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Mechanisms of bacterial virulence in pulmonary infections

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Abstract

Purpose of review—To consider the relevance to severe human lung infections of recently discovered virulence mechanisms of *Staphylococcus aureus* and *Francisella tularensis*

Recent findings—*S. aureus* has long been considered an opportunistic pathogen. However, due to the emergence of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) strains that can readily infect and kill normal hosts, *S. aureus* must now be considered a potentially virulent pathogen. The evolution of *S. aureus* from an organism associated with asymptomatic nasopharyngeal colonization to one associated with community-acquired lethal infections may reflect horizontal acquisition of bacterial genes that enable efficient spread, aggressive host invasion, and effective immune evasion. Alleviating the burden of staphylococcal disease will require better understanding of host susceptibility and of staphylococcal virulence and antibiotic resistance. In contrast to the rapidly evolving staphylococcal virulence strategy, recent genomic analysis of *Francisella tularensis* has revealed a small set of bacterial genes associated with the marked virulence of its North American subspecies. This suggests that a relatively stable strategy of immune evasion underlies this pathogen's ability to establish serious life-threatening lung infections from a very small inoculum.

Summary—Understanding bacterial pathogenesis will require additional research into both host susceptibility factors and bacterial virulence mechanisms, including horizontal gene transfer. Refinements in the molecular detection of bacteria in the clinical setting, as well as whole genome analysis of both pathogens and patients, are expected to aid in the understanding of bacterial-induced lung injury.

Keywords

Staphylococcus aureus; virulence; antibiotic resistance; horizontal gene transfer; pathogen

Introduction

Lung infections associated with bacteria found in the community such as *S. aureus* or *P. aeruginosa* (in contrast to potential bioterror agents such as *F. tularensis*) require large inoculums in normal hosts and can attack the lungs of such hosts via two routes. Infections can occur when bacteria directly invade the airspaces, promoted by bacterial products that damage airway epithelial cells or host phagocytes (neutrophils and macrophages), antibiotic administration that alters the normal respiratory tract flora, or epithelial injury (i.e., after viral

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airspace infections). Bacteria can also damage the lung via the vascular route, though these mechanisms are less clearly established. Interestingly, bacteremia readily promotes interstitial lung edema, but not alveolar epithelial injury.¹⁻³

Part of the problem facing clinicians is that the chest radiograph generally does not indicate which barrier is affected in lung infections;⁴ the interstitial edema caused by bacteremia is not easily distinguished from the alveolar edema caused by airspace infections. Nonetheless, the bacterial products producing epithelial injury within the lung may be different from those that cause bacteremia-induced acute lung injury from the endothelial side. We explore what is understood about these distinct mechanisms for *S. aureus*, which has long been regarded as an opportunistic pathogen but has recently been behaving in increasingly virulent fashion in many clinical settings.

Finally, we briefly review recent findings from experiments with a potential bioterror agent, *F. tularensis*. This bacterium can cause lung injury after inhalation of very few bacterial cells. Mechanisms underlying the differential virulence of this organism and bacteria that more commonly cause lung injury are discussed.

The Role of Pantan-Valentine Leukocidin (PVL) in the Pathogenesis of Community-Acquired Methicillin-Resistant *Staphylococcus aureus* (CA-MRSA) Lung Infections

There has been a worldwide emergence of community-acquired methicillin-resistant *S. aureus* (CA-MRSA);⁵ these strains have often been identified by carriage of genes that encode LukF-PV and LukS-PV, the components of the Pantan-Valentine leukocidin (PVL). Such CA-MRSA strains have been associated with severe respiratory and skin infections.⁵ However, how these organisms spread so efficiently and what gene products enable them to infect the skin and respiratory tract are not clear. Moreover, the role of PVL in disease causation is controversial, as disease-causing CA-MRSA strains that do not carry or express PVL are known to occur.⁶

An important issue confronting investigators of bacterial pathogenesis is that the various experimental animal models differ in their sensitivity to bacterial products; indeed, different strains of genetically inbred mice vary significantly in their response to endotoxin and to other bacterial products. The pathogenesis of lung injury in one inbred mouse strain involves PVL,⁷ but similar experiments performed in other inbred mouse strains have demonstrated that PVL-negative strains are just virulent as wild-type strains.⁸ The role of PVL in the pathogenesis of CA-MRSA provides an explanation for these differences.

