Identification of Anti-Alpha Toxin Monoclonal Antibodies That Reduce the Severity of *Staphylococcus aureus* Dermonecrosis and Exhibit a Correlation between Affinity and Potency

C. Tkaczyk,* L. Hua,* R. Varkey,† Y. Shi,* L. Dettinger,‡ R. Woods,* A. Barnes,* R. S. MacGill,* S. Wilson,* P. Chowdhury,‡ C. K. Stover,* and B. R. Sellman* †

Departments of Infectious Disease* and Antibody Discovery and Protein Engineering,† MedImmune, LLC, Gaithersburg, Maryland, USA

*Staphylococcus aureus* alpha toxin (AT) is an important virulence determinant and may be a valid target for immunoprophylaxis against staphylococcal disease. Here we report the identification of potent inhibitory anti-AT monoclonal antibodies (MAbs) derived using B-cell hybridoma technology from VelocImmune mice engineered to produce IgG with a human variable domain. A small panel of inhibitory MAbs blocked AT-mediated lysis of rabbit red blood cells, A549 human lung epithelial cells, and THP-1 human monocytic cells, in a dose-dependent manner. Binding studies indicated that these MAbs recognize a similar epitope on AT and exhibit dissociation constants ($K_D$) ranging from 0.50 to 15 nM. In an *S. aureus* dermonecrosis model, mice passively immunized with anti-AT inhibitory MAbs exhibited significant reductions of lesion size relative to mice treated with an irrelevant IgG control. Interestingly, there was a correlation between MAb affinity for a single epitope, the 50% inhibitory concentration ($IC_{50}$) in the AT hemolytic assay, and lesion size reduction in the dermonecrosis model. A representative high-affinity MAb, 2A3.1, was demonstrated to significantly reduce lesion size following infection with three different clinical isolates (USA300, CC30, and CC5). Taken together, these results indicate that in vitro potency of anti-AT MAbs predicts in vivo potency in this model, supporting their continued preclinical evaluation as molecules for immunoprophylaxis against staphylococcal skin and soft tissue infections caused by diverse clinical isolates.

Regardless of origin (the community or hospital), methicillin-resistant *Staphylococcus aureus* (MRSA) infections are often difficult to treat, due in part to the limited availability of safe and effective antibiotics. Treatment is further complicated by an increase in the level of vancomycin tolerance in *S. aureus* strains following vancomycin therapy (10). The difficulty of treatment and the slowed progress in the identification of new antibiotics necessitate the development of new approaches to antibacterial prophylaxis and therapy (20, 26, 32). One alternate strategy being explored is the use of monoclonal antibodies (MAbs) directed against a surface determinant on the pathogen and/or virulence factors produced by the invading pathogen to be used in prophylaxis or as adjunctive therapy with antibiotics (13, 14, 22, 30, 36). Antigens for which there are promising preclinical data on the use of MAbs against *S. aureus* include IsaA, IsdB, ClfA, and alpha toxin (AT). Antibodies against IsaA, IsdB, and ClfA bind to the surface of bacteria and can limit disease by either promoting opsonophagocytic killing (OPK) or inhibiting the function of the targeted protein (e.g., fibrinogen binding or heme acquisition). In contrast, passive immunization with MAbs against a soluble protein toxin such as AT does not target the bacteria directly but acts by inhibiting a virulence mechanism important for staphylococcal disease (30). Toxins as targets for immunoprophylaxis have been successful for decades as part of vaccines or passive immunotherapy against bacterial diseases such as diphtheria, tetanus, and botulism (1, 8, 34). Such an approach targeting AT may be possible for *S. aureus* as well.

AT is a cytolysic pore-forming toxin that is conserved among *S. aureus* clinical isolates and has been shown to play a role in pneumonia, dermonecrosis, endocarditis, and sepsis (3, 5, 17, 18). AT is secreted as a 33-kDa soluble monomeric protein that binds to ADAM10 on cell membranes (38). After binding, AT undergoes a conformational change resulting in the formation of a heptameric transmembrane β-barrel leading to cell lysis, inflammation, and tissue damage (2, 9, 28, 38). At sublytic concentrations, AT activates ADAM10 metalloprotease activity on epithelial cells, leading to cleavage of E-cadherin and disruption of the epithelial barrier (16). Inhibition of AT function could therefore limit *S. aureus*-associated pathology and disease. Targeting of AT for prophylaxis of *S. aureus* infections was reported as early as the mid-1900s, with limited success (11, 27, 35). More recently, passive and active immunization against AT has been shown to afford protection in murine models of pneumonia, dermonecrosis, and sepsis, validating its potential as a target for immunoprophylaxis against these infections (6, 17, 24, 30). Herein we describe the identification of anti-AT MAbs that are potent inhibitors of AT function and provide protection against diverse *S. aureus* isolates in a murine dermonecrosis model. Their in vitro 50% inhibitory concentrations ($IC_{50}$) and binding affinities correlate with their potency in the murine dermonecrosis model, suggesting that potency is linked to affinity for these MAbs. Taken together, these results support the continued investigation of potent, high-affinity AT MAbs for the prevention of staphylococcal skin and soft tissue infections.
MATERIALS AND METHODS

Bacterial strains. Strains NRS382 (USA100, clonal complex 5 [CC5]) and NRS261 (CC30) were obtained from the Network on Antibiotic Resistance (NARSAR). Strains Wood (ATCC 10832) and PFR3757 (BAA-1556; USA300) were obtained from the American Type Culture Collection (ATCC). SF8300 (USA300) was generously provided by Binh Diep (University of California at San Francisco).

