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Role of C5 and Recruited Neutrophils in Early Clearance of Nontypable *Haemophilus influenzae* from Murine Lungs

GALEN B. TOEWS,[1]† * WAYNE C. VIAL,[1,3] AND ERIC J. HANSEN[2]

Department of Medicine, Southwestern Medical School,[1] and Department of Microbiology, Southwestern Graduate School of Biomedical Sciences,[2] and Pulmonary Section, Dallas Veterans Administration Medical Center,[3] The University of Texas Health Science Center at Dallas, Dallas, Texas 75235

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We used a mouse model system to investigate the pulmonary defense mechanisms involved in clearance of nontypable *Haemophilus influenzae* from the lower respiratory tract. The importance of the C5 complement protein molecule in polymorphonuclear leukocyte (PMN) recruitment was studied by using congenic C5-sufficient B10.D2/nSn ($C5^+$) and C5-deficient B10.D2/oSn ($C5^-$) mice. The $C5^+$ and $C5^-$ mice were inoculated with saline or nontypable *H. influenzae* via an endobronchial catheter. Clearance of bacteria was studied by using quantitative lung cultures. Bronchoalveolar lavage was performed at several time intervals. The number of cells in the lavage fluid were counted, and chemotactic activity was assayed in lavage fluid by the leading front technique, using human PMN in modified Boyden chambers. Pulmonary clearance of bacteria was significantly impaired in the absence of C5 ($P < 0.05$). The $C5^+$ mice recruited significantly more PMN after challenge with nontypable *H. influenzae* than $C5^-$ mice did ($P < 0.05$), but significant PMN recruitment occurred in $C5^-$ mice. Similarly, although chemotactic activity was present in both $C5^+$ and $C5^-$ mice, significantly more intraalveolar chemotactic activity was noted in $C5^+$ mice than in $C5^-$ mice ($P < 0.05$). The C5 molecule yields important chemotaxins during this early time period, but other chemotaxins are also present within the alveoli, demonstrating the redundancy of the inflammatory response after pulmonary challenge with nontypable *H. influenzae*. Nitrogen mustard-induced neutropenic animals were studied to evaluate the functional importance of PMN in pulmonary clearance of nontypable *H. influenzae*. Pulmonary clearance of nontypable *H. influenzae* was significantly impaired in neutropenic animals ($P < 0.05$). Our results indicate that the prompt appearance of PMN in lungs is crucial for early clearance of nontypable *H. influenzae*.

Nontypable *Haemophilus influenzae* is a common cause of nonbacteremic pneumonia and tracheobronchitis in adults (2, 17) and has recently been reported to be an important cause of pneumonia in the pediatric population of developing countries (22). Few studies have evaluated the pulmonary defense mechanisms involved in the clearance of this organism from the lower respiratory tract, because of the relatively recent recognition of nontypable *H. influenzae* as an important pulmonary pathogen.

The mechanism of clearance of most bacteria from lungs involves phagocytic cells which are capable of killing bacteria before multiplication leads to clinical disease (10). Resident alveolar macrophages alone are capable of clearing low-level inocula of certain bacteria (19, 20), but studies done in our laboratory have shown that inocula of nontypable *H. influenzae* resist early clearance, grow rapidly, and induce recruitment of polymorphonuclear leukocytes (PMN) to alveoli (27). Although bacterial clearance occurs rapidly after the appearance of PMN within the alveoli, the functional importance of these cells could not be evaluated in these previous studies.

The factors responsible for PMN recruitment after challenge with nontypable *H. influenzae* have not been defined. A number of recent studies have suggested that proteins of the complement system, C5 in particular, are involved in producing pulmonary inflammation (5, 11, 13). C5 fragments have been shown to be involved in the early immigration of neutrophils into murine lungs after challenges with *Streptococcus pneumoniae* (26) and *Pseudomonas aeruginosa* (14), but chemotaxins other than C5 are of primary importance after challenges with *Staphylococcus aureus* (25). Thus, the results of previous studies suggest that the inflammatory response within lungs is mediated by different pathways depending in part on the bacterial stimulus.