PVL and leukocytes

PVL protein subunits assemble into cytolytic pore-forming octamers on the surface of susceptible cells.⁹ Compared to human leukocytes, murine leukocytes are relatively insensitive to the leukolytic effects of PVL. Therefore, more recent experiments have used rabbit leukocytes, which are more sensitive to PVL, and thus more closely mimic human leukocytes.^{10,11} Injection of purified PVL into rabbits results in non-lethal transient granulocytopenia followed by a significant granulocytosis.¹² As a consequence of these effects, PVL may play an important role in host defense (discussed below).

Influence of PVL on MRSA gene regulation

To evaluate the effect of PVL on global gene regulation, transcriptional profiling of the clinically relevant USA300 and USA400 CA-MRSA strains and their isogenic Δpvl mutants was performed under conditions known to promote PVL overexpression.¹⁰ Real-time reverse

transcriptase-PCR confirmed that expression of PVL did not alter the abundance of transcripts encoding an accessory gene regulator, AgrA, Agr-regulated virulence factors, including protein A, alpha toxin, beta hemolysin, serine aspartate repeat protein, serine protease, or clumping factor B. Similarly, protein profiles from cell extracts and culture supernatants were identical between each of the wild-type parental and isogenic Δpvl mutant strains when cultured under the same *in vitro* conditions. Therefore, PVL does not contribute to CA-MRSA virulence by altering global gene and/or protein regulatory networks of *S. aureus*.

PVL and bacteremia

Bacteremia accounts for 65% of CA-MRSA invasive infections.⁵ In rabbit experiments in which wild type or isogenic Δpvl mutant strains of CA-MRSA, or a 1:1 mixture of each, were injected intravenously in rabbits, no effect of PVL on the final density of bacterial colonization or persistence was detected.¹³ PVL did play a modest but measurable role in pathogenesis during early stages of bacteremic seeding of the kidney, the target organ from which bacteria were not cleared. This difference in temporal effect may be due to the fact that PVL induces neutrophils to release interleukin-8, leukotriene B₄, and reactive oxygen species, thus priming the host innate immune system.¹⁴⁻¹⁶ Thus, although CA-MRSA strains with PVL may have an early benefit in colonizing the kidney, the priming effect of PVL on the immune system may lead to improved clearance of these strains compared to the PVL null strains.¹³

PVL and lung injury in rats

To evaluate the pathological effects of PVL in a rat model of lung infection, CA-MRSA wild type strain USA300 (also known as the LAC strain) and an isogenic Δpvl mutant were instilled into the tracheas of anesthetized rats.¹⁷ Investigators observed a time-dependent increase in inflammation and in the number of bacteria in the lungs of experimental animals. Cytokine and chemokine expression peaked at six hours. Bacterial counts peaked nine hours after instillation, and severe inflammation, pulmonary edema, and necrosis were present in 60% of animals at that time point, with milder degrees of these pathologies in the remaining animals. Animals dying from lung infection had more marked cytokine and chemokine expression than survivors, which displayed decreasing cytokine and chemokine expression by 24 h. Nearly all of the mortality occurred within the first 24 h. In contrast to humans, rats that survived for 24 h usually went on to full recovery. Animals that received the isogenic Δpvl mutant did not differ from those that received the wild type strain in terms of quantity of bacteria recovered, severity of lung histology, or mortality. The only observed difference was that animals receiving the isogenic Δpvl mutant expressed less CC12 than animals receiving wild type bacteria.¹⁷ PVL did not appear to affect the early host response to CA-MRSA necrotizing pneumonia in this model.

PVL and lung injury in mice

PVL is not required for pathogenesis of *S. aureus* bacteremia and sepsis or skin abscess formation in CD1 Swiss or hairless mice.¹⁸ PVL also does not play a role in pathogenesis of staphylococcal skin abscess formation in BALB/cJ and in BALB/cAnNHsd mice.⁸ Interestingly, experiments in the latter mouse strains indicate that infection with mutants lacking PVL genes (LAC $\Delta lukF$ -PV, LAC $\Delta lukS$ -PV and LAC Δpvl mutants) actually causes more severe lung disease than infection with the wild type LAC strain. This is in contrast to data published by the same authors indicating that the wild type LAC strain and its Δpvl mutant were equally virulent in C57BL/6j mice.^{19,20} These studies suggest that genetic background is an important determinant of disease expression in mice, and that PVL may not cause disease directly but through priming of the host immune system, which prompts a brisk inflammatory response but also facilitates pathogen recognition and clearance. In line with this viewpoint, PVL has been noted to stimulate granulopoiesis in mice and rabbits.⁸