Cloning and expression of wild-type S. aureus AT and nonhemo-
lytic H35L mutant. The wild-type AT gene (hla) was amplified from S. aureus USA300 PFR3757 (BAA-1556; ATCC) genomic DNA by PCR using the primers ATATATGAGCTCGAGATCTGATATAATTTA AAACC and ATATATAAGCTTTTTCTCTTTTTCTTCTTCTTTCC and Hercule II polymerase (Agilent Technologies). The resultant fragment was digested with SacI and HindIII and ligated into the pGold II DNA vector (TaKaRa Bio USA) in frame with an N-terminal 6×His tag. The H35L mu-
tant (AT1513L) was constructed by site-directed mutagenesis of the wild-type gene, using a QuikChange II XL site-directed mutagenesis kit (Agilent Tech-
nologies) and the following mutagenic primers: GATAAGAAAAATTGGGCA TGCTCAAAAAAGTTTTATAGTTTATAGC and GATAAAACATATAA AATACCTTTTTGACAGGCTATTTCTTTATC. The sequences of wild-type AT and the AT1513L mutant were confirmed by automated DNA sequencing. Expression of the wild type and the H35L mutant was accom-
plished with the BL21 strain of Escherichia coli as the host. A 50-ml over-
night culture grown in LB plus carbenicillin was diluted 1:10 in a 500-ml culture and grown at 37°C to an A600 of 0.5. The culture was shifted to 15°C for 30 min, and IPTG (isopropyl-
D-β-D-thiogalactopyranoside; Invit-
rogen) was added to achieve a final concentration of 100 mM. The culture was incubated for an additional 24 h at 15°C. Cells were harvested by centrifugation and stored at −20°C.

Purification of rAT-His. Bacterial cell pellets were thawed on ice and resuspended in Ni-nitrilotriacetic acid (Ni-NTA) buffer A (20 mM so-
dium phosphate [Sigma], pH 7.2, 300 mM NaCl [Sigma]). Cells were lyzed in a microfluidizer (Microfluidics model M-110P) at 20,000 lb/in², and the crude lysate was clarified by centrifugation at 27,000 × g for 10 min at 4°C. Following 0.2-μm filtration, the supernatant was loaded onto a 5-ml Ni-NTA Superflow column (Qiagen) equilibrated with Ni-NTA buffer A. Recombinant, His-tagged alpha toxin (rAT-His) was eluted with a 300 and 500 mM imidazole step gradient (into tubes containing EDTA at a final concentration of 1 mM) and dialyzed into SP buffer A (50 mM sodium phosphate, pH 7.0, 25 mM NaCl, 1 mM EDTA [Sigma]). Dialsyates were loaded onto a 5-ml HiTrap SP Sepharose FF column (GE Healthcare) in SP buffer A, and rAT-His was eluted with a step gradient to 1 M NaCl. Fractions containing rAT-His were dialyzed into 1× phosphate-buffered saline (PBS), pH 7.2, with 1 mM EDTA, and aliquots were frozen at −80°C. The same methods were employed to purify rAT1513L-His.

Purification of nAT from S. aureus. S. aureus Wood was grown over-
night with shaking (250 rpm) at 37°C in tryptic soy broth (TSB). Culture supernatant was harvested by centrifugation and then brought to 75% saturation with solid ammonium sulfate. After stirring for 3 h at 4°C, the precipitate was captured by centrifugation at 12,000 × g for 45 min, res-
suspended in SP buffer A2 (25 mM sodium acetate, pH 5.2, 20 mM NaCl, 1 mM EDTA), and dialyzed against SP buffer A2 overnight at 4°C, with one exchange. The soluble dialysate was filtered (0.2 μm) and loaded onto a 10-ml SP Sepharose FF column (GE Healthcare) equilibrated with SP buffer A2. Bound native alpha toxin (nAT) was eluted with a linear gra-
dient to 0.3 M NaCl, followed by steps to 0.5 and 1 M NaCl. Fractions containing nAT were pooled and dialyzed overnight into PBS, pH 7.2, containing 1 mM EDTA. For final polishing, the dialysate was loaded onto a HiPrep Sephacryl S-200 high-resolution column (GE Healthcare) at a flow rate of 1.3 ml/min in 1× PBS, pH 7.2, with 1 mM EDTA. Fractions containing nAT were pooled and stored at −80°C.

Immunization and hybridoma generation. All in vivo work was re-
viewed and approved by our Institutional Animal Care and use commit-
tee. Eight-week-old VelocImmune mice received 5 rounds of subcutane-
ous injections of rAT1513L-His at multiple sites, following the RIMMS immunization protocol (19). Mice were immunized over a course of 13 days at intervals of 2 to 3 days. For each round of immunization, rAT1513L-His was emulsified in complete Freund’s adjuvant or incomplete Freund’s adjuvant plus TiterMax Gold (Sigma) adjuvant and injected bilaterally at the nape of the neck, axilla, calf, and groin. Test bleeds were collected on day 13 and assayed in an antigen enzyme-linked immunosorbent assay (ELISA). On day 17, mice were given a preboost focus intraperitoneally. Lymph node lymphocytes and splenocytes were fused with a myeloma partner (P3X63) to generate stable hybridomas (20).

Expression and purification of chimeric anti-AT Mabs. Clarified murine hybridoma supernatants (~5 liters at 30 to 50 mg/liter) were concentrated by tangential-flow filtration. The concentrated supernatants were then passed over five 5-ml protein G HiTrap HP columns (GE Healthcare) in sequence, and the bound IgG was eluted with 50 mM sodium bicarbonate, pH 11.0, and neutralized to −ph 7.0 with 1 M phospho-
ric acid. The neutralized material was loaded onto two 1-ml HiTrap Q FF (GE Healthcare) columns in sequence. The IgG-containing flow-
through was collected and dialyzed into PBS, pH 7.2.

Neutralization of hemolytic activity. Anti-AT Mabs were added to a 96-well plate at 80 μg/ml in PBS and the samples serially diluted (2-fold) in PBS to a final volume of 50 μl. A nonspecific human IgG1, R347 (anti-
HIV gp120), was included as a control throughout these studies. Twenty-five microliters of each MAb dilution was mixed with 25 μl of rAT-His in a 96-well round-bottom plate, followed by the addition of 50 μl 5% rabbit red blood cells (RBC; PelFreeze Biological) in PBS, for a final rAT-His concentration of 0.1 μg/ml. Control wells contained RBC with or without AT. Plates were incubated for 1 h at 37°C, the intact cells were pelleted by centrifugation, 50-μl supernatants were transferred to a new 96-well plate, and the A50 was measured in a spectrophotometer (Molecular De-
vices). Neutralizing activity was calculated relative to lysis with RBC plus rAT-His alone, as follows: % inhibition = 100 − [(A50 nAT + MAB)/[(A50 nAT, no MAB)]].

