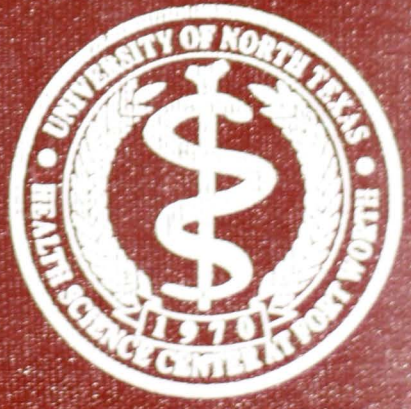
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Overheim, Katie A., Lethality of *Staphylococcus aureus* in Murine Pneumonia is Due to Alpha-Toxin and Other Secreted Factors Regulated by *agr* and *sar*. Doctor of Philosophy (Biomedical Sciences), August, 2003, 91 pp., 6 Tables, 9 illustrations, bibliography, 106

The purpose of these studies was to determine if the *S. aureus* global regulators *agr* and *sar* play a role in staphylococcal pneumonia and if the virulence factors regulated by them contributed to the severity of staphylococcal pneumonia. To determine this, we established a pneumonia model in mice in order to identify if *S. aureus* global regulators *agr* and *sar* play a role in the pathogenesis of staphylococcal pneumonia. As well, we took steps to identify the extracellular factors responsible for the lethality in a murine model of staphylococcal pneumonia and determine if these factors involved in disease process could be used as targets for immune therapy.

My work revealed that lethal pneumonia in a mouse model is dependent on the *S. aureus* global regulators *agr* and *sar*. This study also revealed that the lethality associated with our model is due to secreted factors, regulated by *S. aureus* global regulators *agr* and *sar*. Further investigation demonstrated that alpha-toxin is a major virulence factor involved in the lethality in our model. By generating an alpha-toxin deficient strain in *S. aureus* RN6390, we show a reduced virulence in our disease model. As well, antiserum to alpha-toxin, when administered with a lethal dose of *S. aureus* RN6390 protected animals from death. By evaluating the role of alpha-toxin's ability to contribute to lethality, we assessed numerous strains of *S. aureus* in our pneumonia

model. We discovered that there was a correlation to alpha-toxin production levels and lethality in our pneumonia model. However, our study also demonstrated that alpha-toxin is not the only factor involved in the disease process

LETHALITY OF *STAPHYLOCOCCUS AUREUS* IN MURINE PNEUMONIA

IS DUE TO ALPHA-TOXIN AND OTHER SECRETED

FACTORS REGULATED BY *AGR* AND *SAR*

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LETHALITY OF *STAPHYLOCOCCUS AUREUS* IN MURINE PNEUMONIA IS DUE TO ALPHA-TOXIN AND OTHER SECRETED FACTORS REGULATED BY *AGR* AND *SAR*

DISSERTATION

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DOCTOR OF PHILOSOPHY

By

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CHAPTER I

BACKGROUND AND SIGNIFICANCE

Staphylococcus aureus is a gram-positive, non-motile, none-spore-forming member of the Micrococcaceae family (1). *S. aureus*, a facultative anaerobe, that produces catalase, ferments mannitol and can grow in the presence of high salt (1). *Staphylococcus aureus* is a major cause of disease in humans, despite the advances in antimicrobial regimens. Furthermore, *S. aureus* typically resides on the skin and mucous membranes of up to 25% of the population at a time and up to 90% of health care workers (2 ,3). *S. aureus* is a common cause of hospital and community-acquired infections. Infections caused by *S. aureus* can range from minor skin infections, such as furuncles, to more serious infections such as endocarditis, osteomyelitis, septic shock and pneumonia (2). *S. aureus* is also one of the most causative agents of nosocomial infections that result in hospitalization (4). Thus, *S. aureus* infections are characterized for the variety and seriousness of their disease, and the ability to treat and/or prevent *S. aureus* disease is of critical importance.

Adding to the seriousness of staphylococcal infections is the increasing presence of antibiotic resistant organisms. Currently, methicillin (MRSA) and vancomycin resistant (VRSA) *S. aureus* pose a threat to human health (5, 6). MRSA are resistant to all β-lactam antibiotics (7). These strains tend to pick up other resistances to antibiotics

as well, making them difficult to treat. Historically, vancomycin was the drug of choice to treat MRSA (8). Unfortunately, resistance to vancomycin has now been reported through out the world and in the United States (9,10). Therefore, it is becoming important to find new and better approaches to prevent and treat staphylococcal infections.

As mentioned above, *S. aureus* causes numerous diseases that range from simple skin infections to more life-threatening diseases (2). Infections caused by *S. aureus* usually begin at the site of a skin infection (3). If a skin infection persists; *S. aureus* can spread to the bloodstream and subsequently to other tissues and organs (11). This spread from the bloodstream can result in serious infections such as endocarditis, osteomyelitis, or pneumonia (2). Understanding how *S. aureus* establishes and causes disease is an important aspect of fighting and treating the infections it causes.

S. aureus has been studied for over 100 years, and since then, researchers have isolated over forty extracellular and cell-wall associated proteins (11, 12). Most of these proteins are recognized as being involved in some aspect of disease (13). For example, protein A, assists *S. aureus* in evading the immune system by binding the Fc portion of IgG(14), while collagen and fibronectin binding proteins facilitate adherence to their respective extracellular matrix proteins (12). These proteins are considered to be staphylococcal virulence factors, and over the course of study of *S. aureus* have been classified into groups, in terms of function against the host. These virulence factors are said to aid in 1) Attachment to the host cells, 2) Evasion of the host defense, and 3)

Invasion of the host tissue (12). The purpose of these factors is not to cause disease, but to enhance bacterial survival within the host.

Steps to establishing Staphylococcal infection

Attachment

One essential step in the establishment of infection is the attachment of staphylococci to the host. Attachment may be an important step in aiding *S. aureus* to colonize the host. Lipotechoic acid, a protein in the *S. aureus* membrane aids in the attachment to the host (12). Another set of factors that aid in attachment to extracellular matrix proteins are named microbial surface components recognizing adhesive matrix molecules (MSCRAMMS) (15). Some of these proteins, such as fibronectin-binding protein, collagen-binding protein (cna) and fibrinogen binding protein, bind to the extracellular matrix of the host, aiding in *S. aureus* attachment. Collagen-binding proteins, fibronectin-binding and fibrinogen-binding proteins have all been shown to play a role in the attachment in infective endocarditis (16). These proteins are also important in the attachment in osteomyelitis (15).

Evasion of Host Defenses

Staphylococci have developed several strategies for evading the host defense system. Protein A binds the Fc portion of IgG antibodies (14), rendering staphylococcal cells invisible to phagocytes, by blocking opsonization (17). As well, several extracellular proteases are known to aid staphylococci in evading the host immune system. Proteases can block the action of antibodies by cleaving and inactivating (12) them as well as inactivating antimicrobial peptides such as defensins (18).

Superantigens, another class of extracellular proteins produced by staphylococci, also affect the immune system. Enterotoxins A-F, toxic shock staphylococcal toxin 1(TSST-1) and the exfoliative toxins all bind to the major histocompatability complex class II (MHC II) proteins and activate T-cells (12). This activation of T-cells causes the proliferation of T-cells and the release of high levels of cytokines. This host reaction can ultimately lead to shock.

Invasion of host cells

Several factors produced by staphylococci are involved in tissue invasion. Hemolysins, are the best characterized cytotoxic agents that damage host cells (19). One of the best studied, α-toxin, causes the formation of pores in cell membranes (12, 20). This ability to form pores in cells stimulates the release of nitric oxide in endothelial cells, and initiates apoptosis in lymphocytes (19,21). Strains deficient in α-toxin have been shown to have a reduced virulence in several animal models (22-26).

These three strategies used by *S. aureus* participate in establishing the numerous diseases briefly mentioned before. These factors are all regulated by *S. aureus* in order to survive within the host and in vitro. These factors are expressed under the regulation of at least two global regulatory systems, the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sar*).

The *Agr* and *SarA* loci

The in vitro growth pattern of *S. aureus* is known to have at least 4 distinct stages (lag, exponential, post-exponential and stationary phase) (27,12). Virulence factors of *S. aureus* are differentially expressed during these stages of in vitro growth. Most

exoproteins are produced during post-exponential phase (28). Cell-wall associated proteins are produced during log phase of growth (12). All of the aforementioned virulence factors, as well as others, are regulated by at least two global regulatory systems. The accessory gene regulator (*agr*) and the staphylococcal regulatory system (*sar*). These systems aid *S. aureus* in adapting to specific environmental changes, by sensing the environment and signaling gene expression or repression, allowing the bacterium to survive and persist in the host.

The *agr* locus was originally identified via transposon insertion (29). This insertion affected the expression of several virulence factors (29). In this mutant, expression of most extracellular virulence factors are depressed, while cell-wall associated proteins are over-expressed (30,30, 29).

The *agr* locus consists of two transcriptional units, transcribed from two divergent promoters P2 and P3 (32, 33). The P2 transcript contains 4 genes *Agr* A-D (33). *Agr* A and *Agr*C are considered part of a classical 2-component signal transduction system, while AgrB and AgrD together produce an auto-inducing peptide, AIP (11). AgrC acts as the sensor and is phosphorylated by AIP (11). The activation of the AgrAC signaling pathway leads to transcription from the *agr* promoters, P2 and P3 (RNA II and RNAIII) (31). RNAIII, the P3 transcript, has been shown to be the actual effector molecule that upregulates the transcription of secreted protein genes and downregulates the transcription of cell-surface protein genes (34, 33, 35). RNAIII appears in mid to post-exponential phase of growth, which results in the accumulation of AIP. The genes encoding cell-surface proteins that are induced by RNAIII are therefore typically

activated during early exponential phase of growth, while secreted toxins and enzyme genes are transcribed during late and post-exponential phases of growth (11).

The second regulatory system, *sar*, the staphylococcal accessory regulator was originally identified in a similar manner as *agr* via transposon insertion (36). The product of the SarA locus is a small basic protein. SarA is transcribed from 3 distinct promoters (P1, P2, and P3) and terminates at a common 3' end (37). A transposon insertion in the *sarA* locus results in a similar effect on extracellular and cell wall-associated proteins as *agr*, although the effect of *sar* appears to be strain dependent (38). In addition to its pleiotropic effects on extracellular and cell wall-associated proteins, *sar* also regulates expression of RNAII and RNAIII (39).

The P1 and P2 promoters of *SarA* are recognized by the vegetative sigma factor A, σA, and are mainly expressed during the exponential phase of growth. *SarA* promoter P3 is recognized by sigma factor B, σB, and is induced in cells entering post-exponential growth (37, 40, 41). SarA, appears to regulate the transcription of a number of virulence genes in an *agr*-independent way. Most significantly, expression of several proteases and collagen binding protein is suppressed by SarA (42, 43, 44).

Numerous animal models have been used to study the effects of *agr* and *sar* on virulence. Cheung et al (45) showed in an endocarditis model that a *sar* and *agr/sar* double mutant had decreased infectivity as compared to wild type, suggesting that both loci are involved in initiation and persistence of *S. aureus* in endocarditis. Booth, et al (46), compared the virulence of parental, isogenic *agr* mutant, *sar* mutant, and an *agr/sar* double mutant in endophthalmitis. This study suggested that both *agr* and the *sar* loci

play a role in the pathogenesis of this disease. Other models, including an osteomyelitis model in (42) and a murine arthritis model (47) show that a mutation in *agr* results in a reduction in virulence and severity of disease.

Staphylococcus aureus and Pneumonia

There are approximately two million hospitalizations each year as a result of nosocomial infections(48). As mentioned before *S. aureus* is the most common cause of nosocomial infections and the second leading cause of nosocomial bloodstream infections (4). Colonization of *S. aureus* in the nares can be shed onto the skin or respiratory tract to cause sinusitis, bronchitis or pneumonia (49). Importantly, *S. aureus* is the major cause of bacterial pneumonia in the elderly who are compromised by an influenza viral infection (50). As well, pneumonia and influenza are the sixth leading cause of death among persons 65 years of age or older in the United States. Because of its severity and rapid progression, *S. aureus* pneumonia has a high mortality even with potentially effective antimicrobial therapy.

More than 95% of patients with *S. aureus* infections worldwide do not respond to first line of defense antibiotics, such as penicillin (51), as well methicillin resistant *S. aureus* (MRSA) are common (51). Often, hospital acquired pneumonia's caused by *S. aureus* are complicated by isolates resistant to multiple antibiotics (52). In cases of MRSA infection vancomycin treatment is required (52). However, with the recent appearance of vancomycin-resistant *S. aureus* strains (53), pneumonia and other diseases caused by this organism will increasingly be a greater threat and therefore there is a critical need to

develop appropriate alternative methods to treat staphylococcal pneumonia and other diseases.

My interest in staphylococcal disease stems from the fact that very little is known about staphylococcal pneumonia. Although staphylococcal pneumonia is often times a lethal disease, particularly in the elderly, its prevalence is only seen primarily during an outbreak of influenza. However, as our population ages (it is estimated that the aged population of 65 years or older will double by the year 2030 (54)), the role of *S. aureus* in lung disease will become an increasingly important concern. The rates of hospital acquired pneumonia account for 15% of all nosocomial infections (5); with a mortality rate that exceeds 30% (55, 56, 57, 58, 59, 60). Therefore, it is of the utmost importance that the mechanisms of staphylococcal pneumonia be elucidated in order to identify virulence factors that may serve as potential targets for new and innovative antimicrobial therapies.

Little is known about the role that staphylococcal extracellular proteins play in causing pneumonia. Only one study has looked at the role that *agr* or *sar* play in pneumonia (61), and a few have looked at specific virulence factors, but have not definitively explained the disease process. *Agr* and *sar* were found to be required for invasive infection, but not inflammatory responses in the lung (61). In this investigation of staphylococcal pneumonia, a neonatal (10-14 day old) mouse model of pneumonia was utilized, where mice were intranasally inoculated with *S. aureus* RN6390 and its isogenic mutants defected in expression of the global regulators, *agr* and *sar* (61). In this study, all mice inoculated with *S. aureus* RN6390 were pneumonic and bacteremic while a

significant number of mice inoculated with either the *agr* or *sar* mutant strains were not (61). In addition, no deaths occurred, 18-hours post-inoculation, with mice inoculated with 10^8 cfu of either the *agr* or *sar* mutant strain, while a 30% mortality was observed with mice inoculated with the parent strain, RN6390 (61). Microscopic analysis of lung tissue from mice inoculated with either RN6390 or the mutant strains showed a significant level of inflammation (edema, cellular infiltration and consolidation) as compared to mice administered PBS alone but no significant differences were observed between RN6390 and either mutant strain (61).

