A Differential Effect of C5a and C5a des Arg in the Induction of Pulmonary Inflammation

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Earlier studies have shown that C5 fragments induce an inflammatory reaction when instilled into the rabbit lung. Because C5a is rapidly converted to C5a des Arg *in vivo*, experiments were performed to determine which fragment was most effective in producing pulmonary inflammation in this animal model. C5a des Arg consistently produced marked inflammation. This was characterized by neutrophil accumulation, edema, hemorrhage, fibrin formation, and damage to alveolar epithelium. The time course of the inflammatory reaction initiated by C5a des Arg showed pulmonary vascular sequestration of neutrophils with no intra-alveolar migration at 30 minutes after injection. By 2 hours, interstitial and alveolar neutrophils were numerous, with the accumulation of neutrophils in the alveoli increasing to a maximum at 6 hours. At 24 and 48 hours, the predominant cells were mononuclear (macrophages). By 120 hours, the lesions were resolving. In contrast, at all doses examined, a similar instillation of C5a induced either no inflammation or a milder, more focal response than C5a des Arg. This inability of C5a to initiate inflammation was not apparently due to the generation of inhibitors, since mixtures of C5a and C5a des Arg were phlogistic. A prolonged, intrapulmonary infusion of C5a (20 minutes), in contrast to a bolus instillation (1 minute), did initiate an inflammatory response, which may reflect the conversion of the C5a to C5a des Arg in the lung. This study points out the inflammatory potential of products of complement activation, particularly of the C5 fragment C5a des Arg, when applied to the airway side of the lungs. This inflammatory response raises the possibility that cleavage of intrapulmonary C5 may play an important role in the initiation of pulmonary inflammation. (Am J Pathol 1980, 100:179–192)

NEUTROPHIL ACCUMULATION in the air spaces in the lung appears to be a common event in many diverse forms of pulmonary injury. For example, patients with idiopathic pulmonary fibrosis have been reported to have an increased number and percentage of polymorphonuclear leukocytes (PMNs) in their lavage fluid.[1] Neutrophil accumulation has also been noted in lungs of patients with oxygen toxicity,[2] bacterial pneumonias,[3] hypersensitivity pneumonitis,[1] and in acute adult respiratory distress syndrome (ARDS) due to septicemia.[4] In experimental animals, immune complexes have been shown to cause an acute inflammatory reaction,[5,6] and complement has been shown to be required for the injury to occur.[7] The participation of complement in inflammatory reac-

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tions in the lung is of particular interest because complement may be activated by both immunologic and nonimmunologic reactions. Therefore, complement components and their split products could mediate pulmonary inflammation in diverse lung diseases.

Initial studies by Shaw et al [8] and subsequently by a number of investigators [9-11] demonstrated the ability of C5 and C5 fragments to produce lung injury. These reports have dealt with the action of C5, C5a, or C5 fragments but not with the possible effects of the C5a des Arg molecule alone. Although the anaphylatoxin C5a is known to have highly potent actions on many different cell types,[12] our recent studies suggest that C5a des Arg as well as C5a can induce neutrophil chemotaxis, oxygen radical generation, and neutrophil granule enzyme exocytosis.[13] Although quantitatively more C5a des Arg is required to produce such actions *in vitro*, the fact that serum carboxypeptidase will rapidly convert C5a to C5a des Arg within seconds after C5 cleavage [14] suggests that the prevalent low-molecular-weight C5 fragment *in vivo* may be C5a des Arg, not C5a.

This study was therefore undertaken to distinguish which fragment of C5 is responsible for producing this pulmonary inflammation. Previous experiments in our laboratory have shown that complement-induced neutrophil sequestration (margination) in the pulmonary vasculature is not sufficient to induce an alveolitis [15] and have led to the suggestion that migration of neutrophils out of the pulmonary vascular bed requires a stimulus in the interstitium or alveolus. With the observation in mind that human bronchoalveolar washout fluids have been shown to contain components of the alternative complement pathway,[16] we wanted to determine the effect of having products of complement activation (C5a and C5a des Arg) in the airways and air spaces of the lung in an *in vivo* system.

Materials and Methods

Animals

New Zealand white rabbits, weighing 1.8 to 3.0 kg, were used in the experiments.

