CHAPTER-10

RESULTS

In a previous study, on the *Acinetobacter baumannii* trimeric autotransporter (Ata) was identified as a surface protein important for biofilm formation, binding to various extracellular matrix proteins and the adhesion of A. baumannii cells to collagen type IV. This Ata protein whose production also contributed to the virulence of *A. baumannii* in a mouse model of lethal systemic infection [131]. In the present study antibodies generated in rabbits to recombinant Ata was used, and investigated for their antibacterial functional activity *in vitro*. Specifically, the functional activity of antibody was tested by testing their ability to inhibit the binding of *A. baumannii* to various extracellular matrix proteins, mediate complement dependent bactericidal and opsonophagocytic killing of *A. baumannii* strains, and protect mice in a model of pneumonia in both immunocompetent and neutropenic mice.

10.1. ANTIBODIES TO ATA BLOCK THE BINDING OF A. BAUMANNII TO COLLAGEN TYPE IV

1. Previous findings showed that purified recombinant Ata binds extracellular matrix protein components and mediates the adhesion of whole cells of *A. baumannii* to collagen type IV [131].

2. Then it was planned to determine whether antibodies to Ata could selectively block the binding of whole cells of *A. baumannii* to collagen type IV. *A. baumannii* ATCC 17978 (wild type), ATCC17978 ata, and ATCC 17978 ata-c strains grown in arabinose or glucose were incubated with antisera to Ata or NRS as a control before addition to collagen type IV coated wells.
3. Incubation of *A. baumannii* strains producing Ata with antibodies to Ata resulted in a dramatic decrease in the binding of these strains to collagen type IV compared to NRS controls (Fig. 10.1A). *A. baumannii* ATCC 17978 or arabinose induced ATCC 17978 *ata*-c incubated with NRS versus anti Ata; (P= 0.0001 and P= 0.0007), respectively (unpaired Student *t* test).

4. The antisera to Ata had no effect on the 17978 *ata*-c strain grown in glucose repressing conditions or on the 17978 *ata* strain that does not produce Ata.

5. The ability of antibodies to Ata to block bacterial binding to collagen type IV coated plates was determined using a range of antiserum dilutions (1:100, 1:1,000, 1:10,000, and 1:100,000).

6. Incubation of *A. baumannii* with antisera to Ata at 1:100, 1:1,000, or 1:10,000 dilutions significantly decreased the adhesion of the bacterial cells to collagen type IV in comparison to NRS (Fig.10.1B; anti Ata antibodies versus NRS; (P=0.0072, 0.0001, and 0.0132, respectively; unpaired Student *t* test), but the adhesion blocking properties were lost at the highest dilution tested i.e. 1:100,000 (Fig. 10.1 B; anti Ata antibodies versus NRS, *P* not significant [ns]; unpaired Student *t* test).

7. In addition to collagen type IV, the adhesion blocking properties of antibodies to Ata was analyzed against other ECM/BM proteins reported to bind Ata, including collagen types I, III, and V and laminin [131]. As shown, incubation of wild type *A. baumannii* with antibodies to Ata diluted 1:100, 1:1,000, 1:10,000, and 1:100,000 resulted in measurable reductions in the binding of this bacteria to all ECM/BM coated plates compared to NRS, but the levels of reduction did not reach statistical significance. Taken together, these results demonstrate that antibodies to Ata block the binding of *A. baumannii* to immobilized collagen type IV but not other ECM/BM proteins, including collagen types I, III, and V and laminin.
10.2. ANTIBODIES TO ATA PROMOTE THE OPSONOPHAGOCYTIC KILLING OF A. BAUMANNII

The OPKA activity of antiserum to Ata was used at dilutions of 1:10 and 1:20 in the presence of human complement and PMNs against the A. baumannii ATCC 17978, 17978 ata, and 17978 ata-c strains, the latter strain induced with either arabinose or glucose.

1. Rabbit sera raised to Ata was heat inactivated to eliminate internal complement activity and absorbed with the mutant strain ATCC 17978 ata to remove all non Ata directed antibodies, whereas the human sera used as a source of complement was absorbed with wild type ATCC 17978 in order to remove all antibodies to A. baumannii from this assay component.