Alpha-toxin and coagulase are implicated in the disease process associated with staphylococcal pneumonia (25,62). A role for alpha toxin was shown using a rat model of pneumonia. Damage occurred to the air-blood barrier due to an alpha-toxin producing strain of *S. aureus*, but not its corresponding mutant (25). Bronchoalveolar lavage (BAL) taken from the rats 4 hours post-inoculation revealed a significant increase in protein and hemoglobin levels when rats were inoculated with a high inoculum of the alpha-toxin strain as compared with its mutant. In addition, there was significantly greater damage to the alveolar epithelial cells in rats inoculated with the wild type over the mutant. However, when high concentrations of purified alpha-toxin was administered to rats, necrosis of epithelial cells was not observed (25). In this study, they concluded that alpha-toxin caused damage to the air-blood barrier, but did not directly affect the type I epithelial cells (25). These results suggest that alpha-toxin is an important factor in causing damage to staphylococcal infected lungs and most likely contributes to the disease by damage to the endothelial cells of the lung vasculature.

Coagulase may also play a significant role in *S. aureus* lung disease. Sawai, et al (62) developed a mouse model of hematogenous pneumonia by using *S. aureus*-enmeshed agar beads. These beads were inoculated into the tail vein of mice, and the number of bacteria in the lungs was determined 7 days post-inoculation. Seventeen different strains of *S. aureus* enmeshed in agar were examined, and of these, twelve were found in the lungs of mice in significant numbers ($10^5 - 10^8$ cfu) (62). In contrast, the same strains suspended in saline instead of agar and inoculated in a similar manner were not detected in the lungs after 7 days. The levels of coagulase were significantly higher in those strains recovered from the lung in significant numbers than those strains that were not (62). To verify the role of coagulase, a coagulase positive *S. aureus* strain and its isogenic mutant were compared. The number of bacteria recovered from the lungs was significantly higher with the coagulase positive *S. aureus* strain than with the coagulase negative strain over an inoculum range of $10^2 - 10^6$ cfu (62). This demonstrates that coagulase is an important component for maintaining *S. aureus* in the lung, but is not the only component important for causing pneumonia.

In the present study, I hypothesized that the *S. aureus* global regulators *agr* and *sar* play a role in staphylococcal pneumonia and the virulence factors regulated by them contribute to the severity of staphylococcal pneumonia. To determine this, we established a pneumonia model in mice in order to identify if *S. aureus* global regulators *agr* and *sar* play a role in the pathogenesis of staphylococcal pneumonia. As well, we took steps to identify the extracellular factors responsible for the lethality in a murine

model of staphylococcal pneumonia and determine if these factors involved in disease process could be used as targets for immune therapy.

CHAPTER II

LETHAL PNEUMONIA IN AN ADULT MOUSE MODEL IS DEPENDENT UPON *AGR* AND *SAR* REGULATED FACTORS SECRETED BY *STAPHYLOCOCCUS AUREUS*

Introduction

Staphylococcal pneumonia is a life-threatening disease primarily in the elderly population and in hospital settings where *S. aureus* is the leading cause of nosocomial pneumonia (4). Mortality rates for *S. aureus* hospital-acquired pneumonia can be quite high (63, 52, 49); for example, 25% of the of the nosocomial staphylococcal pneumonias that occurred in the New York City area in 1995 resulted in death (52).

Most strains of *S. aureus* are capable of producing several extracellular proteins that contribute to various types of diseases caused by this bacterium (13, 15, 64). However, little is known about the identity of those proteins or the mechanisms that contribute to the occurrence and subsequent severity of staphylococcal pneumonia. Previous studies have implicated α-toxin and coagulase; for example, damage to the air-blood barrier due to an α-toxin producing strain of *S. aureus* but not its corresponding α-toxin mutant was demonstrated in a rat model in which the *S. aureus* were inoculated directly into the lung (25), and the number of viable cells recovered from lungs of mice hematogenously inoculated with a coagulase-deficient mutant of *S. aureus* was reduced as compared to its

parental strain(62). In addition, several studies involving the installation of purified staphylococcal exotoxins into the lungs of mice and clinical findings from several human case studies indicate the involvement of one or more exotoxins with immunomodulator properties in staphylococcal pneumonia (65, 66 ,67).

Expression of virulence factors in *S. aureus* is controlled by at least two global regulators, the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sar*) (12). These regulons form a network of activators and repressors that result in differential expression of extracellular proteins primarily in response to cell density (12). A mutation in either or both of these regulators results in a pleiotrophic effect upon expression of several extracellular proteins (36, 42, 32) with concomitant reduction in virulence in several models of disease (68, 46, 69, 45, 70, 42, 47).

In an effort to determine which staphylococcal virulence factors are involved in causing pneumonia, we first decided to examine whether mutations in the *agr* or *sar* global regulators result in reduced virulence in an adult mouse model of pneumonia. Results obtained from this study indicate that factors found in *S. aureus* spent media are responsible for the lethality of disease in adult mice intranasally inoculated and that the expression of these factors are dependent upon *agr* and *sar* regulation. Differences between the parent strain and either regulon mutant were not observed with respect, to histopathology and the levels of the proinflammatory cytokine, IL-6 indicating lethality is not dependent upon an immunological response.

(Portions (30) of this work were presented at the 101st General Meeting of the American Society for Microbiology, May 20-24, 2001 in Orlando, Florida.)

Materials and Methods

Bacteria strains and growth conditions. *Staphylococcus aureus* RN6390, the parent strain to RN6911 and ALC488, was kindly provided by Mark Smeltzer (University of Arkansas for Medical Science, Little Rock, Ark.). *S. aureus* RN6911, the *agr*-null mutant (71, 35) was provided by Steve Projan (Wyeth-Ayerst Research, Pearl River, N.Y.) and maintained on tryptic soy agar (TSA; Difco Laboratories, Detroit, Mich.) containing 10 μg/ml of tetracycline (Sigma Chemical Co., St. Louis, Mo.) , *S. aureus* ALC488, the *sar* mutant (72) was provided by Ambrose Cheung (Dartmouth Medical School, Hanover, N.H.) and maintained on TSA containing 4 μg/ml of erythromycin (Sigma). Strains were routinely grown overnight (15-18 h) in flasks containing tryptic soy broth (TSB; DIFCO) without antibiotic. Flasks were incubated at 37°C with a flask to volume ratio of 2.5 and rotary aeration (180 rpm).

Mice. Specific pathogen-free, female BALB/c adult (ages 6 to 10 weeks) mice were obtained from Harlan Sprague Dawley (Indianapolis, Ind.), maintained in sterile microisolator cages, and given sterile food and water ad libitum. For all experimental procedures, mice were anesthetized with an intramuscular injection of a mixture of ketamine and xylazine.

Strain and spent media preparation. *S. aureus* strains grown in TSB were pelleted by centrifugation (10,000 x *g* for 15 min), washed twice in an equal volume of ice-cold, phosphate-buffered saline (PBS), and suspended in PBS. Portions of each cell suspension were diluted in PBS to the appropriate optical density (550 nm) that corresponded to the desired cell concentration (colony forming units (cfu)/ml) as

determined by a standard curve of cfu/ml as a function of optical density. To verify the concentration of each inoculum, portions were diluted in PBS, plated on TSA, and incubated overnight at 37°C prior to enumeration of bacteria.

Cell suspensions of UV-killed RN6390 were prepared by diluting cells to the appropriate concentration and exposing the cells to a lethal dose of UV light. Cell viability was determined by plating undiluted samples onto TSA and incubating at 37°C for 24 hours.

Spent media were prepared from overnight cultures (15-18 h) by collecting the supernatants after centrifugation. Supernatants were filter sterilized through 0.22 μm filters, and quick frozen on dry ice and ethanol, and stored overnight at -85°C. Frozen supernatants were lyophilized to dryness and stored at -85°C until used. Prior to each experiment, lyophilized supernatants were reconstituted in sterile, deionized, glass-distilled water to a concentration of 1X, 5X, and 10X of original volume and stored on ice until used. Spent media from the parent strain were treated with proteinase K beads (Sigma) for two hours at 37° or heat-treated by boiling for ten minutes.

Virulence assay. A portion (25 μl) of either cells or spent media was pipetted on the anterior nares of each anesthetized mouse and inoculation was accomplished by forced inhalation. Inoculated mice were monitored every six hours for signs (lethargy and ruffled fur) of illness and death. Whole lungs and spleens were aseptically removed at various times and placed in sterile, 50-ml, conical centrifuge tubes containing 1 ml of PBS. Tissues were aseptically minced and briefly sonicated prior to dilution and plating on TSA and mannitol salt agar (MSA; DIFCO). Blood was collected retro-orbitally into

tubes containing heparin, diluted in PBS, and briefly sonicated prior to plating on TSA and MSA. All plate media were incubated at 37°C for 24-48 hours prior to enumerate bacteria cfu.

Histopathology. Lungs were removed at various times, fixed in neutral formalin (VWR Scientific Products, Westchester, Pa.), embedded in paraffin, sectioned at 5 μm thickness, and stained with hematoxylin and eosin for light microscopy. Subjective scoring was performed by a blind observer (University of Alabama at Birmingham, Birmingham, Ala.) to quantify each of four major types of lung lesions; airway exudate, airway epithethial hyperplasia, peribronchial inflammatory cell infiltrate, and alveolar exudate. A lesion index was calculated as previously described (73).

IL-6 ELISA. IL-6 levels in mouse serum were determined by enzyme-linked immunosorbent assay (ELISA) using an optEIA™ Mouse IL-6 kit (BD PharMingen, San Diego, Calif.). Briefly, Easy-Wash 96-well flat-bottom microtiter plates (BD Biosciences, Bedford, Maine) were coated with monoclonal antibody specific for IL-6 overnight at 4°C. Wells were washed with PBS/Tween 20 prior to blocking with PBS/Tween 20 containing 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah) at room temperature for 1 hour. Sera or recombinant mouse IL-6 (standard) were placed in the appropriate wells and incubated overnight at 4°C. Wells were extensively washed with PBS/Tween 20 followed by the addition of biotinylated anti-mouse IL-6 antibody and streptavidin-horseradish peroxidase. After a one-hour incubation at room temperature, wells were washed with PBS/Tween 20, and 3,3′ 5,5′-tetramethylbenzidine

(Moss, Inc., Pasadena, Mo.) substrate was added to each well. Plates were incubated for 30 minutes, and the reaction was read using a MX80 plate reader (Dynatech, Chantilly, Va.) at an absorbance of 630 nm. The reaction was terminated by the addition of 0.25 HCl, which also increases the sensitivity and the final color change, was read at 450 nm. Serum IL-6 levels were determined from a standard curve generated with the recombinant mouse IL-6 after log/log quadratic linear regression analysis using Revelation 2.0 software (Dynatech).

Cell Culture. Peritoneal (J774A.1) and alveolar (MH-S) macrophages were obtained from the American Type Culture Collection (Manassas, Va.) and maintained in vented cell culture flasks (75 cm^2; Fisher Scientific) with either Dulbecco's modified Eagle's (DMEM; Hyclone Laboratories) supplemented with fetal bovine serum (FBS; 10%) or RPMI 1640 (Hyclone Laboratories) supplemented with FBS (10%), sodium pyruvate (1 mM), HEPES (10 mM; Mediatech Cellgro, Herndon, Va.) and 2-mercaptoethanol (50 μM), respectively. Cell cultures were incubated at 37°C with 5% CO_2 and passaged no more than 15-20 times or until cell morphology was lost.

Macrophage uptake assay. Macrophages (ca. 10^5 cells) were placed in six-well, flat bottom plates with low evaporation lids (Becton Dickinson and Co., Franklin Lakes, N.J.) and incubated overnight at 37°C with 5% CO_2. Macrophage monolayers from overnight incubations were inoculated with approximately 10^7 cfu of *S. aureus* that had been opsonized for 30 minutes with human AB serum (Atlanta Biologicals, Norcross, Ga.) and incubated for 0 and two hours. Monolayers were washed with either DMEM or RPMI,

depending upon cell line, and treated with gentamicin (100 μg/ml; Sigma Chemical Co.) for 30 minutes to kill extracellular bacteria. After gentamicin removal, monolayers were washed twice with sterile PBS. The appropriate cell culture medium was added, and Triton X-100 (0.01%) was added to lyse the macrophage cells. Cell lysates were collected and briefly sonicated prior to serial dilution and plating on TSA agar plates. Plates were incubated overnight (15-18 h) at 37°C prior to colony enumeration.

Statistical analysis. Survival data were analyzed using Kaplan-Meir, Mantel-Cox, or Chi-squared test. Statistical significance was determined by analysis of variance followed by Fisher protected least significant difference multigroup comparison. All analyses were performed using StatView (SAS Instituted, Inc., Cary N.C.) and a *P* value of ≤ 0.05 was considered statistically significant.

Results

Severity of disease, after pulmonary infection of *S. aureus* is dependent upon a functional *agr* and *sar*. To determine *S. aureus* lethality in a mouse model of pneumonia, overnight cultures (15 to 18 h) of *S. aureus* RN6390 (parent strain) were harvested by centrifugation, washed and suspended in PBS, diluted at various concentrations, and intranasally inoculated into the lungs of age-matched, anesthetized mice (Fig. 1). An inoculum of 10^6-10^7 cfu did not cause death in mice, although animals inoculated with the higher dose (10^7 cfu) consistently demonstrated signs of illness (ruffled coat and lethargy) and lost significantly more weight than either mice given 10^6

cfu or PBS inoculum. In contrast, greater than 80% of mice inoculated with 10^8 cfu died within 24 hours, and mice inoculated with 10^9 cfu did not survive.

To determine the disease impact of the *agr* and *sar* loci, mice were intranasally inoculated with approximately 10^8 cfu of either the parent, the *agr* mutant, or the *sar* mutant strains. The mice were monitored for signs of disease and survival for five days after infection. Similar to above, less than 20% (4/24) of mice inoculated with the parent survived after 24 hours and by 48 hours the cumulative percent total of survivors was only 8% (Fig. 2). No additional deaths were observed through the remaining three days of monitoring. In contrast, all mice inoculated with the *agr* mutant survived throughout the five days of monitoring. As well, only 46% of mice had succumbed to the *sar* mutant by 48 hours.

To further assess the role of *agr* and *sar* in virulence, mice were inoculated with a sublethal dose (10^7 cfu) of either the parent, the *agr*, or *sar* mutant strains. There was a significant weight loss, regardless of strain, by 24 hours when compared to control mice administered PBS alone (Fig. 2). However, weight loss for mice inoculated with the *agr* mutant strain were significantly less than either RN6390 or the *sar* mutant strains.

To determine whether extrapulmonary dissemination of bacteria contributed to the deaths of mice given 10^8 cfu, groups of mice were intranasally inoculated with a lethal dose of 10^8 cfu, and blood samples and spleens were taken at 4 hours post inoculation. Spleens and blood samples were diluted and plated onto bacteriological media. No detectable levels of any of *S. aureus* strains examined were found in either the spleens or blood samples (data not shown).