C5a and C5a des Arg Purification

Human C5a was purified from serum activated by yeast cell walls by a modification of the method of Fernandez and Hugli [17] as previously described.[13] C5a des Arg was generated by the incubation of the purified C5a with insolubilized porcine carboxypeptidase B, or from serum activated by yeast without added epsilon aminocaproic acid. Both C5a and C5a des Arg were shown to be homogeneous by the criteria of single bands on 6% acid and 10% SDS polyacrylamide gels and the complete abrogation of detectable anaphylatoxin activity in the C5a des Arg preparation. Endotoxin in these preparations was determined by the limulus amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA) using as a standard purified preparations of LPS from *E coli* 011:B4 kindly provided by Dr. Sally Betz. The assay was performed in duplicate, and an end point sensitivity of 0.06 ng was

routinely obtained. The results were expressed as the maximum possible amount of LPS contained in 1 ml of the preparation, with all preparations having less than 60 ng of endotoxin per ml of fluid.

Administration of C5 Fragments to the Lungs

C5a, C5a des Arg, or control solutions of diluent (sterile saline) were administered to the rabbits by direct intratracheal instillation. After light sedation with approximately 6–10 mg/kg of intravenous sodium pentobarbital (Abbot Laboratories, North Chicago, Ill), the animal was restrained in a supine position. Under direct laryngoscopic visualization, a 2.5-mm endotracheal tube (Portex Inc., Wilmington, Mass) was inserted into the trachea of the spontaneously breathing animal. A PE 90 polyethylene catheter (Clay Adams, Parsippany, NJ) was then introduced through the endotracheal tube into the rabbit's airway. The catheter was marked so that the distance past the end of the endotracheal tube was known and the catheter could be advanced to a point where the end was distal to the carina. In many experiments, the marker indocyanine green (Hynson, Westcott, and Dunning, Baltimore, MD) was injected with the test material so that the exact location of the injected substance was known. With use of this technique, no questions arose as to the location of the injected material, and the relationship between the dose of C5a or C5a des Arg and the resultant inflammation could be studied with the use of a defined pulmonary distribution. An additional benefit was that treated areas in an animal could be compared to nontreated areas in the same animal. Because the site of injection with the use of a marker was found to be the right lower lobe in approximately 75% of the animals and the left lower lobe in most other rabbits, when a marker was not used, 3–5 sections were obtained from each lower lobe plus 1–2 sections from other lobes of the lung. Both methods proved satisfactory in evaluating the lung after the challenge. The total volume of injected material was 1 ml. In most experiments, the test solution was introduced into the lung over a 1-minute period. In 2 sets of experiments, C5a or saline was infused at an even rate over 15–20 minutes using a Harvard peristaltic pump, model number 600-000 (Harvard Apparatus, Millis, Mass).

Histologic Examination of Rabbit Lung

All rabbits were killed with intravenous sodium pentobarbital. After careful removal from the rabbit, the lungs were inflated with 10% neutral buffered formalin from a height of 25 cm. When the lungs were fully inflated, the trachea was tied off. The inflated lungs were then submerged in 10% formalin solution overnight. Thick sections (5 μ) were taken from selected areas of lower, middle, and upper lobes for embedding and sectioning. Sections were stained with hematoxylin and eosin, periodic acid–Schiff (PAS), Masson trichrome, and Mallory's phosphotungstic acid–hematoxylin stain for connective tissue elements.

Electron-Microscopic Examination of Rabbit Lungs

After removal from the animals, the lungs were inflated at 25 cm of H_2O with 1.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3 (mOsm = 330). The trachea was tied off, and the lungs were left in the fixative solution overnight. Selected areas were taken for electron-microscopic examination. Closely adjacent areas of tissue were also taken for histologic examination into 10% formalin solution.

The tissue was postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.3, at 4 C for 1 hour. *En bloc* 1% uranyl acetate staining was performed, and then tissues were dehydrated in alcohol, cleared in propylene oxide, and infiltrated with Epon/araldite overnight. The tissues were embedded in Epon/araldite and cured for 3 days at 70 C. Sections were cut on an LKB Ultratome III and stained with 2% aqueous uranyl acetate and Reynold's lead stain. The sections were examined in a JEOL JEM 100 B electron microscope at an accelerating voltage of 60 kV.

Grading of Acute Inflammation

All histologic results obtained in the first 6 hours after challenge with saline and/or C5 fragments were scored blindly by one of the authors (PMH) to assess inflammatory changes in the lung, using a histologic scoring system similar to that described by Johnson, Anderson, and Ward.[7] With this system, normal histologic results received a score of 0, while a score of 1 represented the presence of large numbers of neutrophils in the interstitial space but few if any in the alveoli. A score of 2 reflected not only interstitial accumulation of neutrophils, but the regular appearance of several cells in the alveoli. When the neutrophils occupied more than half of the area of most alveoli, a score of 3 was given, while a score of 4 signified complete occlusion of alveolar spaces by these cells.

