Pulmonary Host Defenses and Oropharyngeal Pathogens

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The lower respiratory tract is repetitively inoculated with oropharyngeal bacteria and yet pneumonia is an infrequent event. Efficient mechanisms of antibacterial defense are present in the respiratory tract that eliminate microbes before their presence or multiplication leads to disease in the majority of instances. Resident pulmonary defenses consist of aerodynamic defenses, the mucociliary apparatus, alveolar macrophages, complement, and surfactant. These resident defenses can be augmented by the development of an inflammatory response or the development of specific immunity. Significant species variability exists in the efficiency and mechanisms of clearance for oropharyngeal organisms. Streptococci are cleared promptly, *Branhamella catarrhalis* is cleared slowly, whereas non-typable *Haemophilus influenzae* multiply before being cleared. A dual phagocytic system of alveolar macrophages and recruited polymorphonuclear leukocytes is required for clearance of most oropharyngeal microbes. Systemic immunization can significantly enhance clearance of non-typable *H. influenzae*, suggesting immunoprophylaxis might be possible for this organism.

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The largest epithelial surface exposed to the environment is the lung. Although the lung is continuously exposed to inspired air that contains microbes, early bacteriologic studies of bronchoscopically obtained tracheobronchial secretions and of lung biopsy material cultured at the time of thoracotomy suggested that the lung was a sterile organ [1–3]. This traditional concept of a continuously sterile lung has been challenged by more recent observations. Post mortem cultures of lung tissues obtained from previously healthy persons who died suddenly yielded small numbers of bacteria similar to those cultured from the pharynx [4]. Similarly, microbiologic studies of normal dogs showed that 89 percent of the lungs contained oropharyngeal organisms despite a study design that prevented agonal aspiration of upper respiratory tract secretions [5]. Finally, it has been demonstrated that pharyngeal contents enter the lower respiratory tract of normal persons during sleep. Pharyngeal secretions were noted in the lungs of 45 percent of normal persons and 70 percent of patients with altered consciousness. All normal subjects who slept soundly throughout the night aspirated oropharyngeal secretions. The foci of localized deposits of pharyngeal contents detected in human lungs and the foci of bacteria isolated from normal dog lungs were distributed in both gravity- and non-gravity-dependent areas [5,6] which suggests that both aspiration and aerosolization of pharyngeal secretions are responsible for the deposition of bacteria from the oropharynx into the lung.

The number of bacteria inoculated into the lungs of normal persons is unknown. Two possibilities could account for the small number of bacteria isolated from normal lungs. A small number of bacteria might be deposited in the lung during sleep but cleared slowly, or many organisms might be deposited but cleared rapidly. The high density of bacteria in pharyngeal secretions suggests the latter [7,9]. Small volumes of oropharyngeal material can provide a significant inoculum of bacteria. Oropharyngeal bacteria are present in upper respiratory tract secretions in concentrations of 10^7 bacteria/ml; therefore, aspiration of only 0.01 ml would inoculate 10^5 bacteria into the lower respiratory tract. These data suggest the existence of efficient mechanisms of defense that are capable of eliminating micro-organisms before their multiplication leads to disease.

The components of the pulmonary host defenses are distributed throughout the respiratory tract. Aerodynamic filtration and impaction occur in the nose, which removes large particulates, and cough and neurologic reflexes are important in the prevention of aspiration. In the lower respiratory tract, defenses include the mucociliary apparatus, alveolar macrophages, complement, and surfactant. All of these defenses are present at all time in normal persons and comprise the res-