***S. aureus* persistence in the lung is dependent upon *agr* and *sar*.** To determine whether the differences in lethality that exist between the parent and mutant strains were a function of bacterial persistence in the lungs, mice were intranasally inoculated with a sub-lethal dose (10^7 cfu) of *S. aureus* as previously determined. The lungs were harvested from mice euthanized at 4, 24 and 48 hours, post-inoculation. Lungs were macerated, diluted in PBS, and plated on bacteriological media. No significant difference in the number of viable cells recovered from the lungs was observed between the parent and either mutant strain at 4 hours post-inoculation. However, by 24 hours the number of viable cells recovered from the lungs of mice inoculated with the parent strain was about twice the number recovered from mice inoculated with either the *agr* or *sar* mutant strains (Fig. 3).

The severity of pulmonary inflammation is not dependent upon a functional *agr* or *sar*. Mice were inoculated with a sublethal dose (10^7 cfu) of parent or either mutant strain and euthanized 24 hours post inoculation. Lungs were removed, fixed, and prepared for light microscopy. Sections of the each lung lobe, as well as the trachea were examined and scored with respect to four major types of lung lesions. While significant differences were observed between lungs administered PBS alone and lungs inoculated with *S. aureus*, for all four types of indices, no significant differences were observed between parent and either mutant strain (Fig. 4). Infected lungs demonstrated a substantial infiltration of inflammatory cells around airways and vessels with alveolar consolidation regardless of the inoculating strain.

To further assess the severity of inflammatory disease, we monitored the levels of IL-6 in serum. In a previous study (17), we found that IL-6 levels in serum corresponded to the severity of inflammatory disease due to infection with another respiratory pathogen, *Mycoplasma pulmonis*. Groups of mice were inoculated with a sublethal dose (10^7 cfu) of parent or either mutant strain and blood samples were taken at four and 24 hours either retro-orbitally or by femoral laceration upon euthanasia, respectively. Serum IL-6 levels were assayed by ELISA and reported in Table 1. By four hours post-inoculation, infected mice exhibited a significant increase in serum IL-6 levels as well as incidence when compared to control animals. However, no significant differences were observed between parent and either mutant strain. By 24 hours post-inoculation, the serum IL-6 levels for infected animals had fallen to below significant levels. These results are consistent with the histopathological findings in that a significant level of inflammation occurs upon nasal-pulmonary inoculation of mice with *S. aureus* irrespective of a functional *agr* or *sar* operon.

The *S. aureus agr* and *sar* mutant strains are equally phagocytosed as the parent strain by in vitro macrophages. To further investigate the differences of *S. aureus* parent and mutants observed in lethality, we examined whether mutations in either the *agr* or *sar* locus affected the susceptibility of *S. aureus* to macrophage phagocytosis. Overnight monolayers of either murine alveolar (MH-S) or peritoneal (J774A.1) macrophages were inoculated with approximately 10^7 cfu of either the parent or mutant strains of *S. aureus*. Monolayers at 0 and 2 hours post-inoculation were treated with or without gentamicin for an additional 30 minutes. Monolayers were then lysed, and

briefly sonicated prior to serial dilution and plating on TSA agar plates. Results from these studies suggest that both cell lines were able to internalize *S. aureus*, although the peritoneal macrophages appear to be more efficient than the alveolar macrophages (Fig. 5). No significant differences in phagocytosis were observed between parent and either mutant strain regardless of cell line. This would suggest that neither *agr* nor *sar* affected any significant level of resistance to phagocytosis (Fig. 5). In addition, while both cell lines appear to be efficient in phagocytosis, no killing of ingested *S. aureus* was observed by two hours.

Lethality of *S. aureus* is not due to cell-wall components. To determine if there were any effects due to staphylococcal cell wall components, mice were intranasally inoculated with UV-killed parent at a lethal concentration of 10^8 cfu (data not shown). While these mice exhibited signs of illness including significant weight loss (data) as compared to PBS controls, all six mice survived. No growth was observed on TSA plates with undiluted portions of either the UV-killed parent inoculum or lung homogenates from mice 24 hours post inoculation with UV-killed parent (data not shown).

Lethality is dependent upon *agr* and *sar* regulated exoproteins found in spent media. The *agr* and *sar* loci control the expression of numerous *S. aureus* secreted proteins, many of which are involved with the disease process (32). Therefore, we decided to determine if spent media alone when administered intranasally into the lungs of mice could cause death. Various concentrations (1X, 5X, and 10X) of spent media isolated from overnight cultures (15-18 h) of parent and the mutant strains were each intranasally administered to mice. As a control, groups of mice were administered sterile 1X, 5X, and

10X TSB. Mice were monitored for signs of illness and death over a five-day period, and the cumulative percent mortality after three days is presented in Table 2. Results from these studies clearly indicate that lethality is most likely due to one or more components found in the spent media of the *S. aureus* parent strain. Regardless of concentration, spent media isolated from the parent strain and administered to mice resulted in death of all mice. The time required to achieve 100% mortality with RN6390 was concentration dependent (data not shown). In contrast, there was no significant decrease in survival of mice inoculated with spent media isolated from either the *agr* or *sar* mutants and mice administered TSB alone did not exhibit any signs of illness.

To further investigate if the lethal components in spent media were proteinaceous, we decided to treat the parent spent media with proteinase K and heat. After treatment with heat or proteinase K, spent media were intranasally inoculated into BALB/c mice as done previously. Mice were monitored for weight loss, illness and death over a five day period. Treatment with proteinase K and heat completely ablated the lethal effects of the spent media (Table 3). These results indicate that an exoprotein contributes to the lethality in our model.

Discussion

Hospital-acquired staphylococcal pneumonia is a serious threat to human health particularly among the elderly where pneumonia is the sixth leading cause of death in the United States (74). However, with the exception of studies, which implicate α-toxin (25) and coagulase (62) in staphylococcal pneumonia, little is known about the factors that are responsible for this life-threatening disease.

Recently, an investigation of staphylococcal pneumonia was undertaken using a neonatal (10-14 day old) mouse model of pneumonia where mice were intranasally inoculated with *S. aureus* RN6390 and its isogenic mutants defected in expression of the global regulators, *agr* and *sar* (61). In this study, all mice inoculated with *S. aureus* RN6390 were pneumonic and bacteremic while a significant number of mice inoculated with either the *agr* or *sar* mutant strains were not (61). In addition, no deaths occurred, 18-hours post-inoculation, with mice inoculated with 10^8 cfu of either the *agr* or *sar* mutant strain, while a 30% mortality was observed with mice inoculated with the parent strain, RN6390(61). Microscopic analysis of lung tissue from mice inoculated with either RN6390 or the mutant strains showed a significant level of inflammation (edema, cellular infiltration and consolidation) as compared to mice administered PBS alone but no significant differences were observed between RN6390 and either mutant strain(61).

Results obtained with our study indicate that mortality in adult mice intranasally inoculated with *S. aureus* RN6390 is dependent upon an intact *agr* locus and to a lesser extent upon *sar*. All mice inoculated with the *agr* mutant survived while mice inoculated with the *sar* mutant demonstrated a 50% survival rate after two days post-inoculation. Interestingly, lethality doesn't appear to be due to induction of an exaggerated proinflammatory response by host cells in the lung. Microscopic analysis of lung tissue isolated from mice inoculated with either RN6390 or either mutant strain demonstrated an equal amount of inflammation, regardless of whether the *sar* or *agr* loci were intact. These results are similar to those observed by Heyer et al. (61) in the neonatal mouse model of pneumonia. In our studies, both the levels of the proinflammatory cytokine, IL-

6 and the incident in mice were significantly elevated as compared to the PBS controls but were essentially the same regardless of the inoculating strain. Given that peptidoglycan and lipoteichoic acid are potent inducers of TNF-α and IL-10 as well as IL-6 (75), it is not surprising that IL-6 levels were elevated. What these data collectively suggest is that the lethality associated with an intranasal inoculation of *S. aureus* in mice does not appear to rely on an elevated host cell response in the lung.

Results from our study indicate that clearance of *S. aureus* in the mouse lung is extremely efficient; most likely attributed to the phagocytic activity of resident alveolar macrophages and recruited polymorphonuclear leukocytes. An intranasal inoculum of 10^7 cfu resulted in a drastic reduction in the total number of *S. aureus* cells recovered from lungs by 24 hours (Fig. 3) and by 48 hours no viable cells were recovered (data not shown). A significant difference in the total number of cells recovered from the lung was observed between RN6390 and either mutant strain albeit just a half log difference. Whether this small but significant difference in cells recovered from the lung between RN6390 and either mutant strain represents the contributing factor for lethality in mice is debatable. However, because lethality is dependent upon a secreted factor(s) found in the medium, it is possible differences in the numbers of actively metabolizing cells may contribute to the levels of the secreted factor(s) that accumulate, promote disease, and ultimately lead to death. An inadequate number of cells or low level expression of the secreted factor(s) allow clearance by the host phagocytic cells before a sufficient amount of factor(s) are produced to result in death.

Results obtained with our study also suggest that lethality is not due to dissemination of *S. aureus*. No detectable levels of RN6390 or either mutant strain at a lethal intranasal inoculum of 10^8 cfu were found in blood and spleen samples taken at 4 hours post-inoculation. This is in contrast to what Heyer et al.(61) observed in the neonatal mouse model of pneumonia. Using an intranasal inoculum of 10^8 cfu and spleen samples taken at 18 hours post-inoculation, bacteremia was observed in all mice inoculated with *S. aureus* RN6390 while only 41 and 16% of mice inoculated with the *agr* or *sar* mutant strains exhibited bacteremia, respectively (61). In contrast to neonatal infections, intranasal inoculation of adult mice with *S. aureus* did not result in disseminated infection. It is likely that the difference observed between our study and that of Heyer et al (61) is a function of the age of mice. In support, a study conducted with Swiss mice demonstrated that pulmonary clearance of aerosolized *Pseudomonas aeruginosa* was age dependent and shown to be due to diminished PMN migration to the airways in mice 20 days or less in age (76).

Results obtained with the *sar* mutant were somewhat surprising since the primary product of the *sar* locus, SarA, is known to positively regulate expression of the RNAII and RNAIII transcripts of the *agr* operon (72, 38, 77). In fact, in a *sar* mutant of *S. aureus* RN6390, RNAII and RNAIII message levels are reported to be significantly reduced (38). Because *S. aureus sar* mutants have reduced levels of the *agr* effector molecule RNAIII, it is conceivable that the intermediate level of virulence displayed by the *sar* mutant represents low level expression of *agr*-dependent virulence factors as a result of a nonfunctional *sar* locus. However, the *sar* locus is also known to modulate the

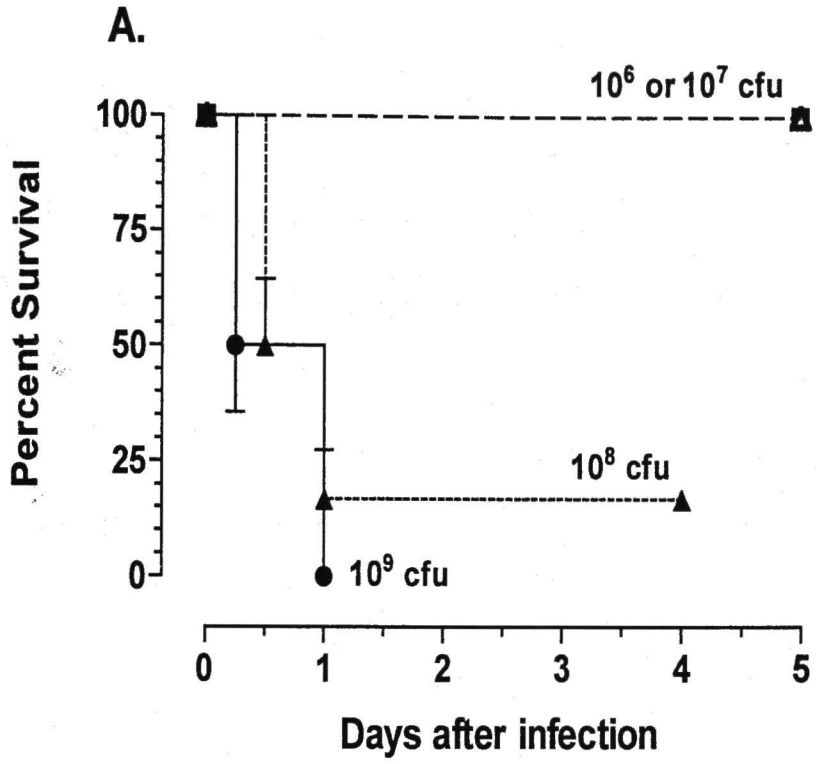
expression of several virulence factors in an *agr*-independent manner (78, 79, 72, 77, 80,81). For example, at least four extracellular proteases (79, 81) and the collagen binding protein (78) are up-regulated in *sar* mutants of *S. aureus*. Therefore, it is quite possible that the intermediate level of virulence displayed by the *sar* mutant is due to derepression of certain virulence factors which partially compensate for less than optimal levels of certain *agr*-dependent factors whose expression is reduced due to the loss of the activating affect of SarA on *agr*. Whether the intermediate level of virulence displayed by the *sar* mutant in our study is the result of the compensatory action of derepressed virulence factors or is the result of reduced expression of *agr*-regulated factors as a result of a mutation in the *sar* locus is not known at present.

Previous studies comparing *S. aureus agr* and *sar* mutants in the RN6390 parental background indicate a significant reduction in disease when both regulons were rendered dysfunctional. For example, Cheung et al. (45) demonstrated that a low inoculum (10^3 and 10^4 cfu) of either the *agr* or *sar* mutant was significantly reduced in causing endocarditis in rabbits as compared to RN6390. Interestingly, induction of endocarditis was consistently less with the *sar* mutant than with the *agr* mutant, even though no statistical significant difference was observed between mutants (45). More importantly, the double (*agr sar*) mutant displayed a significant reduction in its ability to induce endocarditis as compared to either single mutant or the parent strain and regardless of the inoculum size (45). These data would suggest that factors critical to the formation of endocarditis in rabbits are independently regulated by *agr* and *sar* and inactivation of both regulons is necessary for maximal reduction of endocarditis in rabbits. In another

comparative study, Booth et al. (69) determined the ability of *S. aureus* RN6390 and its isogenic *agr* and *sar* mutants to cause endophthalmitis in rabbits. In this case, while the *agr* mutant was significantly impaired in its ability to cause endophthalmitis when compared to RN6390, the *sar* mutant was not (69). Likewise, as in the study by Cheung et al. (45), the double (*agr sar*) mutant was significantly reduced in its ability to cause endophthalmitis more so than either RN6390, or the single mutant strains (69). Again, these data indicate that staphylococcal disease, in this case, endophthalmitis in rabbits, most likely requires virulence factors regulated by both *agr* and *sar*, independently of one another. In contrast, our studies would suggest that the complete loss of lethality in a mouse model of pneumonia appears to require only that the *agr* regulon of *S. aureus* be disrupted. Furthermore, the factor or factors critical for lethality are found in the spent media of metabolically active cells of *S. aureus*. Taken together, these comparative studies indicate that the involvement of one or more virulence gene regulators is most likely dependent upon the particular site of infection.

In summary, results obtained from this study clearly indicate that lethality in a mouse model of pneumonia is dependent upon *S. aureus agr*-regulated factors that are secreted into the environment. Furthermore, these factors were shown to be protein secreted into the environment. Efforts are now focused on isolating and identifying factors in spent media that contribute to the lethality observed in the mouse model of pneumonia.