Results

A total of 90 animals were studied histologically. For any one dose of C5a or C5a des Arg, or for any time point with a fixed amount of injected material, an average of 4 animals were examined. In all animals, sections were taken as described above.

C5a Instillation

Experiments were initiated to examine the effect of the potent phlogistic agent C5a. Surprisingly, instillation of C5a in doses of 0.01, 0.05, 1.0, 10.0, and 20.0 μg in 1 ml total volume with sterile saline produced very little inflammatory response when given as a bolus over 1 minute (Table 1). With the lower doses (0.01, 0.05, or 1.0 μg), mild inflammation was noted in only 1 of 8 animals studied, the other 7 animals showing no inflammation. These low doses produced no significant edema or hemorrhage, and the walls of the alveolus appeared normal in thickness and cellularity. With the higher doses of C5a (10 or 20 μg), inflammation was variably observed, ranging from a completely normal histologic picture in 6 of 15 animals (Figure 1A) to one in which affected areas had up to 50% of the alveolar area occupied by the neutrophil infiltrate (Figure 1B). It was unusual, however, to see an alveolus with confluent neutrophils from

Table 1—Induction of Pulmonary Inflammation by C5 Fragments

Stimulus*	Dose	Inflammatory response†
Saline	1 ml	0 (10)
C5a	0.01, 0.05, or 1.0 μg	0.2 ± 0.1 (8)
C5a	10 or 20 μg	0.8 ± 0.2 (15)
C5a des Arg	0.01 or 1.0 μg	1.2 ± 0.2 (6)
C5a des Arg	10 or 20 μg	2.4 ± 0.3 (20)
C5a—slow	10 μg	2.6 ± 0.4 (6)
C5a and C5a des Arg	10 μg of each	2.7 ± 0.3 (3)

* All instillations were intratracheal as a bolus given over 1 minute except for "C5a—slow," where the infusion was at a constant rate over 15–20 minutes.

† Histologic features were scored 0–4 and are expressed as the mean ± SEM. The number of animals studied is in parenthesis. All histologic results are from a 6-hour time point.

one wall to another. While more inflammation was present with the higher doses of C5a, as shown by the histologic scores in Table 1, it is apparent from the low scores that the inflammation induced was mild, even after 10 or 20 μg of C5a. In addition, the highest dose of C5a (20 μg) appeared to produce no more inflammation than the 10-μg dose. In all experiments where C5a was given as a bolus over 1 minute, the C5a was found to be less phlogistic than a comparable dose of C5a des Arg, the response being more variable and ranging from normal lung to sections showing moderate inflammation.

C5a des Arg Instillation

In marked contrast to the C5a, C5a des Arg produced a constant inflammatory response within the lung. With increasing dosages from 0.01 to 10 or 20 μg in a 1-ml total volume with sterile saline, a gradation in inflammation was seen (Table 1). At the higher doses, at 6 hours after instillation, marked neutrophil infiltration commonly occupying 50–100% of the area of the air spaces was noted (Figure 1C). In addition, a proteinaceous edema fluid within the air spaces was commonly seen, with a PAS-positive material suggesting that fibrin was included in this fluid. The comparison of the pulmonary inflammation induced by saline, C5a, or C5a des Arg shown in Table 1 also demonstrates that low doses of C5a des Arg produced more inflammation than high doses of C5a.

The Effect of Slow Infusion of C5a

Studies by Desai et al [9] demonstrated that instillation of whole human C5 into hamster lungs would induce inflammation. The C5 would presumably have been cleaved slowly to generate phlogistic fragments. Accordingly, a slow infusion of C5a (10 μg in 1 ml) into 6 rabbit lungs was examined to determine whether this would now permit C5a to induce inflammation. The histologic characteristics were compared with those of control animals slowly given injections of saline and with animals given the same dose and volume of C5a over a 1-minute period (see above and Table 1). Marked inflammation consisting of edema formation, hemorrhage, and neutrophil accumulation was noted in all 6 animals treated in this way. The histologic characteristics and histologic scores were similar to animals given 10 μg of C5a des Arg by fast infusion (Figure 1C). Slow infusion of saline into 4 rabbits over 15–20 minutes failed to invoke an inflammatory reaction.