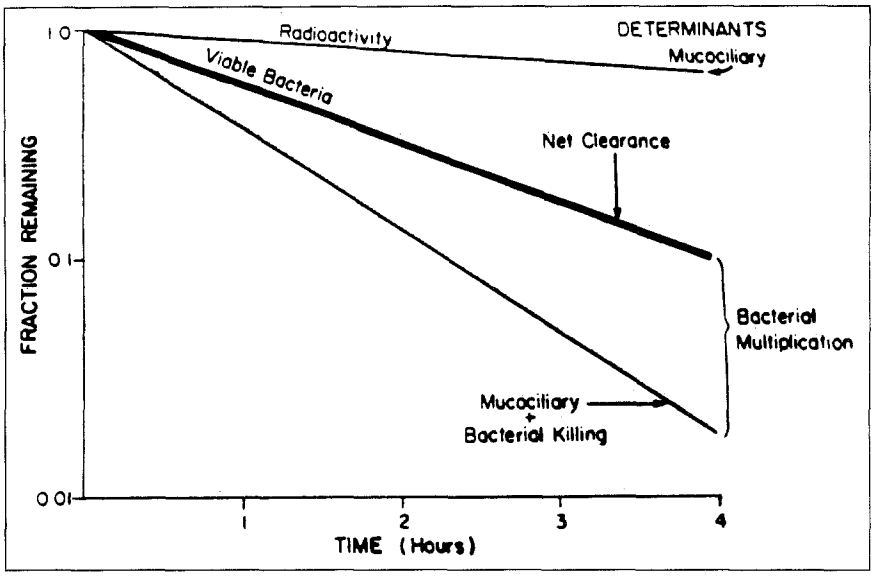

Figure 1. Determinants of bacterial clearance. The fraction of bacteria remaining in the lung are shown at various time points. The **heavy black line** depicts net bacterial clearance, which is the variable measured in most clearance experiments. A small portion of the total decrease is due to physical removal by the mucociliary apparatus. The **lower line** represents the combined effects of mucociliary clearance and *in situ* killing in the absence of bacterial multiplication. Reprinted with permission from [49].

ident defenses of the lungs. These resident defenses deal with day-to-day challenges and effectively clear small inocula of bacteria. However, the combination of cough, mucociliary clearance, and alveolar macrophage phagocytosis is inadequate for clearance of large inocula of virulent bacteria. Under these circumstances, resident defenses are augmented by the rapid development of a pulmonary inflammatory response [10,11]. Additionally, specific immune responses may be generated leading to the production of specific immunoglobulins and to cell-mediated immunity.

ANIMAL MODEL SYSTEMS

The concept of an antibacterial defense system intrinsic to the pulmonary parenchyma was established utilizing an animal model at the turn of the century [12]. Animal models continue to be used extensively because the lower respiratory tract is unique with regard to phagocytic populations, levels of both immunoglobulin and complement [13–15], and the induction of immunity [15–20]. A well-characterized murine model system has been utilized to study the clearance of bacteria from the lung. Utilizing this model, the entire sequence of events involved in bacterial clearance can be studied *in vivo*.

Bacteria can be deposited in the lower respiratory tract by exposure to a bacterial aerosol or by an intratracheal or intrabronchial injection of fluid containing micro-organisms [21,22]. The foci of localized deposits of pharyngeal contents detected in human lungs and the foci of bacteria isolated from normal dog lungs were distributed in both gravity- and non-gravity-dependent areas [5,6], which suggests that both aspiration and aerosolization of pharyngeal secretions are responsible for the deposition of oropharyngeal bacteria into the lung. Either technique delivers a sufficiently precise and reproducible inoculum of bacteria to allow quantitation of bacterial clearance over extended time intervals and neither inoculation alters lung architecture. Following aerosol exposure, bacteria are widely dispersed throughout the lung, and the ratio of bacteria to resident phagocytes is low, whereas bolus inoculation deposits bacteria in a localized area of parenchyma and results in a higher bacteria-to-phagocyte ratio. At various time points after inoculation, the lungs of infected animals are removed and homogenized, and serial dilutions of the homogenates are plated on agar in order to quantify the number of viable bacteria remaining in the lung. In most experiments, several time points after inoculation are evaluated. The decline in bacteria (clearance) is the net result of three processes: mucociliary clearance, bacterial killing, and bacterial multiplication (**Figure 1**). Net clearance is the variable evaluated in *in vivo* experiments. Although bacterial clearance can be altered by changes in any of these processes, bacterial killing appears to be the rate-limiting process. In circumstances where killing is exceeded by bacterial multiplication rates, net increases in bacteria isolated from the lung will occur [23-25].

This animal model can also be used to evaluate the development of a pulmonary inflammatory response or the development of pulmonary immunity. Utilizing bronchoalveolar lavage, the number and types of cells present in the air spaces can be determined. The lavage fluid can be assayed for the presence of chemotaxins and immunoglobulins. Adequate numbers of cells are obtained for the performance of functional cellular studies or for the performance of *in vitro* studies of secreted cell products. A particularly attractive feature of this animal model is the ability to assess both the induction of the response (inflammation, immunity) and its expression in the lung. The model allows determination of whether the effectors (antibodies, cells) are delivered to the site of the host-bacterial interaction and whether they are functional (i.e., protective) in the lung.

