Anti-Alpha-Hemolysin Monoclonal Antibodies Mediate Protection against Staphylococcus aureus Pneumonia

Brook E. Ragle and Juliane Bubeck Wardenburg

Departments of Microbiology1 and Pediatrics,2 University of Chicago, 920 E. 58th Street, Chicago, Illinois 60637

Received 30 January 2009/Returned for modification 3 March 2009/Accepted 9 April 2009

Staphylococcus aureus pneumonia is one of the most common invasive diseases caused by this human pathogen. S. aureus alpha-hemolysin, a pore-forming cytoxin, is an essential virulence factor in the pathogenesis of pneumonia. Vaccine-based targeting of this toxin provides protection against lethal staphylococcal pneumonia in a murine model system, suggesting that a monoclonal antibody-based therapy may likewise prove to be efficacious for prevention and treatment of this disease. We report the generation of two distinct anti-alpha-hemolysin monoclonal antibodies that antagonize toxin activity, preventing human lung cell injury in vitro and protecting experimental animals against lethal S. aureus pneumonia. Each of these two monoclonal antibodies recognized an epitope within the first 50 amino acid residues of the mature toxin and blocked the formation of a stable alpha-hemolysin oligomer on the target cell surface. Active immunization with the first 50 amino acids of the toxin also conferred protection against S. aureus pneumonia. Together, these data reveal passive and active immunization strategies for prevention or therapy of staphylococcal pneumonia and highlight the potential role that a critical epitope may play in defining human susceptibility to this deadly disease.

Staphylococcus aureus is a gram-positive human pathogen that causes a myriad of diseases ranging from minor skin infections to life-threatening deep tissue infections and toxicoses (19). Pneumonia is among the most prominent S. aureus-mediated diseases, accounting for approximately 15% of documented invasive staphylococcal infections (14), and there are an estimated 50,000 cases per year in the United States alone (17). In addition to being one of the most common causes of ventilator-associated pneumonia, S. aureus is increasingly recognized as an important cause of community-acquired pneumonia, affecting previously healthy adults and children (8, 16). This is particularly notable in association with influenza infection, where concomitant staphylococcal pneumonia is often a lethal complication (7, 8, 12). Up to one-half of staphylococcal pneumonia isolates are classified as methicillin (meticillin)-resistant S. aureus (MRSA), confounding the delivery of appropriate treatment and resulting in reported mortality as high as 56% (1, 17, 24). The combination of an increasing disease burden and declining potency of traditional antimicrobials to susceptible cells (2, 26). This chromosomally encoded toxin is secreted as a water-soluble monomer by the majority of S. aureus strains (22). Membrane binding of the monomer permits a series of well-defined intermolecular interactions between neighboring monomers, resulting in the formation of a barrel-shaped oligomeric pore that penetrates the membrane (9, 13). Residues located at the N terminus of the mature toxin are essential for assembly of the lytic oligomer, as point mutations or truncations within this region disrupt the formation of an active toxin (21, 27, 28).

In addition to its role in the lung, Hla is central to pathogenesis in other tissues, as hla mutants are less virulent in animal models of intraperitoneal (i.p.), intramammary, and corneal infection (3, 6, 23). Supporting this role for Hla in disease, immune sera generated against a single point mutant with a mutation that disrupts pore formation, termed Hla1351, provide a high degree of protection against pneumonia, i.p. infection, and challenge with purified active toxin (5, 20). We therefore built upon these observations by generating mouse monoclonal antibodies (MAbs) following immunization with inactive Hla1351, to investigate whether an antibody with a single specificity could provide protection against S. aureus pneumonia.