2. The results presented in Fig. 10.2 demonstrate that antisera to Ata were highly opsonic against both the Ata producing wild type strain ATCC 17978 (90 and 93.5% killing at 1:10 and 1:20 dilutions, respectively) and the arabinose induced ATCC 17978 ata-c strain (99.3 and 98.3% killing at 1:10 and 1:20 dilutions, respectively) and not against the ATCC 17978 ata strain (2.2 and 3.4% killing at 1:10 and 1:20 dilutions, respectively) and had low opsonic activity against ATCC 17978 ata-c strain grown in glucose repressing conditions (20 and 10.8% killing at 1:10 and 1:20 dilutions, respectively).

3. The low OPK activity seen against the 17978 ata-c strain grown with glucose (20%) might be explained by a low level of Ata expression due to leaky expression of the ata gene under the repressive effects of the glucose.

4. Overall, these results demonstrate the high OPK activity of antibodies to Ata against A. baumannii in the presence of human PMN cells and complement.

5. The next step was to test whether antiserum to Ata had OPK activity against unrelated heterologous A. baumannii strains. These strains had been
previously characterized by fluorescence activated cell sorting (FACS) for Ata production [131].

6. Two MDR, high Ata producing strains (S8 and S11; mean fluorescence intensity [MFI] values [determined by FACS] and percent Ata positive cells, 598.8 and 97% and 731.3 and 99.8%, respectively) and two low Ata producing strains (I38 and S25; MFI values [determined by FACS] and percent Ata positive cells, 17.2 and 61.5% and 6.6 and 26%, respectively; see Fig. 10.3 were tested for OPK activity with antibody to Ata and NRS at 1:10, 1:20, 1:40, and 1:80 dilutions.

7. As shown in Fig. 10.3, in the presence of human complement and PMNs, antibodies to Ata exhibited low to moderate OPK activity against all A. baumannii S8, S11, I38, and S25 strains in comparison to NRS.

8. However, a clear positive correlation was not observed between the amount of surface Ata produced by a particular strain and its susceptibility to opsonic killing by antibodies to Ata, suggesting that once a threshold of expression was obtained the antibodies could be functional in the OPK assay.

10.3. ANTISERUM TO ATA MEDIATES THE COMPLEMENT DEPENDENT BACTERICIDAL KILLING OF A. BAUMANNII

1. To measure the antibody dependent serum bactericidal activity, dilutions of rabbit antisera to Ata and NRS (1:10 and 1:20) were heat inactivated to eliminate internal complement activity and absorbed with A. baumannii ATCC17978 ata to remove all non Ata directed antibodies.

2. The bactericidal activity was tested against all A. baumannii isogenic strains. The final concentration of complement in the bactericidal assay was increased to 80% from the 25% used for the OPKA, and the bactericidal activity of antisera to Ata was calculated as the difference in killing activity between immune and control NRS.
3. It is important to note that the concentration of human serum used in the bactericidal assay as a source of complement (80%), while comparable to the 90% serum previously used by others [139,140,141,142], is higher than that of human blood, which contains 45% serum. This excess amount of sera was used to guarantee that a sufficient supply of complement components would be available in the in vitro bactericidal assay.

4. As shown in Fig.10.4, wild-type *A. baumannii* ATCC 17978 cells and arabinose induced ATCC 17978 *ata*-c cells were highly susceptible to killing by antibodies to Ata in the presence of human complement (92 and 72% killing and 95 and 95% killing at 1:10 and 1:20 dilutions, respectively).

5. Conversely, *A. baumannii* producing little to no Ata protein, such as the glucose grown ATCC 17978 *ata*-c and ATCC 17978 *ata* strains, were resistant to the bactericidal effect of anti Ata antibodies (14% killing at 1:10 and 1:20 dilutions).

6. As with the OPKA, the low levels of bactericidal killing of the 17978 *ata* strain and the 17978 *ata*-c strain grown with glucose by antisera to Ata may be due to antibodies not directed to the Ata protein and/or to low expression of Ata in the 17978 *ata*-c strain grown under glucose repression.

