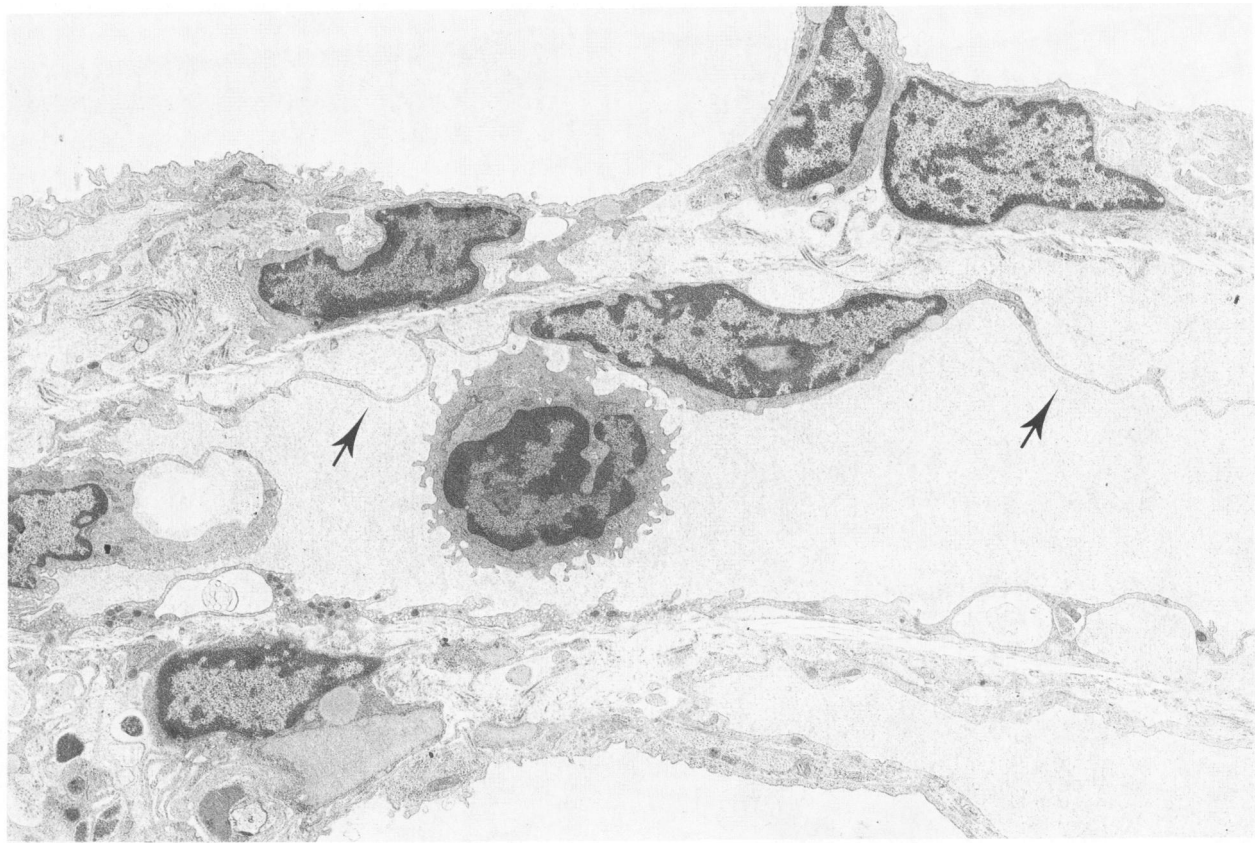

Figure 3—Four days after intravenous injection of oleic acid there is thickening of alveolar septa with an infiltration of chronic inflammatory cells in interstitial and subpleural locations. Numerous alveolar macrophages with foamy cytoplasm are prominent. (H&E, ×320) **Figure 4**—Ten minutes after injection of oleic acid, pulmonary vascular endothelial cells can be seen in electron-microscopic examination to be separating from the basement membrane (*arrows*). (Uranyl acetate and Sato's lead citrate, ×3850)

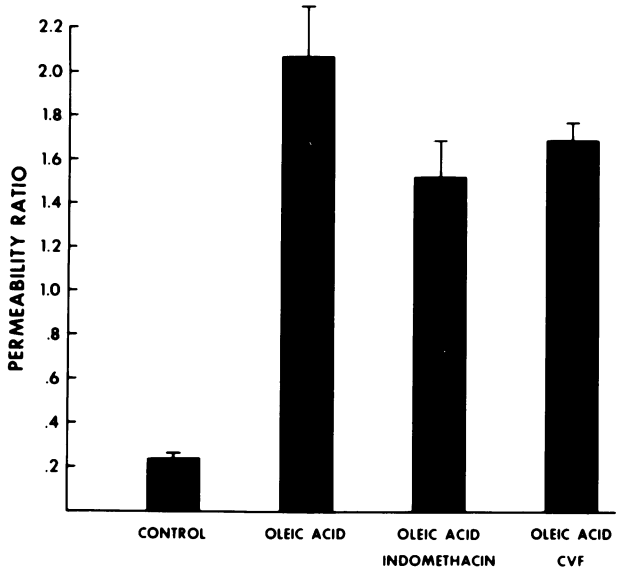

Figure 5—Inability to modify oleic-acid-induced lung injury by pretreatment of animals with indomethacin or cobra venom factor. An approximate 7-fold increase in lung permeability, used as an index of lung injury, was observed in all treatment groups over controls at 1 hour after oleic acid administration. No significant differences ($P > .05$) were observed among treatment groups. Vertical bars represent the mean (± SEM) of 5 animals except the control group, which includes 15 animals.

