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Brief report

Immunization with lipopolysaccharide-free outer membrane complexes protects against *Acinetobacter baumannii* infection

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ABSTRACT

Outer membrane complex (OMC) vaccines, which contain antigens from the bacterial outer membrane, have been developed for multiple Gram-negative bacteria. However, OMC vaccines demonstrate high endotoxin activity due to the presence of lipopolysaccharide in the bacterial outer membrane, thus precluding their use in humans. We isolated OMCs from an LPS-deficient strain of *A. baumannii* (IB010) which completely lacks LPS due to a mutation in the *lpxD* gene. OMCs from IB010 demonstrated a more than 10,000-fold reduction in endotoxin activity compared to OMCs from wild type *A. baumannii*. Vaccination with IB010 OMCs produced similar levels of antigen-specific IgG and IgM after two administrations compared to wild type OMCs, and resulted in a similar reduction in post-infection spleen bacterial loads and serum pro-inflammatory cytokine levels. Vaccination with IB010 OMCs provided significant protection against infection compared to control mice, indicating the LPS-free OMCs could contribute to vaccine strategies for preventing infection by *A. baumannii*.

1. Introduction

Outer membrane complex (OMC) vaccines have been developed for multiple Gram-negative bacteria species including *Pseudomonas aeruginosa* [1], *Neisseria meningitidis* [2], *Francisella tularensis* [3], *Burkholderia multivorans* [4] and *Acinetobacter baumannii* [5]. These studies have shown that OMC vaccines can induce an antibody response against multiple outer membrane antigens, and provide protection against infection in animal models. However, the use of OMC vaccines for preventing human disease is precluded by the high endotoxin levels in OMC preparations due to the presence of lipopolysaccharide (LPS) in the outer membrane of Gram-negative species. Our group previously described an *A. baumannii* OMC vaccine that elicited a robust antibody response against multiple outer membrane antigens, and provided protection against infection in a mouse model [5]. The OMC vaccine consisted of OMCs isolated from wild type bacteria, and in spite of detergent treatment, contained high levels of endotoxin [5]. Recently, we isolated strains of *A. baumannii* that completely lack LPS due to mutations in genes involved in lipid A biosynthesis, *lpxA*, *lpxC* and *lpxD* [6,7]. The goal of the present study was to characterize the protective capacity of OMC vaccines prepared using LPS deficient strains of *A. baumannii*, and compare the immune response generated by these OMCs with the response produced after immunization with OMCs from wild type *A. baumannii* and purified *A. baumannii* LPS.

2. Materials and methods

2.1. Bacterial strains and OMC preparation

A. baumannii ATCC 19606 is an antibiotic susceptible reference strain. IB010 is an LPS-deficient derivative of ATCC 19606 that is completely deficient in LPS biosynthesis due to a 462 base pair deletion in the *lpxD* gene [7]. OMCs from ATCC 19606 and IB010 were prepared as described previously [5]. Endotoxin levels were determined in three independent assays using the QCL-1000 Limulus Amebocyte Assay (Lonza).

2.2. LPS extraction and antisera production

LPS was extracted from approximately 3.5×10^9 ATCC 19606 cells with the LPS Extraction Kit (iNtRON Biotechnology, Inc). The absence of protein in LPS extracts was confirmed by separating

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10 μl of LPS samples on 12.5% polyacrylamide gels followed by Coomassie blue staining (Simply Blue Safestain, Thermo Scientific). LPS polyclonal antisera were produced by immunization of C57BL/6 mice with the purified LPS as detailed below. All animal procedures were approved by the Ethics Committee of the University Hospital Virgen del Rocío/Biomedical Institute of Seville.

2.3. SDS-PAGE and western blotting

Purified OMCs were resuspended in 50 μl 50 mM Tris-HCl pH 7.2, 8 M urea and 2 M thiourea, and 10 μg were resolved on a 12% polyacrylamide gel. Proteins were visualized by Coomassie staining (Simply Blue Safestain, Thermo Scientific). For western blots, proteins were transferred to a nitrocellulose membrane and probed with a 1:2000 dilution of the LPS antisera before developing with chemiluminescence.

2.4. Immunization, ELISAs, and infection

Purified OMCs were resuspended in PBS to a concentration of 2.5 μg/μl and combined 1:1 (v/v) with the aluminum-based adjuvant, Alhydrogel 2% (InvivoGen). Six to 8-week-old, female C57BL/6 mice (University of Seville; n = 8/group) were immunized with 100 μl of the mixture in each quadriceps muscle (total volume 200 μl; vaccine dose 250 μg protein) on days 0 and 14. For LPS immunization, mice were similarly immunized with 30 μg purified LPS/mouse. One group of mice was immunized with a mixture of IB010 OMCs (250 μg protein) plus LPS (30 μg). Control mice were immunized with a mixture of PBS and adjuvant.