Neutralization of A549 and THP-1 cell lysis. THP-1 and A549 cells were maintained in RPMI medium (In vitrogen) supplemented with non-
esential amino acids, 2 mM glutamine, and 10% fetal bovine serum in a 5% CO2, 37°C incubator. Anti-AT Mabs were added to a 96-well plate at 80 μg/ml in RPMI medium, and the samples were serially diluted (2-fold) in RPMI medium. A nonspecific IgG, R347, was included as a negative control. Twenty-five microliters of each MAb dilution was mixed with 25 μl nAT (final concentrations for THP-1 and A549 cells, 1.5 and 20 μg/ml, respectively), followed by the addition of 50 μl RPMI medium-washed cells (10 cells/ml in RPMI medium with 10% fetal bovine serum [FBS]) in a 96-well plate, for a final volume of 100 μl. Control wells of cells with and without nAT were included. Plates were incubated at 37°C with 5% CO2, for 3 h and then centrifuged, and 50 μl of supernatant was transferred to a new 96-well plate. Cell lysis was measured as the release of lactate dehydrogenase (LDH), using a Cytotox 96 nonradioactive assay kit (Pro-
mega) following the manufacturer’s protocol. Background LDH was sub-
tracted from each well, and the inhibition of LDH release was calculated as follows: % inhibition = 100 − [(A50 nAT + MAB)/[(A50 nAT, no MAB)]].

Erythrocyte ghost receptor binding and oligomerization. Erythrocyte ghosts were prepared by incubating 5 ml of washed and packed RBC in 500 ml of lysis buffer (5 mM phosphate, 1 mM EDTA, pH 7.4) over-
night at 4°C, with constant stirring (31). The ghosts were removed by centrifugation at 15,000 × g, washed three times with lysis buffer, washed in PBS, and resuspended in a final volume of 3 ml PBS.

To assess binding of nAT to cell membranes, RBC ghosts were diluted to an optical density at 600 nm (OD600) of approximately 0.2 in PBS, and 50 μl was used to coat 1/2-well 96-well flat-bottom plates (Costar) and incubated overnight at 4°C. The liquid was removed from the plates, and the wells were blocked with 100 μl of 1% bovine serum albumin (BSA) in PBS, pH 7.4, for 2 h at 4°C and then washed three times with PBS. A 10-fold molar excess of IgG was mixed with nAT at 3 μg/ml, and 50 μl...
was added to the blocked plates. The plates were incubated at 4°C for 2 h and then washed three times with PBS. Biotinylated rabbit anti-AT IgG was added to the wells at 1 μg/ml, incubated at 4°C for 1 h, washed three times, and incubated for 1 h at 4°C with streptavidin peroxidase conjugate (1:30,000; Jackson ImmunoResearch). The wells were washed three times and developed with Sure Blue Reserve (KPL, Inc.). The A_{405} was read using a plate reader (Molecular Devices), and the % AT bound was calculated as follows: % AT bound = 100 × (A_{405} AT + IgG/A_{405} AT0).

Inhibition of AT heptamer formation was measured by mixing purified IgG with purified nAT (0.5 μg) at a 5:1 molar ratio, along with 5 μl RBC ghosts and PBS to a final volume of 22 μl, and incubating the reaction mix at 37°C for 45 min. The samples were then solubilized in 5 μl SDS-PAGE sample buffer (Invtrogen) for 5 min at 37°C, and 10 μl was subjected to SDS-PAGE on a 4 to 12% precast polyacrylamide gel (Invitrogen). The separated proteins were then transferred to nitrocellulose, blocked for 10 min with casein blocker in PBS (Thermo Scientific), and probed with rabbit anti-AT IgG (2 μg/ml) for 2 h at room temperature with constant shaking. The AT bands were detected following a 1-h incubation with an alkaline phosphatase-labeled goat anti-rabbit IgG (Caltag Laboratories) and developed with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (BCIP/NBT) membrane phosphatase substrate system (KPL, Inc.).

**Measurement of kinetic rate and binding constants.**

Kinetic rate constants (k_{on} and k_{off}) for binding of the anti-AT IgGs to purified nAT were measured by employing an IgG capture assay on a BIAcore 3000 instrument. Rat anti-mouse IgG was immobilized on a CM5 sensor chip with a final surface density of ~2,500 resonance units (RU). A reference flow cell surface was also prepared on this sensor chip by use of the identical immobilization protocol, but without nAT. Anti-AT IgGs were prepared at 20 nM in instrument buffer (HBS-EP buffer; 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% P-20), along with 2-fold serial dilutions of nAT (0.78 nM to 50 nM) in instrument buffer. A sequential approach was utilized for kinetic measurements. Each anti-AT IgG was first injected over the capture and reference surfaces, at a flow rate of 50 μl/min. Once the binding of the captured IgG stabilized, a single concentration of the nAT protein was injected over both surfaces, at a flow rate of 50 μl/min. The resulting binding response curves yielded the association phase data. Following the injection of nAT, the flow was then switched back to instrument buffer for 10 min to permit the collection of dissociation phase data, followed by a 1-min pulse of 10 mM glycine, pH 1.5, to regenerate the IgG capture surface on the chip. Binding responses from duplicate injections of each concentration of nAT were recorded against all anti-AT IgGs. In addition, several buffer injections were interspersed throughout the injection series. Select buffer injections were used along with the reference cell responses to correct the raw data sets for injection artifacts and/or nonspecific binding interactions, commonly referred to as “double referencing” (26). Fully corrected binding data were then globally fit to a 1:1 binding model (BIAevaluation 4.1 software; BIAcore, Inc.) that included a term to correct for mass transport-limited binding, should it be detected. These analyses determined the kinetic rate constants k_{on} and k_{off} from which the apparent dissociation constant (K_{D}) was calculated as k_{off}/k_{on}.

**Epitope competition experiments.**

Epitope competition assays were performed with a ForteBio Octet 384 instrument using streptavidin sensors and 384-well slanted-bottom plates (ForteBio, Menlo Park, CA). All reagents were diluted in ForteBio kinetics buffer (PBS plus 0.002% Tween 20 plus 0.1 mg/ml BSA). Native AT, biotinylated with a FluorReporter mini-biotin–XX protein labeling kit (Invitrogen) and retaining full hemolytic activity, was captured at 1 μg/ml, and then MAb 1 (500 nM) was allowed to bind. Finally, binding was measured with MAb 2 (500 nM) in combination with MAb 1, and 500 nM, so as to maintain MAb 1 equilibrium. All steps were performed for 400 s at 1,000 rpm, with a 3-mm sensor offset and a 0.6-Hz sensitivity.

