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ORIGINAL ARTICLE

Recombinant outer membrane protein A fragments protect against *Escherichia coli* meningitis

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Background: Although the mortality rates have decreased over the past few decades, neonatal meningitis is still a severe disease with high morbidity. Moreover, approximately 40% of survivors exhibit neurological sequelae. *Escherichia coli* is the major Gram-negative bacterial pathogen in neonatal meningitis. The N-terminal β-barrel domain of the outer membrane protein A (OmpA) of *E. coli* is essential for effective protein conformation and function and contains four surface-exposed hydrophilic loops. In this study, we expressed different fragments of the four ring structures of the N-terminal domain, and investigated whether these recombinant OmpA fragments can protect mice from death after *E. coli* infection.
Methods: We expressed the recombinant proteins of the following OmpA fragments by using molecular cloning of Loop 1—2, Loop 1—3, Loop 1—4, Loop 2—3, Loop 2—4, and Loop 3—4. Animal experiments were subsequently performed to investigate the effects of these recombinant *OmpA* fragments on the survival of C57BL/6 mice after intracerebral *E. coli* RS218 administration.
Results: This study demonstrated that the recombinant Loop 1—3, Loop 2—3, and Loop 2—4 fragments of *OmpA* can protect mice from intracerebral *E. coli* infection.

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Conclusion: In bacterial meningitis, although antibiotic therapy is the first choice for management, neurological complications can seldom be averted. Based on the results of the present study, we intend to establish an effective therapeutic application for *E. coli* meningitis.

Introduction

Despite the availability of advanced antibiotic therapy and patient care for bacterial meningitis, it is still associated with a relatively high morbidity and mortality. Nearly 40% of surviving patients experience various neurological complications.[1–3] The pathological complications of bacterial meningitis include cerebritis, brain abscess, empyema, and ventriculitis in the acute phase and the sequelae of cerebral atrophy, hydrocephalus, seizure, and hearing impairment. Seizures are the most common complication, followed by hydrocephalus and hearing impairment.[3,4] A neuronal injury associated with bacterial infection of the central nervous system involves multiple microbial and host factors; *Escherichia coli* strains with the K1 capsular polysaccharide are the most predominant Gram-negative bacteria associated with neonatal bacterial meningitis.[5] Severe bacteremia and invasion through brain microvascular endothelial cells (BMECs) are the determining factors contributing to central nervous system infection.[6] Several K1-associated components participate in BMEC binding and invasion, including Fim H, K1 capsule, and outer membrane protein A (OmpA).[7–11] OmpA is a major outer membrane protein of *E. coli* and is essential in maintaining the integrity of the outer membrane and in bacterial conjugation.[12–14] This protein is also the receptor for several bacteriophages.[15–18] OmpA is encoded using a 1,038-bp open reading frame consisting of a 21-amino acid leader peptide and a mature 325-amino-acid protein. Moreover, the N-terminal membrane-anchoring domain of OmpA forms an antiparallel β-barrel, which has eight transmembrane β-strands connected by three short periplasmic turns and four relatively large surface-exposed hydrophilic loops; the OmpA C-terminal domain interacts with the peptidoglycan layer in the periplasm to maintain outer membrane integrity.[19] Furthermore, OmpA is highly conserved through the evolution of Gram-negative bacteria and is crucial for *E. coli* binding and the invasion of BMECs and astrocytes.[20,21] We previously reported that the recombinant full-length OmpA protein can protect mice from death after *E. coli* infection.[21] This has therefore encouraged study into a new therapeutic approach to improve the prognosis of bacterial meningitis. In this study, we expressed different fragments of the four ring structures of the N-terminal domain that were exposed on the surface of the OmpA protein, including Loop (L) 1–2, L1–3, L1–4, L2–3, L2–4, and L3–4 (Fig. 1), and investigated whether these recombinant OmpA fragments can protect mice from death after *E. coli* infection.

