Vaccination with Outer Membrane Complexes Elicits Rapid Protective Immunity to Multidrug-Resistant \textit{Acinetobacter baumannii} \textsuperscript{V1}\textsuperscript{†}

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\textit{Acinetobacter baumannii} causes different types of infections, including, among others, pneumonia, bacteremia, meningitis, and skin and soft tissue infections (24). Over the last 3 decades, \textit{A. baumannii} has emerged as a pathogen of increasing clinical importance due to the global increase in the incidence of infections caused by this organism. \textit{A. baumannii} was shown to be the causative agent in 6.9% of nosocomial pneumonias in 2003 in a national surveillance study performed in the United States, which represents a 72% increase compared to data collected in 1986 (14). In addition, \textit{A. baumannii} was the causative agent in 2.4% of bacteremias and 6.2% of bacteremias in intensive care units (14, 39). Infections caused by this pathogen have been especially problematic in patients receiving mechanical ventilation, in burn patients, and in military personnel sustaining war-related trauma in Iraq and Afghanistan. \textit{A. baumannii} can cause outbreaks in intensive care units and trauma/burn units, which are presumably caused by passage of the organism from infected or colonized individuals and contaminated hospital equipment to uninfected patients. Although \textit{A. baumannii} causes primarily nosocomial infections, recent reports have described community-acquired pneumonias caused by this pathogen (16, 25). Mortality rates associated with \textit{A. baumannii} have been reported to be between 35 and 70% for nosocomial pneumonias (37) and between 20 and 60% for bacteremic infections (14, 39).

The treatment of infections caused by \textit{A. baumannii} has become difficult due to the emergence of multidrug-resistant strains. \textit{A. baumannii} has demonstrated the ability to acquire resistance to diverse classes of antibiotics via multiple resistance mechanisms (40). Carbapenems have been the standard treatment for \textit{A. baumannii} infections; however, increasing resistance rates have limited their efficacy. Surveillance studies performed in 2007 showed that resistance rates to imipenem were between 38 and 71%, whereas 20 years ago resistance to imipenem was exceedingly rare (37). Due to increasing resistance to commonly used antibiotics, clinicians have relied increasingly upon the use of the polymyxin antibiotic colistin (15). However, the emergence of strains resistant to colistin has now been reported (1, 5). Of particular concern are recent reports describing outbreaks of panresistant strains, which are resistant to all standard antimicrobials (33, 34). Given these trends, the development of new strategies for preventing and treating infections caused by this pathogen is necessary.

Immunization represents a potentially effective strategy for preventing infections caused by \textit{A. baumannii}; however, to date, there have been no vaccines developed for this pathogen. Vaccines that elicit antibodies against bacterial outer membrane proteins are attractive candidates due to the role of these proteins in interacting with the host and their availability for antibody neutralization due to their localization on the cell surface.

Vaccines based on inactivated whole cells and attenuated strains are able to elicit antibodies against multiple surface proteins; however, the administration of whole organisms raises potential safety concerns. One approach that has been used to elicit antibodies against multiple bacterial surface proteins without the administration of whole organisms is the development of vaccines based on proteins extracted from the...
bacterial outer membrane, termed outer membrane complex (OMC) vaccines (4, 10, 17). OMC vaccines have been shown to elicit antibodies against multiple proteins of the bacterial outer membrane and to induce protective immunity against infection and have given promising results in early human clinical trials (4, 10, 17–19). The objective of the present study was to develop an OMC vaccine against A. baumannii and to characterize its efficacy in a murine sepsis model. In addition, the ability of antisera from vaccinated mice to passively protect and treat naive mice was tested.

MATERIALS AND METHODS

Bacterial strains. The A. baumannii ATCC 19606 strain is an antibiotic-susceptible reference strain (31). Clinical isolates were confirmed as A. baumannii by amplified ribosomal DNA restriction analysis, and clonal relationships were determined by pulsed-field gel electrophoresis (13, 35). Ab-154, Ab-26, and Ab-1 are clonally distinct clinical strains, whereas 113-16 has the same pulotype as Ab-1, suggesting that they arose from the same clone (9, 34). Relevant characteristics of the strains used in this study are shown in Table 1.