On days 7 and 21 sera were collected. For ELISAs, 96-well plates were coated with 5×10^7 bacteria cells/well of the ATCC 19606 strain in PBS by incubating at 4 °C overnight. ELISAs were performed as described previously [7].

Seven days after the second immunization (day 21), mice were infected with a lethal dose of the ATCC 19606 strain using a mouse intraperitoneal sepsis model [5,8], and monitored for 7 days. Post-infection spleen bacterial loads and serum levels of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6 were determined (n = 8/group) at 12 h post-infection as described previously [7].

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.01 (GraphPad Software Inc., San Diego California, USA). Antibody titers were compared using the Kruskal-Wallis test, followed by the Dunn Multiple Comparison test. Tissue bacterial loads and serum cytokine levels were compared by ANOVA followed by the Newman-Keuls Multiple Comparison Test. Survival was analyzed using the log-rank test. p values of <0.05 were considered significant.

3. Results

3.1. Characterization of OMCs

Measurement of endotoxin levels in OMCs from ATCC 19606 and IB010 revealed a dramatic reduction in endotoxin activity of more than 10,000-fold in IB010 OMCs (Fig. 1A). Furthermore, western blotting of purified OMCs with polyclonal LPS antisera demonstrated multiple reactive species at a wide range of molecular weights in OMCs obtained from ATCC 19606, whereas these species were not detected in IB010 OMCs (Fig. 1B). Qualitative analysis of the protein content of OMCs demonstrated a similar pattern of Coomassie staining (Fig. 1C).

3.2. Antibody response after immunization with OMCs

After two immunizations (Fig. 2A; day 21), total IgG levels were in mice immunized with IB010 OMCs compared to ATCC 19606 OMCs. Mice immunized with purified LPS showed specific IgG both days 7 and 21. Interestingly, mice immunized with IB010 OMCs plus purified LPS demonstrated lower levels of IgG than mice immunized with either ATCC 19,606 OMCs or IB010 OMCs alone.

Antigen specific IgM was detected in all vaccinated groups at day 21 (Fig. 2B). Mice immunized with ATCC19606 and IB010 OMCs showed significantly higher IgM levels than mice immunized with purified LPS or with IB010 OMCs and purified LPS. Antigen specific IgG1 and IgG2c were present in all immunized mice at day 21 (Fig. 2C and D).

3.3. Effect of vaccination on post-infection bacterial loads, cytokine levels, and survival

Seven days after the second immunization (day 21), groups of mice were infected with 1.6 x 10^6 cfu of the ATCC 19606 strain ($242.2 \times LD_{50}$) using a disseminated sepsis model [9]. All groups had significantly fewer bacteria in spleens compared to control mice (Fig. 3A). There was no difference in bacterial load between

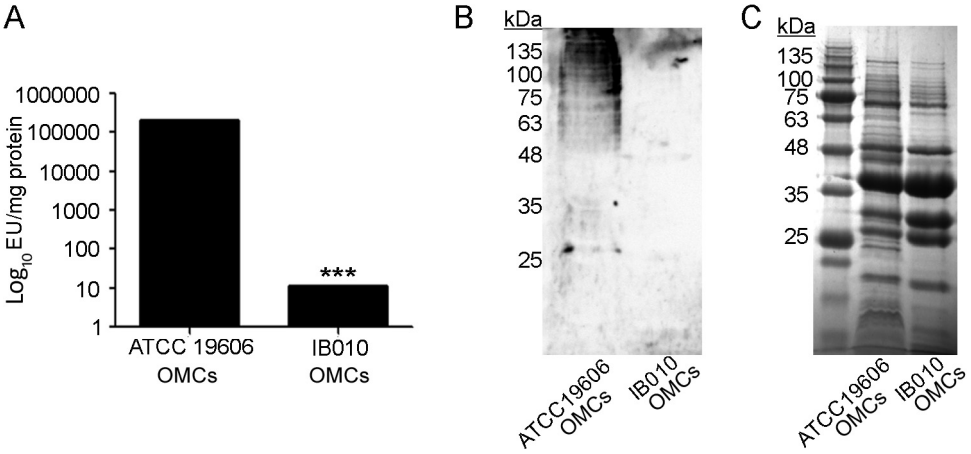

Fig. 1. Characterization of OMCs. (A) Endotoxin levels of ATCC 19606 and IB010 determined by the Limulus Amebocyte Assay. Bars represent the mean values of three independent cultures, and error bars represent the standard error of the mean (***p < 0.001). (B) western blot of OMCs from ATCC19606 and IB010 using an anti-LPS polyclonal sera. (C) Coomassie staining of OMCs from ATCC19606 and IB010. EU: endotoxin units.

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