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

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Staphylococcus aureus pneumonia is one of the most common invasive diseases caused by this human pathogen. S. aureus alpha-hemolysin, a pore-forming cytotoxin, 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 toxinoxemia (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 (mecillinam)-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 treat S. aureus pneumonia heightens the need for novel prophylactic and therapeutic strategies.

We have defined an essential role of alpha-hemolysin (Hla) in S. aureus pneumonia, as strains lacking this pore-forming cytotoxin are avirulent in a murine model of disease (4). Drawing on this knowledge, we have demonstrated that vaccine-based approaches targeting Hla provide protection from lethal pneumonia in experimental animals (5). The ability of Hla to injure the lung and other tissues rests on the ability of the toxin to form a 2-nm heptameric pore in the plasma membrane of 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 HlaH35L, 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 HlaH35L 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 × 10^6 to 4 × 10^6 CFU per 30 µl), while LAC/USA300 was resuspended in 1,250 µl (2 × 10^6 CFU per 30 µl). For bacterial load and histopathology experiments, S. aureus Newman was resuspended in 1,250 µl (2 × 10^6 CFU per 30 µl). For cytotoxicity studies, 5 ml of a culture prepared as described above was resuspended 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 of the plasmids was sequenced for verification. A plasmid encoding glutathione S-transferase (GST)-Hla1231 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/D3E3.

**MAbs.** MAbs to Hla were generated by the Frank W. Fitch Monoclonal Antibody Facility at the University of Chicago. Spleenocytes derived from mice immunized with full-length Hla1231 were utilized to generate hybridsomas. 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 with ketamine 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 and supervised by the Institutional Review Board at the University of Chicago.

**Histological analysis.** At the end of the experiment, the left lung was placed in 10% formalin, embedded in paraffin, sectioned, and thin sections were stained with hematoxylin-eosin and analyzed by microscopy.

**Anti–Hla MAbs prevent injury to cultured alveolar epithelial cells (data not shown).** Infected mice were examined with a live (green)/dead (red) staining reagent 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 trypan blue staining. The left lung was placed into a formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin and analyzed by microscopy.

**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 Hla1231 mutant. Immunoreactive MAbs were examined to determine their ability to prevent injury to cultured alveolar epithelial cells (data not shown). Two MAbs, MAbs 7B8 and 1A9, provided a high degree of protection against Hla-mediated injury. Uninfected human A549 alveolar epithelial cells retain a green fluorescent 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 trypan blue staining. The left lung was placed into a formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin and analyzed by microscopy.

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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 \(\mu\)g/ml, while that for 1A9 was 1.92 \(\mu\)g/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 \(3 \times 10^8\) to \(4 \times 10^9\) 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 \(\times 10^6\), 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 \(\times 10^6\), 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 heptamer 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 panel 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). Dot blot analysis of these fusion proteins 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 (Fig. 4C) in the context of the full-length monomer of Hla can be appreciated by examination of these residues (Fig. 4C) in the context of the full-length monomer (based on a predicted structure) and the assembled heptamer derived from the known crystal structure (26).

To investigate whether the interaction of each MAb with Hla disrupts the formation of fully assembled Hla, we utilized a RRBC hemolysis assay in which 

$$[35S]$$methionine-labeled Hla is visualized by SDS-PAGE in its monomeric and heptameric forms (27). To confirm the functional ability of 7B8 and 1A9 to protect against hemolysis, we incubated 3 × 10⁸ cells with 100 nM purified, active Hla and each MAb at concentrations of 0.1 to 5 μM. Dose-dependent prevention of hemolysis was observed for both MAbs (Fig. 5A); a significant reduction in hemolysis was observed with concentrations of 7B8 as low as 0.5 μM (P < 0.005) and with concentrations of 1A9 as low as 0.1 μM (P < 0.033). An examination of the radiolabeled monomeric toxin (Hla) (Fig. 5B) demonstrated that the binding of Hla to RRBC was not impaired by addition of 7B8 or 1A9. Importantly, only Hla that is bound to the RRBC membrane was revealed in the autoradiogram. Thus, the similar signal intensities observed for the monomer in lanes treated with the MAbs and in lanes without antibody treatment or in the presence of control antibody strongly suggest that the
MAbs 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 MAb, however, prevented the assembly of Hla into an SDS-stable oligomer (Hla7) in a concentration-dependent fashion (Fig. 5B). The inhibition of heptamer formation in the presence of 7B8 and 1A9 correlates with prevention 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, 7B8 prevents the formation of the heptamer more effectively than 1A9 does, which correlates with enhanced in vivo protection.

Active vaccination with Hla1-50 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 adjuvant and then were boosted on day 10 with antigen emulsified in incomplete Freund’s adjuvant. On day 21, animals were infected via the i.n. route with 3 × 10^8 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 Hla 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 Hla1-50 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 Hla1-50, 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-
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 proteic 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.

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REFERENCES