Preparation of the OMC vaccine. A. baumannii OMCs were prepared based on a protocol previously described for the purification of OMCs from Neisseria meningitidis (10). A. baumannii ATCC 19606 was grown in 1 liter of Mueller-Hinton broth to an optical density at 600 nm (OD600) of 0.8, and pelleted by centrifugation at 20,000 g for 1 h and then washed with 2 ml of 62.5 mM Tris-Cl, pH 6.8. Endotoxin was extracted from the preparation by use of a cold detergent wash step in which OMCs were resuspended in 5% SDS and incubated at 4°C for 10 min. SDS and endotoxin were subsequently removed by precipitating OMCs as described previously (38). Isolated proteins were then resuspended in phosphate-buffered saline (PBS) and dialyzed extensively against PBS. Endotoxin levels in OMC preparations before and after detergent extraction were determined using the QCL-1000 LAL limulus amoebocyte lysate assay (Lonza, Basel Switzerland). Coomassie blue staining (Safestain; Invitrogen, Carlsbad, CA) and silver staining (Plus One silver staining kit; GE Healthcare, Pittsburgh, PA) were performed according to the manufacturers’ instructions on proteins separated by SDS-PAGE on 12% gels.

LC-MS/MS. A solution containing 50 g of the OMC preparation and an in-gel sample containing 6 mg of protein were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS). Samples were reduced with 10 mM dithiothreitol (DTT) and 55 mM iodoacetamide in 50 mM ammonium bicarbonate buffer before digestion with 1 mg of trypsin at 37°C overnight. Peptides were separated on a PepMap C18 column (LC Packings, Amsterdam, Netherlands), and spectra were obtained on a model 4700 proteome analyzer (Applied Biosystems, Carlsbad, CA). Spectra were obtained in MASCOT format for SwissProt database queries, using a 100-ppm tolerance as the maximum mass error, with allowances for oxidation, carbamidomethylation, and deamidation modifications. Proteins included in subsequent analyses were those for which at least two unique peptides were identified in one run or those for which a single peptide was identified in two independent runs.

Mice and vaccination. Experiments involving mice were approved by the Committee on Ethics and Experimentation at the University Hospital Virgen del Rocío. Six- to 8-week-old female C57BL/6 mice were purchased from the University of Seville (Seville, Spain) and housed under specific-pathogen-free conditions. For vaccination, proteins were resuspended in PBS and combined (vol/vol) with the aluminum phosphate adjuvant Adjuphos (Brenntag Biosector, Frederikssund, Denmark) to a final concentration of 125 µg/ml. Mice were immunized by intramuscular injection of 100 µl of the vaccine into each quadriceps muscle (total dose = 25 µg/mouse) on days 0 and 21, unless otherwise stated. All control mice were immunized similarly with a mixture of PBS and adjuvant.

ELISA, Western blots, and LPS staining. Blood samples were collected from the retro-orbital plexus of anesthetized mice. For indirect enzyme-linked immunosorbent assays (ELISAs), 96-well plates were coated with 0.3 µg outer membrane proteins/well, and ELISAs were performed as described previously (23). For Western blots, 3 µg of whole bacterial lysate from A. baumannii ATCC 19606 and 3 µg of outer membrane proteins were resolved in a 12% gel, and proteins were transferred to a nitrocellulose membrane. Mouse serum collected 1 week after the second immunization was used at a dilution of 1:2,000 to probe the blot before its development with chemiluminescence. Proteins K digestion experiments were performed as described previously (26). For analysis of lipopolysaccharide (LPS), whole bacterial lysates were resolved by SDS-PAGE, and the resulting gel was stained with the LPS/glycoprotein-specific stain in a Pro-Q Emerald 300 lipopolysaccharide gel stain kit (Invitrogen, Carlsbad, CA).

IFN-γ secretion from spleenocytes. Splenocytes were isolated as described previously (22) from unvaccinated and vaccinated mice 1 week after the second immunization dose. Splenocytes were resuspended to a concentration of 1 x 10^6 cells/ml, and 200 µl of the cell suspension was added to each well of a 96-well plate and either stimulated with 0.2 µg/well of OMCs or left unstimulated. After incubation for 48 h, supernatants were collected and levels of gamma interferon (IFN-γ) were determined, using a BD OptEIA mouse IFN-γ kit (BD Biosciences, San Jose, CA).