In the present study, we used congenic C5-sufficient ($C5^+$) and C5-deficient ($C5^-$) mice to systematically evaluate the role of the C5 molecule in the recruitment of PMN to alveolar spaces in response to nontypable *H. influenzae*. In addition, we used a granulocytopenic murine model to further evaluate the importance of granulocytes in the early pulmonary clearance of nontypable *H. influenzae*. Our results suggest that PMN are important in the early cellular defense of lungs against nontypable *H. influenzae*. Additionally, our results indicate that C5 is involved in the early influx of PMN into the alveoli after nontypable *H. influenzae* challenge but that other chemotaxins are also involved at later times.

MATERIALS AND METHODS

Animals. Congenic $C5^+$ B10.D2/nSn mice and $C5^-$ B10.D2/oSn mice weighing 20 to 24 g (Jackson Laboratories, Bar Harbor, Maine) were used in all experiments. Only male mice were used because of reports of intermediate C5 concentrations in female mice of this strain (18).

Bacterial strains and culture media. Nontypable *H. influenzae* strain TN100, which was isolated from a transtracheal aspirate from a patient with acute nontypable *H. influenzae* pneumonia, was used in all experiments. This strain has been described previously (27). Strain TN100 was grown in brain heart infusion broth (Difco Laboratories, Detroit,

* Corresponding author.
† Present address: Internal Medicine-Pulmonary Division, University of Texas Health Science Center at Dallas, Dallas, TX 75235.

Mich.) supplemented with Levinthal base (1) as a source of hemin and nicotinamide adenine dinucleotide. Before each experiment, a flask containing 50 ml of brain heart infusion broth supplemented with Levinthal base was inoculated with a 5-ml sample of a mid-logarithmic-phase culture and incubated at 37°C until the bacteria were again in the mid-logarithmic phase of growth. The nontypable *H. influenzae* cells were then harvested by centrifugation at 7,000 × *g* for 10 min at 4°C, and the resulting cell pellet was suspended in a volume of sterile cold phosphate-buffered saline (pH 7.2) to yield the desired concentration of organisms. The bacterial suspension was kept on ice throughout the experiment. Each final bacterial suspension was quantitated by serial 10-fold dilution followed by plating on chocolate agar plates.

Method of bacterial inoculation. The method used to deposit bacteria into lungs has been described previously (19). Briefly, mice were anesthetized by intraperitoneal injection of 82.5 mg of sodium pentobarbital per kg. The trachea of each mouse was surgically exposed and then intubated transorally with a blunt 20-gauge needle, which was advanced to the thoracic inlet under direct observation through the translucent tracheal wall. A type PE10 polyethylene catheter (Clay Adams, Div. Becton Dickinson and Co., Parsipany, N.J.) previously filled with 5 μl of bacterial slurry was passed through the needle into the lung to a predetermined depth based on the weight of the animal. The bacterial slurry was expressed from the catheter by injecting 0.1 ml of air. This technique reproducibly delivered the inoculum to a localized peripheral segment of the lung. Approximately 85% of the inocula were injected into the lower third of the left lung, with the remaining usually injected into the right inferior lobe. The animals recovered from anesthesia within 1 h of administration.

Clearance. To determine the deposition and clearance of nontypable *H. influenzae*, equal numbers of $C5^+$ and $C5^-$ mice were killed by cross-clamping of the trachea immediately after inoculation (zero time) and at 4, 6, and 24 h after bacterial challenge. The lungs were removed aseptically, added to 4 ml of sterile phosphate-buffered saline, and homogenized in a tissue homogenizer (model 45; The VirTis Co., Inc., Gardiner, N.Y.). After further disruption in a Broeck tissue grinder (Corning Glass Works, Corning, N.Y.), a sample of the homogenate was serially diluted in brain heart infusion broth supplemented with Levinthal base, plated onto chocolate agar, and incubated at 37°C in a candle extinction jar. Colony counts were determined with a dark-field Quebec colony counter after 24 h. The cultures of each lung homogenate were expressed as CFU per lung. To compare test groups, a percentage was obtained by dividing the number of CFU from each mouse at each time point by the mean CFU in the lungs of all zero-time mice for that experiment and then multiplying by 100. The resulting number represented the percentage of viable bacteria that remained in the lung at each time point.