7. It was then tested whether a complement dependent bactericidal activity could be engendered by anti Ata antibodies against the four heterologous *A. baumannii* strains S8, S11, I38, and S25 previously evaluated in the OPK assay. The results presented in Fig. 10.5 depict the bactericidal killing of all four strains by antisera to Ata over that obtained with control NRS. Antibodies to Ata exhibited high bactericidal activity against both high Ata producing heterologous strains (73 and 77% killing for S8 and S11, respectively) and high to moderate killing (73 and 52%) against the low Ata producing *A. baumannii* strains I38 and S25, respectively.
8. Taken together, these results suggest that bactericidal activity against \textit{A.\ baumannii} clinical strains can be engendered upon immunization with purified recombinant Ata.

10.4. \textbf{ATA IS A TARGET OF PROTECTIVE IMMUNITY}

1. To determine whether antibody to Ata could have a beneficial effect on bacterial levels in a mouse pneumonia model, groups of mice were immunized intravenously 16 and 4 hour before an intranasal infection with various \textit{A.\ baumannii} strains using either a heat inactivated antiserum to Ata or heat inactivated control NRS.

2. Animals were sacrificed 24 h post infection, and the number of CFU/g of lung tissue was calculated. Immunization of immunocompetent mice with antibodies to Ata followed by intranasal infection with \textit{A.\ baumannii} ATCC 17978 or two MDR clinical isolates, S8 and S11, resulted in a significant decrease in the number of bacteria/g of lung tissue compared to animals given control NRS (P= 0.0001 for ATCC 17978, P = 0.0004 for S8, and P = 0.009 for S11 [Mann-Whitney U test]; Fig. 10.6A to C).

3. In order to investigate the role of neutrophils in the protective response of antisera to Ata against \textit{A.\ baumannii} infections, similar protection studies were conducted in mice rendered neutropenic by the administration of 250g of MAb RB6 24 h before infection, as described elsewhere [143]. Antiserum to Ata was also protective in neutropenic mice infected intranasally against the same \textit{A.\ baumannii} strains, i.e., ATCC 17978, S8, and S11 (P=0.0028 for ATCC 17978 and S8 and P=0.016 for S11 [Mann-Whitney U test]; Fig.10. 6E to G).

4. For specificity control experiments, we used the same pneumonia model in both immunocompetent and neutropenic mice but challenged animals with the ATCC 17978 ata strain at doses similar to the ones used with wild type ATCC 17978 strain. As shown in Fig.10.6, either
immunocompetent or immunocompromised mice infected with ATCC 17978 ata had significant differences in CFU/g of lung tissue between animals given antiserum to Ata or NRS ($P = 0.97$ and 0.88, respectively [Mann-Whitney U test]).

5. Notably, the levels of the *A. baumannii* ATCC 17978 ata strain in mice given NRS were significantly lower than the levels of wild type *A. baumannii* ATCC 17978 in the lungs of NRS treated mice, a result indicative of a loss of virulence caused by deletion of the *ata* gene.