MATERIALS AND METHODS

Bacterial strains and culture. For mouse lung infection, S. aureus strains Newman and LAC/USA300 were grown at 37°C in tryptic soy broth to an optical density at 660 nm of 0.5. Culture aliquots (50 ml) were centrifuged and washed in phosphate-buffered saline (PBS) prior to resuspension. For mortality studies, S. aureus Newman was resuspended in 750 μl (3 × 108 to 4 × 108 CFU per 30 μl), while LAC/USA300 was resuspended in 1,250 μl (2 × 109 CFU per 30 μl). For bacterial load and histopathology experiments, S. aureus Newman was re-suspended in 1,250 μl (2 × 108 CFU per 30 μl). For cytotoxicity studies, 5 ml of a culture prepared as described above was re-suspended in 10 ml F12K medium (Invitrogen). A 100-μl suspension was used for each assay well.
Plasmid construction. PCR products encoding serial 50-amino-acid fragments of Hla, amplified from S. aureus Newman chromosomal DNA, were cloned into pGEX-6P-1 (GE Healthcare) and transformed into Escherichia coli. Each construct was sequenced for verification. A plasmid encoding glutathione S-transferase (GST)-HlaGST was generated as previously described (5). To generate a polyhistidine-tagged version of full-length, active Hla, a PCR product encoding the mature polypeptide was amplified from S. aureus Newman chromosomal DNA, cloned into pET24b (Novagen), and then transformed into E. coli BL21/D3.

MAbs. MAbs to Hla were generated by the Frank W. Fitch Monoclonal Antibody Facility at the University of Chicago. Splenocytes derived from mice immunized with full-length HlaGST were utilized to generate hybridomas. Control MAbs of isotypes immunoglobulin G2a (IgG2a) and IgG2b were purified and supplied by the Frank W. Fitch Monoclonal Antibody Facility.

Animals and procedures. Animal experiments were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee at the University of Chicago. For lung infection, 7-week-old C57BL/6j mice (The Jackson Laboratory) were anesthetized before inoculation of 30 µl of an S. aureus suspension prepared as described above into the left naris. Animals were placed into a cage in a supine position for recovery and were observed for the time courses indicated below. Routinely, a small percentage of animals died within the first 6 h after inoculation, likely from the combined effects of aspiration and anesthesia. These animals were not included in subsequent statistical analyses.

For passive immunization studies, 7-week-old mice received i.p. injection 420 µl of either IgG2a, IgG2b, MAb 7B8, or MAb 1A9 at the concentrations indicated below 24 h before S. aureus challenge. For active immunization, 4-week-old mice received 20 µg of either GST, GST-HlaGST, or GST-Hla1-50 in complete Freund's adjuvant on day 0 via the intramuscular (i.m.) route, followed by a boost with 20 µg of each protein antigen in incomplete Freund's adjuvant on day 10. Animals were challenged with S. aureus on day 21. Sera were collected before immunization and on day 20 to assess specific antibody production.

To evaluate the pathological correlates of pneumonia, infected animals were killed via forced CO2 inhalation before removal of both lungs. The right lung was homogenized for enumeration of the lung bacterial load using serial dilution and plate counting techniques. The left lung was placed in 1% formalin, embedded in paraffin, and sectioned, and thin sections were stained with hematoxylin-eosin and analyzed by microscopy.

Enzyme-linked immunosorbent assay (ELISA). Serum antibody titers were determined with immunoplates (MaxiSorp; Thermo Fisher Scientific) coated with 1 µg/ml purified HlaGST or GST-Hla. Dilutions of either mouse or human sera prepared in PBS were incubated in the appropriate plates, which were developed with horseradish peroxidase-conjugated secondary antibodies and a 3,3'-5,5'-tetramethylbenzidine substrate kit (Thermo Scientific) and ex- amined with a spectrophotometer (GENios; Tecan). All human sera were collected in accordance with a human subject protocol that was reviewed, approved, and supervised by the Institutional Review Board at the University of Chicago.