Figure 1. Percent survival (A) and weight loss (B) for mice inoculated with various concentrations of *S. aureus* RN6390. Each value represents the mean of two independent determinations with six mice per group. Asterisks denote significant ($P \leq 0.05$) difference in weight loss between mice inoculated with 10^7 cfu and either 10^6 cfu or PBS alone.


A.
10^6 or 10^7 cfu
100
75
50
25
0
Percent Survival
10^8 cfu
10^9 cfu
0
1
2
3
4
5
Days after infection

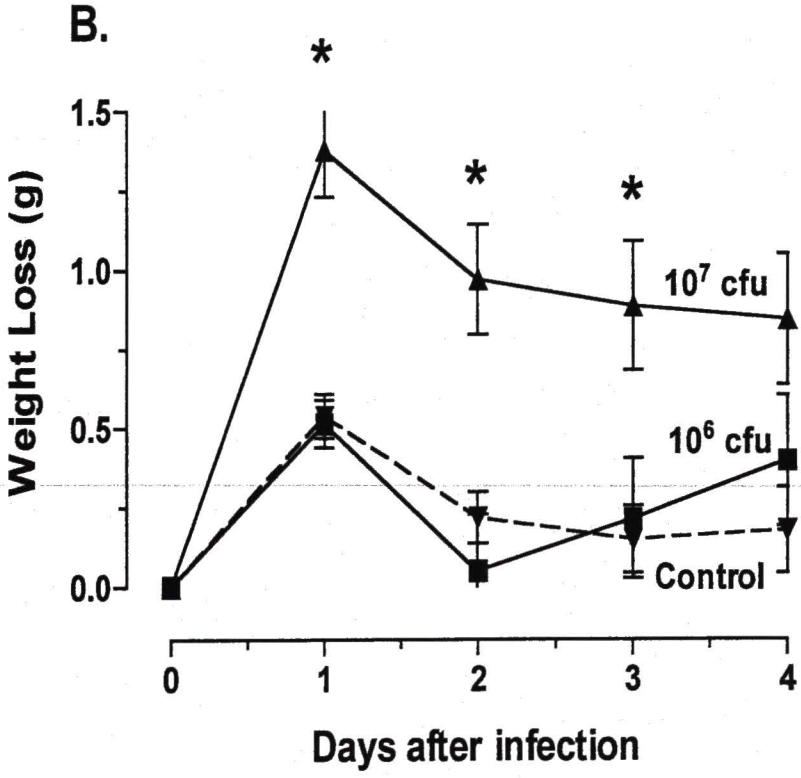
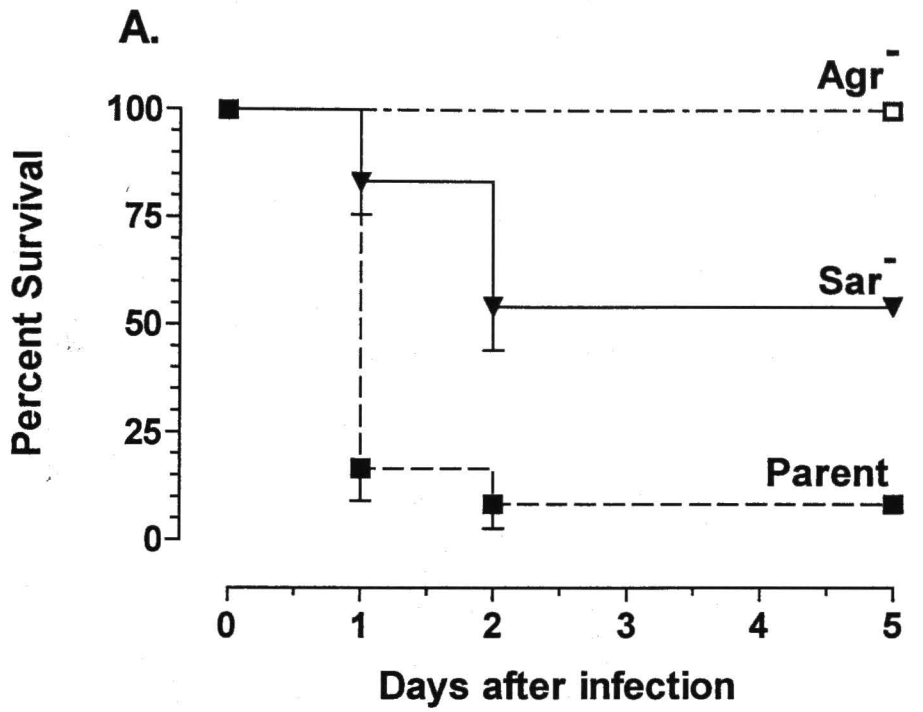

B.
*
*
*
1.5
1.0
0.5
0.0
Weight Loss (g)
10^7 cfu
10^6 cfu
Control
0
1
2
3
4
Days after infection

Figure 2. Percent survival (A) and weight loss (B) for mice inoculated with *S. aureus* RN6390, the *sar* mutant, and the *agr* mutant at 10^8 cfu and 10^7 cfu, respectively. Each value represents the mean of two independent determinations with twelve mice per group. Asterisks denote significant ($P \leq 0.05$) difference in weight loss between mice inoculated with *S. aureus* and PBS alone.


A.
Agr-
Sar-
Parent
100
75
50
25
0
Percent Survival
0
1
2
3
4
5
Days after infection

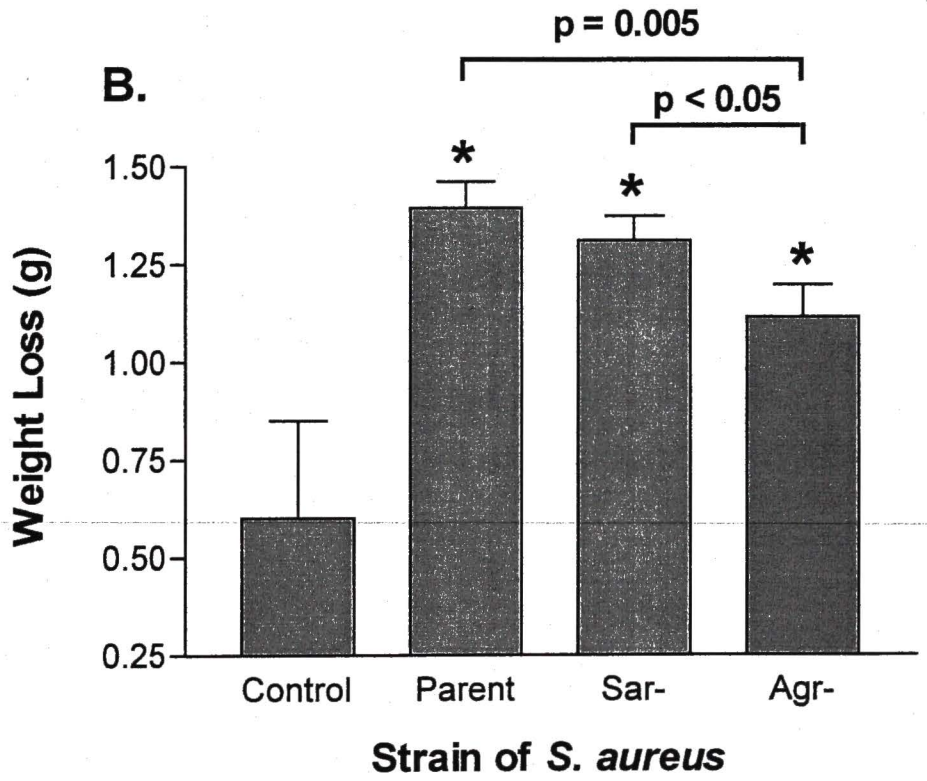

p = 0.005
p < 0.05
B.
*
*
*
1.50
1.25
1.00
0.75
0.50
0.25
Weight Loss (g)
Control
Parent
Sar-
Agr-
Strain of S. aureus

Figure 3. Number (cfu) of *S. aureus* RN6390, *sar*, and *agr* mutant strains recovered from lungs of mice inoculated with 10^7 cfu and harvested 24 hours post-inoculation. Each value represents the mean of three independent determinations with three mice per group. Asterisks denote significant ($P \leq 0.05$) difference between cfu recovered from lungs inoculated with *S. aureus* RN6390 and either mutant strain.

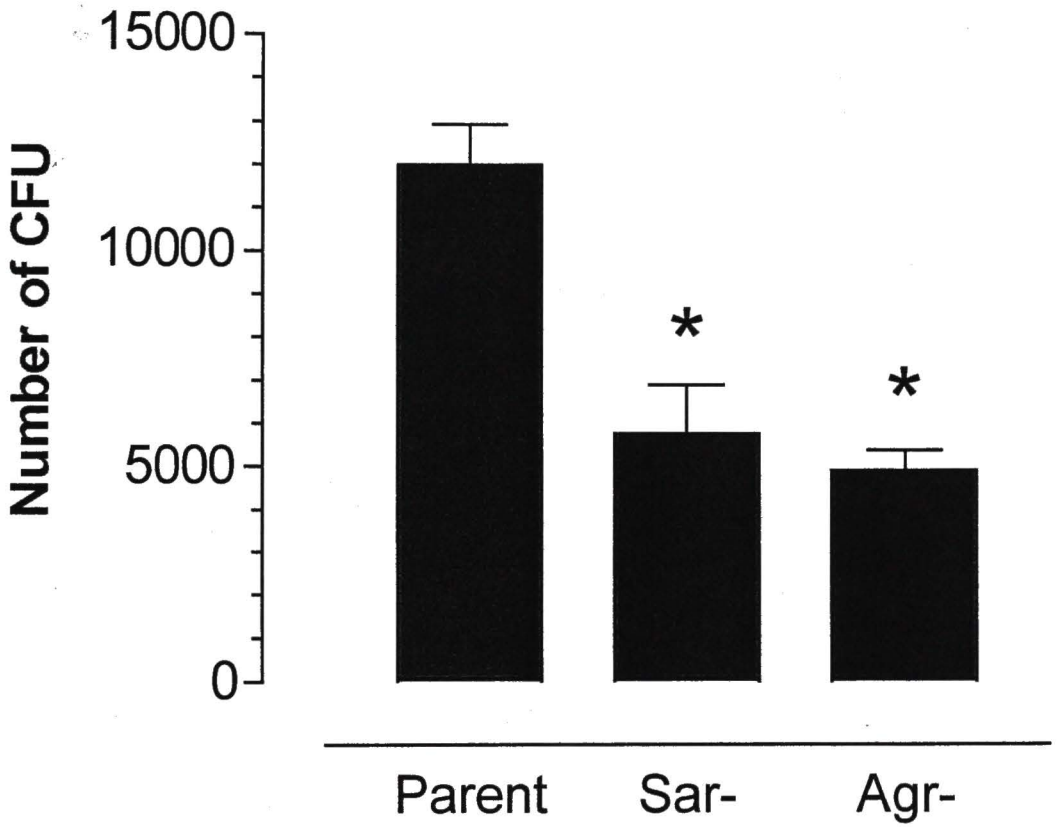

15000
10000
5000
0
Number of CFU
*
*
Parent
Sar-
Agr-

Figure 4. Lesion index for lungs isolated from mice inoculated with *S. aureus* RN6390, the *sar* mutant, and the *agr* mutant at 10^7 cfu and harvested 24 hours post-inoculation. Each value represents the mean of three mice per group. Asterisks denote significant ($P \leq 0.05$) difference in lesion index between lungs inoculated with *S. aureus* and PBS alone.

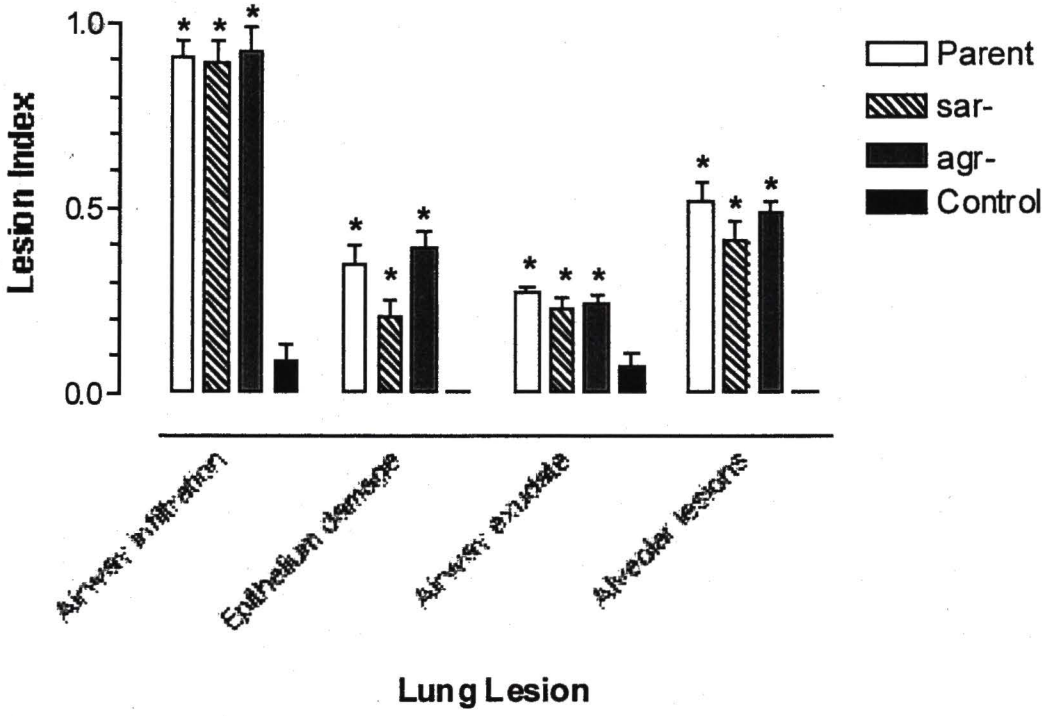

Lesion Index
1.0
0.5
0.0
Parent
sar-
agr-
Control
Airway inflammation
Epithelium damage
Airway exudate
Alveolar lesions
Lung Lesion

TABLE 1. Serum IL-6 levels and incidence in mice infected with *S. aureus*.

Strain	IL-6 levels and incidence at			
	4 hours[a]		24 hours	
	IL-6[b]	Incidence[c]	IL-6	Incidence
RN6390	209 ± 41	6/6	9 ± 6	2/6
sar (ALC488)	100 ± 36	5/6	10 ± 7	2/6
agr (RN6911)	136 ± 63	6/6	50 ± 40	3/6
PBS control	15 ± 15[d]	1/6[d]	0 ± 0	0/6[e]

[a] Time post-inoculation with *S. aureus* at 10^7 cfu.

[b] Mean IL-6 levels (pg/ml) ± SE.

[c] Number of mice with detectable IL-6/total number of mice.

[d] Four hours post-inoculation, IL-6 levels and incidence of control mice were significantly lower ($P \leq 0.05$) than groups infected.