BAL. The presence of intraalveolar chemotaxins and the recruitment of PMN to alveolar spaces were assessed by bronchoalveolar lavage (BAL), using a modification of a procedure described previously (30). Unilateral BAL was performed in both $C5^+$ and $C5^-$ mice. Five mice were killed immediately after inoculation, and the lungs were cultured quantitatively to determine the bacterial deposition. Bronchopulmonary lavage was performed on separate groups of eight mice at 0, 4, 6, and 24 h after bacterial challenge. The mice in each group were killed by intraperitoneal injection of pentobarbital (160 mg/kg of body weight). The thorax was opened aseptically, the lungs were inspected, and the main bronchus of the noninoculated lung was ligated. The trachea was cannulated with type PE50 polyethylene tubing (Clay Adams), and the inoculated lung was lavaged with 0.3-ml portions of heparinized normal saline until a final volume of 2.5 ml was recovered. The lavage fluid was collected on ice and centrifuged at 4°C for 10 min at 150 × *g*. The supernatant was frozen at −70°C until it was assayed for chemotactic activity. The cell pellet was suspended in Hanks balanced salt solution without calcium or magnesium, and the total number of cells was determined with a Coulter Counter (model ZBI; Coulter Electronics, Inc., Hialeah, Fla.). Differential cell counts of 200 cells were done on cytocentrifuged, Wright-stained preparations.

Assay of chemotactic activity. The chemotactic activities of BAL fluids were assayed in modified Boyden chambers (4) by using human PMN which were obtained from heparinized normal human blood by Ficoll-Hypaque centrifugation (3), followed by sedimentation in Plasmagel for 30 min. The supernatant containing PMN was centrifuged at 200 × *g* for 10 min, and the residual erythrocytes were lysed with 0.2% NaCl. The cells were then washed twice in Hanks balanced salt solution supplemented with 1% bovine serum albumin and suspended in Hanks balanced salt solution containing 1% bovine serum albumin at a concentration of 2.0×10^6 cells per ml. This procedure yielded 98% PMN with 95% viability as determined by trypan blue exclusion. The cell suspension (0.5 ml) was placed in the upper well of a modified Boyden chamber separated from the chemotactic stimulant in the lower compartment by a cellulose nitrate filter with 3.0-μm pores (Sartorius, Hayward, Calif.). The BAL fluid or negative control (heparinized saline) was added to the lower compartment. After incubation for 30 min at 37°C in an atmosphere containing 5% CO_2, the chambers were emptied, and the filters were removed, fixed, and stained with hematoxylin. Cell migration was quantitated by using the leading front technique (34). The deepest penetration of the leading two PMN was determined with a micrometer incorporated in the fine focus of a microscope at five separate sites, and the mean for each filter was calculated. Chemotactic activity was expressed as the mean migration in duplicate samples. Filters were coded and counted without knowledge of the solution being assayed.

Induction of granulocytopenia. Granulocytopenia was induced by intravenous injection of nitrogen mustard (Mustargen; Merck Sharp & Dohme, West Point, Pa.), using a modification of a previously described method (21). Nitrogen mustard was dissolved in sterile water according to the directions of the manufacturer and was diluted to a concentration of 200 μg/ml in 0.9% saline. To induce neutropenia, 60 μg of nitrogen mustard per day was injected into the lateral tail vein on 3 consecutive days before challenge.

Hematological studies. All peripheral blood leukocyte counts were done with a hemacytometer. Differential counts of 200 peripheral blood leukocytes were done by using Wright-stained blood films of orbital sinus blood.