Live/dead and cytotoxicity assays. A549 cells were washed and plated in F12K medium supplemented with 10% fetal bovine serum at a density of 1.5-2.0 × 10^4 cells per well in a 96-well plate. For both live/dead and cytotoxicity assays, washed A549 cells were cultured with 100 µl of staphylococcal suspension per well in F12K medium with or without antibody in triplicate wells. After 4 h of incubation at 37°C, either cells were treated with a live (green)/dead (red) reagent (Invitrogen), or lactate dehydrogenase (LDH) activity was determined (Roche) according to the manufacturer's recommendations. Microscopic images of stained cells were obtained using a microscope (Eclipse TE2000U, Nikon). LDH activity was measured with a spectrophotometer and was expressed as the percentage of maximal lysis obtained after detergent treatment of the A549 cells. The results are representative of a minimum of two independent experiments.

Protein preparation and immunoblot analysis. All GST- and His-tagged fusion proteins were prepared and purified using standard protocols. For dot blot analysis, each antigen was spotted on a nitrocellulose membrane that was then blocked with 5% milk in Tris-buffered saline containing 0.1% Tween 20. Each MAb of interest was used at a final concentration of 1 µg/ml. Blots were developed using goat anti-mouse horseradish peroxidase-conjugated secondary antibody and SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). For molecular modeling, atomic coordinates were retrieved from the Protein Data Bank (PDB ID 7ahl) based on the data of Song et al. (26). Models were generated using PyMol (http://www.pymol.org/).

Oligomerization and binding assay. Radiolabeled Hla was synthesized by in vitro transcription and translation in an E. coli S30 extract (Promega) supplemented with T7 RNA polymerase, rifampin (rifampicin), and [35S]methionine according to the manufacturer's instructions. One hundred twenty microlitres of 12.5% rabbit red blood cells (RRBC) in K-PBSA/JME (20 mM potassium phosphate, monobasic), 150 mM NaCl [pH 7.4], 1 mg/ml bovine serum albumin, 1 mM β-mercaptoethanol) was incubated with 30 µl of the radiolabeled Hla mixture in the presence of 0.1 to 5 µM MAb 7B8 or 1A9 for 1 h at 20°C. The controls were analyses performed in the absence of added antibody or in the presence of 5 µM isotype-matched MAbs as indicated below. Following incubation, samples were centrifuged at 13,000 rpm for 5 min and then washed with 500 µl K-PBSA/JME and centrifuged as described above. Samples were then resus- pended in 90 µl 1× Laemmli buffer and incubated at 37°C for 10 min before 12-µl portions of the samples were loaded onto 10% sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE) gels for electrophoresis. The gels were dried, and then the results were visualized using a phosphorimager.

RRBC hemolysis assay. Purified, active full-length Hla was added to 900 µl of 12.5% RRBC in PBS to a final concentration of 100 nM. The MAbs indicated below or PBS was added to the reaction mixture (100 µl), and the cells were then incubated at 20°C for 1 h. The reaction mixtures were centrifuged at 13,000 rpm for 5 min, and the absorbance at 475 nm of the supernatants was measured. Percentages of hemolysis were calculated using the supernatant value for an equivalent number of cells that had been lysed in 1% Triton X-100.

Surface plasmon resonance (SPR). The affinity and rates of association and dissociation between 7B8 and Hla and between 1A9 and Hla were measured using a BLAcore 3000. The carboxyl groups on the sensor surface of a CM5 chip were activated with 0.2 M N-ethyl-N-[(3-dimethylamino-propyl)carbodiimide and 0.05 M N-hydroxysuccinimide. Antibody was bound to the chip by passing 1 µM antibody in HBS-P buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 0.005% [vol/vol] surfactant P20) over the activated chip. Free amines were then neutralized using 1 M ethanolamine hydrochloride. The control surface was prepared similarly, except that running buffer was injected instead of Hla. We measured the rates of association and dissociation of purified Hla at concentrations ranging from 5 to 100 nM. All measurements were performed in triplicate. After each binding sensor the chip was regenerated using 10 mM NaOH. Derived sensorgrams were analyzed using BIAevaluation 4.1. Affinity constants were estimated by curve fitting using a 1:1 binding model.