A. baumannii sepsis model. A mouse sepsis model was used in order to characterize the efficacy of the OMC vaccine and of antibody treatment. In this model, mice are infected via intraperitoneal injection of a bacterial suspension containing 5% porcine mucin, which has previously been shown to increase the infectivity of A. baumannii and other bacteria in different experimental models (2, 28). A. baumannii strains were grown for 18 h at 37°C in Mueller-Hinton broth and then adjusted to the appropriate concentration in physiologic saline. Inocula were prepared by mixing the bacterial suspensions 1:1 (vol/vol) with a 10% solution (wt/vol) of porcine mucin (Sigma, St. Louis, MO). Bacterial concentrations of the inocula were determined by plating on blood agar. Mice were infected by intraperitoneal injection with 0.5 ml of the bacterial suspension and were carefully monitored for survival for 7 days. Fifty percent lethal dose (LD50) values for the strains used in challenge studies were determined by infecting groups of mice with 10-fold dilutions of bacteria and analyzing survival data using the Probit method (7) (Table 1). For antibody treatment studies, mice were treated with 200 µl of 4-week sera from vaccinated mice or naive serum by intravenous injection via the tail vein 1 h after infection with the indicated inoculum.

Postinfection tissue bacterial loads were determined for vaccinated and control mice euthanized with an overdose of thiopental 12 h after infection. Spleens, kidneys, and lungs were removed aseptically. Tissues were weighed and then homogenized in 2 ml of physiologic saline before plating of serial log dilutions on blood agar plates for bacterial quantification.

Serum levels of interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and IL-6 were determined in mice at 12 h postinfection, using BD OptEIA mouse kits (BD Biosciences, San Jose, CA).

Statistical analyses. Statistical data were performed using SPSS, version 15.0 (SPSS Inc., Chicago, IL). Antibody titers were compared using Student’s t test. Cytokine levels and bacterial loads were compared using the Mann-Whitney U test. Survival data were compared using the log rank test.

### Table 1. Characteristics of Acinetobacter baumannii strains used for this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>LD50 (log10 CFU)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 19606</td>
<td>Reference strain isolated from urine, United States, 1948</td>
<td>3.8 (3.6–4.1)</td>
<td>31</td>
</tr>
<tr>
<td>Ab-154</td>
<td>Susceptible clinical isolate, Spain, 2000</td>
<td>5.6 (5.2–6.1)</td>
<td>9</td>
</tr>
<tr>
<td>Ab-26</td>
<td>Multidrug-resistant clinical isolate, Spain, 2000</td>
<td>ND</td>
<td>9</td>
</tr>
<tr>
<td>Ab-1</td>
<td>Multidrug-resistant clinical isolate from a hospital outbreak in Seville, Spain, 2002</td>
<td>ND</td>
<td>This study</td>
</tr>
<tr>
<td>113-16</td>
<td>Panresistant clinical isolate from a hospital outbreak in Seville, Spain, 2002</td>
<td>4.7 (4.4–5.2)</td>
<td>34</td>
</tr>
</tbody>
</table>

*LD50 Values for C57BL/6 mice, determined using the Probit method. ND, not determined.*
RESULTS

Characterization of A. baumannii OMCs. OMCs prepared from the ATCC 19606 strain were subjected to Coomassie blue and silver staining in order to characterize both protein and lipopolysaccharide components present in the preparation (Fig. 1A and B, respectively). As shown in Fig. 1A and B, the OMC preparation contained multiple protein components at a broad range of molecular sizes. LC-MS/MS analysis of the OMC preparation identified 61 protein components in the OMC preparation (see Table S1 in the supplemental material). In order to verify that the preparation contained predominantly proteins of the bacterial outer membrane, an in silico analysis of protein subcellular localization was performed using PSORTb software (12). Of the 61 proteins identified, the subcellular locations of 41 were able to be predicted. Thirty-one of the 41 proteins were predicted to be outer membrane proteins, 8 were predicted to be cytoplasmic, and 2 were predicted to be inner membrane proteins (Fig. 1C). Interestingly, of the 8 proteins predicted to be cytoplasmic proteins, 3 have previously been identified in outer membrane fractions of A. baumannii (20), and 2 are ribosomal subunits which have been shown to be associated with bacterial outer membranes (29). These results suggest that the OMCs consisted primarily of proteins from the bacterial outer membrane.

In order to assess the reproducibility of the method used for preparation of the OMC vaccine, three independent samples were prepared. The protein concentrations of the independent preparations after resuspension in PBS were 0.938, 1.10, and 0.999 μg/μl, resulting in a standard deviation of 0.082 μg/μl. In addition, the three preparations were resolved by SDS-PAGE and stained by Coomassie blue in order to compare protein components. As shown in Fig. 1D, the banding patterns observed for the three preparations were almost identical. Taken together, these data suggest that the OMC preparation method is highly reproducible.