Statistical analysis. All data represent pooled results of at least two separate experiments. The clearance and phagocytic cell data were analyzed by using the Mann-Whitney U test for nonparametric analysis. A probability value of less than 0.05 was considered significant (33).

RESULTS

Clearance. In previous studies we showed that an inoculum of 10^5 strain TN100 cells elicited a large PMN response and net bacterial clearance in BALB/c mice (27). The pulmonary clearance of strain TN100 by $C5^+$ and $C5^-$

mice is shown in Fig. 1. Initial deposition did not differ between $C5^+$ and $C5^-$ mice. At 4 and 6 h after inoculation with 2.9×10^5 nontypable *H. influenzae* cells, the bacteria had multiplied in both strains; however, significantly more bacteria were recovered from the $C5^+$ mice than from the $C5^-$ mice at both times ($P < 0.05$). By 24 h, both strains of mice had cleared the bacteria.

Recruitment of PMN. Similar numbers of PMN were present in the unilateral BAL fluids of normal, saline-challenged, and bacterium-inoculated mice at zero time ($0.02 \times 10^5 \pm 0.001 \times 10^5$ cells). PMN represented less than 1% of the total cells recovered by lavage from the $C5^+$ and $C5^-$ mice ($4.1 \times 10^5 \pm 0.3 \times 10^5$ and $3.9 \times 10^5 \pm 0.34 \times 10^5$ cells, respectively). The percentage of PMN recovered by BAL increased to $1.4 \pm 0.3\%$ in $C5^+$ mice and to $1.2 \pm 0.2\%$ in $C5^-$ mice at 4 h after challenge of both strains with normal saline (the diluent for nontypable *H. influenzae*), but no significant difference was noted between the strains. After challenge with 2.1×10^5 nontypable *H. influenzae* cells, the number of PMN increased at 4, 6, and 24 h in both $C5^+$ and $C5^-$ mice compared with zero time ($P < 0.05$) (Fig. 2). Although both strains exhibited increases in the numbers of PMN, $C5^+$ mice had significantly more PMN at 4 and 6 h after challenge with nontypable *H. influenzae* than $C5^-$ mice did ($P < 0.05$). However, by 24 h, the numbers of PMN in the lavage fluids from $C5^-$ mice did not differ significantly from the numbers found in $C5^+$ mice. This same pattern of response was noted in two other sets of experiments. In each experiment significant differences in the PMN response were noted at 4 and 6 h postchallenge, with $C5^+$ mice exhibiting 2.1 to 3.1 times as many neutrophils as the $C5^-$ mice at these early times, whereas no differences existed between the two strains at 24 h postchallenge.

Peripheral leukocyte counts. To show that the difference in BAL neutrophil counts between $C5^+$ and $C5^-$ mice after nontypable *H. influenzae* challenge was not due to relative neutropenia in $C5^-$ mice, blood was obtained for leukocyte counts and differential counts before and after challenge. Base-line neutrophil counts (as well as total leukocyte counts and differential counts [data not shown]) did not differ between $C5^+$ mice (976 ± 104 cells per mm^3) and $C5^-$ mice (990 ± 98 cells per mm^3). At 6 h after exposure to nontypable *H. influenzae*, the percentage and absolute number of neutrophils in circulation had increased in both $C5^+$ mice ($2,538 \pm 220$ cells per mm^3) and $C5^-$ mice ($3,750 \pm 178$ cells per mm^3). The increases from zero time were significant in both strains.