Results and Discussion

Anti-Hla MAbs prevent Hla-mediated cell injury. The ability to neutralize Hla-mediated S. aureus lung injury through the development of MAbs offers an exciting new strategy for the prevention and treatment of pneumonia. To this end, we generated murine MAbs to the nontoxic HlaGST mutants. Immunoreactive MAbs were examined to determine their ability to prevent injury to cultured alveolar epithelial cells (data not shown). Two MAbs, 7B8 and 1A9, provided a high degree of protection against Hla-mediated injury. Uninfected human A549 alveolar epithelial cells retain a green fluorescence when they are examined with a live (green)/dead (red) staining reagent (Fig. 1A). Infection of cells with S. aureus Newman, a methicillin-sensitive clinical isolate, resulted in cell death after 3 h of coculture in the presence of PBS, as demonstrated by an increased number of red fluorescent cells (Fig. 1B). Treatment of A549 cells with IgG2a, an isotype-matched control for 7B8, was similar to treatment with PBS (Fig. 1C). In contrast, treatment with 7B8 provided nearly complete protection from S. aureus-induced death (Fig. 1D). Nonspecific IgG2b did not confer protection (Fig. 1E), while isotype-matched MAb 1A9 provided robust protection (Fig. 1F). The percentages of cell death are shown in Fig. 1.

To quantify the ability of MAbs 7B8 and 1A9 to antagonize the effects of Hla on A549 cells, we examined concentration-dependent protection using an LDH release assay. Both MAbs conferred a significant degree of protection at concentrations ranging from 0.5 to 5 µg/ml (3 to 33 nM) when they were...
added to cocultures of A549 cells with live *S. aureus* (Fig. 1G and H) (*P* < 0.02 for all conditions). The calculated 50% inhibitory concentration for 7B8 was 2.83 µg/ml, while that for 1A9 was 1.92 µg/ml. These results reveal the in vitro protective efficacy of two novel anti-Hla MAbs which protect human lung epithelial cells from *S. aureus*-mediated injury. The low concentrations at which these antibodies have protective effects in vitro highlight their potential therapeutic relevance.

**Passive immunization with anti-Hla MAbs protects mice from *S. aureus* pneumonia.** To examine the ability of anti-Hla MAbs to prevent *S. aureus*-induced pneumonia, we passively immunized 7-week-old C57BL/6J mice with 7B8 or 1A9 delivered via i.p. injection 24 h prior to intranasal (i.n.) infection with 3 × 10^8 to 4 × 10^8 CFU of *S. aureus* Newman. Groups of 15 mice received 10, 5, 1, or 0.1 mg/kg of 7B8 (Fig. 2A) or 1A9 (Fig. 2B); control animals received 10 mg/kg of the corresponding control MAb. Mice were observed for acute lethal disease secondary to *S. aureus* pneumonia. For animals receiving as little as 1 mg/kg of 7B8 there was a significant reduction in mortality (Fig. 2A) (*P* = 0.015) compared to animals receiving the isotype control. The protective efficacy of 1A9 was less prominent; a reduction in mortality over 72 h was evident with a minimum MAb dose of 5 mg/kg (Fig. 2B) (*P* = 0.039). Concentrations of 7B8 that resulted in a significant decrease in mortality resulted in half-maximal serum antibody titers greater than 1:547 ± 218, similar to anti-Hla titers that we have reported to confer protection in prior immunization studies (5). Interestingly, 1 mg/kg of 1A9 resulted in a half-maximal titer of 1:606 ± 143, yet it was not protective, suggesting that 1A9 has either a lower affinity for Hla or a diminished capacity for functional neutralization.

To evaluate the pathological correlates of 7B8 and 1A9 protection, we examined *S. aureus* recovery from lungs 24 h after infection for mice that received 10 mg/kg of each MAb or a control. Immunization with 7B8 and 1A9 led to marked reductions in the number of CFU recovered from the right lung compared to controls (Fig. 2C) (*P* = 0.027 and *P* = 0.02, respectively). Gross inspection of infected lungs revealed more focal disease in animals that received 7B8 or 1A9, as indicated by a reduction in the dense, red appearance of the tissue (Fig. 2D). These observations were confirmed by histopathologic analysis, which showed that the majority of the airspace in control mice was obliterated by inflammatory cell infiltrates or aggregates of *S. aureus* (Fig. 2E, left panels); in contrast, in animals treated with 7B8 or 1A9 the lung tissue was predominately unaffected (Fig. 2E, right panels).