In order to reduce endotoxin levels in the OMCs prepared from A. baumannii, a cold detergent wash step was employed to dissociate protein-associated endotoxin before selective precipitation of proteins. Detergent extraction removed >99.4% of endotoxin, resulting in endotoxin levels in OMCs of 15.6 ± 5.4 endotoxin units (EU)/μg protein (mean ± standard deviation [SD]) after extraction.

Immune response to OMC vaccine. In order to evaluate the humoral response to immunization with the OMC vaccine, mice were immunized at 0 and 3 weeks, and serum was collected before immunization and at 2 and 4 weeks (n = 10 mice/group). ELISAs demonstrated that vaccination elicited significant levels of IgG against A. baumannii outer membrane proteins, whereas control mice had no detectable antigen-specific IgG (P < 0.001; Student’s t test) (Fig. 2A). Antibody subtype ELISAs for IgG1 and IgG2c, the IgG2a homolog in C57BL/6 mice (21), demonstrated that immunization with the OMC vaccine produced antibodies of both subtypes, suggesting that both the Th1 and Th2 arms of the immune response had been activated (Fig. 2B).

To further characterize the response produced by vaccination with the OMC vaccine, A. baumannii whole bacterial lysates and outer membrane proteins were probed in a Western blot with sera collected from vaccinated mice at 4 weeks. The results show that the sera contained antibodies that recognized multiple proteins in both whole-cell lysates and outer membrane samples, indicating that immunization with the OMC vaccine produced antibodies against multiple bacterial antigens (Fig. 2C). The Western blot demonstrated that 4-week sera recognized approximately 12 to 15 proteins, while the mass spectrometry experiments described for Fig. 1C detected 61 protein components in the OMC preparation. This discrepancy could be explained by a number of factors. First, it is possible that some of the proteins identified by LC-MS/MS were not present in sufficient quantities to stimulate a detectable antibody response. Second, some of the proteins identified by LC-MS/MS may not be sufficiently immunogenic to produce an antibody response. Finally, it is possible that the OMC preparation contained immunodominant antigens that masked the response to less dominant antigens in the mixture.

It was next determined if the antibodies elicited by immunization could recognize outer membrane proteins from the clinical isolates Ab-154, Ab-26, Ab-1, and 113-16. ELISAs performed using 4-week sera demonstrated that vaccination produced antibodies that were able to recognize outer membrane antigens from clonally distinct clinical isolates (Fig. 2D).

The ability of the OMC vaccine to stimulate a cell-mediated
Immune response was evaluated by isolating splenocytes from vaccinated and control mice (n = 5 mice/group) at 4 weeks and measuring the secretion of IFN-γ after stimulation with OMCs. Splenocytes from vaccinated mice secreted significantly more IFN-γ after stimulation than did splenocytes from control mice (P < 0.01; Mann-Whitney U test), indicating that immunization was able to elicit a cell-mediated immune response (Fig. 2E). Throughout these studies, no toxicity was observed in mice after immunization with the OMC vaccine.

A. baumannii sepsis model. Intraperitoneal infection with an inoculum of 1.3 × 10⁶ CFU of the ATCC 19606 strain produced an acute sepsis in which bacteria rapidly disseminated throughout the body to different organs as early as 1 h postinfection, as shown in Fig. 3A, reaching bacterial concentrations at 12 h of 9.2 ± 0.31, 8.4 ± 0.55, and 8.6 ± 0.54 CFU/g of tissue in the spleen, kidneys, and lungs, respectively. Furthermore, mortality was dependent on the concentration of bacteria in the inoculum (Fig. 3B), ranging from 0% with 4.8 × 10⁶ CFU to 100% with 5.0 × 10⁷ CFU.

Immunization with the OMC vaccine reduces postinfection bacterial loads and serum proinflammatory cytokine levels. Using the A. baumannii sepsis model, the effect of vaccination on postinfection tissue bacterial loads was determined by measuring the quantities of viable bacteria in the spleens, kidneys, and lungs in vaccinated and control mice (n = 10 mice/group) 12 h after infection with 1.0 × 10⁶ CFU (151.3 × LD₅₀) of the ATCC 19606 strain. Vaccination resulted in a reduction in tissue bacterial loads of approximately 10⁵-fold compared to those in control mice for all tissues tested (P < 0.001 for all tissues; Mann-Whitney U test) (Fig. 4A).