**Figure 10.1.** Antibodies against Ata block the binding of *A. baumannii* to collagen type IV. *A. baumannii* ATCC strains 17978, 17978 *ata*, and 17978 *ata-c* (the latter strain induced with 2% arabinose or 0.2% glucose) were incubated for 30 min with NRS or antibody to Ata (1:1,000 dilution) and tested for binding to immobilized collagen type IV. (B) Concentration dependent evaluation of anti Ata antibodies and NRS binding inhibition of *A. baumannii* ATCC 17978 to collagen type IV. The bars indicate the means of at least three independent experiments the standard error of the mean (SEM). The $P$ values were determined by unpaired Student $t$ test.
Figure 10.2. Antibodies to Ata mediate the opsonophagocytic killing of A. baumannii. Opsonophagocytic killing of A. baumannii strains 17978, 17978 ata, and 17978 ata-c, with the latter strain induced with 2% arabinose or 0.2% glucose, mediated by rabbit sera raised to recombinant Ata in the presence of human PMNs and complement. The results represent the percentage of bacteria killed in the assay by antibodies to Ata after 90 min incubation at 37°C compared to NRS. Controls lacking complement or antibodies were included in every assay, and only bacterial growth (i.e., no killing) was achieved. Bars represent the average of six independent experiments ± the SEM. The P value was determined by one way ANOVA with Tukey’s post hoc analysis, not significant.
Figure 10.3. Opsonophagocytic killing activity of antibodies to Ata against four *A. baumannii* strains. The results represent the percentage of bacteria killed in the assay by antibodies to Ata compared to control NRS. Controls lacking complement or antibodies were included in every assay, and only bacterial growth (i.e., no killing) was achieved. Bars represent the average of three independent experiments the SEM.
**Figure 10.4.** The Ata protein is a target for bactericidal antibodies. Killing by antisera to Ata, used at 1:10 and 1:20 dilutions in the presence of human complement of wild type *A. baumannii* ATCC 17978, its isogenic 17978 Δata strain, or the 17978 Δata-c complemented strain induced for 2 h with either 2% arabinose or 0.2% glucose. The results represent the percentage of bacteria killed in the assay after 1 h of incubation at 37°C compared to NRS. The bars indicate the means of at least three independent experiments the SEM. The P value was determined by one way ANOVA with Tukey’s post hoc analysis, not significant.
Figure 10.5. Antisera to Ata mediate the complement depended bactericidal killing of heterologous strains of *A. baumannii*. Bactericidal activity of sera raised to Ata used at 1:10, against four unrelated clinical strains of *A. baumannii*, two high Ata producers (black columns) and two strains producing medium to low levels of Ata (white columns) in the presence of human sera as a source of complement. The results represent the percentage of bacteria killed in the assay after 1 h of incubation at 37°C compared to control NRS. Bars represent the averages of three independent experiments the SEM.
Figure 10.6. The Ata protein is a target for protective antibodies. Antisera to Ata reduced the levels of *A. baumannii* in the lungs of immunocompetent (A to C) and neutropenic (E to G) mice in a pneumonia model of *A. baumannii* infection in comparison to NRS immunized animals (n=8 to 12 mice per group). The infectious doses for studies using immunocompetent mice were $1.9 \times 10^8$ CFU of strain ATCC 17978, $5.2 \times 10^7$ CFU of strain S8, or $7.4 \times 10^6$ CFU of strain S11. The infectious doses for neutropenic studies were as follows: $6.3 \times 10^6$ CFU of *A. baumannii* ATCC 17978, $6.5 \times 10^6$ CFU of strain S8, or $5 \times 10^5$ CFU of strain S11. For a specificity control, the same pneumonia models were used but animals were challenged with the *A. baumannii* ATCC17978 ata strain at doses similar to the ones used with wild type strain ATCC 17978: doses of the ata strain $1.2 \times 10^8$ or $4.6 \times 10^6$ CFU/mouse for immunocompetent (D) or immunocompromised (H) studies, respectively. Bars indicate the means the SEM. The P values were determined by using the Mann Whitney U test, not significant.
In this study the potential of Ata was investigated as a vaccine target against *A. baumannii* infections. Previous studies have shown that Ata was surface exposed on the *A. baumannii* outer membrane, mediated adherence to ECM/BM proteins and the adhesion of whole *A. baumannii* cells to collagen type IV, and was important for biofilm formation and needed for full virulence of *A. baumannii* in mice [131]. The study also demonstrated that Ata is a target for both opsonic killing and bactericidal antibodies, and passive administration to mice leads to significant reductions in bacterial burdens in the lung at 24 h post infection. Therefore, Ata represents a classic bacterial vaccine target in that expression is needed for full virulence and antibodies to Ata mediate killing.

Antibody to Ata blocked the adherence of *A. baumannii* to collagen type IV in vitro, suggesting another protective mechanism for these antibodies. Despite the fact that bacteria elaborate high numbers of different types of adhesins, which puts a constraint on their use as vaccines, several adhesins have been shown to give protection in animal models of infection. Some of them include the FimH adhesin in a complex with the FimC chaperone and the fimbrial antigen of *Escherichia coli* [144], the SafD/F adhesin chaperone complex of *Salmonella enterica serovar Enteritidis* [145], or pertactin of *Bordetella pertussis* [146]. Therefore, we speculate that the adhesion blocking activity of antisera to Ata that reduces the binding of *A. baumannii* to collagen type IV may work in vivo by decreasing the levels of bacterial tissue invasion and/or colonization.

Rabbit antibodies to Ata also mediated both phagocyte dependent and phagocyte independent killing of *A. baumannii*, indicating that immunity to Ata could be operative in both immunocompetent and immunosuppressed patients. A previous study by Goe et al. [147] reported that an IgM raised against iron regulated outer