Materials and methods

Chemicals, bacteria, and culture media

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated. The *E. coli* strains used in the present study were kindly provided by Dr K.S. Kim (Division of Pediatric Infectious Diseases, School of Medicine, Johns Hopkins University, Baltimore, MD, USA); RS218 (O18:K1:H7) was isolated from the cerebrospinal fluid of a neonate with meningitis.[20] *E91* is an RS218 mutant that lacks the entire *ompA* gene. The bacteria were grown in brain–heart infusion broth with appropriate antibiotics (Difco Laboratories, Detroit, MI, USA). For infection experiments, overnight cultures were expanded in brain–heart infusion broth and incubated at 37°C for 2–3 hours to the midlog phase. The bacteria were centrifuged at 10,000 × g for 5 minutes and resuspended in a cell culture medium without antibiotics.

Mouse strain

C57BL/6 mice were obtained from the National Laboratory Animal Center of Taiwan, Nangang, Taipei, Taiwan and maintained under pathogen-free conditions. All animal procedures were performed according to the approved institutional protocol (LAC-99-0009) of Taipei Medical University, Taipei, Taiwan.

Expression and purification of OmpA fragments

The DNA fragments of the *ompA* L1–2, L1–3, L1–4, L2–3, L2–4, and L3–4 were amplified using polymerase chain reaction with restriction enzyme sites containing primers. These fragments were subsequently digested with SacI and XhoI and ligated into the pET-21a expression vector (Novagen, Darmstadt, Germany). The resultant plasmid was

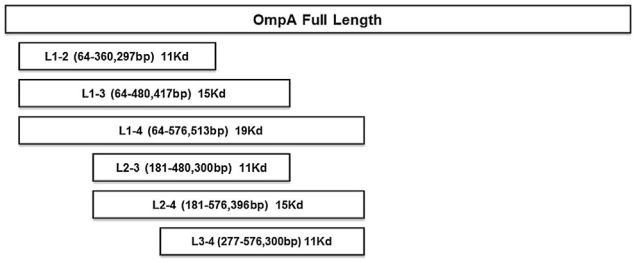

Figure 1. N-terminal, surface-exposed, OmpA fragments: Loop 1–2, Loop 1–3, Loop 1–4, Loop 2–3, Loop 2–4, and Loop 3–4.

transformed into *E. coli* BL21 (DE3). Protein expression was induced by administering 0.5 mmol/L of isopropyl-β-D-thiogalactopyranoside. His-recombinant proteins were then purified using Ni^{2+}-charged sepharose according to the manufacturer's instructions (Amersham plc, Amersham, UK). The His-recombinant proteins were eluted using an elution buffer (20 mmol/L of sodium phosphate, 500 mmol/L of NaCl, and 500 mmol/L of imidazole). The samples were massively dialyzed against the elution buffer without imidazole and then into phosphate-buffered saline (PBS; pH 7.4) to reduce the salt concentrations.

Animal experiments

In this experiment, 8–12-week-old C57BL/6 mice were randomly distributed into groups. The experimental protocol developed by Tsao et al[22] was followed to assess the survival of mice after intracerebral bacterial administration. Each group containing five mice was anesthetized with pentobarbital sodium salt (50 mg/kg) by intraperitoneal injection; each brain was then infected with *E. coli* RS218 (5×10^5 in 20 μL of PBS). Concurrently, PBS (20 μL) was used as a negative control. The mice were monitored for survival every 12 hours for 8 days. To investigate the effects of the recombinant OmpA fragments on the survival of the C57BL/6 mice after intracerebral *E. coli* RS218 administration, each brain was infected with *E. coli* RS218 (5×10^5 in 20 μL of PBS) by intracerebral injection in the presence of each recombinant OmpA fragment (20 μg); the survival of these mice was observed for up to 8 days after administration. To detect remnant bacteria in the brain, groups of three C57BL/6 mice were infected with *E. coli* RS218 by intracerebral injection in the presence or absence of each recombinant OmpA fragment. The mice were euthanized at various time points after the challenge. The brains were aseptically removed and homogenized with 3% gelatin in PBS. These samples were serially diluted, and the colony-forming unit for each sample was determined on a blood agar plate.

Statistical analysis

The results were expressed as the mean ± standard deviation of independent experiments. Quantitative and qualitative variables were analyzed using the Kruskal–Wallis one-way analysis of variance or Student *t* tests, when appropriate. The results were considered significant when the calculated $p < 0.05$.