In order to determine if immunization with the OMC vaccine was able to protect infected mice from the proinflammatory cytokine release produced by bacterial sepsis, serum levels of IL-1β, IL-6, and TNF-α were measured in vaccinated and control mice at 12 h postinfection (n = 10 mice/group). Levels of all three cytokines were significantly lower in vaccinated mice than in control mice (P < 0.001 for IL-1β, IL-6, and TNF-α; Mann-Whitney U test), suggesting that vaccinated mice did not experience the proinflammatory cytokine release associated with the development of septic shock (Fig. 4B).

Immunization with the OMC vaccine protects mice from A. baumannii infection. It was next determined if the response produced by immunization with the OMC vaccine was sufficient to provide protection from infection with A. baumannii. Mice immunized twice, at weeks 0 and 3, were challenged 2 weeks after the final immunization and monitored for survival over 7 days (n = 10 mice/group). Vaccinated mice challenged with 1.0 × 10⁶ CFU (151.3 × LD₅₀) of the ATCC 19606 strain...
were completely protected from challenge, whereas all control mice died within 24 h ($P < 0.001$; log rank test) (Fig. 5A).

Similar results were obtained when mice were challenged with $1.0 \times 10^7$ CFU ($20.9 \times LD_{50}$) of the clinical isolate Ab-154.

FIG. 3. *A. baumannii* sepsis model. (A) Bacterial loads in spleens, kidneys, and lungs of C57BL/6 mice after intraperitoneal injection of $1.3 \times 10^6$ CFU of the ATCC 19606 strain ($n = 3$ mice/group). (B) Survival of mice after intraperitoneal injection of the indicated inocula of the *A. baumannii* ATCC 19606 strain ($n = 8$ mice/group).

FIG. 4. Immunization with the OMC vaccine decreases postinfection tissue bacterial loads and serum proinflammatory cytokine levels. Twelve hours after infection with $1.0 \times 10^6$ CFU of *A. baumannii* ATCC 19606, tissues and sera were collected from vaccinated and control mice ($n = 10$ group) for determination of tissue bacterial loads and serum cytokine levels. (A) Bacterial loads in spleens, kidneys, and lungs from vaccinated and control mice at 12 h postinfection. *, $P < 0.001$ for all tissues in vaccinated versus control mice (Mann-Whitney U test). (B) Serum levels of IL-1β, IL-6, and TNF-α in vaccinated and control mice at 12 h postinfection. *, $P < 0.001$ for IL-1β, IL-6, and TNF-α in vaccinated versus control mice (Mann-Whitney U test).

FIG. 5. Immunization with the OMC vaccine protects against diverse *A. baumannii* strains. Mice were vaccinated with the OMC vaccine or mock vaccinated ($n = 10$ group) at 0 and 3 weeks and then challenged 2 weeks after the second immunization with $1.0 \times 10^6$ CFU ($151.3 \times LD_{50}$) of the ATCC 19606 strain (A), $1.0 \times 10^7$ CFU ($20.9 \times LD_{50}$) of the clinical isolate Ab-154 (B), or $1.5 \times 10^6$ CFU ($27.3 \times LD_{50}$) of the clinical isolate 113-16 (C). *, $P < 0.001$ for all panels (log rank test).
and 1.5 × 10⁶ CFU (27.3× LD₅₀) of the panresistant clinical isolate 113-16, with 80% and 100% survival of vaccinated mice, respectively (P < 0.001 for both strains; log rank test) (Fig. 5B and C). These results demonstrate that immunization with the OMC vaccine can provide protective immunity against infection with diverse A. baumannii strains.

Role of antibodies against LPS in cross-protection. Given that the OMC vaccine contained residual endotoxin, it is possible that immunization produced antibodies against LPS and that these antibodies played a role in the protection and cross-protection seen in Fig. 5. In order to characterize the LPS chemotypes for each strain used for challenge studies, LPSs from whole bacterial lysates were resolved by SDS-PAGE and visualized using an LPS/glycoprotein-specific stain. As shown in Fig. 6A, the three strains used for challenge studies demonstrated distinct banding patterns, particularly at lower molecular masses. These data suggest that LPSs from these strains are of distinct chemotypes.