CLEARANCE OF OROPHARYNGEAL BACTERIA FROM THE LUNGS

Bacteria are normally present in the oropharynx and in saliva of humans and animals. Viridans streptococci (*Streptococcus salivarius* and *Streptococcus sangius*) and aerobic gram-negative organisms (*Branhamella catarrhalis* and nontypable *Haemophilus influenzae*) are normally present in the oropharynx

TABLE I

Bacterial Clearance*

	Deposition (CFU × 10^5) 0 Hours	Percentage of Bacteria Remaining 1 Hour	2 Hours	4 Hours
S. sanguis	4.7 ± 0.3	24.0 ± 2.0	8.0 ± 1.0	1.0 ± 0.0
S. salivarius	3.9 ± 0.2	49.0 ± 3.0	24.0 ± 3.0	5.0 ± 0.0
B. catarrhalis	4.5 ± 0.2	69.0 ± 9.0	49.0 ± 4.0	22.0 ± 2.0

CFU = colony-forming units.
*Each value represents a mean of 15 to 18 animals at each interval; data are mean ± SEM.
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TABLE II

Phagocytic Cell Response*

	Time after Aerosol 0 Hours	2 Hours	4 Hours
S. sanguis			
Alveolar macrophages × 10^5	9.3 ± 1.3	18.4 ± 1.9	196. ± 1.4
Granulocytes × 10^4	0.3 ± 0.2	0.1 ± 0.1	0.4 ± 0.4
S. salivarius			
Alveolar macrophages × 10^5	10.3 ± 0.6	13.2 ± 0.9	12.3 ± 1.0
Granulocytes × 10^4	0.4 ± 0.2	7.6 ± 2.3	8.4 ± 2.4
B. catarrhalis			
Alveolar macrophages × 10^5	10.1 ± 0.8	12.7 ± 1.4	11.7 ± 1.1
Granulocytes × 10^4	0.1 ± 0.1	3.4 ± 0.8	40.9 ± 5.5

*Data are mean ± SEM.
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and in the saliva of humans and animals [7–9]. There are significant species differences in the clearance of these organisms. Ninety-five percent of aerosolized streptococci were eliminated within four hours, whereas aerosolized *B. catarrhalis* was cleared at a slower rate (**Table I**) [26]. Following bolus inoculation of non-typable *H. influenzae* (NTHI), a biphasic pattern was observed. During the first six hours after inoculation with NTHI, bacterial multiplication exceeded killing and the number of bacteria increased fourfold (395 ± 32 percent). During the second phase, killing exceeded multiplication and the bacteria were eradicated within 24 hours (2 percent bacteria remaining) [27]. These results indicate that resident defenses in mice are unable to effect eradication of oropharyngeal organisms and must be augmented for clearance to occur.

PHAGOCYTIC CELL RESPONSE TO OROPHARYNGEAL BACTERIA

Phagocytic cells are importantly involved in bacterial killing in the lung. Bronchoalveolar lavage of normal, water-aerosolized and water-bolus-injected mice resulted in similar numbers of phagocytes (approximately 9 to 10 × 10^5). Alveolar macrophages appear to be the only phagocyte involved in the clearance of *S. sangius*. A doubling in the number of alveolar macrophages was noted within four hours after inoculation of *S. sangius*. Studies of changes in alveolar cell populations after challenge with *S. salivarius*, *B. catarrhalis*, and NTHI suggest that recruitment of polymorphonuclear leukocytes (PMN) is required to augment resident host defenses [26,27]. Challenge with *S. salivarius* resulted in a 20-fold increase in the number of PMN at four hours, and challenge with *B. catarrhalis* resulted in a 400-fold increase in PMN (**Table II**). Challenge with NTHI resulted in 100-fold increase in total cells at six hours. No PMNs were present in alveolar lavage at zero hours, whereas 1.7 × 10^6 PMN were isolated at six hours. At six hours, PMN constituted 63 percent of the phagocytic cells in the alveolar lavage. Thus, both alveolar macrophages and PMN are apparently important in the clearance of most oropharyngeal bacteria.

Studies of neutropenic mice have demonstrated the importance of recruited PMN to clearance of NTHI [28]. Systemic treatment of mice with nitrogen mustard resulted in profound neutropenia but did not alter the numbers of resident alveolar macrophages. Nitrogen mustard-treated mice were unable to recruit PMN into the lungs after NTHI challenge, and pulmonary clearance of NTHI was significantly impaired in these neutropenic animals. These data demonstrate that recruited PMN are a major component of the early defense against NTHI and are crucial for effective clearance of this oropharyngeal organism. Although similar studies have not been performed following challenges with other oropharyngeal organisms, these data suggest that PMN are important to early clearance of those organisms that result in their recruitment.