The final MAb 2-plus-MAb 1 binding step was analyzed using ForteBio’s analysis software to determine R equilibrium (change in nanometers [nm] at equilibrium). Percent relative binding was calculated using the following formula: % binding = [(MAb 2 + MAb 1 binding – 0% control binding)/(100% MAb 1 control binding – 0% control binding)) × 100. The 100% and 0% controls were the binding of the R347 negative-control MAb and MAb 1, respectively, followed by R347 plus MAb 1, both at 500 nM. For the five MAbs (four anti-AT MAbs plus R347 negative control), all possible combinations of MAb 1 and MAb 2 were tested, and the assay was repeated three times.

**Murine dermatomycosis model.** Groups of five 6- to 8-week-old female BALB/c mice (Harlan) were shaved on the back and administered 0.5 ml IgG by intraperitoneal injection at the concentrations indicated. Twenty-four hours later, the mice were infected with 50 μl of a bacterial suspension (1 × 10^6 CFU S. aureus Wood, NRS382, NRS261, or SF8300 in PBS) by intradermal injection. The animals were monitored twice daily for signs of infection, and abscess size was measured once daily. The areas of the lesions were calculated using the following formula: area = length × width. Statistical significance was determined using analysis of variance and Dunnert’s posttest.

**RESULTS**

**Monoclonal antibody generation.** Anti-AT MAbs were generated in VelocImmune mice immunized with His-tagged, recombinant, nonhemolytic ATH35L (rAT-H35L–His). Hybridomas produced from VelocImmune mice express human-mouse chimeric antibodies with fully human variable regions and murine constant domains. These chimeric antibodies are easily converted to fully human IgG by genetic replacement of the murine sequences with human constant domains (4). Initially, >1,800 hybridoma supernatants were found to contain IgGs that bound rAT-His by anti-GEN ELISA. The hybridoma supernatants which exhibited binding to rAT-His were screened for inhibition of rAT-His-mediated lysis of rabbit RBC, whereby the pool of hybridomas expressing functional IgGs was reduced to ~250. The hybridoma supernatants were then normalized for IgG levels and their inhibitory activities compared. The 12 most potent rAT-His inhibitors were selected for limited dilution cloning and small-scale IgG expression and purification.

**Inhibition of cytolytic activity.** The inhibitory activities of the 12 purified anti-AT IgG MAbs were compared in a hemolytic assay. The MAbs were each titrated from 80 to 0.07 μg/ml in the presence of constant amounts of nAT and RBC. Surprisingly, only 6 (2A3.1, 10A7.5, 11D12.1, 12B8.19, 15B6.3, and 28F6.1) of the 12 purified MAbs inhibited nAT-mediated RBC lysis (Fig. 1). All 6 inhibitory MAbs were determined by ELISA to be IgG1, with the exception of 15B6.3, which was IgG3 (data not shown). The 6 MAbs that did not inhibit nAT lysis were confirmed by ELISA to retain binding to rAT-His but not to nAT or another 6×His-tagged protein (data not shown). These results suggest that the IgGs that were inactive on nAT bound an epitope unique to rAT-His. Of the antibodies capable of binding nAT, three (2A3.1, 10A7.5, and 12B8.19) were found to be potent inhibitors and exhibited ~80% inhibition of nAT-mediated RBC lysis at a 1:1 (IgG: AT) molar ratio, indicating that these MAbs inhibit pore formation in rabbit RBC and that the interaction is nearly stoichiometric.

AT has been reported to lyse a variety of mammalian cell types in addition to RBC (e.g., platelets, monocytes, and epithelial and endothelial cells). In fact, it has been proposed that one or more of these cell types, not RBC, may be the primary target for AT lysis in human S. aureus infections (7, 15, 38). Therefore, we tested the purified MAbs for the ability to prevent nAT-mediated lysis of the
human cell lines A549 (alveolar epithelial cell line) and THP-1 (monocytic cell line). Again, the MAbs were titrated against a constant level of nAT, giving ~90% lysis of A549 or THP-1 cells, and cell lysis was quantified by LDH release. The MAbs which inhibited rabbit RBC lysis also inhibited nAT-mediated lysis of both human A549 and THP-1 cells (Fig. 2A and B), with the exception of 11D12.1, which had no effect on nAT-mediated lysis of THP-1 cells.

**Inhibitory MAbs block formation of SDS-resistant heptamer.** AT lyases cells in a multistep process whereby a soluble monomeric AT molecule binds to a cell surface receptor, oligomerizes into a heptameric prepore on the cell surface, and undergoes a conformational change leading to formation of a 14-stranded transmembrane β-barrel (33). Experiments were conducted to determine at which step in this mechanism the inhibitory MAbs block AT function. First, the ability of the most potent inhibitors to prevent binding of AT to rabbit RBC ghosts was examined. At a 10-fold molar IgG excess, there was little or no inhibition of nAT binding to rabbit RBC membranes (Fig. 3), indicating that these inhibitory MAbs were not acting at the step of receptor binding.

The effect of anti-AT IgG on AT heptamer formation on erythrocyte membranes was tested next. Following incubation of nAT with erythrocyte ghosts in the presence of a 5-fold molar excess of IgG, heptamer formation was examined by Western blot analysis. The SDS-resistant high-molecular-weight heptamer was readily apparent in the presence of the control MAb (Fig. 4). However, SDS-resistant heptamers were not observed following incubation with any of the four AT MAbs, providing evidence that these inhibitory MAbs prevent AT-mediated cell lysis through a blockade of SDS-resistant heptamer formation.

**Binding kinetics and competition.** As a means to further characterize and possibly differentiate the nAT-inhibitory MAbs, affinity measurements were carried out using surface plasmon resonance (SPR). In these studies, different concentrations of nAT were allowed to bind purified IgGs that were immobilized on sensor chips. The association and dissociation rate constants were measured and used to determine the apparent binding constants. MAbs 2A3.1, 10A7.5, and 12B8.19 had similar affinities, with $K_D$ values of 601, 504, and 485 pM, respectively, whereas 28F6.1 exhibited a $K_D$ value of 13 nM, due largely to a lower $k_{on}$ rate (Table 1).