[e] Twenty-four hours post-inoculation, incidence of IL-6 detection was significantly lower ($P \leq 0.05$) than mice inoculated with the *agr* mutant.

Figure 5. Number (cfu) of *S. aureus* RN6390, *sar*, and *agr* mutant strains recovered after 0 and 2 hour exposures to alveolar (MHS) and peritoneal (J774) macrophages with and without treatment with gentamicin. Each value represents the mean of four independent determinations. Asterisks denote significant ($P \leq 0.05$) difference between cells with and without gentamicin treatment.

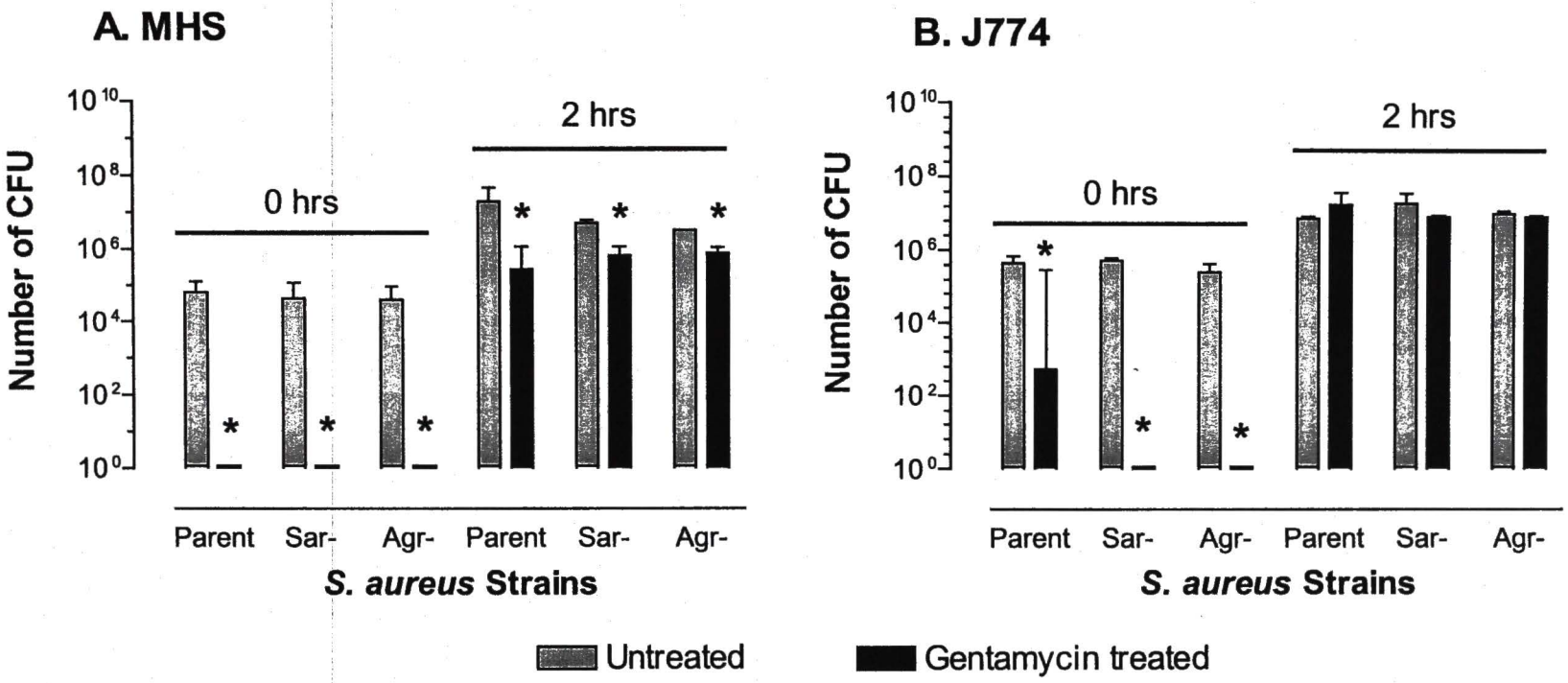

A. MHS
B. J774
Number of CFU
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
0 hrs
2 hrs
*
Parent
Sar-
Agr-
Parent
Sar-
Agr-
S. aureus Strains
Untreated
Gentamycin treated

TABLE 2. Percent mortality and weight loss due to intranasal inoculation with staphylococcal spent media.

Inoculum type[a]	Mortality		
	spent media[b]		
	1X[d]	5X	10X
RN6390	56 (5/9)*	100 (9/9)*	100 (9/9)*
sar (ALC 488)	0 (0/6)	0 (0/6)	17 (1/6)
agr (RN6911)	0 (0/6)	0 (0/6)	0 (0/6)
TSB control	0 (0/9)	0 (0/9)	0 (0/9)

[a] Mice were inoculated with either spent media from overnight (15-18 h) cultures of RN6390, *sar*, or *agr* mutant strains.

[b] Percentage (numbers of deaths/number inoculated) of mice that died within 3 days post-inoculation with various concentrations of spent media or TSB.

[c] Concentration of spent media or TSB relative to the original culture volume (e.g., 1X is equivalent to the original culture volume while 5X and 10X are 5 and 10 times more concentrated than the original culture volume).

*Significantly higher mortality ($P \leq 0.05$) than other groups.

CHAPTER III

ALPHA-TOXIN IS A KEY VIRULENCE FACTOR IN AN ADULT MOUSE MODEL OF PNEUMONIA, AND A POTENTIAL TARGET FOR IMMUNOTHERAPY

Introduction

Soon after *Staphylococcus aureus* was discovered in 1878 (20), isolates were observed to produce soluble substances that evoked an inflammatory response in animal models (20, 82). It was later discovered that alpha-toxin was among these factors. Alpha-toxin, or alpha-hemolysin, is an exotoxin secreted by *S. aureus* with hemolytic, cytotoxic, dermonecrotic and lethal properties (20). Alpha-toxin has the molecular weight of 34 kDa, with an isoelectric point of 8.5 to 8.6 (83). It was demonstrated that *S. aureus* alpha-toxin causes pore formation, which damages the membranes of host cells (19) and can result in cell death (21).

Due to alpha-toxin's properties of causing damage to cells, numerous studies established a role for alpha-toxin in relationship to *S. aureus'* virulence in disease. For example, Bayer, et al (22), studied the in vivo role of alpha-toxin in the induction and progression of infective endocarditis. In these studies, they revealed that staphylococcal strains which were deficient in a functional alpha-toxin secretion or were hypersecreting strains exhibited a reduced virulence. Alpha-toxin deficient strains had a reduced ability to induce infection and persist, but were not completely avirulent (22), suggesting that

alpha-toxin makes a significant contribution to the virulence, but is not the only contributing factor to disease. Alpha-toxin deficient mutants were shown to have similar effects in a brain abscess model (24) and a rat pneumonia model (25). Kielian, et al, (24) demonstrated that staphylococci strains that lack alpha-toxin exhibit reduced virulence in vivo. Alpha-toxin deficient mutants could not establish well defined brain abscesses, had an impaired ability to replicate and were cleared quickly from the area by host immune cells. However, the alpha-toxin deficient strain was not completely avirulent, again suggesting the participation of another factor(s) in infection. In a rat pneumonia model, it was demonstrated that an alpha-toxin deficient *S. aureus* strain had a reduced ability to cause damage to lung epithelial cells (25). This study not only supports that alpha-toxin has a role in damaging the air-blood barrier, but also suggests other factors are involved in the disease process. Thus, alpha-toxin is a significant factor among several that contribute to staphylococcal diseases.

We believe that staphylococcal exotoxins would be effective targets for immunotherapy in staphylococcal disease. Most recent attempts to design immune therapies for *S. aureus* have been to utilize live and killed bacteria and *S. aureus* cell wall and toxoids (84). Numerous studies have demonstrated the value of Staphylococcal virulence factors as possible immune therapy targets (85, 86, 87, 88, 89, 90, 91, 92) and (93). For example, Joseffson, et al (89), demonstrated that the use of recombinant clumping factor A, a surface protein, provided protection against *S. aureus* arthritis and sepsis induced death. As well, Nillson et al (88) demonstrated that immunization with recombinant staphylococcal enterotoxin A (rSEA) devoid of superantigenic properties

provided protection against staphylococcal sepsis. Two studies utilized alpha-toxin as a specific target (93, 91) for immune therapy. Menzies and Kernodle (93), demonstrated that anti-serum raised against a non-toxic alpha-toxin mutant provided protection in a murine model. As well, Hume, et al (91) demonstrate that rabbits immunized with alpha-toxin toxoid are protected from damage to the cornea associated with staphylococcal keratitis. These studies further suggest that virulence factors produced by *S. aureus* could serve as potential immune target.

In support, our study in chapter 2 demonstrates that a functional *agr* and *sar* regulator are needed to cause severe pneumonia in mice. This study as well demonstrates that some secreted factor(s) are contributing to the lethality in disease. This was demonstrated by the fact that spent media from *S. aureus* RN6390 cultures killed mice when intranasally inoculated, while spent media from an *agr* or *sar* mutant did not. We show that when heat or proteinase K treated, spent media from *S. aureus* RN6390 does not cause death. These data suggest that a proteinaceous substance contributes to the lethality in our pneumonia model.

As a way to establish what virulence factors may be contributing to this lethality, we decided to examine the role of alpha-toxin in our mouse model of pneumonia. First, an alpha-toxin (*hla*) deficient mutant was generated via transduction into *S. aureus* RN6390, and subsequently used in this study. We demonstrate that a *hla* deficient mutant has a reduced virulence in this model, and antiserum against alpha-toxin protects mice from the lethal effects of *S. aureus* RN6390. However, we demonstrate that although alpha-toxin contributes to the lethality, other factors play a role in the ability to cause disease.

Materials and Methods

Bacteria strains and growth conditions. *S. aureus* RN6390 (35), was kindly provided by Mark Smeltzer (University of Arkansas for Medical Science, Little Rock, Ark.) and *S. aureus* 8325-4 provided by John Iandolo (University of Oklahoma Health Science Center, Oklahoma City, O.K.) were maintained on tryptic soy agar (TSA; DIFCO, Sparks, MD). *S. aureus* DU1090 (*hla* deficient in 8325-4)(94) provided by John Iandolo (University of Oklahoma Health Science Center, Oklahoma City, O.K.) and *S. aureus* NTH 373 (*hla* deficient in RN6390) a strain generated in this study via transduction were maintained on TSA containing 5 μg/ml of erythromycin (Sigma). Strains were routinely grown overnight (15-18 h) in flasks containing tryptic soy broth (TSB; DIFCO) without antibiotic. Flasks were incubated at 37°C with a flask to volume ratio of 2.5 and rotary aeration (180 rpm).

Transduction of *Hla* mutation from *S. aureus* 8325-4 (DU1090) into *S. aureus* RN6390. Transduction of the *hla* mutation from *S. aureus* 8325-4 (DU1090) to RN6390 was mediated by the bacteriophage ϕ11 as described by McNamara et al (95). Transduction was performed using the transducing lysate of ϕ11 containing *hla*::erm at a multiplicity of 1:1. The transducing lysate was added to TSB containing $CaCl_2$ (5mM) and 10^8cfu/ml of RN6390. After a five-minute incubation at room temperature, cells were incubated at 37°C for 20 minutes at high aeration (250 rpm) to carry out transduction. Ice-cold sodium citrate (20mM) was added to chelate $CaCl_2$ and stop transduction. Cells were pelleted by centrifugation, resuspended in ice-cold sodium

citrate (20 mM) and plated onto TSA containing sodium citrate (0.5 mg/ml) and erythromycin (5 μg/ml). Plates were incubated at 37°C for up to 48 hours to allow for transductants to grow. Erythromycin resistant transductants were subcultured to selective media, and then further analyzed for hemolytic activity, subjected to southern and western analysis. Transductants containing the *hla*::erm mutation were procured for further analysis.

Southern Analysis

Chromosomal DNA was isolated from *S. aureus* RN6390, 8325-4, DU1090 (*hla* deficient) and NTH 373 (*hla* deficient) via the CTAB (hexadecyltrimethyl ammonium bromide, Sigma) method. Briefly, overnight cultures of *S. aureus* were pelleted and bacterial cells were suspended in Tris-EDTA buffer (TE buffer, pH 8.0). Bacterial cells were treated with recombinant lysostaphin (5 μg/ml; Applen and Barrett LTD., England), RNase A (5μg/ml, Sigma) prior to cells lysis with sodium dodecyl sulfate (SDS (10%), Fisher) and proteinase K (10 μg/ml; Fisher). Cell lysates were then incubated with 10%CTAB in 0.7 M NaCl and then DNA was isolated by phenol/chloroform (1:1, Fisher) extractions. The DNA was ethanol precipitated and then subsequently suspended in TE buffer. DNA was digested with *Eco*RI (Promega Corp., Madison, Wi.), resolved by agarose gel electrophoresis, and transferred by passive diffusion onto neutral nylon membranes (MagnaGraph; Micron Separations Inc., Westborough, Mass.). Membranes were hybridized overnight (18-24 h) at 65°C with an alpha-toxin fragment labeled with digoxygenin-11-UTP (Roche Molecular Biochemicals, Indianapolis, Ind.) as described

by Smeltzer et al. (16) and Hart, et al. (96). Hybridizing probes were detected by autoradiography with alkaline phosphatase-conjugated, antidigoxygenin $F(ab')_2$ antibody fragments (Roche Molecular Biochemicals, Indianapolis, IN) and the chemiluminescent substrate CDP-*Star* (Roche Molecular Biochemicals).

SDS-PAGE and Western Analysis. Culture supernatants were isolated from overnight cultures, filter sterilized and concentrated using a YM-3 centricon (Millipore, Billerica, MA) overnight at 4°C. Protein concentrations were quantified using the BCA assay kit (Pierce, Rockford, Il.). Culture supernatants and purified alpha-toxin (Sigma) were separated by SDS-polyacrylamide gel (12%) electrophoresis according to Laemmli (97). Gels were stained with Rapid Coomassie stain to visualize protein bands. For Western analysis, proteins were transferred to nitrocellulose membranes (Invitrogen) by semi-dry electroblotting (Bio-Rad, Hercules, Ca.). Following blotting, membranes were blocked in 5% skim milk (DIFCO) in Tris Buffered Saline with 0.1% Tween 20 (Fisher) (TBS-T) overnight at 4°C. Membranes were then incubated for 1 hour at room temperature with whole antiserum anti-alpha-toxin (Sigma, 1:25,000) in TBS-T, followed by an incubation for 1 hour in anti-rabbit secondary (Amersham Pharmacia, England, 1:10,000). Blots were developed using ECL substrate (Amersham Pharmacia) and blots were scanned using Epson Scanner.

Mice. Specific pathogen-free, female BALB/c adult (ages 6 to 10 weeks) mice were obtained from Harlan Sprague Dawley (Indianapolis, Ind.), maintained in sterile microisolator cages, and given sterile food and water ad libitum. For all experimental

procedures, mice were anesthetized with an intramuscular injection of a mixture of ketamine and xylazine.