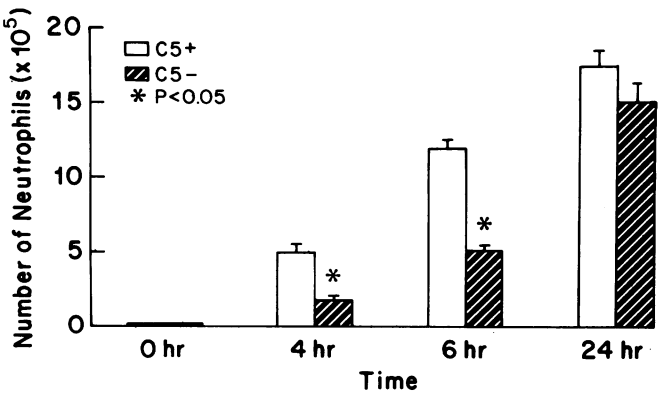

FIG. 2. Number of PMN recovered by BAL from $C5^+$ and $C5^-$ mice at 0, 4, 6, and 24 h after challenge with 2.1×10^5 nontypable *H. influenzae* cells. Each bar represents the mean ± standard error of the mean for eight animals. Significantly more PMN were observed in $C5^+$ mice than in $C5^-$ mice at 4 and 6 h ($P < 0.05$), but no difference was observed at 24 h.

Chemotactic activity in BAL fluids. Chemotactic activity was assayed in BAL supernatants of both $C5^+$ and $C5^-$ mice after inoculation with 2.1×10^5 nontypable *H. influenzae* cells (Fig. 3). In BAL fluids obtained immediately after injection of bacteria, the chemotactic activities were similar in $C5^+$ and $C5^-$ mice. As early as 2 h, there was a significantly greater amount of chemotactic activity in the

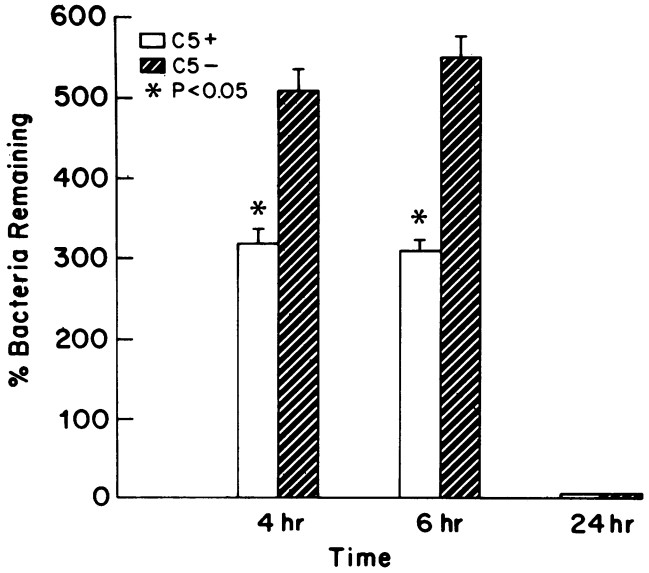

FIG. 1. Percentage of originally deposited bacteria remaining in the lungs of $C5^+$ and $C5^-$ mice 4, 6, and 24 h after challenge with 2.9×10^5 nontypable *H. influenzae* cells. Each bar represents the mean ± standard error of the mean for eight animals. The bacteria multiplied in both strains at 4 and 6 h, but significantly more bacteria were recovered from $C5^-$ mice at these times ($P < 0.05$). Both strains had cleared the bacteria by 24 h.

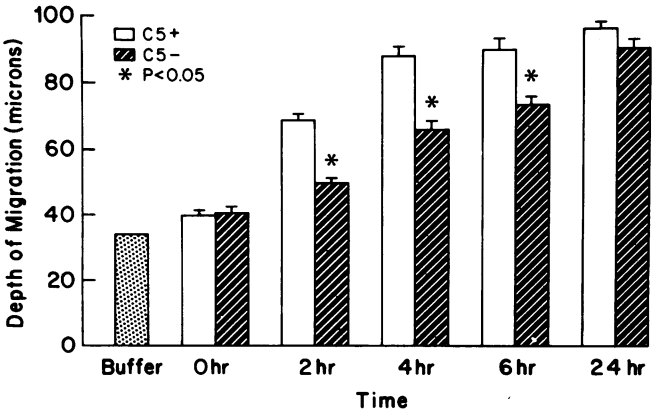

FIG. 3. Chemotactic activity of buffer and BAL supernatants obtained 0, 2, 4, 6, and 24 h after inoculation of 2.1×10^5 nontypable *H. influenzae* cells into $C5^+$ and $C5^-$ mice. Chemotactic activities are expressed as migration distances. Each bar represents the mean ± standard error of the mean for eight animals. Significantly more chemotactic activity was observed in $C5^+$ mice than in $C5^-$ mice at 2, 4, and 6 h ($P < 0.05$).