Epitope competition assays were performed using Octet 384 biolayer interferometry to determine if the inhibitory MAbs compete for binding to biotinylated nAT. In this experiment, biotinylated nAT was immobilized on a streptavidin-coated sensor. A single AT MAb was then bound to the sensor, followed by an equimolar quantity of a second AT MAb, and the % binding of the second MAb was calculated. In Fig. 5, data from a representative epitope competition assay with MAb 2A3.1 are shown. Epitope competition assay results for the four MAbs with the most potent in vitro nAT-inhibitory activities (2A3.1, 10A7.5, 12B8.19, and 28F6.1) are provided in Table 2. Antibodies 2A3.1, 10A7.5, and 12B8.19 prevented each other, and 28F6.1, from binding nAT. The failure of 28F6.1 to inhibit binding of 2A3.1, 10A7.5, or 12B8.19 was most likely the result of the ~20-fold difference in $K_D$ between 28F6.1 and the other three MAbs. At equilibrium, when 28F6.1 vacates an AT binding site, the other three higher-affinity MAbs are able to occupy the vacated site. Overall, the data suggest
reported as means

Binding detected with rabbit anti-AT IgG, and % binding was calculated as follows: %

without a 10-fold molar excess of anti-AT IgG. Binding of nAT was then

and blocked with 1% BSA. The ghosts were then incubated with nAT, with or

erythrocyte ghosts. Ninety-six-well ELISA plates were coated with RBC ghosts

a high-AT-producing strain,

5 mg/kg of body weight, 24 h prior to intradermal challenge with

istered by intraperitoneal injection to groups of 5 mice, at a dose of

inhibitors could also limit the severity of a staphylococcal skin

of staphylococcal disease (17). To determine if our potent AT

passive delivery of AT-specific immune serum limits the severity

tissue infections in mice, and active immunization with AT or

crotic lesions.

that the four potent AT MAbs bind similar, overlapping epitopes

on AT.

Passive immunization with anti-AT MAbs reduces dermonecrotic lesions. AT has been shown to play a role in skin and soft

tissue infections in mice, and active immunization with AT or

passive delivery of AT-specific immune serum limits the severity

of staphylococcal disease (17). To determine if our potent AT inhibitors could also limit the severity of a staphylococcal skin

infection, anti-AT or the irrelevant R347 IgG control was admin-

istered by intraperitoneal injection to groups of 5 mice, at a dose of

5 mg/kg of body weight, 24 h prior to intradermal challenge with

a high-AT-producing strain, S. aureus Wood. The sizes of the dermonecrotic lesions were measured daily and documented photo-

graphically. The four most potent in vitro inhibitors of nAT func-

tion, 2A3.1, 10A7.5, 12B8.19, and 28F6.1, significantly reduced

lesion size relative to that with the R347 control, whereas the least

potent MAbs in vitro (11D12.1 and 15B6.3) did not (Fig. 6A

and B).

To further differentiate these MAbs, 2A3.1, 10A7.5, 12B8.19, and 28F6.1 were titrated in the dermonecrosis model (5 mg/kg to

0.05 mg/kg) (Fig. 7), and the concentration required for 50% ef-

ficacy (EC50) 1 day after S. aureus challenge was determined (Table

3). The EC50s for 2A3.1, 10A7.5, and 12B8.19 were calculated to be

9- to 11-fold lower than that for 28F6.1. Table 3 shows a compari-

son of the in vivo EC50s for these MAbs, their IC50s determined in

the RBC lysis assay, and their calculated binding affinities for nAT.

Interestingly, 2A3.1, 10A7.5, and 12B8.19 exhibited 9- to 14-fold

differences in both in vivo and in vitro potencies relative to 28F6.1,

correlating with their relative binding affinities. Thus, for this

group of AT-specific MAbs, binding affinity for AT is predictive of

both in vitro and in vivo anti-AT activity.

The in vivo studies described above were all carried out using a

single strain of S. aureus (Wood). To test whether the anti-AT

MAbs could limit disease caused by more clinically relevant iso-

lates, groups of 5 mice were treated with 5 mg/kg 2A3.1 or control

MAb (R347) 24 h prior to intradermal infection with 1e8 CFU of

NRS382 (USA100; CC5), SF8300 (USA300), or NRS261 (CC30).

Prophylaxis with 2A3.1 resulted in a significant reduction (P <

0.0001) in lesion size over the course of the 6-day experiment for
each infecting strain, indicating that the potency of this MAb, and
likely others with similar epitope specificities, is not limited to S.

aureus Wood and this MAb could potentially provide protection

da against disease caused by diverse clinical isolates (Fig. 8).

Anti-AT MAbs with Efficacy against S. aureus

TABLE 1 Association and dissociation rate constants and apparent

binding constants for MAbs in this studya

| IgG     | k_{on} (1/M-s) | k_{off} (1/s) | K_D (nM) | χ²  |
|---------|---------------|---------------|----------|-----|
| 2A3.1   | 13.7          | 8.21          | 0.601    | 0.433 |
| 10A7.5  | 6.92          | 3.49          | 0.504    | 0.345 |
| 12B8.19 | 5.75          | 2.78          | 0.485    | 0.288 |
| 28F6.1  | 0.67          | 8.81          | 13.1     | 0.906 |

a Association (k_{on}) and dissociation (k_{off}) rate constants were measured using a BIAcore instrument, and the apparent binding constants (K_D) were calculated as k_{off}/k_{on}.

Anti-AT Epitope Competition

FIG 5 Epitope competition assays. Biotinylated AT was captured on strepta-

vidin sensors (step not shown), MAb 2A3.1 (500 nM) was allowed to bind and

come to equilibrium, and then a second MAb (500 nM) was introduced while

maintaining 2A3.1 at 500 nM. 100% control = R347 followed by 2A3.1 plus

R347; 0% control = 2A3.1 followed by 2A3.1 plus R347. R equilibrium was fit

with Octet analysis software, and percent binding was calculated as follows: %

binding = [(MAb 2 + MAb 1 binding − 0% control binding)/(100% MAb 1

control binding − 0% control binding)] × 100. Results are representative of 3

independent experiments.