Results

Expression and purification of recombinant ompA fragments

The DNA fragments of *ompA* L1–2, L1–3, L1–4, L2–3, L2–4, and L3–4 were amplified with polymerase chain reaction and then incorporated into the pET-21a expression vector and subsequently transformed into the *E. coli* BL21 (DE3) strain. The His-recombinant proteins were expressed and purified. Moreover, homogeneities of the purified His–OmpA fragments were identified using the Coomassie blue staining method and confirmed using immunoblotting (Fig. 2A and B). The purified His–OmpA fragment L1–2, L1–3, L1–4, L2–3, L2–4, and L3–4 exhibited the molecular weights of 11 kDa, 15 kDa, 19 kDa, 11 kDa, 15 kDa, and 11 kDa, respectively, on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, as predicted.

Recombinant His–OmpA fragments L1–3, L2–3, and L2–4 protect C57BL/6 mice against *E. coli* meningitis

We challenged the mice by intracerebral injection to avoid interference of the blood–brain barrier, according to the protocol presented by Tsao et al.[22] An infectious inoculum of 5×10^5 colony-forming units/mL was used in this study. Groups containing five C57BL/6 mice were anesthetized and then infected with intracerebral injection of *E. coli* RS218 or E91 and assessed for 8 d. As shown in Fig. 3, the mice died within 36 h of intracerebral injection of the $OmpA^+$ *E. coli* strain RS218, with an average survival time of 22 h (Fig. 3); by contrast, the mice challenged with the $OmpA^-$ *E. coli* strain E91 survived for 8 days before they were euthanized (data not shown). This suggests that OmpA-mediated *E. coli* infection subsequently results in the death of the mice.

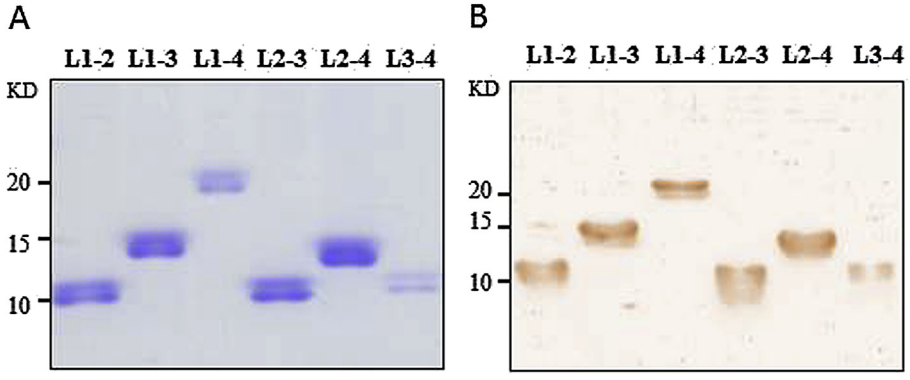

Figure 2. Purified recombinant OmpA fragments (A) displayed using 10% SDS-PAGE and stained with Coomassie blue; (B) immunoblotted using chicken anti-OmpA IgY (1:5000) and HRP-conjugated donkey anti-chicken IgY antibody (1:10000). Immunoreactive bands were visualized with DAB. OmpA = outer membrane protein A.