PVL and human infections

Whole-genome sequencing was recently performed to compare ten epidemic USA300 strains.²¹ The strains were found to group into nine closely related isolates; eight of these contained the same *SCCmecIVa* subtype. However, the virulence of the closely related isolates was variable in animal infection models and caused different disease syndromes in patients. Thus, host factors clearly play a role in infection severity.^{6,21}

Immunization against *S. aureus*

Vaccines have been sought to improve outcomes in patients who are subject to chronic *S. aureus* infections [ie: renal failure patients on dialysis, patients with chronic skin conditions]. The question has been which protective antibodies are essential to protect patients against MRSA-induced pneumonia and other diseases.

Immunization with PVL components

A group of BALB/c mice was immunized intranasally with recombinant LukF-PV, recombinant LukS-PV, a combination of LukF-PV and LukS-PV, alpha toxin, DbpA (*Borrelia burgdorferi* adhesin used as a control antigen), or adjuvant alone (cholera toxin) once weekly for five weeks prior to intradermal or respiratory infection with *S. aureus* wild type strain LAC on day 35.²² A second group was immunized subcutaneously with the same antigens twice prior to infection on day 35; the first immunization on day 0 included complete Freund's adjuvant (CFA), boosted on day 14 with a second immunization that included incomplete Freund's adjuvant. Intranasal administration of the combination of LukF-PV and LukS-PV was not tolerated due to excessive inflammation of the respiratory epithelium. Both intranasal and subcutaneous immunization led to antibody responses against the *S. aureus* antigens. However, only intranasal immunization provided significant protection against experimental pneumonia, with LukS-PV providing greater protection than LukF-PV or alpha toxin. Conversely, intranasal immunization did not provide significant protection against subcutaneous infection.

Immunization with alpha-hemolysin

S. aureus alpha-hemolysin (Hla) is a member of a family of pore-forming beta-barrel toxins. *S. aureus* mutants lacking *hla* display reduced virulence in animal models of invasive infection.²³ Passive immunization with anti-Hla antisera protects animals from challenge with purified toxin and from intraperitoneal *S. aureus* infection.²⁴ Further evidence of the importance of Hla in lung infection comes from experiments in which the virulence of *S. aureus* strains was shown to be directly proportional to the quantity of Hla expressed.²³ In other experiments by the same investigators, C57BL/6j mice injected intramuscularly with Hla H₃₅₁ (a mutant form of Hla that cannot form pores) emulsified in CFA had a robust antibody titer compared to control animals injected with PBS and CFA. Immunization decreased the recovery of *S. aureus* CFUs from the lungs of infected animals, including animals infected with the LAC strain of CA-MRSA. Administration of rabbit anti-Hla serum also protected against both MSSA and CA-MRSA (LAC strain) lung infections. In these experiments, anti-PVL serum did not protect the animals.²³ These results indicate that intramuscular immunization with a mutant Hla which lacks toxin activity can provide broad protection against *S. aureus* lung infections.

Antibiotic resistance of CA-MRSA strains

Multidrug resistance has now been reported for CA-MRSA strains.¹³ Sixty clinical MRSA isolates that were tetracycline resistant on the basis of broth microdilution testing were found to carry the *tetK* and *tetM* resistance genes.²⁵ Exposure of these strains to doxycycline induced TetK-mediated resistance and raised the MIC of doxycycline above the CLSI susceptibility breakpoint of 4 mg/L. However, susceptibility to minocycline was not affected, even after

incubation in sub-inhibitory concentrations of this agent.²⁵ When CA-MRSA strains susceptible to fluoroquinolones are exposed to sub-inhibitory concentrations of these agents, an alteration in the SOS gene response is associated with an increased proportion of CFUs displaying methicillin resistance (so-called “heteroresistance”).²⁶ These studies suggest the need for caution when using fluoroquinolones or doxycycline to treat CA-MRSA, and imply that minocycline may be a reasonable alternative to the latter; however, clinical confirmation of these *in vitro* observations is needed.