FIG 4 AT heptamer formation is inhibited by MAbs. AT was incubated with

erythrocyte ghosts at 37°C in the presence or absence of anti-AT IgG. The

samples were then solubilized in SDS-PAGE, and heptamer formation was detected by Western blot analysis.

FIG 3 Anti-AT MAbs do not inhibit nAT binding to its receptor on rabbit

erythrocyte ghosts. Ninety-six-well ELISA plates were coated with RBC ghosts

and blocked with 1% BSA. The ghosts were then incubated with nAT, with or

without a 10-fold molar excess of anti-AT IgG. Binding of nAT was then

detected with rabbit anti-AT IgG, and % binding was calculated as follows: %

without a 10-fold molar excess of anti-AT IgG. Binding of nAT was then

and blocked with 1% BSA. The ghosts were then incubated with nAT, with or

without a 10-fold molar excess of anti-AT IgG. Binding of nAT was then

detected with rabbit anti-AT IgG, and % binding was calculated as follows: %

without a 10-fold molar excess of anti-AT IgG. Binding of nAT was then

detected with rabbit anti-AT IgG, and % binding was calculated as follows: %

without a 10-fold molar excess of anti-AT IgG. Binding of nAT was then

detected with rabbit anti-AT IgG, and % binding was calculated as follows: %

without a 10-fold molar excess of anti-AT IgG. Binding of nAT was then
DISCUSSION

This report describes the characterization of anti-AT MAbs generated from VelocImmune mice that inhibit AT-mediated lysis, reduce lesion size in a murine model of staphylococcal dermonecrosis, and exhibit a correlation between affinity and in vitro and in vivo potency. Following immunization with rAT H35L-His, anti-AT IgGs from B-cell hybridoma supernatants were screened and triaged based on the ability to inhibit rAT-His-mediated rabbit RBC lysis. Surprisingly, six of the selected MAbs exhibited no AT-neutralizing activity when nAT instead of rAT-His was used in the RBC lysis assay. This was unexpected, since His-tag-specific antibodies were screened out by removing those that bound to an unrelated His-tagged protein. The fact that some of the antibodies bound to rAT-His and not to nAT illustrates the importance of using either native or untagged recombinant proteins, when possible, to generate a functional antibody response. Six MAbs were identified as exhibiting a 100-fold range of potencies in vitro, inhibiting AT-mediated lysis of rabbit RBC, human alveolar epithe-

TABLE 2 Percent relative binding in epitope competition assays

| 1st MAb | 2A3.1 | 10A7.5 | 12B8.19 | 28F6.1 | R347 (0% control) |
|---------|--------|--------|---------|--------|------------------|
| 2A3.1   | 2.2 ± 2.8 | 3.7 ± 1.8 | 3.6 ± 2.1 | 4.8 ± 2.3 | 0                |
| 10A7.5  | 1.8 ± 4.8 | −2.1 ± 3.1 | 1.8 ± 4.1 | 1.2 ± 4.2 | 0                |
| 12B8.19 | 2.7 ± 2.7 | 1.8 ± 3.3 | 1.4 ± 1.2 | 3.1 ± 3.5 | 0                |
| 28F6.1  | 63.0 ± 2.7 | 62.4 ± 4.9 | 66.9 ± 7.5 | 0.9 ± 4.2 | 0                |
| R347 (100% control) | 100 | 100 | 100 | 100 |

Data are means ± standard deviations for three replicate assays.

FIG 6 Passive immunization with the most potent inhibitory MAbs reduces severity of dermonecrosis. Groups of 5 BALB/c mice were passively immunized with anti-AT MAbs (0.05 to 5 mg/kg) and infected with S. aureus Wood, and lesion size was monitored for 6 days. (A) Photographs of lesion sizes at 6 days postinfection. (B) Lesion sizes over the time course of infection (lesion size area = length × width). Statistical significance was determined using analysis of variance and Dunnett’s posttest.

FIG 7 Lesion size comparison for the most potent MAbs. Groups of 5 BALB/c mice were passively immunized with anti-AT MAbs (0.05 to 5 mg/kg) and infected with S. aureus Wood, and the lesion size was measured 24 h after infection. The means ± SD for three experiments were plotted, and the EC_{50}s were determined using GraphPad Prism.
lial cells (A549), and human monocytic cells (THP-1). The three most potent MAbs exhibited similar activities, resulting in \( \frac{80\%}{\text{H11011}} \) reductions in nAT-mediated RBC lysis at a 1:1 MAb:toxin molar ratio, indicating that the mode of action is likely stoichiometric in nature. The four MAbs with the greatest \textit{in vitro} anti-AT activities were shown to bind with high affinity to overlapping/shared epitopes on nAT. Thus, it is not surprising that all of these MAbs act through a similar mechanism, namely, inhibition of SDS-resistant AT heptamer formation. Potent \textit{in vitro} activity was mirrored by \textit{in vivo} prophylactic potency, as indicated by reduced skin lesion size in an \textit{S. aureus} murine dermonecrosis model using various clinical isolates, including USA300 (SF8300), an \textit{S. aureus} strain for which there is some debate about the virulence determinants involved in disease. Our observations further substantiate AT’s role in staphylococcal skin and soft tissue infections and exemplify its potential as a therapeutic target. In a recent publication, Kennedy et al. reported that active and passive immunization (using AT-specific immune sera) against AT reduces lesion size in a dermonecrosis model \cite{17}. Our findings support these results and demonstrate that reductions in disease severity for infections with diverse clinical isolates can be accomplished with a single high-affinity MAb in a murine dermonecrosis model.