MECHANISMS OF PMN RECRUITMENT

The ability to develop an inflammatory response is a fundamental part of the pulmonary response to bacteria. The initiation of an inflammatory response involves the generation of intra-alveolar chemotaxis [29]. The specific chemotactic factors responsible for PMN recruitment following challenges with oropharyngeal organisms have been partially characterized using congenic C5-sufficient B10.D2/nSn (C5+) and C5-deficient B10.D2/nSn (C5−) mice. The C5− mice recruited significantly fewer PMN following challenges with NTHI than C5+ mice. The impaired influx of PMN into the lungs of C5− mice paralleled impaired clearance of NTHI. Thus, the C5 molecule and its fragments are important chemotaxins during the first six hours after exposure to oropharyngeal organisms [28].

Although PMN recruitment was significantly impaired during the first six hours following NTHI challenge in C5− mice, the C5− mice recruit near-normal numbers of PMN to the lung at 24 hours. The nature of the non-C5 chemotactic factors is speculative but probably includes alveolar macrophage-derived cytokines [30–33] and macrophage-generated products of the lipoxygenase pathway such as leukotriene B_4, and 5- or 11-monohydroxyeicosatetraenoic acid [34,35]. Alveolar macrophages have the capacity to metabolize endogenous arachidonic acid, mobilized by agonist stimulation, into substantial amounts of 5-lipoxygenase (5-LO) products. Monocytes are unable to metabolize endogenous arachidonic acid into 5-LO products. This inability to generate 5-LO products is due to a lack of coupling between arachidonic acid deacylated by phospholipase and the 5-LO enzyme. By contrast, the degree of coupling between arachidonic acid release and 5-LO metabolism increases dramatically with differentiation of monocytes to alveolar macrophages in the lung. This alteration in 5-LO metabolism of endogenous arachidonic acid, which occurs with differentiation of monocytes to al-

veolar macrophages, is important in equipping alveolar macrophages with the ability to mount an inflammatory response in the alveolar space [36].

Alveolar macrophages also secrete numerous cytokines, which are important in neutrophil accumulation. Stimulated alveolar macrophages produce both interleukin-1 (IL-1) and tumor necrosis factor (TNFα) [37,38]. Initial reports suggested these cytokines were chemotactic for neutrophils [39], but recent studies have shown that neither recombinant IL-1 nor TNF has direct chemotactic activity *in vitro* for neutrophils [40]. Alveolar macrophages have also been shown to generate a low molecular weight chemotactic factor after phagocytosis of microbes that preferentially attracts neutrophils [31–33]. This alveolar macrophage-derived neutrophil chemotactic factor is similar to a recently isolated and cloned monocyte NCF [41]. The complete nucleotide sequence of the complementary deoxyribonucleic acid of monocyte-derived NCF has recently been published. This complementary deoxyribonucleic acid encodes a 99-amino acid protein with an estimated molecular weight of 8,000 daltons for the active species. The amino acid sequence of monocyte-derived NCF shows a high degree of homology with other molecules involved in inflammation and cell growth such as beta-thromoglobulin and platelet factor 4 [42,43], suggesting this factor may be a member of a supergene family. Lipoploysaccharide, interleukin-1, and tumor necrosis factor have all been shown to increase monocyte-derived NCF steady state m-RNA within one hour and cause the production of biologically active NCF by monocytes [41]. Recent studies have shown that alveolar macrophages increase cellular steady-state NCF m-RNA and produce biologically active NCF in response to LPS (Strieter RM, *et al*, submitted for publication).

Recent studies have demonstrated that non-immune cells may participate in the generation of a pulmonary inflammatory response by the synthesis of a chemotactic factor similar to monocyte-derived NCF. Human endothelial cells produce a NCF with molecular and physiochemical characteristics consistent with monocyte-derived NCF following stimulation with tumor necrosis and interleukin-1B or lipopolysaccharide [44]. More recently, fibroblasts have been shown to have dose-dependent increases in steady-state m-RNA and to secrete a NCF similar to monocyte-derived NCF following exposure to TNFα, IL-1B, or IL-1α. In contrast, LPS and interleukin-6 failed to induce fibroblast-derived NCF [45]. Finally, IL-1α, IL-1B, and TNFα have been shown to induce the expression of high levels of NCF m-RNA in a type II-like epithelial cell line (A549). Interestingly, LPS had no effect on the induction of NCF in epithelial cells (Strieter RM, *et al*, submitted for publication).

The entry of bacteria or bacterial products into the lower respiratory tract initiates a complex series of events that results in the recruitment of PMN to the lung (**Figure 2**). The initiation of the inflammatory response involves several mechanisms including C-5-derived chemotactic peptides, a cascade of macrophage-derived cytokines, and arachidonic acid metabolites and cytokines produced by non-immune cells of the alveolar-capillary membrane. It seems likely that different chemotaxins are generated in sequence such that some factors are involved in the early phases, whereas others are important at later time points.