Strain and spent media preparation. *S. aureus* strains grown in TSB were pelleted by centrifugation (10,000 x *g* for 15 min), washed twice in an equal volume of ice-cold, phosphate-buffered saline (PBS), and suspended in PBS. Portions of each cell suspension were diluted in PBS to the appropriate optical density (550 nm) that corresponded to the desired cell concentration (colony forming units (cfu)/ml) as determined by a standard curve of cfu/ml as a function of optical density. To verify the concentration of each inoculum, portions were diluted in PBS, plated on TSA, and incubated overnight at 37°C prior to enumeration of bacteria.

Spent media were prepared from overnight cultures (15-18 h) by collecting the supernatants after centrifugation. Supernatants were filter-sterilized through 0.22 μm filters, and quick frozen on dry ice and ethanol, and stored overnight at -85°C. Frozen supernatants were lyophilized to dryness and stored at -85°C until used. Prior to each experiment, lyophilized supernatants were reconstituted in sterile, deionized, glass-distilled water to a concentration of 10X of original volume and stored on ice until used.

Virulence assay. A portion (25 μl) of either cells (10^8 cfu) or spent media was pipetted on the anterior nares of each anesthetized mouse and inoculation was accomplished by forced inhalation. In experiments using whole antiserum for alpha-toxin (Sigma) mice were inoculated with a portion (25 μl) of cells suspended in the

antibody and subsequently given another portion (25 μl) of antisera alone. Inoculated mice were monitored once a day for signs (lethargy and ruffled fur) of illness and death.

Statistical analysis. Survival data were analyzed using Kaplan-Meir, Mantel-Cox, or Chi-squared test. Statistical significance was determined by analysis of variance followed by Fisher protected least significant difference multigroup comparison. All analyses were performed using StatView (SAS Instituted, Inc., Cary N.C.) and a *P* value of ≤ 0.05 was considered statistically significant.

Results

Alpha-toxin contributes to the lethality in an adult mouse model of pneumonia. To assess if alpha-toxin made a contribution to the lethality of disease, we decided to intranasally challenge mice with the alpha-toxin (*hla)* deficient mutant and its parent strain. *S. aureus* RN6390, was transduced using ϕ11 transducing lysate containing the alpha-toxin mutation from DU1090, to contain a mutation in alpha-toxin. This mutation was confirmed via southern analysis (Figure 1) which shows a 1.4kb increase in the chromosome and by Western analysis (Figure 2) to confirm that the mutant was deficient in alpha-toxin production. When intranasally inoculated into mice, the *hla* deficient strain had a reduced virulence as compared to it parent strain RN6390. All mice inoculated with the *hla* deficient strain survived over a 5 day period, whereas all mice intranasally inoculated with the parent died within 24 hours (Table 1). However, mice inoculated with the *hla* mutant did become ill and had a significant weight loss as compared to mice inoculated with PBS (Figure 3).

To determine further if alpha-toxin was associated with the lethality associated with our model, we collected spent media from the parent and alpha-toxin deficient strain. Lyopholized spent media were then suspended in sterile water to 10X concentration (based on original spent media volume) in sterile glass distilled water. Spent media were then intranasally inoculated and mice were monitored for weight loss, illness and death over time. Our data demonstrate, as with live cells, that the *hla* deficient mutant has a reduced virulence as compared to the parent strain. All mice survived when inoculated with the *hla* deficient spent media as compared to the parent strain (Table 2,). However, mice given the *hla* deficient spent media did become ill as demonstrated by a significant weight loss over the infection period of 3 days (data not shown), suggesting that perhaps other factors contribute to disease in this model.

Whole antiserum to alpha-toxin provides protection to lethal dose *of S. aureus* RN6390. To further investigate the role of alpha-toxin in our model of pneumonia we decided to explore if antiserum against alpha-toxin could provide protection from a lethal dose of *S. aureus* RN6390, by reducing its virulence. When mice were intranasally inoculated with *S. aureus* RN6390 (10^8 cfu) suspended in antiserum to alpha-toxin mice survived longer, than those given *S. aureus* RN6390 alone or suspended in normal rabbit serum (Figure 4). A majority of the mice given antisera were protected from lethality; however, they still showed signs of illness as indicated by a significant weight loss (Figure 4). This again suggested that alpha-toxin is a major factor that contributes to pneumonia, but that it is not the only factor involved in the disease process.

Discussion

It has previously been demonstrated that alpha-toxin causes damage to host cells and in several animal models, *S. aureus* strains deficient in alpha-toxin have a reduced capacity to cause disease (22, 24,25). As well in our previous study, we demonstrate that a secreted factor in the spent media of *S. aureus* RN6390 contributes to the virulence in our mouse pneumonia model. In this study, we examined whether alpha-toxin had a major contribution to virulence in our pneumonia model and is a reasonable target for immunotherapy in staphylococcal disease.

We show that an *hla* deficient strain of *S. aureus* RN6390 has a reduced virulence when intranasally inoculated into mice (Table 1) and that when antisera to alpha-toxin is administered with a lethal dose of RN6390 mice are protected from the lethal effects of the disease (Table 3). This data suggests that alpha-toxin has a significant role in the virulence in our mouse model of pneumonia. However, mice had significant weight loss when inoculated with the *hla* deficient strain suggesting that it is not completely avirulent. This observation suggests that alpha-toxin is not the only virulence factor contributing to the disease. A recent study also demonstrates alpha-toxin's role in disease (24)). Keilan, et al (24), show that an *hla* deficient strain had a reduced capacity to form brain abscesses. The replication of this strain was severely attenuated at the site of infection, and proinflammatory cytokine expression was reduced. However, this strain was not completely avirulent, suggesting other factors participate in brain infection.

We also demonstrate in our study that antiserum for alpha-toxin provides protection from a lethal dose of *S. aureus* RN6390 when administered together. The lethal effects

of disease are diminished as compared to mice inoculated with *S. aureus* RN6390 alone or with non-immune rabbit serum (Figure 4). This data suggests that alpha-toxin could be a potential target for immune therapy against Staphylococcal diseases. Menzies and Kernodle (93), demonstrate in a murine model that antiserum to a nontoxic alpha-toxin mutant provides protection from disease. As well, Hume, et al (91), show reduced corneal damage in staphylococcal keratitis when rabbits were immunized with alpha-toxin toxoid. These studies further suggest that alpha-toxin could serve as a possible immune therapy target.

In summary, this study suggests the importance of alpha-toxin in our mouse model of pneumonia as well as being a possible target for immune therapy. Our findings may be attributed to the fact that alpha-toxin production is lacking in our mutant, allowing more phagocytic cells to survive in the lung and reduce bacterial loads. The *hla* deficient strain may also cause less of an immune response (cytokine expression), than *S. aureus* RN6390, preventing the lethal effects seen with the parent strain. As well, the addition of antiserum to alpha-toxin with a lethal dose of *S. aureus* RN6390 reduces virulence, by blocking alpha-toxin from interacting with the host cells. However, this study also suggests that other factors also contribute to staphylococcal pneumonia suggesting that the disease process is complex.

Figure 1. Southern Analysis of *S. aureus hla* deficient mutant. Chromosomal DNA was restriction digested with EcoRI, electrophoresed and transferred passively to a neutral nylon membrane. Southern blot was probed with alpha-toxin specific chemiluminescent probe. Lane 1, *S. aureus* RN6390, Lane 2, *S. aureus* RN6390 *hla* deficient, Lane 3, *S. aureus* 8325-4, and Lane 4, *S. aureus*8325-4 *hla* deficient (DU1090).

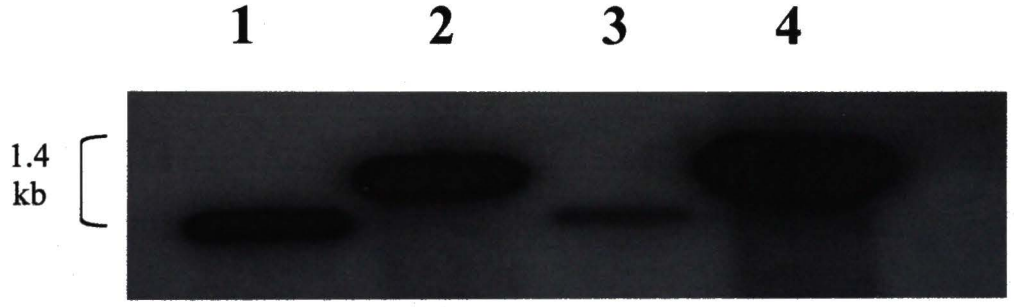


Figure 2. Western analysis of *S. aureus* RN6390 alpha-toxin (*hla)* deficient strain. A total of 7 μg of protein of culture supernatants was loaded onto an SDS-PAGE prior to western blotting. Western blots were scanned using the Epson CX2300 scanner (Epson). Lane 1, Purified alpha-toxin (50 ng), Lane 2, *S. aureus* RN6390, Lane 3, *S. aureus* RN6390 *hla* deficient, Lane 4, *S. aureus* DU1090 (*hla* deficient)

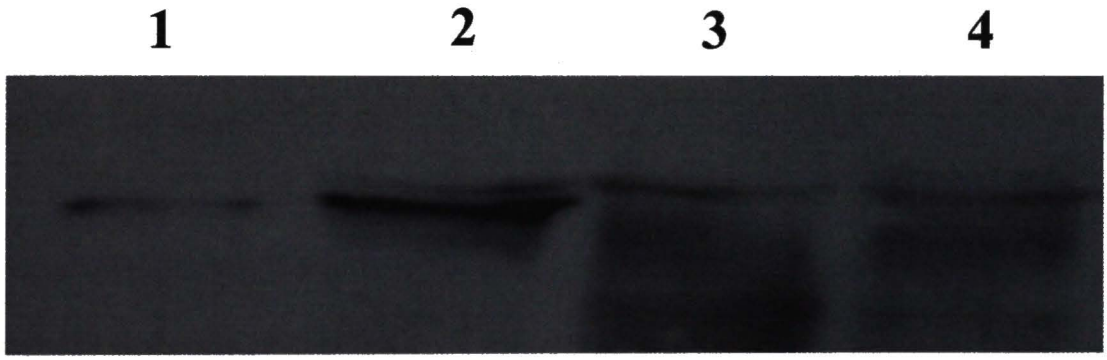


Table 1. Percent Mortality due to intranasal inoculation with *S. aureus* RN6390 *hla* deficient strain.

Inoculum	Mortality
RN6390	100 (12/12)*
RN6390 alpha-toxin (*hla*) deficient	0 (0/12)
PBS	0 (0/12)

* Significantly higher mortality ($P \leq 0.05$) than other groups.

Figure 3. Percent weight loss due to intranasal inoculation with *S. aureus* RN6390 *hla* deficient strain. Each value represents the mean of three independent determinations with three mice per group. Asterisks denote significant ($P \leq 0.05$) difference in weight loss between mice inoculated with 10^8cfu and PBS alone.

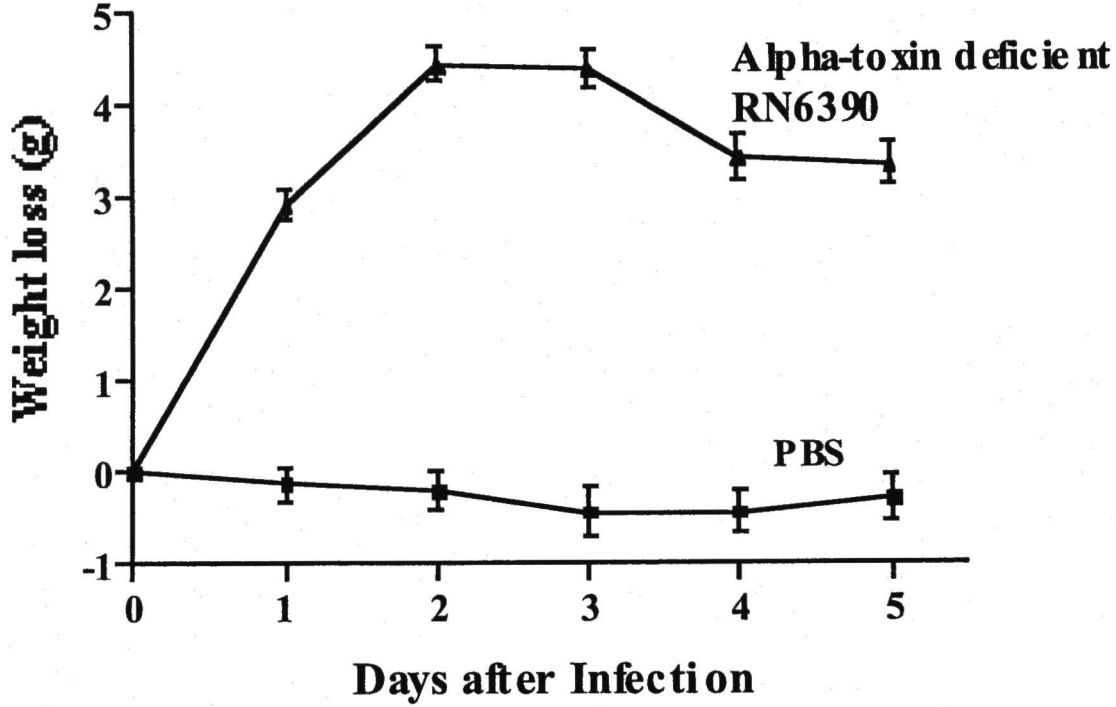

Weight loss (g)
5
4
3
2
1
0
-1
0
1
2
3
4
5
Days after Infection
Alpha-toxin deficient
RN6390
PBS

Table 2. Percent mortality associated with intranasal inoculation of *S. aureus* RN6390 *hla* deficient strain spent media.

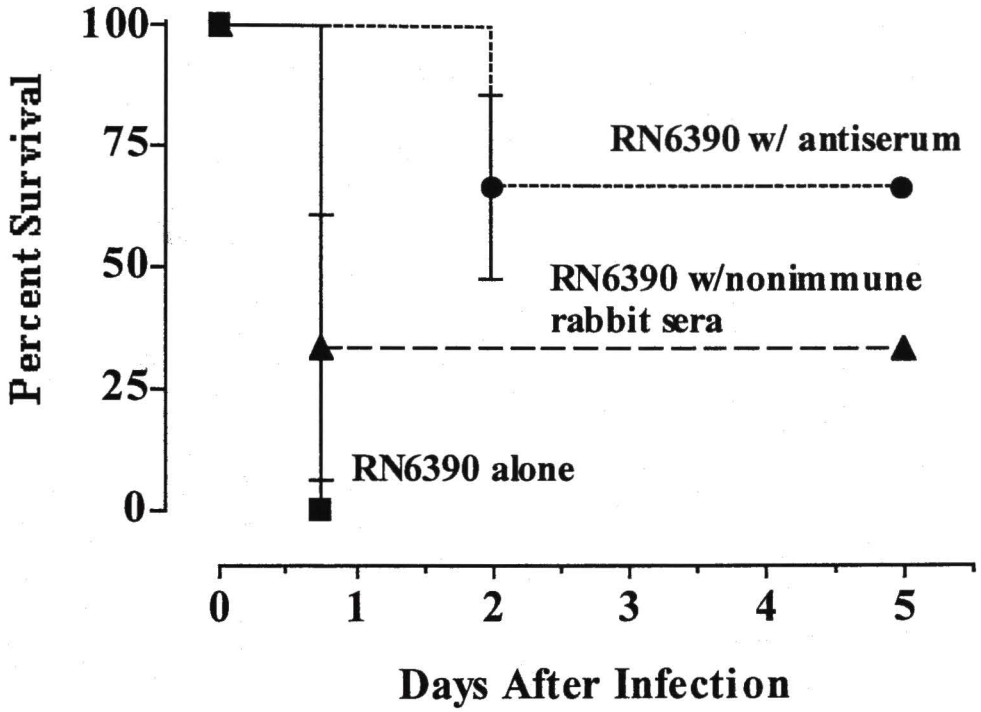
Strain	Mortality
S. aureus RN6390 10X[a]	100 (6/6)*
S. aureus RN6390 *hla* deficient 10X	0 (0/6)

[a] Represents spent media suspended to 10X of the original culture volume.