TABLE 1. Checkerboard assay of $C5^+$ BAL fluids 4 h after challenge with nontypable *H. influenzae*[a]

% of BAL fluid above filter	% of BAL fluid below filter			
	0	20	50	80
	Migration (μm)			
0	55	72 (61)[b]	79 (61)	90 (65)
20	54 (61)	67	78 (70)	80 (70)
50	58 (70)	68 (70)	73	83 (69)
80	63 (70)	68 (69)	66 (69)	65

[a] The inoculum used was 2.2×10^5 nontypable *H. influenzae* cells.
[b] The values in parentheses are predicted values assuming only a chemokinetic effect.

TABLE 3. Effect of nitrogen mustard treatment on numbers of phagocytic cells recovered by BAL of $C5^+$ mice after challenge with nontypable *H. influenzae*

Time (h)	Treatment	Total no. of cells ($\times 10^5$)	No. of alveolar macrophages ($\times 10^5$)	No. of PMN ($\times 10^5$)
0	Saline	4.12 ± 0.3[a]	4.10 ± 0.5[a]	0.02 ± 0.001[a]
	Nitrogen mustard	4.00 ± 0.4	4.00 ± 0.4	0
6	Saline	14.16 ± 0.3[b]	4.09 ± 0.6	10.07 ± 0.3[b]
	Nitrogen mustard	3.96 ± 0.5[c]	3.81 ± 0.5	0.15 ± 0.2[c]

[a] Mean ± standard error of the mean ($n = 8$).
[b] $P < 0.05$ compared with the zero-time value.
[c] $P < 0.05$ compared with the saline value.

BAL supernatant from the $C5^+$ mice than in the BAL supernatant from the $C5^-$ mice ($P < 0.05$). Significant differences in chemotactic activity were also noted at 4 and 6 h ($P < 0.05$). Chemotactic activity continued to increase up to 24 h, but no significant difference existed between $C5^+$ and $C5^-$ mice.

To determine whether BAL supernatants from $C5^+$ and $C5^-$ mice contained chemotactic activity as well as chemokinetic activity, checkerboard assays were performed. Pooled BAL fluids obtained 4 h after inoculation of 2.2×10^5 nontypable *H. influenzae* cells from $C5^+$ and $C5^-$ mice were studied (Tables 1 and 2). These BAL fluids from both $C5^+$ and $C5^-$ mice demonstrated a chemokinetic effect. Migration was augmented at concentrations as high as 50% in $C5^+$ (Table 1) and $C5^-$ (Table 2) mice when the concentrations of the BAL fluids above and below the filter were equal, indicating stimulated random motion. However, true chemotactic activity was noted in both $C5^+$ and $C5^-$ mice. In both tables all experimental values for migration toward the higher concentration exceed the values predicted by the chemokinetic effect. Conversely, all experimental values for migration away from the higher concentration are less than the values predicted by chemokinetic effect.

Effects of nitrogen mustard administration. To further evaluate the importance of granulocytes in pulmonary clearance of nontypable *H. influenzae*, neutropenia was induced with nitrogen mustard. Nitrogen mustard treatment of $C5^+$ mice reduced drastically the number of peripheral blood PMN from 940 ± 89 to 136 ± 17 cells per mm^3. Furthermore, these animals were unable to mobilize neutrophils to their lungs in response to nontypable *H. influenzae* compared with saline-treated mice (Table 3). In normal mice more than 70% of the cells in the BAL fluid were PMN, compared with approximately 3% for neutropenic animals.