It possible, however, that antibodies generated against one chemotype of LPS cross-react with LPS molecules from a distinct chemotype and therefore play a role in cross-protection. In order to characterize the LPS chemotypes for each strain used for challenge studies, LPSs from whole bacterial lysates were resolved by SDS-PAGE and visualized using an LPS/glycoprotein-specific stain. As shown in Fig. 6A, the three strains used for challenge studies demonstrated distinct banding patterns, particularly at lower molecular masses. These data suggest that LPSs from these strains are of distinct chemotypes.

Sera reacted strongly with multiple antigens in the undigested lysates from all strains. Conversely, after digestion with proteinase K, the sera did not react with any antigens in lysates from any of the strains. These data indicate that the antibodies produced by immunization with OMC are directed primarily against protein antigens, not against other components that may be present in the OMC preparation, such as lipopolysaccharide.

A single immunization with the OMC vaccine elicits rapid protective immunity. The induction of rapid protective immunity against A. baumannii may be beneficial in clinical scenarios where it is not possible to use a prolonged vaccination schedule prior to bacterial exposure. In order to characterize the humoral response after a single administration of the OMC vaccine, IgG and IgM were measured in sera collected from groups of vaccinated mice every 3 days after vaccination. As shown in Fig. 7A, antigen-specific IgG and IgM were detectable in some mice (60%) as soon as 3 days after a single immunization with the OMC vaccine or after mock vaccination (n = 5/time point) after a single immunization. Data are presented as mean values, with error bars representing standard deviations. The horizontal dashed line represents the limit of detection of the assay. Six days after a single immunization with the OMC vaccine or after mock vaccination (n = 10/group), mice were challenged with 1.3 × 10⁶ CFU (196.8× LD₅₀) of the ATCC 19606 strain (B) or 1.0 × 10⁶ CFU (18.2× LD₅₀) of the clinical isolate 113-16 (C). * P < 0.001 for vaccinated versus control mice (log rank test).
mice had detectable levels of both IgG and IgM. These levels increased until approximately 9 days postimmunization and remained at these levels through day 21.

We next determined if the response elicited 6 days after a single immunization was sufficient to provide protection against infection. On day 6 after a single administration of the vaccine, mice were challenged with either $1.3 \times 10^6$ CFU (196.8×LD$_{50}$) of the ATCC 19606 strain or $1.0 \times 10^6$ CFU (18.2×LD$_{50}$) of the panresistant clinical isolate 113-16 (B). All vaccinated mice challenged with the ATCC 19606 strain survived challenge, whereas 90% of control mice succumbed to infection ($P < 0.001$; log rank test) (Fig. 7B). Similar results were obtained with the 113-16 clinical isolate, where 90% of vaccinated mice surviving challenge and 100% of control mice succumbing to infection ($P < 0.001$; log rank test) (Fig. 7C). These results indicate that a single administration of the OMC vaccine can produce protective immunity in as few as 6 days after a single administration.

**Treatment of established infection with anti-OMC antisera.** It was next determined if vaccine antisera could be used to therapeutically rescue infected mice. Naive mice were injected with either $2 \times 10^5$ CFU (30.2×LD$_{50}$) of the ATCC 19606 strain or $1.9 \times 10^6$ CFU (34.6×LD$_{50}$) of the panresistant clinical isolate 113-16. At 1 h postinfection, sufficient time for bacteria to disseminate to distant organs (Fig. 3A), mice were treated by intravenous administration of either anti-OMC antisera or naive sera ($n = 7$ group). *P* < 0.001 for mice treated with anti-OMC serum versus naive serum (log rank test).

**FIG. 8.** Vaccine antisera can therapeutically rescue mice infected with *A. baumannii*. Naive mice were infected with $2 \times 10^5$ CFU (30.2×LD$_{50}$) of the ATCC 19606 strain (A) or $1.9 \times 10^6$ CFU (34.6×LD$_{50}$) of the clinical isolate 113-16 (B). At 1 h postinfection, mice were treated by intravenous administration of either anti-OMC antisera or naive sera ($n = 7$ group). *P* < 0.001 for mice treated with anti-OMC serum versus naive serum (log rank test).

with the panresistant clinical isolate 113-16 survived challenge, compared to none of the controls receiving naive serum ($P < 0.001$; log rank test) (Fig. 8B). These results demonstrate that vaccine antisera can effectively treat established infections, including those caused by a panresistant clinical isolate.