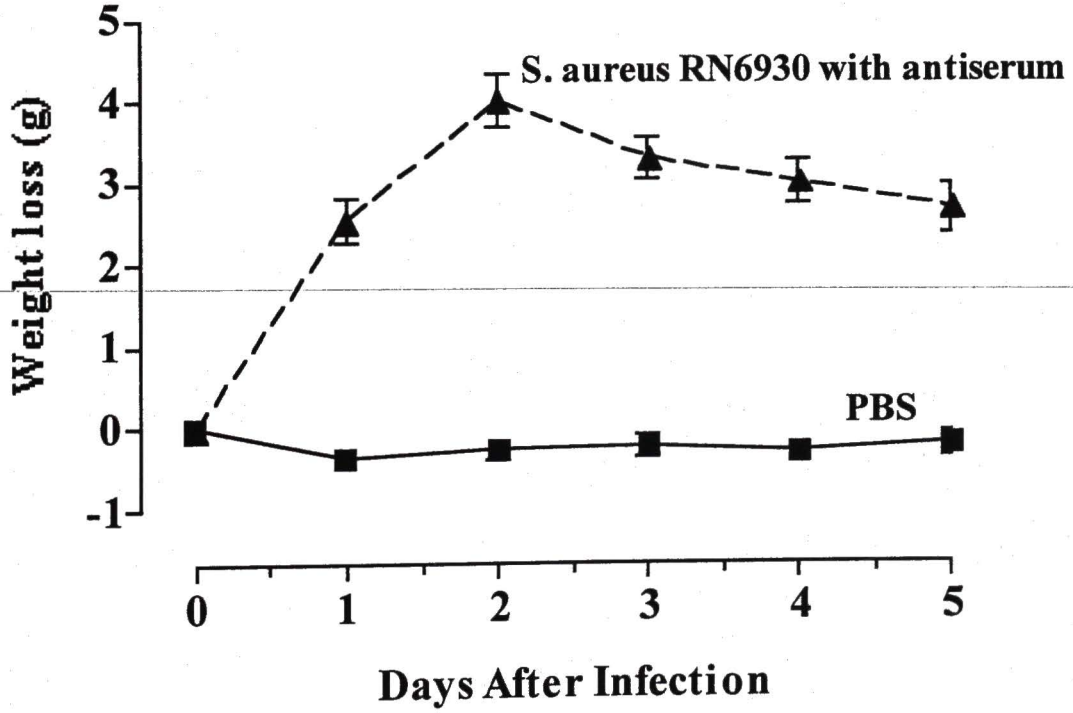
* Significantly higher mortality ($P \leq 0.05$) than other groups.

Figure 4. Percent survival (A) and weight loss (B) for mice inoculated with a lethal dose (10^8 cfu) of *S. aureus* RN6390 with and without antiserum to alpha-toxin. Each value represents the mean of two independent determinations with six mice per group. Asterisks denote significant ($P \leq 0.05$) difference in weight loss between mice inoculated *S. aureus* RN6390 with antiserum and PBS alone.

A.


100
75
50
25
0
Percent Survival
RN6390 w/ antiserum
RN6390 w/nonimmune rabbit sera
RN6390 alone
0
1
2
3
4
5
Days After Infection

B.


5
4
3
2
1
0
-1
Weight loss (g)
S. aureus RN6930 with antiserum
PBS
0
1
2
3
4
5
Days After Infection

CHAPTER IV

THE VARIABILITY OF *S. AUREUS* ISOLATES LETHALITY IN A MOUSE MODEL OF PNEUMONIA IS CORRELATED TO ALPHA-TOXIN PRODUCTION

Introduction

Our previous studies and other published studies indicate that alpha-toxin (*hla*) alone is not completely responsible for all of the effects associated with Staphylococcal lung disease (25). We demonstrated in chapter 3, that a *hla* deficient mutant was not lethal in our mouse model of pneumonia, but mice did become ill as noted by a significant weight loss over the course of infection. As well, when antiserum to alpha-toxin was administered with a lethal dose of *S. aureus* RN6390, mice were protected against its lethal effects, but not illness. McElroy, et al (25), also demonstrated a role for alpha-toxin in a rat model of pneumonia. Their study demonstrated that alpha-toxin caused damage to the air-blood barrier, but was not the only contributing factor to pneumonia in their model. As demonstrated previously, Menzies and Kernodle (93) found that antiserum raised against a non-toxic alpha-toxin mutant provided protection in a murine model of lethality. Hume, et al (91) demonstrated that rabbits immunized with alpha-toxin toxoid were protected from damage to the cornea associated with staphylococcal keratitis. However, in both of these studies, *S. aureus* strains were not rendered avirulent.

Previous studies indicated that *S. aureus* isolates can vary in the types and amounts of expression of virulence factors. For example, Coia et al (98), demonstrated that 50 to 58% of clinical isolates produced alpha-toxin and delta-toxin. In a study evaluating the cytotoxicity of clinical isolates against in vitro cells, Krut, et al (99) demonstrated that intracellular survival and the ability to cause death was correlated to the pathogenicity in mice. Seven of the twenty-three isolates evaluated were cytotoxic in cells and pathogenic in the mouse (99). This study also evaluated the role of alpha-toxin expression and it relationship to cytotoxicity. These studies suggest that alpha-toxin expression is not related to a strains ability to cause cell death.

The purposes of these studies were to assess the ability of *S. aureus* strains lethality in our mouse model of pneumonia, as well as whether their hemolytic abilities and alpha-toxin production correlated with lethality of infection. Our data demonstrate that there is variability in *S. aureus* strains lethality in our mouse model of pneumonia. These studies also suggest a correlation of alpha-toxin production to lethality. As well, we demonstrate that alpha-toxin is a key virulence factor in lethality of murine staphylococcal pneumonia, but it is not the only factor that contributes to disease outcome.

Materials and Methods

Bacteria strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Strains were maintained at - 85 °C as frozen stocks in brain heart infusion borth (DIFCO) plus 25 % glycerol. Working stocks were routinely cultured on tryptic soy agar (TSA, DIFCO,). Strains were routinely grown overnight (15-18 h) in flasks containing tryptic soy broth (TSB; DIFCO, Detroit, Mich.). Cultures were

incubated at 37°C with a flask to volume ratio of 2.5 and rotary aeration (180 rpm). For virulence assays, *S. aureus* strains grown in TSB were pelleted by centrifugation (10,000 x *g* for 15 min), washed twice in an equal volume of ice-cold, phosphate-buffered saline (PBS), and suspended in PBS. Portions of each cell suspension were diluted in PBS to the appropriate optical density (550 nm) that corresponded to the desired cell concentration (colony forming units (cfu)/ml) as determined by a standard curve of cfu/ml as a function of optical density. To verify the concentration of each inoculum, portions were diluted in PBS, plated on TSA, and incubated overnight at 37°C prior to enumeration of bacteria.

Hemolysis. Hemolytic profiles of strains were determined by plating *S. aureus* strains of TSA containing 5% sheep blood (Remel, Lenexa, KS). Zones of hemolysis (clearing) were measured and subjectively quantified into relative values represented by (-) no hemolytic activity and (+++++) high hemolytic activity.

SDS-PAGE and Western Analysis. Culture supernatants isolated from overnight cultures were filter sterilized through a 0.22 μm filter and concentrated overnight at 4°C using a YM-3 centricon (Millipore, Bedford, Ma.). Protein concentrations were determined using the BCA assay kit (Pierce, Rockford, IL). Culture supernatants and purified alpha-toxin (Sigma) were separated by SDS-polyacrylamide gel (12%) electrophoresis according to Laemmli (97). Gels were stained with Rapid Coomassie stain to visualize protein bands. For Western analysis, proteins were transferred to nitrocellulose membranes (Invitrogen,) by semi-dry electroblotting (Bio-Rad Laboratories, Richmond, Ca.). Following blotting, membranes were blocked overnight at

4°C in Tris Buffered Saline with 0.1% Tween 20 (Fisher, TBS-T) containing 5% skim milk (DIFCO). Membranes were then incubated for 1 hour at room temperature with whole antiserum raised against purified alpha-toxin (1:25,000, Sigma) in TBS-T, followed by an incubation for 1 hour with anti-rabbit secondary antibody (1:10,000,Amersham Pharmacia, England). Blots were developed using ECL substrate (Amersham Pharmacia) and alpha-toxin production was quantified by densitometry of bands using the AlphaImager 2000 (Alpha Innotech Corp., San Leandro, Calif.) imaging system.

Mice. Specific pathogen-free, female BALB/c adult (ages 6 to 10 weeks) mice were obtained from Harlan Sprague Dawley (Indianapolis, Ind.), maintained in sterile microisolator cages, and given sterile food and water ad libitum. For all experimental procedures, mice were anesthetized with an intramuscular injection of a mixture of ketamine and xylazine.

Virulence assay. A portion (25 µl) of each cell suspension approximately 10^8 cfu was pipetted on the anterior nares of each anesthetized mouse and inoculation was accomplished by forced inhalation. Inoculated mice were monitored every six hours for signs of illness (lethargy and ruffled fur) and death over time.

Statistical analysis. Survival data were analyzed using Kaplan-Meir, Mantel-Cox, or Chi-squared test. Statistical significance was determined by analysis of variance followed by Fisher protected least significant difference multigroup comparison. All analyses were performed using StatView (SAS Instituted, Inc., Cary N.C.) and GraphPad

Prism (GraphPad Software,Inc., San Deigo, C.A.) and a *P* value of ≤ 0.05 was considered statistically significant.

Results

Differences in lethality are strain dependent.

To determine if *S. aureus* isolates listed in table 1 had the same lethality in our mouse model of pneumonia mice, as *S. aureus* RN6390, overnight cultures of each strain were centrifuged, washed and resuspended in sterile PBS. Groups of mice were intranasally inoculated with individual strains at a concentration of 10^8 cfu (10^8 cfu represents a lethal dose for *S. aureus* RN6390). Mice were monitored for death and illness over time. We demonstrated that only three of the strains (NTH 48, NTH 50, and Newman) evaluated were moderately lethal in our model (Table 2), as compared to *S. aureus* RN6390, while the other strains were not. This data suggests that variability exist in *S. aureus* strains ability to be lethal in our model. However, all strains evaluated did cause illness (ruffled fur and lethargy) in the mice over the infection period. This data suggests that the *S. aureus* strains evaluated in this study have different virulence determinants in which they use to establish and maintain disease.

Alpha-toxin production and hemolytic activity are correlated to lethality.

To determine if the hemolytic ability of *S. aureus* was related to the disease process hemolytic activity and alpha-toxin production we analyzed each strain. To determine hemolytic activity, *S. aureus* strains used in this study, were plated onto sheep blood agar plates and incubated at 37°C overnight. After incubation, zones of hemolysis (lysis of blood cells, noted by clearing) were measured and quantified into relative

amounts. We determined that 4 of the 8 (NTH 48, NTH49, NTH 50, and W704) clinical isolates used in this study had varying abilities of hemolysis (Table 2). NTH 48 and NTH50 had larger zones of hemolysis as compared to *S. aureus* RN6390. NTH 49 and W704 had considerably smaller zones of hemolysis as compared to *S. aureus* RN6390. These data suggest that hemolytic activity may be a factor involved in respiratory disease.

To determine if alpha-toxin production was correlated to disease, alpha-toxin production was assessed for each strain using Western analysis. Culture supernatants were isolated from overnight cultures, filter-sterilized and then concentrated using a YM-3 centricon. Protein determinations were carried for each concentrated spent media using a BCA assay kit. Equal amounts of protein for each spent media were loaded and electophoresed on a 12% SDS-PAGE gel. The proteins were then transferred to a nitrocellulose membrane and evaluated for alpha-toxin production. Western analysis demonstrated alpha-toxin production (data not shown) and was quantified by densitometry as shown in table 2. We observed variable production of alpha-toxin in the clinical isolates as compared to *S. aureus* RN6390. *S. aureus* NTH48, NTH 50, and *S. aureus* W704 (a respiratory isolate), had similar levels of alpha-toxin production as *S. aureus* RN6390. However, when correlated to lethality only 2 of the high alpha-toxin producers were lethal (Table 2). As well, *S. aureus* NTH 49 produced a small amount of alpha-toxin and *S. aureus* COL, and UAMS-1 did not produce any detectable levels of alpha-toxin strains which were not lethal. (Table 2). These data suggest that the production of alpha-toxin is correlated to disease. However, *S. aureus* Newman, had a

moderate virulence in our model, did not produce alpha-toxin, suggesting that alpha-toxin may not be the only factor involved in respiratory disease.

Discussion

S. aureus' ability to cause disease is largely based on the production of virulence factors. In our previous studies we showed that alpha-toxin is a major factor involved in a mouse model of pneumonia using *S. aureus* RN6390, but not the sole factor responsible for disease. As well, McElroy, et al (25), describe that alpha-toxin is an important factor, that causes damage to the air-blood barrier, in a rat pneumonia model. However, this study also suggests other factors are involved in the disease process. Previous literature also highlights the diversity of *S. aureus* strains ability or lack there of the produce virulence factors. In this study, we examined eight clinical isolates in our mouse pneumonia model. We also examined whether hemolytic activity and/or alpha-toxin production are linked to disease.

Our data demonstrates that *S. aureus* clinical isolates are not as lethal as *S. aureus* RN6390 in our mouse model of pneumonia. The three strains with the highest mortality (RN6390, NTH 48 and NTH 50) had the highest level of alpha-toxin production. However, infection two of the *S. aureus* strains (W704 and NTH49), that produced lower levels of alpha-toxin, did not result in mortality of mice when inoculated with the infectious dose suggesting alpha-toxin production levels are correlated with lethal disease. Interestingly, there was one strain (Newman) that showed some lethality without evidence of alpha-toxin production, indicating other factors can lead to lethal disease. These data support that alpha-toxin is a key virulence factor in lethality of murine

staphylococcal pneumonia, but it is not the only factor that contributes to the lethality as the levels of alpha-toxin production and other unknown factors contribute to disease outcome. We also demonstrate that hemolytic and alpha-toxin production is variable among strains. When strains were cultured on blood plates, four of the eight strains exhibited hemolysis activity (table 2).