As an increasing number of severe *S. aureus* pneumonias are caused by MRSA isolates, we examined the efficacy of 7B8 and 1A9 for protecting mice against the highly virulent MRSA strain LAC/USA300. We have previously shown that LAC/USA300 secretes approximately twice as much Hla as *S. aureus*...
Newman, which directly correlates with increased virulence in the lung (5). Treatment of mice with 7B8 or 1A9 24 h prior to i.n. infection with $2 \times 10^8$ CFU of LAC/USA300 resulted in a marked reduction in mortality compared to the controls (Fig. 3A) ($P = 0.013$ and $P = 0.002$, respectively), confirming the ability of these MAbs to protect against both methicillin-sensitive *S. aureus* and MRSA isolates upon prophylactic administration.

The clinical utility of MAbs that prevent *S. aureus* pneumonia, especially disease caused by virulent strains such as LAC/USA300, is readily appreciated when surgical and intensive care patients that are highly susceptible to staphylococcal ventilator-associated pneumonias are considered (15, 25). A more far-reaching impact, however, would be possible if these MAbs confer protection following the onset of infection. To examine therapeutic efficacy, mice were immunized via the i.p. route with 10 mg/kg control IgG2a or 7B8. Anti-Hla MAb-treated animals received doses 24 h prior to infection or at 4, 8, or 12 h after infection with *S. aureus* Newman. Treatment up to 8 h following infection, when mice exhibit prominent signs of disease, conferred significant protection from 24-h mortality ($P = 0.021$) (Fig. 3B). This protection was not durable, however, as the late mortality was similar to that observed for the control. While the absolute 24-h mortality was reduced in mice vacci-
nated 12 h after infection, the results were not statistically significant. These data suggest that Hla may be essential for an early stage of pathogenesis when the toxin may be crucial for inciting lung injury. Once this injury has occurred, antagonism of the toxin may be of little benefit. An extrapolation of these findings to human disease implies that treatment with such MAbs early in the course of S. aureus pneumonia has the potential to delay disease progression, providing a much-needed window of opportunity to enhance the utility of antimicrobial and supportive therapies.

SPR reveals similar affinities of MAbs for Hla. To quantify the affinity of 7B8 and 1A9 for Hla, we performed SPR studies. Purified toxin (5 to 100 nM) was passed over each MAb, which was amino coupled to a CM5 chip, allowing determination of the kinetic rate constants for association and dissociation and the affinity constant. Both 7B8 and 1A9 have affinities in the low-nanomolar range (8.54 nM and 6.49 nM, respectively) (Fig. 4A), indicating that these MAbs are able to form a tight association with Hla, a feature that likely enhances their capacity to neutralize toxin activity.

MAb recognition of an N-terminal epitope prevents Hla oligomerization. An appreciation of the role of specific amino acids and regions of Hla in toxin assembly and function has been obtained through studies of the crystal structure of the heptameric toxin and a series of elegant biochemical studies (21, 26, 27). To examine the mechanism by which 7B8 and 1A9 neutralize Hla, we generated a series of GST fusion proteins comprised of overlapping 50-amino-acid segments of the mature toxin to map the epitope(s) recognized by each MAb (Fig. 4B). Overlapping, serial 50-amino-acid segments of Hla were purified as GST fusion proteins and used in a dot blot analysis to map the epitopes recognized by 7B8 and 1A9, which revealed that both MAbs bind within the first 50 amino acids. The Coomassie blue-stained SDS-PAGE gel shows the integrity of each fusion protein. (C) Structure of Hla as a monomer (left structure) and as a heptamer (middle and right structures), in which the first 50 amino acids recognized by the MAbs are indicated by black lines.