C5a and C5a des Arg Instilled Together

In order to determine whether C5a would have an inhibitory effect on the inflammation normally seen with C5a des Arg, C5a was instilled along

with C5a des Arg. In one experiment, 3 animals were given injections of a combination of 10 μg of C5a and 10 μg of C5a des Arg. Examination of the lungs 6 hours after the instillation again showed a marked neutrophil infiltrate with hemorrhage and exudate. When compared with the inflammation produced when the complement fragments were administered alone, the findings were similar to the C5a des Arg reaction (Table 1). No evidence of an inhibitory effect of C5a was demonstrated.

Time Course

Ten micrograms of C5a des Arg was instilled into rabbit lungs, and the animals were killed at 1/2, 2, 4, 6, 24, 48, 72, and 120 hours after injection. At 30 minutes, an increased cellularity of the alveolar walls was noted, but no neutrophils were observed in the alveoli. By 2 hours, however, neutrophils could be found free in the alveoli. Between 2 and 6 hours, the number of inflammatory cells in the alveoli increased, and edema fluid and hemorrhage became noticeable. At 24 and 48 hours, the predominant cell type in the air spaces had changed and, as can be seen in Figure 1D, was a mononuclear cell (macrophage). Also prominent at this time point was marked thickening of the alveolar walls. At 72 hours, the cellularity of the air spaces was decreasing, and while the lung was not completely normal at 120 hours—ie, the walls were still thickened and more cellular than normal—the air spaces were nonetheless free of inflammatory infiltrate.

Ultrastructural observations revealed that at 30 minutes after C5a des Arg administration neutrophils were adhering to endothelial cells. In addition, in some examples, endothelial cells exhibited blebbing, the cell lifted off the basement membrane (Figure 2). When the neutrophils were found in the interstitium, interstitial edema was common. In the interstitium, neutrophils were often found in contact with collagen fibers, or adherent to the epithelial basement membrane beneath a Type II cell (Figure 3). When found in the air spaces, neutrophils were usually found with fibrin or adherent to alveolar macrophages. Evidence of epithelial damage was also obtained (Figure 4). At later time points (24 and 48 hours) neutrophil debris was noted in the phagocytic vacuoles of the macrophages. Also of note was the fact that eosinophils could be frequently identified in the air and interstitial spaces at these late time points.

Control Areas of Lung in Animals Given Injections of Saline

Animals given injections of saline alone or saline and indocyanine green had normal histologic characteristics at all time points examined (6, 24, and 48 hours), except for an occasional neutrophil in an alveolus. Areas of

lung not given injections of saline, C5a, or C5a des Arg, were routinely normal histologically, with no neutrophils observed in the alveoli.

Endotoxin

As noted above, the preparations used in this study were shown by the limulus lysate assay to contain less than 60 ng of endotoxin/ml of injected material. Previous studies by Shaw et al [11] have shown that microgram, and not nanogram, quantities of endotoxin are needed to induce significant pulmonary inflammation in rabbits. In addition, Desai and co-workers [9] found that intratracheal instillation of up to 100 ng of bacterial endotoxin into hamster lungs failed to induce significant inflammatory changes in the lung. In addition, the complete lack of a phlogistic response to C5a in over half the animals studied also makes it unlikely that endotoxin contamination was responsible for the observed changes.

Discussion

This study has demonstrated C5a des Arg is a potent phlogistic agent when introduced in purified form into the rabbit lung. While the inflammatory potential of C5, C5a, and C5 fragments has been appreciated in previous work,[8-11] the *in vivo* potential for inflammation by the C5a des Arg molecule has not been evaluated. This is in part due to the fact that while the anaphylatoxic and chemoattractant properties of C5a are well known,[12] the inflammatory potential of C5a des Arg in an *in vitro* system has only recently been appreciated.[13,18,19]

The relative inability of C5a to produce an inflammatory response when compared with the marked inflammation produced by C5a des Arg may be due to several reasons. First, when injected as a bolus, the anaphylatoxic properties of the C5a molecule may increase vascular permeability, causing loss of the C5a into the vasculature, leaving less material to be converted into C5a des Arg. Edema fluid produced from the increased permeability would also decrease the effective concentration of the remaining C5a on a dilutional basis. In addition, fluid derived from the vascular space may contain inhibitors of C5a such as chemotactic factor inactivator.[7] Histamine release caused by the action of C5a on mast cells could also lead to an H_2 receptor-induced inhibition of neutrophil chemotaxis.[20] Rapid neutrophil desensitization to C5a might also account for some of the observations, since we have shown C5a to be a more potent inducer of desensitization than C5a des Arg.[13] Each of these mechanisms, however, would permit a prediction that mixing C5a with C5a des Arg would inhibit the inflammatory effect of the latter. This did not occur, as noted above.