***Francisella tularensis*-induced Lung Injury**

Tularemia is considered an emerging disease and due to the highly infectious nature of *F. tularensis* it is also considered a potential biological weapon. Also, *F. tularensis* is a facultative intracellular bacterium that lives in macrophages,²⁷ whereas wild-type *S. aureus* lives outside of cells. Transmission and entry of the bacteria occurs via aerosolization and inhalation when infected carcasses of rabbits or other animals are disturbed, ingestion of contaminated food or water, or inoculation by bites from infected ticks or through skin abrasions.²⁷ In contrast to the experiments with MRSA in which intratracheal instillation of at least 10⁸ CFUs are required to cause lung injury, as few as 10 CFUs of *F. tularensis* can cause respiratory tularemia, and intraperitoneal injection of as few as 160 CFU of *F. tularensis* subsp. *holarctica* is sufficient to cause sepsis in BALB/c mice.²⁸ As with MRSA, experiments using *F. tularensis* show that different rodent strains and species have different vulnerabilities to this organism.^{28,29}

Why does *F. tularensis* lung infection require so few bacteria?

The ability of *F. tularensis* to target and suppress host dendritic cells (DC) and macrophages allows it to replicate, disseminate, and cause overwhelming infection, even after inoculation with few bacteria, similar to sequence of pathogenic events that occur after infection with Ebola or Marburg virus.³⁰ In fact, murine experiments utilizing Schu S4, a type A strain of *F. tularensis*, showed that aerosols of this organism do not induce secretion of pro-inflammatory cytokines. In those experiments, resident DC did not undergo activation; rather, the immunosuppressive cytokine TGF-beta was detected in the lung, and administration of anti-TGF-beta antibodies was associated with increased production of TNF-alpha and decreased bacterial load in the lungs.³¹ Thus, the ability of few bacterial cells to causing overwhelming lung infection in humans may be attributable to active suppression of the host immune response.

Genomic analysis of *Francisella* species

The most virulent subspecies, *F. tularensis* subsp. *tularensis* (from the Type A lineage of *Francisella*), which is found exclusively in North America,²⁷ is genetically similar to *F. tularensis* subsp. *mediasiatica* (a member of the Type B lineage of *Francisella*), which is found in central Asia and causes less disease in similar experimental hosts.³² Five strains of *F. tularensis*, including a strain of *F. tularensis* subsp. *tularensis*, two strains of the less virulent subspecies *F. tularensis* subsp. *holarctica* (a Type B lineage that is associated with milder disease), and two strains of the nonpathogenic subspecies *F. tularensis* subsp. *novicida*, were recently subjected to whole genome sequencing and comparative genomic analysis, including comparison to a previously sequenced strain of *F. tularensis* subsp. *mediasiatica*.²⁷ All *Francisella* genomes were shown to consist of a circular chromosome ~2 Mb in size. The average genome contains 1730 genes, of which a mean of 1574 encode proteins. A total of 14 genes were found only in human pathogenic strains of *Francisella* (Type A and B lineages).

Immunization against *Francisella* respiratory infections

Immunization against *Francisella* utilizes an attenuated live vaccine strain of *F. tularensis* subsp. *holarctica*, LVS that was created in the 1950s. The vaccine is given via scarification

and does not provide optimal protection against lung infection. Recent experiments in BALB/c mice showed that oral immunization with the LVS strain results in rapid control after a challenge inoculum of virulent *F. tularensis* (Type A), but not complete lung sterilization.³³ Moreover, the immunity provided by the oral LVS immunization lasted only about 4 weeks. Notably, LVS oral immunization of C57BL/6 mice was not protective; the reasons for this are not clear but highlight the importance of genetic background in determining bacterial virulence and immunological protection.

Conclusions

The evolution of *S. aureus* from an opportunistic, antibiotic-sensitive, often hospital-acquired infection to a virulent, antibiotic-resistant, community-acquired pathogen has occurred over the past 60 years.⁶ To understand this evolution, we need more data that shed light on bacterial mechanisms of virulence and horizontal gene transfer, as well as defense mechanisms and physiological variation in the host that influence lung infection severity and outcome. In contrast, throughout recent human history *Francisella tularensis* has remained a virulent pathogen that can cause lung infection after exposure to a minute quantity of bacteria, with a relatively narrow spectrum of disease severity. Existing clinical tools and therapies appear inadequate to the task of preventing the lung infections that these disparate pathogens cause. In addition to the development of new therapies, molecular methods of bacterial identification that are used in clinical practice need to be refined so that the relevant genetics of an individual patient's pathogen can be brought to bear on treatment decisions. In addition, more comprehensive genomic analyses of human hosts are needed to define susceptibility factors that put individual patients at risk for more severe lung infections.

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