SPR reveals similar affinities of MAbs for Hla. To quantify the affinity of 7B8 and 1A9 for Hla, we performed SPR studies. Purified toxin (5 to 100 nM) was passed over each MAb, which was amino coupled to a CM5 chip, allowing determination of the kinetic rate constants for association and dissociation and the affinity constant. Both 7B8 and 1A9 have affinities in the low-nanomolar range (8.54 nM and 6.49 nM, respectively) (Fig. 4A), indicating that these MAbs are able to form a tight association with Hla, a feature that likely enhances their capacity to neutralize toxin activity.

MAb recognition of an N-terminal epitope prevents Hla oligomerization. An appreciation of the role of specific amino acids and regions of Hla in toxin assembly and function has been obtained through studies of the crystal structure of the heptameric toxin and a series of elegant biochemical studies (21, 26, 27). To examine the mechanism by which 7B8 and 1A9 neutralize Hla, we generated a series of GST fusion proteins comprised of overlapping 50-amino-acid segments of the mature toxin to map the epitope(s) recognized by each MAb (Fig. 4B). Overlapping, serial 50-amino-acid segments of Hla were purified as GST fusion proteins and used in a dot blot analysis to map the epitopes recognized by 7B8 and 1A9, which revealed that both MAbs bind within the first 50 amino acids. The Coomassie blue-stained SDS-PAGE gel shows the integrity of each fusion protein. (C) Structure of Hla as a monomer (left structure) and as a heptamer (middle and right structures), in which the first 50 amino acids recognized by the MAbs are indicated by black lines.
M Abs do not function merely by preventing toxin binding to target cells. The monomeric form of Hla produced in vitro migrated as two bands, as seen in previous studies (13). Each MA b, however, prevented the assembly of Hla into an SDS-stable oligomer (Hla-) in a close-dependent fashion (Fig. 5B). The inhibition of heptamer formation in the presence of Hla and IA9 correlates with protection from hemolysis, defining a potent molecular mechanism by which these MAbs exert their protective effects. While the affinity constants of these MAbs for Hla were similar, Hla prevents the formation of the heptamer more effectively than IA9 does, which correlates with enhanced in vivo protection.

Active vaccination with Hla prevents mortality due to S. aureus pneumonia. These observations led to the hypothesis that active immunization with a purified antigen consisting of the first 50 amino acids of Hla may prevent disease. Groups of 15 4-week-old mice were vaccinated via the i.m. route on day 0 with 20 μg of GST, GST-HlaH35L, or GST-Hla1-50 emulsified in Freund’s adjvant and then were boosted on day 10 with antigen emulsified in incomplete Freund’s adjvant. On day 21, animals were infected via the i.n. route with 3 × 108 S. aureus Newman CFU and evaluated for protection from lethal pneumonia. In agreement with our previously published observations, immunization with GST-HlaH35L led to complete protection, in contrast to immunization with GST alone, after which the majority of mice succumbed to infection (P < 0.001) (Fig. 6) (5). Immunization with Hla1-50 resulted in significant protection over the entire course of the assay, and full protection was evident at 24 h (P < 0.022). The half-maximal serum antibody titers for each immunogen at the time of infection were 1:3,178 ± 1:468 (GST-HlaH35L) and 1:692 ± 669 (GST-Hla1-50). These observations, along with our description of protective MAbs, have important implications for the development of clinically relevant immunologic tools to combat S. aureus pneumonia. First, these studies highlight the potential to utilize only a portion of Hla as a component of a multisubunit S. aureus vaccine. This may simplify vaccine design, allowing generation of a chimeric antigen in which a segment of Hla can be fused to a second candidate vaccine antigen. Further, a greater margin of safety can be achieved by avoiding the use of a full-length variant of a potent human toxin. Second, the humanization of MAbs, notably MAb 7B8, with specificity for the N-terminal region of Hla may provide an efficient means by which to translate these observations to the clinical arena.