Effect of nitrogen mustard on clearance of nontypable *H. influenzae*. The pulmonary clearance of two nontypable *H. influenzae* inoculum sizes by saline- and nitrogen mustard-treated $C5^+$ animals is shown in Table 4. Neutropenic animals were significantly impaired in their ability to clear nontypable *H. influenzae* from their lungs. After inoculation with 1.1×10^4 nontypable *H. influenzae* cells, most of the initially deposited bacteria remained in the lungs of the saline-treated animals after 6 h, whereas in the nitrogen mustard-treated animals the bacteria had multiplied fivefold in the lungs ($P < 0.05$). After challenge with 1.2×10^5 nontypable *H. influenzae* cells, the bacteria multiplied in both saline-injected and neutropenic animals, but significantly more bacteria were recovered from neutropenic animals ($P < 0.05$).

DISCUSSION

Nontypable *H. influenzae* is a common pulmonary pathogen in both children and adults (2, 17, 22, 31), but little information exists regarding pulmonary host defenses against this organism. In this study we used the technique of endotracheal inoculation, which probably mimics the actual mechanism (i.e., aspiration) by which nontypable *H. influenzae* gains access to the lower respiratory tract, to evaluate host defense mechanisms involved in the early pulmonary clearance of this bacterium. We chose a bacterial inoculum size that could eventually be cleared from the lungs in order to study the importance of the recruitment of PMN to the lungs as a component of the pulmonary antibacterial defense.

In this study we found that early pulmonary clearance of nontypable *H. influenzae* depends on effective PMN recruitment. In a model of defective PMN recruitment due to C5

TABLE 2. Checkerboard assay of $C5^-$ BAL fluids 4 h after challenge with nontypable *H. influenzae*[a]

% of BAL fluid above filter	% of BAL fluid below filter			
	0	20	50	80
	Migration (μm)			
0	42	51 (47)[b]	57 (46)	65 (48)
20	40 (47)	51	56 (55)	60 (54)
50	45 (55)	47 (55)	58	59 (57)
80	47 (57)	48 (56)	53 (57)	55

[a] The inoculum used was 2.2×10^5 nontypable *H. influenzae* cells.
[b] The values in parentheses are predicted values assuming only a chemokinetic effect.

TABLE 4. Clearance of nontypable *H. influenzae* in nitrogen mustard-treated and control $C5^+$ mice

Inoculum size (no. of cells)	Treatment	% of bacteria remaining after 6 h
$1.1 \times 10^4 \pm 0.2 \times 10^4$	Saline	97 ± 10[a]
	Nitrogen mustard	516 ± 32[b]
$1.2 \times 10^5 \pm 0.3 \times 10^5$	Saline	289 ± 25
	Nitrogen mustard	$1{,}170 \pm 104$[b]

[a] Mean ± standard error of the mean for six to eight animals.
[b] $P < 0.05$ compared with saline treatment.

deficiency, impaired influx of PMN into the lungs paralleled impaired clearance of nontypable *H. influenzae*. The following observations suggest strongly that poor PMN recruitment and resultant poor bacterial clearance occur because of a failure to generate intraalveolar chemotaxins: (i) both $C5^+$ and $C5^-$ mice developed neutrophilia in peripheral blood, but $C5^+$ mice recruited significantly more PMN to the lungs; (ii) $C5^+$ mice had significantly more chemotactic activity in BAL fluids than $C5^-$ mice at 4 and 6 h postchallenge; (iii) in both $C5^+$ and $C5^-$ mice, the number of PMN in BAL fluids correlated closely with chemotactic activity; and (iv) chemotactic activity appeared in BAL fluids before an influx of PMN.