A more likely explanation of the differential effect of the two molecules probably relates to other *in vivo* characteristics. For example, C5a des Arg may bind differently to structures in the lung in an active form, permitting a prolonged phlogistic effect. Differences in C5a and C5a des Arg distribution could also be important. In addition, the animals that exhibited a mild response to C5a may have had higher levels of carboxypeptidase B in the lung, therefore effecting a more rapid conversion to C5a des Arg. These possibilities are under study.

As noted above, C5a is usually rapidly converted into C5a des Arg because of serum carboxypeptidase B.[14] Thus, the half-life of C5a in the blood, and probably other tissues, is very transient. It is therefore possible that the biologically active C5 fragment that is found in highest concentration for the longest period of time *in vivo* would be the C5a des Arg molecule. Recently, our laboratory has found carboxypeptidase B in the lavage fluid of normal rabbit lungs.[21] Therefore, if C5a is introduced into or formed in the lung at a rate where the molecule can be effectively converted to C5a des Arg, a fragment is formed that lacks anaphylatoxic properties but retains the ability to induce neutrophil chemotaxis, form oxygen radicals, and produce exocytosis of neutrophil granule enzymes.

Although C5a des Arg is documented to be quantitatively less active *in vitro* in these actions than C5a, a helper factor has been found in serum that causes an enhancement of neutrophil chemotaxis to C5a des Arg.[18,22] Therefore, *in vivo*, the potential exists for C5a des Arg to be chemotactically more active than it is *in vitro*. The marked phlogistic potential of C5 fragments noted by Shaw et al [8,11] may be explained by the presence of this helper factor in the preparations he used.

The change in cell population from a predominantly polymorphonuclear infiltrate in the first 6 hours to a mononuclear infiltrate at 24 hours and later time points was noted above. A similar change in cell population was also noted by Scherzer and Ward [6] in their model of immune complex pulmonary injury in rats. Their model was also found to be complement-dependent. This change in cell populations with time is a common finding in inflammatory reactions resulting from diverse stimuli.[23] In our model, these changes were evaluated over a 5-day period. While the mononuclear infiltrates were largely gone at the last time point, the alveolar walls remained thick in many areas. The physiologic consequences of these changes in the rabbits are as yet unknown. While no rabbit appeared to have any significant respiratory difficulty at any time point during the experiment, it must be recalled that the inflammatory response was produced in a localized area of lung. It is expected that a more uniform administration of a sufficient quantity of the complement com-

ponents to the lungs of the test animals could cause acute and chronic physiologic changes in the lung.

The data suggest that cleavage of C5 in the lung by immunologic and/or nonimmunologic mechanisms may provide an important trigger to the initiation of inflammation. However, several questions have to be addressed before the possible clinical significance of observations such as these can be assessed. Of central importance is the question of whether the precursor molecule, C5, is present in the lung. Evidence from Robertson and co-workers suggests that normal humans have an intact alternative complement pathway in respiratory secretions.[16] Observations in our own lab would also suggest the presence of C5 in rabbit lungs.[24] Thus, while it appears likely that the substrate for generation of C5a or C5a des Arg is present in the lung, more critical analysis of this question is in order.

If C5 is present in the lung in quantities sufficient to produce significant inflammation, then a question that arises centers around whether C5 can be cleaved in the lung. Desai and co-workers have observed marked inflammation in normal hamsters after intrapulmonary instillation of intact C5, suggesting that cleavage of C5 occurred after intratracheal administration.[9] The protease that could act on the C5 to produce the fragments is unknown, but it has been suggested that rabbit alveolar macrophages generate chemotactic fragments from C5.[25] Other possibilities that could lead to C5 cleavage include inhalation of complement-activating materials such as lipopolysaccharides, other bacterial products, including many of those implicated in hypersensitivity pneumonitis,[26,27] or particulate material. Further analysis of these questions is being undertaken.