In summary this data suggests that alpha-toxin, although involved in our mouse model of pneumonia and other diseases caused by *S. aureus* (25), is not the only factor involved in causing disease. As well, our data supports the diversity of *S. aureus* strains in their production of virulence factors and ability to cause disease. We demonstrate that there is a correlation of high alpha-toxin production levels to lethal disease. However, our data also suggests that although alpha-toxin is a major factor involved in disease it may not be the best immune therapy target alone. Therefore, it is important to further study the complexity of *S. aureus* disease in order to understand what other virulence factors are contributing to disease, in hopes of formulating an immune therapy that has a mixture of common virulence factors to treat *S. aureus* diseases.

TABLE 1. Bacterial strains used in this study.

Strain	Genotype and/or relevant characteristics	Reference and/or source
S. aureus		
RN6390	Prototypic strain	M.S. Smeltzer, University of Arkansas for Medical Sciences
NTH48	S6C, Heme and catalase positive	R.Crum, University of North Texas Health Science Center at Fort Worth
NTH 49	S6C, Heme and catalsae negative	R.Crum, University of North Texas Health Science Center at Fort Worth
NTH50	S6C, Heme postive and catalase negative	R.Crum, University of North Texas Health Science Center at Fort Worth
NTH 51	S6C, Heme negative and catalase positive	R.Crum, University of North Texas Health Science Center at Fort Worth
UAMS-1	Osteomyelitis isolate	M.S. Smeltzer, University of Arkansas for Medical Sciences
Newman	Osteomyelitis isolate	O. Schneewind, University of Chicago
W704	Pneumonia isolate, MRSA	K. Ohlsen, Institut fur Molekulare Infektionsbiolgie, Wurzburg, Germany
COL	MRSA	J.J. Iandolo, University of Oklahoma, Health Science Center

Table 2. Percent mortality due to intranasal inoculation with *S. aureus* strains and their hemolytic activities and alpha-toxin production profiles.

Strain	Hemolytic phenotype[a]	Mortality[b]	Alpha-toxin production[c]
RN6390	++++	83 (10/12)*	+++++
W704	+	0 (0/12)	+++
NTH 48	++++	66 (4/6)*	+++++
NTH 49	-	0 (0/6)	++
NTH 50	++++	50 (3/6)*	+++++
NTH 51	-	0 (0/6)	+
UAMS-1	-	0 (0/6	-
COL	-	0 (0/6)	-
Newman	+	33 (2/6)*	-

[a]Hemolytic phenotype based on zones of hemolysis on sheep blood agar.

[b]Percentage (numbers of deaths/number inoculated) of mice that died within 5 days post-inoculation with 10^8 cfu of various *S. aureus* strains.

[c]Alpha-toxin production determined by densitometric analysis of western analysis. Pluses and minuses represent relative values based on area of alpha-toxin banding pattern.

*Significantly higher mortality ($P \leq 0.05$) than other groups.

CHAPTER V

DISCUSSION

In the present study, I hypothesized that the *S. aureus* global regulators *agr* and *sar* play a role in staphylococcal pneumonia and the virulence factors regulated by them contribute to the severity of staphylococcal pneumonia. To determine this, we established a pneumonia model in mice in order to identify if *S. aureus* global regulators *agr* and *sar* play a role in the pathogenesis of staphylococcal pneumonia. As well, we took steps to identify the extracellular factors responsible for the lethality in a murine model of staphylococcal pneumonia and determine if these factors involved in disease process could be used as targets for immune therapy.

My work revealed that lethal pneumonia in a mouse model is dependent on the *S. aureus* global regulators *agr* and *sar*. This study also revealed that the lethality associated with our model is due to secreted factors, regulated by *S. aureus* global regulators *agr* and *sar*. Further investigation demonstrated that alpha-toxin is a major virulence factor involved in the lethality in our model. By generating an alpha-toxin deficient strain in *S. aureus* RN6390, we show a reduced virulence in our disease model. As well, antiserum to alpha-toxin, when administered with a lethal dose of *S. aureus* RN6390 protected animals from death. By evaluating the role of alpha-toxin's ability to contribute to lethality, we assessed numerous strains of *S. aureus* in our pneumonia model. We discovered that there is a correlation to lethality and alpha-toxin production levels, but that it is not the only factor involved in disease.

Numerous studies have revealed the role of *S. aureus* global regulators *agr* and *sar* in disease models. More specifically, studies have demonstrated that mutations in *agr* and *sar* reduce virulence in animal models (45, 46). In an endocarditis model Cheung et al, (45) demonstrated that a *sar* and *agr/sar* double mutant had decreased infectivity as compared to wild type, suggesting that both loci are involved in initiation and persistence of *S. aureus* in endocarditis. Booth, et al (46), compared the virulence of parental, and isogenic *agr* mutant, *sar* mutant, and an *agr/sar* double mutant in endophthalmitis. This study suggested that both *agr* and the *sar* loci play a role in the pathogenesis of this disease. Other models, including an osteomyelitis model in and a murine arthritis model show that a mutation in *agr* results in a reduction in virulence (42, 47).

Our study revealed that *S. aureus* global regulators *agr* and *sar* were both important in causing lethality in our disease model. We showed a complete reduction in virulence to *S. aureus agr* mutant and a partial reduction in virulence to a *sar* mutant when they are intranasally inoculated into mice. We also demonstrate that these differences are not due to pro-inflammatory response or bacterial dissemination. It is unclear why the *sar* mutant showed only partial reduction in virulence. The primary product of the *sar* locus, SarA, is known to positively regulate expression of the RNAII and RNAIII transcripts of the *agr* operon (100, 38, 80). In fact, in a *sar* mutant of *S. aureus* RN6390, RNAII and RNAIII message levels are reported to be significantly reduced (38). Because *S. aureus sar* mutants have reduced levels of the *agr* effector molecule RNAIII, it is conceivable that the intermediate level of virulence displayed by the *sar* mutant represents low level expression of *agr*-dependent virulence factors as a result of a nonfunctional *sar* locus. However, the *sar* locus is also known to modulate the expression of several virulence factors in an *agr*-independent manner (78, 79, 77, 80, 81). For example, at least four extracellular proteases (79, 81) and the collagen binding

protein (78) are up-regulated in *sar* mutants of *S. aureus*. Therefore, it is possible that the intermediate level of virulence is due to the different virulence factors expressed by the *sar* mutant. In fact, preliminary work involving the proteomic characterization of *S. aureus* RN6390 and its isogenic mutants has revealed a great difference in the proteins found in the spent media of the *sar* mutant as compared to its parent strain (data not shown). It is expected that these studies may reveal what proteins are exactly regulated and expressed by the *agr* and *sar* regulators.

Our study agrees with Heyer, et al (61), who also demonstrate a reduced virulence of *S. aureus agr* and *sar* mutants in a neonatal mouse pneumonia model. Furthermore, they demonstrated that a reduced virulence is observed with an *agr* mutant is not due to differences in pro-inflammatory cytokines or inflammatory response within the lung, which is consistent with what we observed. However, Heyer, et al (61) demonstrate that the severe disease in their model is associated with invasiveness, our study does not.

We also reveal that a secreted factor(s) is responsible for the lethality associated with murine staphylococcal respiratory disease. When spent media from overnight cultures of *S. aureus* RN6390 were intranasally inoculated into mice, it caused death. However, neither the *sar* or *agr* mutant spent medium was lethal. This data suggests that these lethal factor(s) are regulated by *agr*. This data also suggests that the lethal factor(s) are secreted into the extracellular environment. To further support this data, spent media from *S. aureus* RN6390 were heat- or proteinase K-treated and then intranasally inoculated into mice, spent media lost its ability to be lethal. This data further support that lethality in our model was due to a secreted protein(s) regulated by *agr*.

Further experiments determined that the secreted product, *S. aureus* alpha-toxin is a major virulence factor involved in our mouse model of pneumonia. An alpha-toxin (*hla*) deficient mutant in *S. aureus* RN6390 when intranasally inoculated into mice had

reduced virulence as compared to its parent. However, mice still became ill as demonstrated by a significant weight loss over time. As well, we demonstrated that when whole antiserum to alpha-toxin was administered with *S. aureus* RN6390 at a lethal dose, it provided protection from death, suggesting that alpha-toxin may be a potential target for immune therapy.

Several previous studies explored the value of alpha-toxin as a possible immunotherapy target (84, 91 ,92). Menzies and Kernodle (93) demonstrated that when mice were immunized to an alpha-toxin mutant strain, mice were protected in a mouse model of peritoneal infection often used to determine lethality. As well, Hume, et al (91) demonstrated that when rabbits were immunized with alpha-toxin toxoid, rabbits were protected from corneal damage in staphylococcal keratitis. Other studies involving in vitro work demonstrate that when alpha-toxin is neutralized with anti-alpha-toxin antibody, it reduces the damage to epithelial cells (84). These studies demonstrated a reduction in virulence when toxoid or antiserum to alpha-toxin was administered. However, S. *aureus* strains were not completely avirulent in these studies. We observe the same phenomenon, as demonstrated with our *hla*-deficient strain and antiserum experiments. Although, a *hla*-deficient strain of *S. aureus* had a reduced lethality in our pneumonia model, we still observed signs of clinical illness in mice. Likewise, when antiserum for alpha-toxin is administered with *S. aureus* RN6390, mice become ill, as demonstrated by a significant weight loss, although lethality is reduced; further supporting that other virulence factors attribute to the disease process.

Additional investigation into alpha-toxin's potential as an immunotherapy target further supports the thought that several virulence factors are involved in staphylococcal disease. Western analysis, along with virulence assessment using our pneumonia model, demonstrated that there is a correlation to alpha-toxin production and lethality. *S. aureus*

strains RN6390, NTH 48, and NTH 50 all produce high levels of alpha-toxin, and are lethal in when intranasally inoculated. These data suggest that alpha-toxin production correlates to lethality in our model of pneumonia. Interestingly, *S. aureus* W704 and NTH50 had a reduced virulence at the lethal dose, even though they produced moderate amounts of alpha-toxin. These data suggest that perhaps alpha-toxin production alone is not hallmark of lethality, but that the amount of alpha-toxin produced is a more important factor influencing lethality. However, *S. aureus* Newman, a non-alpha-toxin producer showed some lethality when intranasally inoculated. These data therefore, suggest that alpha-toxin is not the only factor involved in the disease process. These strain differences highlight that the presence of more than one virulence factors, specifically a secreted factor is contributing to disease. Therefore this data suggests that an appropriate immune therapy might be a cocktail of antibodies to several *S. aureus* virulence factors.

The relevance of work using *S. aureus* RN6390 in studies characterizing global regulation by *agr* and *sar* and virulence has been questioned in the past years as it relates to the actual regulation in clinical strains of *S. aureus*. A study by Kullik, et al,(101) demonstrated that *S. aureus* 8325-4, a laboratory strain used in numerous virulence and regulation studies, is an RsbU mutant, of which *S. aureus* RN6390 is a derivation. RsbU is a positive regulator of sigma B (σ^B), is essential for the activation of sigma B during exponential growth after environmental stress (102). It is part of the *Rsb* operon, which controls the expression of RsbU (103). A deletion in RsbU causes σ^B to not be expressed (104). *S. aureus* RN6390 is a natural RsbU mutant (105). This deletion causes a hypersecretion of alpha-toxin in this strain (104). This difference is suggested to not be representative of clinical isolates of *S. aureus* and therefore may not be an ideal strain to study the regulation of virulence.

These differences are highlighted by Blevins, et al (106), who demonstrate that regulatory events in *S. aureus* RN6390 are not similar to clinical isolates. In these studies they used UAMS-1, is an osteomyelitis isolate that encode collagen binding protein (*cna*), but not fibronectectin binding protein (*fnbB*), produces a limited amount of exoproteins and has a high capacity to bind to host proteins. All of these characteristics differentiate UAMS-1 from RN6390. When *agr* and *sar* mutations were introduced into UAMS-1 an increased capacity to bind collagen was seen in the *sarA* mutant, while mutation of *agr* had little impact. Mutation of *sarA* resulted in increased production of proteases and a decreased capacity to bind fibronectin. Mutation of *agr* had the opposite effect. This is in contrast to *S. aureus* RN6390, which produces a significant amount of protease as compared to UAMS-1. Mutation of *agr* in RN6390, resulted in a dramatic decrease in protease production. In UAMS-1 a mutation in *agr* had little impact on protease production. Thus, suggesting that studies characterizing regulation of virulence done with *S. aureus* RN6390 may not reflect events that occur with clinical isolates.

Therefore, our studies using *S. aureus* RN6390 may not be completely representative of the process of disease in clinical isolates. However, we showed that although there are strain differences in lethality of *S. aureus* isolates as compared to *S. aureus* RN6390 in our pneumonia model, high alpha-toxin producing strains of *S. aureus* were the most lethal. The variability in alpha-toxin expression could be related to *rsbU*, but require further investigation to determine the role that *rsbU* may play in virulence among *S. aureus* strains.

Through this study and McElroy, et al (25), we demonstrate that alpha-toxin is an important virulence factor in staphylococcal pneumonia albeit a mouse or rat model of pneumonia. When protected from the lethal effects of alpha-toxin, mice survive infection. However, mice still become ill. As well, we show that alpha-toxin is not

necessary to cause lethality in our model of pneumonia, as demonstrated with *S. aureus* Newman. These observations suggest that alpha-toxin is not the only virulence factor associated with causing lung disease. Therefore, it would appear that alpha-toxin alone could serve as a target for immunotherapy. However, I can envision that some type of cocktail of antibodies raised against several staphylococcal virulence factors would provide a reasonable immunotherapy. Therefore, it is of utmost importance that further examination of staphylococcal virulence factors are carried out. By determining what other virulence factors are associated with alpha-toxin in staphylococcal pneumonia, a potential immunotherapy could be developed which would reduce the severity and length of infections.

Further studies could be done to identify the other factors involved in murine respiratory disease. Preliminary proteomic analysis of spent media from *S. aureus* RN6390 and its isogenic mutants has already demonstrated the differences in protein expression between the parent and mutants (data not shown). Further identification of peptides could aid in identifying the other factor(s) involved in staphylococcal respiratory disease. By identifying protein differences between the parent and mutant, knock-out mutants of the identified factors could be generated. These mutants could then be used in our virulence assay to determine the factor(s) influence on lethality in murine pneumonia. Once a specific factor(s) is identified as effecting the lethality, a combination of knock-out mutants could be made with an alpha-toxin deficient strain, which could then be further assessed, for lethality. As well, antiserum or antibodies could be raised against the factor(s) identified and administered intranasally or immunized into mice to assess their protective effects. It plausible that these future experiments could lead to the creation of a new therapeutic agent, used to treat and reduce the detrimental effects of staphylococcal disease.

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