**DISCUSSION**

Subunit vaccines consisting of single protein antigens have been the focus of recent research efforts. However, the use of a single antigen may result in reduced vaccine efficacy if there is antigenic variation across strains or if there are differences in expression of the target antigen. For these reasons, vaccines capable of eliciting a response against multiple bacterial antigens may be desirable. In a recent study by Stranger-Jones et al., it was shown that vaccination with four *Staphylococcus aureus* surface antigens individually provided only partial protection from infection, whereas vaccination with a combination of the four antigens conferred complete protection from bacterial challenge (32). Importantly, this combination of antigens also protected against infection with five clinical isolates. In the case of *A. baumannii*, the downregulation of surface protein expression during the acquisition of antibiotic resistance has been well documented (36), possibly complicating the development of a single-subunit-based vaccine with activity against multiple strains. In the present study, we show that immunization with an OMC vaccine produces antibodies against multiple bacterial proteins that are able to recognize surface antigens from diverse *A. baumannii* strains. Additionally, vaccination with proteins prepared from the ATCC 19606 strain was able to provide protection not only against homologous challenge but also against challenge with two clonally distinct clinical isolates.

Vaccines composed of multiple proteins extracted from the bacterial outer membrane have been tested for the ability to prevent infection in animal models for a number of bacteria (4, 10, 17). Importantly, an initial human trial with a vaccine consisting of outer membrane proteins from *Pseudomonas aeruginosa* showed that the vaccine was immunogenic and well tolerated (18). A subsequent study using the same vaccine in burn patients again demonstrated its safety and showed that the vaccine was effective in reducing the prevalence of *P. aeruginosa* in blood samples (19), indicating that vaccines consisting of multiple bacterial outer membrane proteins can be safe and effective in humans.

An important issue that must be addressed in order to most effectively utilize a vaccine against *A. baumannii* is to determine which populations should be immunized. Unlike the case for widespread diseases such as influenza or hepatitis B, *A. baumannii* causes predominately nosocomial infections in a comparatively small number of individuals. Risk factors for infection with *A. baumannii* have been described, such as mechanical ventilation, previous treatment with antibiotics, and previous stays in intensive care units (11). However, identifying these risk factors with enough time before bacterial exposure to complete a vaccination schedule involving priming and boosting injections would likely be complicated in many cases. The data presented here demonstrate that immunization with an OMC vaccine elicits a protective immune response within 6 days of a single administration. Inducing rapid protective im-
munity may be desirable for patients who are suddenly at risk for acquiring infection, such as patients who have sustained burns or trauma and patients admitted to an intensive care unit during an *A. baumannii* outbreak.

The treatment of *A. baumannii* infections has been complicated by drug-resistant strains, including recent reports describing outbreaks of panresistant strains, which are resistant to all standard antimicrobials (33, 34). One approach that is being developed for the treatment of other highly resistant bacteria, such as *S. aureus* and *P. aeruginosa*, is the use of antibody-based therapies (3, 8). In the present study, we show that antisera produced by immunization with the OMC vaccine can be used to treat established *A. baumannii* infections, including infections caused by a panresistant clinical isolate. Hyperimmune sera from vaccinated individuals have been evaluated in clinical trials for treating and preventing infections caused by *S. aureus* and *P. aeruginosa* (8, 30). The use of polyclonal antibody-based therapies may be advantageous over the use of monoclonal antibodies targeting a single epitope on a single antigen in that it may avoid the emergence of escape mutants that arise via mutation of targeted epitopes. Taken together, the data presented here indicate that antibody-based therapies may be an alternative strategy for treating *A. baumannii* infections.

Prior to the data presented here, the type of immune response necessary for conferring protection to *A. baumannii* was unknown. For the related organism *P. aeruginosa*, it has been demonstrated that antibodies against bacterial antigens are sufficient to provide protection, although experimental animal models have suggested that a T-cell response may also play a role in immunity (8, 27). In this study, we show that immunization with an OMC vaccine produces antigen-specific humoral and cellular responses. The data presented here from experiments involving treatment with vaccine sera suggest that antibodies are sufficient to provide immunity to *A. baumannii*. However, it cannot be ruled out that a cellular response plays a role in protective immunity. The importance of a cell-mediated response is conceivable given recent data describing the invasion of *A. baumannii* into epithelial cells (6). These results provide important information regarding the protective immune response to *A. baumannii* and have implications for the development of future vaccines and antibody-based therapies.

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