The fact that 2A3.1, 10A7.5, 12B8.19, and 28F6.1 bind overlapping epitopes provides an opportunity for comparison of the potencies of the individual MAbs relative to their binding affinities. This comparison could not be made if they bound different epitopes, since it would be difficult to differentiate the impacts of affinity and epitope on the MAb-mediated toxin inhibition. 28F6.1 exhibits a \( K_D \) of 13 nM and was substantially less potent in the cell lysis assays and the murine dermonecrosis model than 2A3.1, 10A7.5, and 12B8.19, each with a \( K_D \) of \( \sim 500 \) pM. This lower affinity is primarily the result of a \( \sim 10\)-fold lower \( k_{\text{on}} \) rate for 28F6.1 than for 2A3.1, 10A7.5, and 12B8.19. Comparisons of apparent affinity to the IC\(_{50}\) for RBC lysis and the EC\(_{50}\) in the dermonecrosis model indicated a correlation between affinity and potency for these MAbs. Two anti-AT MAbs were previously identified and characterized by Ragle and Bubeck Wardenburg \cite{30}. These MAbs were murine IgGs and functioned as potent inhibitors of AT function \textit{in vitro} and in a murine pneumonia model. They exhibited similar affinity constants for AT, thus precluding a similar comparison between affinity and potency. One example where affinity correlated with antitoxin potency was reported for MAbs against staphylococcal enterotoxin B (SEB); however, the potencies of the different MAbs were not examined \textit{in vivo} \cite{12}. The number of MAbs compared in our study and the Drozdowski study \cite{12} is small, yet the results provide support for the hypothesis that in some instances an increase in affinity will result in increased potency.

This study provides further evidence for the role of AT in \textit{S. aureus} skin infections and a rationale for anti-AT IgG as an immunotherapeutic for staphylococcal disease. Although there is only one anti-infective MAb currently approved for use in humans, there are other MAbs targeting pathogens being tested in

| MAb     | \( K_D \) (nM) | Mean IC\(_{50}\) for RBC hemolysis (mg/ml) | Mean EC\(_{50}\) for dermonecrosis (mg/kg) |
|---------|--------------|---------------------------------|----------------------------------|
| 2A3.1   | 0.60         | 0.30                            | 0.107                            |
| 10A7.5  | 0.50         | 0.30                            | 0.088                            |
| 12B8.19 | 0.48         | 0.44                            | 0.082                            |
| 28F6.1  | 13.1         | 4.15                            | 0.906                            |

\( a \) The differences in \( K_D \), mean IC\(_{50}\), and mean EC\(_{50}\) between 28F6.1 and the other three MAbs were 22- to 27-fold, 9- to 14-fold, and 9- to 11-fold, respectively.

FIG 8 Anti-AT MAb reduces disease severity caused by diverse clinical isolates. Groups of 5 BALB/c mice were passively immunized with 5 mg/kg of 2A3.1 or R347 and infected with either \textit{S. aureus} NRS261, NRS382, or SF8300, and lesion size was monitored for 6 days. (Top) Lesion sizes over the time course of infection (lesion size area = length \( \times \) width). Lesion sizes in the 2A3.1-treated animals were significantly reduced at all time points compared to those in R347-treated animals, with \( P \) values of \( <0.0001 \). Statistical significance was determined using analysis of variance and Dunnett’s posttest. (Bottom) Photographs of lesion sizes at 6 days postinfection.
human clinical trials (21, 23, 25, 37). These anti-infective MAbs, which are specific for pathogen and not human antigens, are anticipated to have relatively few off-target toxicities. In addition, the long serum half-life of human IgG ( ~20 days) offers an advantage over small-molecule antibacterials in a prophylactic setting. Passive administration of MAbs also offers certain advantages over active vaccination for protection against disease caused by *S. aureus*. Unlike active vaccination, passive immunophylaxis does not require an active immune response and thus could be utilized in a population of persons with an impaired immune system. In addition, unlike the case with vaccines, the protective qualities of passively administered MAbs are available immediately. MAbs also have a high specific activity, since every molecule in the preparation functions as a potent inhibitor. In contrast, not all antibodies in a polyclonal response elicited by a vaccine are active, which could have contributed in part to the somewhat limited success of earlier attempts at anti-AT active and passive (immune serum) immunization (11, 27, 35). This is exemplified by the fact that the initial pool of >1,800 AT-reactive hybridoma supernatants generated via vaccination of VeloImmune mice with rAT-His contained only 250 (14%) with any AT-inhibitory activity and that only 12 (0.6%) exhibited potent inhibition of AT-mediated lysis. This may have been due in part to low expression levels by some of the hybridomas; however, it is more likely because the majority of the IgGs elicited during vaccination target noninhibitory epitopes on AT or are of low affinity and do not function as potent inhibitors. Thus, the features embodied in a highly potent, fully human anti-AT IgG1 might provide an addition to the available antibacterial armamentarium for *S. aureus* disease prevention and therapy.

**ACKNOWLEDGMENTS**

We thank J. Suzich and M. McCarthy for critical reviews of the manuscript and J. Hilliard for assistance with figures.