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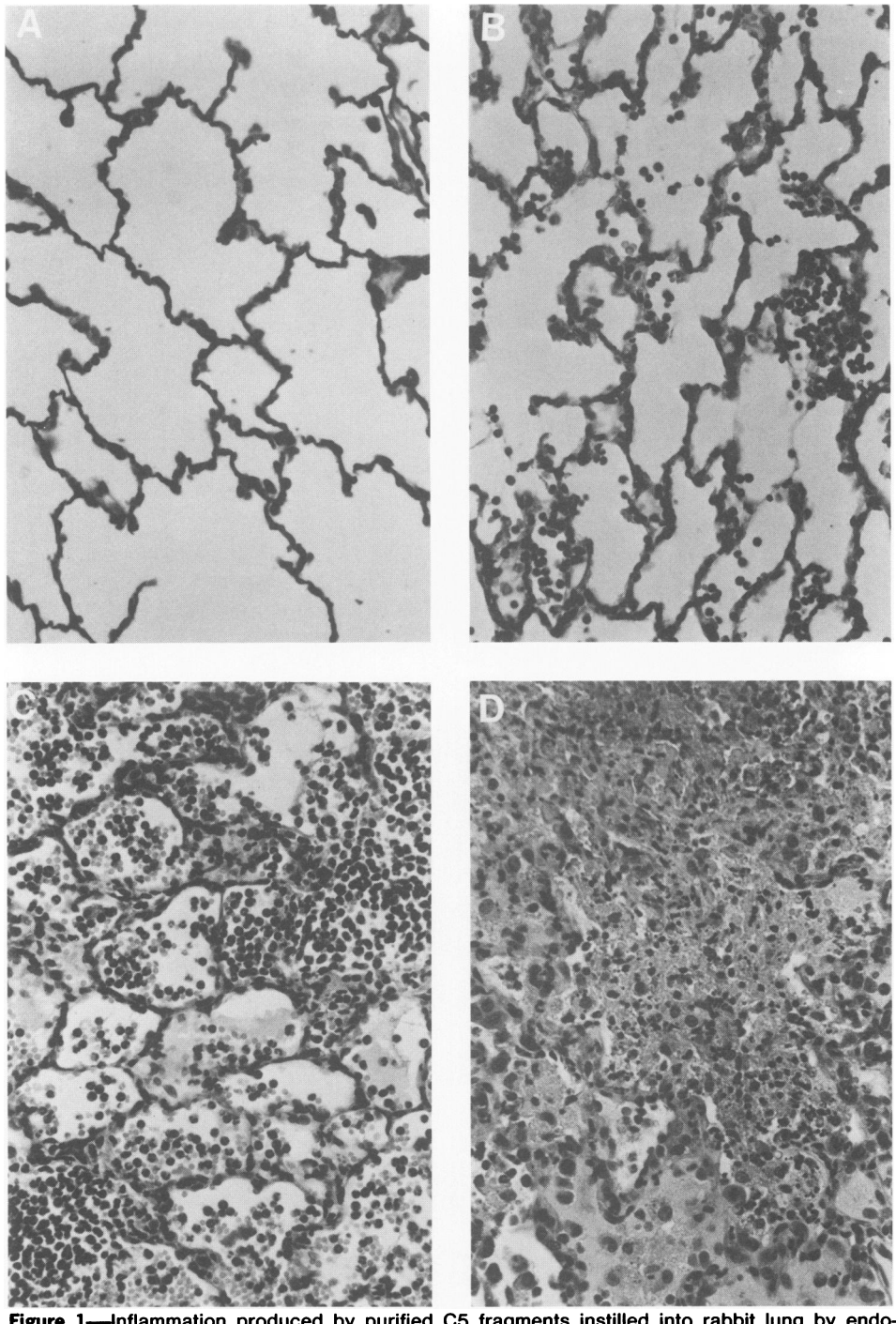

Figure 1—Inflammation produced by purified C5 fragments instilled into rabbit lung by endotracheal catheter. The treated area was defined with the use of indocyanine green as a marker. **A**—C5a in the amount of 10 μg produced no inflammation when given as a bolus (1 minute) in 6 of the 12 animals studied. Six hours. (H&E, ×200) **B**—When inflammation occurred after the rapid injection of 10 μg of C5a, the reaction was characterized by mild neutrophil accumulation without significant hemorrhage or exudate. Six hours. (H&E, ×200) **C**—C5a des Arg in the amount of 10 μg consistently produced a more severe inflammatory response, characterized by neutrophil accumulation, edema, and hemorrhage. When 10 μg C5a was given slowly over 15–20 minutes, a similar histologic picture was seen. Six hours. (H&E, ×200) **D**—C5a des Arg in the amount of 10 μg instilled 48 hours before death caused thickening of alveolar walls as well as a mononuclear cell (macrophage) accumulation in the alveoli. Although the histologic picture was similar at 24 hours, by 5 days, the alveoli were clear, only mild thickening of the alveolar walls remaining. (H&E, ×200)