Serum antibody titers to Hla vary within the human population. Implementation of an effective Hla-based immunotherapy for S. aureus pneumonia is predicated on the notion that some humans do not harbor a preexisting, effective host response to this toxin, rendering them more susceptible to disease. Previous studies have clearly documented that serum antibodies to Hla are commonly found within populations of individuals with invasive S. aureus disease; however, large-scale investigations of preformed anti-Hla responses in subjects without such disease have not been reported (10, 11, 18). To evaluate the human antibody response to epitopes within the first 50 amino acids of Hla, we examined sera from 25 healthy volunteers using an ELISA. While all individuals had detectable antibodies to Hla, the coefficient of variation within the population was calculated to be 50.3% when a 1:1 dilution of serum was analyzed (Fig. 6B). This observation has two important implications. First, it suggests that there is a defined population of humans with relatively lower levels of antibody to at least one epitope of Hla shown in this work to be pro-

FIG. 6. Active immunization with the first 50 amino acids of Hla prevents S. aureus pneumonia. C57BL/6J mice were vaccinated by i.m. injection with GST, GST-HlaH35L, or GST-Hla1-50 and challenged with S. aureus Newman via the i.n. route. Mortality was recorded 24, 48, and 72 h after infection (P < 0.022; 15 animals per group). Statistical significance is indicated by an asterisk. (B) ELISA was performed using the indicated dilutions of serum from 25 human volunteers to determine the titer of anti-Hla1-50 antibodies, expressed as raw units of optical density at 450 nm (OD450). The bottom and top of each box indicate the lower and upper quartiles, respectively, and the horizontal bar indicates the median for the group of samples. The whiskers indicate the lowest and highest values within a distance of 1.5 times the interquartile range from the lower and upper quartiles, respectively; circles indicate outliers in each set.
tective. These individuals may benefit from vaccine strategies targeting Hla, perhaps especially in clinical situations such as critical illness, major surgical procedures, and influenza infection that confer risk for the development of *S. aureus* pneumonia. Second, the data highlight the importance of understanding the human immune response to *S. aureus* disease and the immunologic correlates of protection from invasive disease. In order to fully appreciate the role of preexisting antibodies to Hla in conferring protection from pneumonia, it will be necessary to conduct prospective clinical studies in which the anti-Hla antibody profile of “at-risk” subjects can be correlated with the development of disease. A definitive link between anti-Hla antibody levels in general or levels of an antibody to a specific protective epitope and risk for pneumonia would make it possible to stratify risk for the development of disease. The capacity to prospectively identify at-risk individuals would facilitate highly focused application of developing immunotherapies.

The clinical complexity of *S. aureus* infection and the fact that there is no single virulence factor central to the progression of all disease manifestations pose a challenge for development of a universal vaccine. The essential role of Hla in pneumonia, coupled with the clinical burden of this disease, highlights the potential of the observations described here to have a significant impact upon ongoing and future efforts to design novel immunotherapies to specifically combat lung infection. Further, these observations shed light on a new strategy with which to conceptualize the targeting of Hla in a multifaceted universal *S. aureus* vaccine.

ACKNOWLEDGMENTS

This work was supported by an NCI Cancer Center award (grant SP50CA014599-33) to the Frank W. Fitch Monoclonal Antibody Facility, by the Pediatric Scientist Development Program (grant K12-HD00850 to J.B.W.), and by the Departments of Pediatrics and Microbiology at the University of Chicago. This work was sponsored by the NIH/NIAID Regional Center of Excellence for Bio-defense and Emerging Infectious Diseases Research (RCE) Program.

We acknowledge membership in and support from the Region V Great Lakes RCE (NIH award 1-U54-AI-057153). We have no conflicting financial interests.

We thank D. Missiakas, A. DeDent, and O. Schneewind for critical discussions and comments on the manuscript, the Department of Pathology at the University of Chicago for histology support, K. Alexander for pharmacologic calculations, S. Bond for microscopy support, M. Davis, C. Mulligan, and C. McShan for MAb support, and T. Karrson for statistical support.

REFERENCES