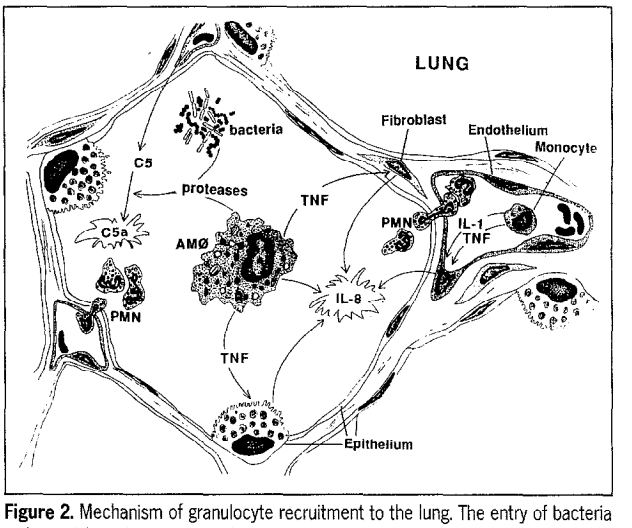

Figure 2. Mechanism of granulocyte recruitment to the lung. The entry of bacteria or bacterial products into the lower respiratory tract initiates a complex series of events that results in the recruitment of PMN to the lung.

TABLE III

Effect of Systemic Immunization with an Outer Membrane Protein/LPS Complex on Pulmonary Clearance of NTHI

Immune Status	Percent Bacteria Remaining at 6 Hours Post Challenge
Control	358 ± 30
Immune	16 ± 1

TABLE IV

Effect of Intravenous Administration of a LPS-Specific Monoclonal Antibody on Pulmonary Clearance of NTHI

Immunization Agent	Percent Bacteria Remaining at 6 Hours Post Challenge
Phosphate-buffered saline	396 ± 37
Monoclonal antibody 6A10	43 ± 3

C-5-derived peptides are important in the first six hours following bacterial entry, whereas macrophage and non-immune cell-derived NCFs are likely important at later time points. Although a variety of cellular components in the lung can express NCF, this expression is stimulus-specific. Both endothelial cells and alveolar macrophages can express NCF m-RNA and functionally active NCF in response to primary bacterial products such as LPS. Alternatively, fibroblasts and type II epithelial cells only produce NCF in response to alveolar macrophage- and monocyte-induced cytokines, IL-1 and TNF. The coordinated participation of mononuclear phagocytes, endothelial cells, fibroblasts, and epithelial cells is likely required for the generation of the chemotactic gradient required for PMN recruitment.

SPECIFIC HUMORAL IMMUNITY

Normal bronchoalveolar washings contain immunoglobulin G (IgG) and IgA [46], but it is uncertain what role immunoglobulin plays in resident defenses within the alveolus. Normal mice have been shown to have no

specific serum antibodies against normal oropharyngeal flora [26,47]. Although the role of immunoglobulin in resident pulmonary antibacterial defenses is uncertain, it is clear that immunization can enhance early clearance of bacteria from the lower respiratory tract. Systemic immunization with NTHI resulted in NTHI-specific antibody in both serum and bronchoalveolar lavage. The concentration of NTHI-specific antibody in serum of immunized animals was 1,000-fold higher than in serum of controls, whereas the concentration of NTHI-specific antibody in bronchoalveolar lavage was at least 200-fold greater than in bronchoalveolar lavage obtained from control animals. Western blot analysis revealed that the specificities of antibodies in bronchoalveolar lavage were identical with those of antibodies in the serum demonstrating that serum IgG can enter the alveolar spaces of the uninflamed lung, probably by transudation. Thus, direct airway immunization is not required to generate protective, specific antibody in the air spaces of the lung [47].

An acellular NTHI antigen preparation has also been shown to generate protective antibodies in the air spaces. Mice immunized with an outer membrane protein-lipo-oligosaccharide complex produced antibodies directed against both immunogens and the presence of these antibodies was correlated with a marked enhancement of pulmonary clearance of NTHI (**Table III**). Although these data do not allow a determination of the role of cellular versus humoral immunity, passive intravenous immunization of mice with a LPS-specific monoclonal antibody resulted in markedly enhanced lower respiratory tract clearance of NTHI (**Table IV**) [48]. Taken together, these data suggest that immunoprophylaxis or immunomodulation of NTHI disease in the lungs is feasible.

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