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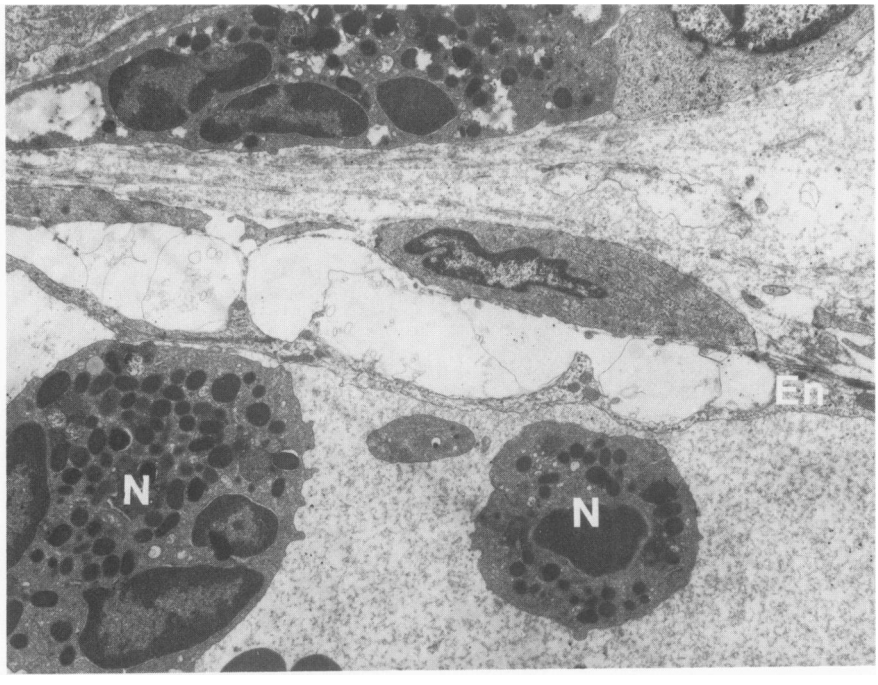

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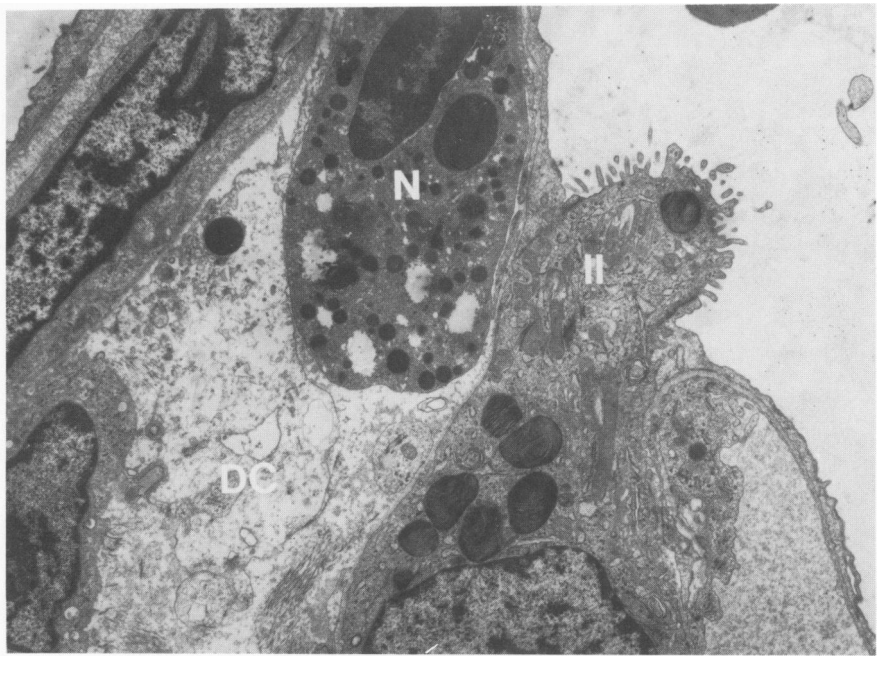