**REFERENCES**

1. Arnon SS, Schechter R, Maslanka SE, Jewell NP, Hatheway CL. 2006. Human botulism immune globulin for the treatment of infant botulism. N. Engl. J. Med. 354:462–471. doi:10.1056/NEJMoa051926.
2. Bartlett AH, Foster TJ, Hayashida A, Park PW. 2008. Alpha-toxin facilitates the generation of CXC chemokine gradients and stimulates neutrophil homing in Staphylococcus aureus pneumonia. J. Infect. Dis. 198:1529–1535. doi:10.1086/592758.
3. Bayer AS, et al. 1997. Hyperproduction of alpha-toxin by Staphylococcus aureus results in paradoxically reduced virulence in experimental endocarditis: a host defense role for platelet microbial proteins. Infect. Immun. 65:4652–4660.
4. Boulliane GL, Hozumi N, Shulman MJ. 1984. Production of functional chimeric mouse/human antibody. Nature 312:643–646.
5. Bubeck-Wardenburg J, Bae T, Otto M, Deleo FR, Schneewind O. 2006. Pore-forming toxins: a powerful weapon in bacterial pathogenesis. Mol. Microbiol. 60:1–10. doi:10.1111/j.1365-2958.2006.05662.x.
6. Bubeck-Wardenburg J, Schneewind O. 2008. Vaccine protection against Staphylococcus aureus pneumonia. J. Exp. Med. 205:287–294. doi:10.1083/jem.20072208.
7. Cassidy PS, Harshman S. 1973. The binding of staphylococcal 125I-alpha-toxin (B) to erythrocytes. J. Biol. Chem. 248:5343–5346.
8. Centers for Disease Control and Prevention (CDC). 2011. Updated recommendations for use of tetanus toxoid, reduced diphtheria toxoid and acellular pertussis (Tdap) vaccine from the Advisory Committee on Immunization Practices, 2010. MMWR Morb. Mortal. Wkly. Rep. 60:13–15.
9. Craven RR, et al. 2009. Staphylococcus aureus alpha-hemolysin activates the NLRP3 inflammasome in human and mouse monocytic cells. PLoS One 4:e7446. doi:10.1371/journal.pone.0007446.
10. David MZ, Daum RS. 2010. Community-associated methicillin-resistant *Staphylococcus aureus* epidemiology and clinical consequences of an emerging epidemic. Clin. Microbiol. Rev. 23:616–687.
11. Dolman CE. 1934. Staphylococcus antitoxin serum in the treatment of acute staphylococcal infections and toxaemias. Can. Med. Assoc. J. 30:601–610.
12. Drozdzowski B, et al. 2010. Generation and characterization of high affinity human monoclonal antibodies that neutralize staphylococcal enterotoxin B. J. Immune Based Ther. Vaccines 8:9. doi:10.1186/1476-8518-8-9.
13. Ebert T, et al. 2010. A fully human monoclonal antibody to Staphylococcus aureus iron regulated surface determinant B (IsdB) with functional activity in vitro and in vivo. Hum. Antibodies 19:113–128. doi:10.3323/humab.2010-0235.
14. Hall AE, et al. 2003. Characterization of a protective monoclonal antibody recognizing Staphylococcus aureus MSCRAMM protein clumping factor A. Infect. Immun. 71:6864–6870.
15. Hildebrand A, Pohl M, Bhakdi S. 1991. Staphylococcus aureus alpha-toxin. Dual mechanism of binding to target cells. J. Biol. Chem. 266:17195–17200.
16. Inoshima I, et al. 2011. A Staphylococcus aureus pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice. Nat. Med. 17:1310–1314. doi:10.1038/nm2451.
17. Kennedy AD, et al. 2010. Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model. J. Infect. Dis. 202:1050–1058. doi:10.1086/656043.
18. Kernodle DS, Voladri RK, Menzies BE, Hager CC, Edwards KM. 1997. Expression of an antisense hla fragment in Staphylococcus aureus reduces alpha-toxin production in vitro and attenuates lethal activity in a murine model. Infect. Immun. 65:179–184.
19. Kilpatrick KE, et al. 1997. Rapid development of affinity matured monoclonal antibodies using RIMMS. Hybridoma. 16:381–389.
20. Kohler G, Milstein C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497.
21. Lopez EL, et al. 2010. Safety and pharmacokinetics of ertuxizumab, a humanized monoclonal antibody, against Shiga-like toxin 2 in healthy adults and in pediatric patients infected with Shiga-like toxin-producing *Escherichia coli*. Antimicrob. Agents Chemother. 54:239–243. doi:10.1128/AAC.00343-09.
22. Lorenz U, et al. 2011. Functional antibodies targeting IsAa of *Staphylococcus aureus* augment host immune response and open new perspectives for antibacterial therapy. Antimicrob. Agents Chemother. 55:165–173. doi:10.1128/AAC.01144-10.
23. Lowy I, et al. 2010. Treatment with monoclonal antibodies against *Clostridium difficile* toxins. N. Engl. J. Med. 362:197–205. doi:10.1056/NEJMoa0907635.
24. Menzies BE, Kernodle DS. 1996. Passive immunization with antiserum to a nontoxic alpha-toxin mutant from *Staphylococcus aureus* is protective in a murine model. Infect. Immun. 64:1839–1841.
25. Migone TS, et al. 2009. Raxibacumab for the treatment of inhalational anthrax. N. Engl. J. Med. 361:135–144. doi:10.1056/NEJMoa0810601.
26. Myszka DG. 1999. Improving biosensor analysis. J. Mol. Recognit. 12:279–284.
27. Parish HJ, Cannon DA. 1960. *Staphylococcus* infection: antitoxic immunity. Br. Med. J. 1:743–747.
28. Peacock SJ, et al. 2002. Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. Infect. Immun. 70:4989–4996.
29. Projan SJ, Shlaes DM. 2004. Antibacterial drug discovery: is it all downhill from here? Clin. Microbiol. Infect. 10(Suppl 4):18–22. doi:10.1111/j.1465-0969.2004.1006.x.
30. Ragel BE, Bubeck Wardenburg J. 2009. Anti-alpha-hemolysin monoclonal antibodies mediate protection against Staphylococcus aureus pneumonia. Infect. Immun. 77:2712–2718. doi:10.1128/IAI.00115-09.
31. Sellman BR, Kagan BL, Tweten RK. 1997. Generation of a membrane-bound, oligomerized pre-pore complex is necessary for pore formation by *Clostridium septicum* alpha toxin. Mol. Microbiol. 23:551–558.
32. Shlaes DM. 2003. The abandonment of antibacterials: why and wherefore? Curr. Opin. Pharmacol. 3:470–473.
33. Song L, et al. 1996. Structure of staphylococcal alpha-hemolysin, a heptamic transmembrane pore. Science 274:1859–1866.
34. Wagner KS, et al. 2009. A review of the international issues surrounding
the availability and demand for diphtheria antitoxin for therapeutic use. Vaccine 28:14–20. doi:10.1016/j.vaccine.2009.09.094.

35. Weise EC. 1930. Staphylococcus toxin in the treatment of furunculosis. JAMA 95:743–747.

36. Weisman LE, et al. 2009. Safety and pharmacokinetics of a chimerized anti-lipoteichoic acid monoclonal antibody in healthy adults. Int. Immunopharmacol. 9:639–644. doi:10.1016/j.intimp.2009.02.008.

37. Weisman LE, et al. 2009. Phase 1/2 double-blind, placebo-controlled, dose escalation, safety, and pharmacokinetic study of pagibaximab (BSYX-A110), an antistaphylococcal monoclonal antibody for the prevention of staphylococcal bloodstream infections, in very-low-birth-weight neonates. Antimicrob. Agents Chemother. 53:2879–2886. doi:10.1128/AAC.01565-08.

38. Wilke GA, Bubeck Wardenburg J. 2010. Role of a disintegrin and metalloprotease 10 in Staphylococcus aureus alpha-hemolysin-mediated cellular injury. Proc. Natl. Acad. Sci. U. S. A. 107:13473–13478. doi:10.1073/pnas.1001815107.