Taken together, our data support the concept that C5 fragments are important alveolar chemotaxins generated in response to nontypable *H. influenzae*, and the absence of C5 results in poor PMN recruitment and poor early clearance. The cleavage of C5 to its chemotactic fragments in lungs could have occurred in several ways. It might have occurred as a result of classical or alternative pathway activation. Nontypable *H. influenzae* are capable of activating the alternative pathway which could have generated chemotactic C5 fragments (C5a and C5a des Arg) (24). Alternatively, the C5 protein may have been cleaved by proteinases from macrophages rather than from activation of the whole complement cascade. Previous studies have shown that a proteinase derived from rabbit alveolar macrophages which was incubated with C5 produced chemotactic activity for macrophages and PMN (23). Granule lysates from neutrophils also generate chemotactic activity when they are incubated with C5 (32). Thus, the generation of chemotactic factors could either depend on or be independent of activation of the whole complement cascade.

It is important to note that $C5^-$ mice exhibited considerable neutrophil immigration, particularly at late time intervals. Indeed, by 24 h there were as many PMN in BAL fluids of $C5^-$ mice as in BAL fluids of $C5^+$ mice. The nature of the chemotactic activity in the absence of C5 is speculative, but may include alveolar macrophage chemotactic factor (8, 9, 12, 15) or products of the lipoxygenase pathway (28). Additionally, the release of a neutrophil chemotactic factor from neutrophils that have already migrated into the lungs could have augmented the influx of neutrophils in $C5^-$ animals (7). These observations indicate the multiplicity of mechanisms for PMN recruitment to the alveoli.

Our results document that C5 is involved in the early recruitment of granulocytes to the alveoli after nontypable *H. influenzae* challenge, but this may not be the only mechanism by which C5 participates in early clearance. C5 fragments have many nonchemotactic properties, and it is possible that reduced clearance of nontypable *H. influenzae* in $C5^-$ mice is due in part to the lack of these effects. Examples of nonchemotactic properties of C5 fragments which could affect bacterial clearance include mobilization of C3b receptors to the surface of PMN (6), enhanced bactericidal activity of PMN (29), and direct bacteriolytic activity of the terminal complement components (16). Our studies did not address these other potential effects of C5 deficiency on clearance of nontypable *H. influenzae* from lungs.

Although clearance was significantly impaired in $C5^-$ animals at early times when fewer neutrophils were recruited, $C5^-$ mice did recruit neutrophils to the lungs. To further assess the importance of intraalveolar neutrophils in the clearance of nontypable *H. influenzae*, we used another model of defective PMN recruitment namely, systemic depletion with nitrogen mustard. Nitrogen mustard treatment resulted in profound, sustained neutropenia, as previously described (21). The resident alveolar macrophage pool was not decreased in size by this treatment. These cells were morphologically intact, and a normal fraction were viable as assessed by trypan blue exclusion. Additionally, this treatment almost completely abolished PMN recruitment to the alveoli after challenge with nontypable *H. influenzae*.

The importance of recruited neutrophils in the clearance of nontypable *H. influenzae* was clearly documented by the observation that clearance of nontypable *H. influenzae* was markedly impaired in neutropenic animals. Alveolar macrophages and recruited neutrophils were able to prevent net multiplication of bacteria in the lungs of normal animals challenged with 10^4 nontypable *H. influenzae* cells, whereas bacteria multiplied in nitrogen mustard-treated animals, which were not able to recruit PMN to their lungs. When a larger inoculum was used, bacterial multiplication was observed in normal animals, as previously reported (27), but neutropenic animals contained fourfold more bacteria than normal animals. Thus, neutrophils recruited from the circulation represent a major component of the normal pulmonary defense against this bacterium.

In summary, in this paper we show that early clearance of nontypable *H. influenzae* from lungs requires PMN. In the absence of C5, alveolar chemotactic activity is deficient, PMN recruitment is reduced, and nontypable *H. influenzae* cells are not cleared well at 4 and 6 h postchallenge. In a different model of reduced PMN recruitment in which systemic depletion of PMN with nitrogen mustard was used, a similar defect in clearance was observed. Taken together, these data indicate that the early appearance of PMN in lungs is crucial for early clearance of nontypable *H. influenzae*.

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