Figure 2—Two neutrophils (*N*) adjacent to an endothelial cell (*EN*) 30 minutes after intrapulmonary instillation of 10 μg C5a des Arg. Blebbing of the endothelial cell is apparent. A neutrophil (unlabeled) is also noted in the interstitium above the endothelial cell. (×6500)

Figure 3—A neutrophil (*N*) adherent to a dead cell (*DC*) in the pulmonary insterstitium after instillation of 10 μg C5a des Arg. The neutrophil is adherent to the epithelial basement membrane beneath a Type II cell (*II*). (×6500)

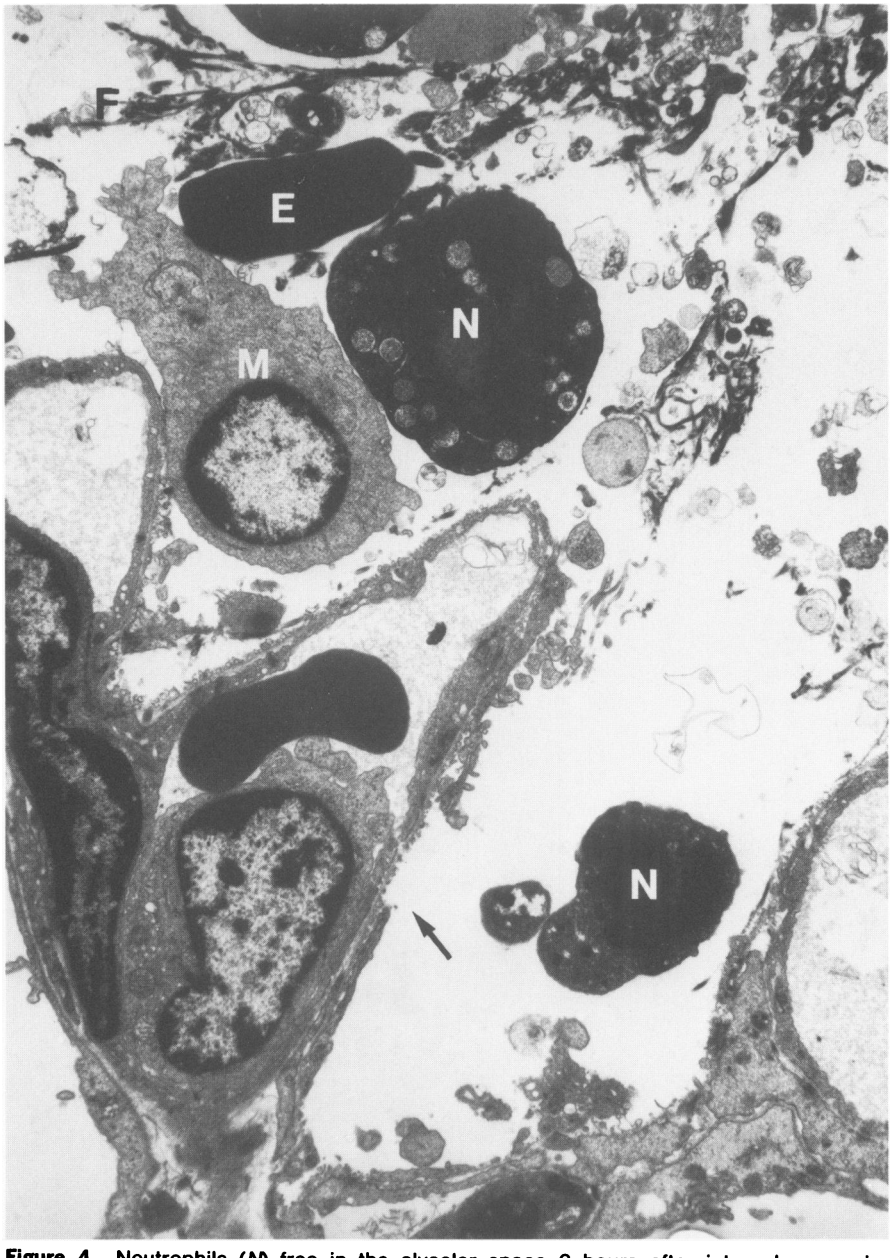

Figure 4—Neutrophils (*N*) free in the alveolar space 6 hours after intrapulmonary instillation of 10 μg C5a des Arg. Epithelial disruption (*arrow*) is apparent. Also present in the air space is an erythrocyte (*E*), a mononuclear cell (*M*), and fibrin (*F*). (×5400)

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