observation of platelet thrombi in experimental and in clinical fat embolism, and of thrombocytopenia occurring during the acute phase of oleic acid injury, has led to conjecture about the role of platelets in the etiology of this disorder.[13-15,26,27] Platelet thrombi have been invoked as the precipitating agents of fat emboli forming in the blood during trauma;[6] vasoactive platelet amines have been suspected of playing a role in increasing right-sided pressures and hypoxemia by causing pulmonary vasoconstriction and bronchospasm;[26,28-30] and platelet plugging of capillaries has also been thought to be a cause of elevated pulmonary artery pressure and hypoxemia.[13,31,32] The development of thrombocytopenia was felt by Bradford et al[26] to parallel the occurrence of hypoxemia following fat embolism, and Kay et al[15] found that experimental animals with the greatest ventilatory and hemodynamic derangements had the most pronounced hematologic alterations. It has recently been demonstrated that platelets are sequestered in the lung during oleic acid injury.[33] Our study of the effects of indomethacin was undertaken in part to clarify the platelet's role in lung injury. Indomethacin, a nonsteroidal drug known to inhibit platelet aggregation and release phenomena through its inhibition of prostaglandin synthesis,[34-36] was given to rats in an attempt to modify oleic acid lung injury through interference with platelet function. The fact that no significant decrease in lung permeability or in histologic damage was demonstrated suggests that the platelet may not play a direct role in acute oleic acid lung injury.

Jacob et al[16] and others[37,38] have proposed that the granulocyte may play an important but previously unsuspected role in the pathogenesis of ARDS. They suggest that activated complement can aggregate granulocytes and cause leukoembolization that could damage pulmonary tissues and cause pulmonary dysfunction through vascular stasis. (Vascular congestion is a prominent feature of acute oleic acid toxicity.) Granulocyte aggregation and degranulation in response to chemotactic stimuli such as C5a can be inhibited *in vitro* by indomethacin,[39] and the reversed passive Arthus reaction in rat lung can be ablated by pretreatment with indomethacin.[40] The inability of indomethacin to modify lung damage in our study mitigates against a pathogenetic role for granulocytes during the acute phase of oleic acid lung injury. Further evidence against complement-induced granulocyte aggregation as a mechanism of injury in this model is provided by the failure of pretreatment with CVF to protect against lung injury. It should be noted that indomethacin has effects on prostaglandin synthesis in a variety of cells other than granulocytes and platelets, and any conclusions regarding the effects of indomethacin cannot necessarily be restricted to granulocytes and platelets. Also, indomethacin treatment does block the synthesis of some prostaglandins, but not all. It is conceivable that prostaglandin synthesis that is not inhibited by indomethacin plays an important role in the pathogenesis of this disease.

Another theory of the pathogenesis of respiratory failure caused by fat embolism involves a complex cycle in which free fatty acids cause a severe local inflammatory reaction leading to vascular congestion, hypoxia, and acidosis. These in turn cause further cell damage and inflammation.[6,12,13] As an important mediator of inflammation, the complement system might be expected to play a role in this cycle. Several studies have indicated that the complement system is activated in shock states, particularly those incited by sepsis, hemorrhage, and trauma.[16,41-44] The fact that complement levels did not significantly decline during the acute phase of lung injury obviates a general activation of the complement system in the oleic acid model. The inability of C3 depletion to modify permeability changes or histologic damage indicates that permeability changes may not be mediated by anaphylatoxin and that a complement-mediated local inflammatory reaction may not play a major role in oleic-acid-induced lung injury.

Other proposals for the mechanism of injury have included the binding of calcium at intracellular junctions,[12,45] derangements of the lipids in cell membranes,[26,29] increased resistance in small pulmonary

veins,[4,30] and tissue necrosis from local acidity.[12] Eludication of the precise mechanism of oleic acid injury may be important in advancing our understanding and management of ARDS.

To facilitate controlled studies of oleic acid pneumopathy in a manipulatable laboratory animal and one in which immune mediator systems have been well studied, we developed the oleic acid model in the rat. We made the suspension in albumin solution to attenuate the high toxicity of pure oleic acid. It is well known that fatty acids are generally bound to albumin in the circulation and that they are not toxic in this condition.[46] However, the concentration of oleic acid we used in our suspension far exceeded the tight binding sites for fatty acids in the albumin solution.[47] The insolubility of oleic acid in commonly used injectable solvents prevented us from making a solution with the oleic acid.

The acute gross and histologic changes in our model are similar to those reported by others in the dog,[10] rabbit,[11,12] and sheep.[9] Thus the rat appears to be a suitable animal for the study of acute oleic acid lung damage. The chronic model of oleic-acid-induced pneumopathy has been reported by Derks et al[10] as a useful model of pulmonary fibrosis in the dog. We have exposed our rats to an equally long course of treatment at levels of oleic acid more than twice as high on a milligram per kilogram basis as those reported with the dog; yet we have not been able to produce significant pulmonary fibrosis in the rats. In this regard the rat animal model of ARDS appears distinctly different from the models in larger animals, which progress to pulmonary fibrosis. It would be interesting to explore the reasons for the species difference.

In summary, we conclude from this study that 1) we have a unique animal model of ARDS that does not progress to pulmonary fibrosis and that is in an animal in which the immune system and inflammatory responses are well characterized, 2) pretreatment of the rats with indomethacin or cobra venom factor does not inhibit the development of lung injury, and 3) serum complement levels do not change during the early stages of oleic-acid-induced lung injury.

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