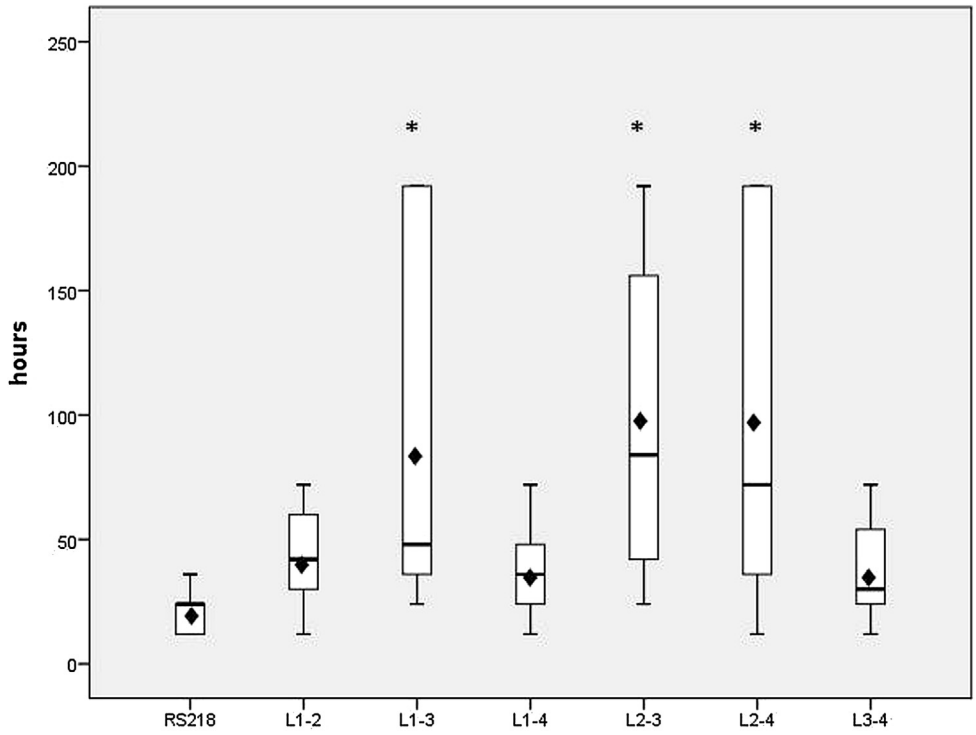

Figure 3. Survival of C57BL/6 mice after infection with *Escherichia coli* RS218. Groups of 5 C57BL/6 mice were anesthetized and challenged with 5×10^5 bacteria alone or a premixture of 20 μg of each His-OmpA fragment Loop 1—2, Loop 1—3, Loop 1—4, Loop 2—3, Loop 2—4, and Loop 3—4 by intracerebral injection. The mice were monitored for survival for up to 8 days. Results were analyzed using the Kruskal—Wallis one-way analysis of variance. ◆ = indicates the average survival time of the mice (43.5 hours, 85.7 hours, 38.0 hours, 99.0 hours, 96.0 hours, and 37.0 hours for Loop 1—2, Loop 1—3, Loop 1—4, Loop 2—3, Loop 2—4, and Loop 3—4 respectively). *$p < 0.05$.

Next, we determined whether the presence of His—OmpA fragments protected the mice from the *E. coli* RS218 infection. Groups of five C57BL/6 mice were challenged by intracerebral injection with a mixture of *E. coli* RS218 and 20 μg of each His—OmpA fragment (L1—2, L1—3, L1—4, L2—3, L2—4, and L3—4), and the survival of these mice was assessed for 8 days. The average survival times of the mice challenged with *E. coli* RS218 premixed with 20 μg of each of the His—OmpA fragments L1—2, L1—3, L1—4, L2—3, L2—4, and L3—4 were 43.5 hours, 85.7 hours, 38.0 hours, 99.0 hours, 96.0 hours, and 37.0 hours, respectively (Fig. 3). These findings suggest that the recombinant His—OmpA fragments L1—3, L2—3, and L2—4 significantly prolonged the survival of the C57BL/6 mice after intracerebral *E. coli* RS218 infection ($p < 0.05$).

Recombinant His—OmpA fragments L1—3, L2—3, and L2—4 protect the C57BL/6 mice against infection, correlating with bacterial clearance

We examined the remnant bacteria in the brain after *E. coli* RS218 infection, which increased up to $(2.4 \pm 0.2) \times 10^7$ at 24 hours after infection and up to $(6.0 \pm 0.3) \times 10^7$ at 36 hours after infection (Table 1); the mice died within 36 hours of intracerebral *E. coli* RS218 infection. In addition, for mice infected with *E. coli* RS218 premixed with recombinant OmpA fragments L1—2, L1—4, and L3—4, the remnant bacteria in the brains increased up to $(4.0 \pm 0.2) \times 10^6$, $(4.5 \pm 0.4) \times 10^6$, and $(5.0 \pm 0.3) \times 10^6$ at 24 hours after infection, respectively, and up to $(8.6 \pm 0.1) \times 10^7$, $(9.2 \pm 0.0) \times 10^7$, and $(8.8 \pm 0.1) \times 10^7$ at 60 hours after infection, respectively (Table 1); all mice died within 72 hours after bacterial infection. By contrast, the remnant bacteria in the brains infected with *E. coli* RS218 premixed with recombinant OmpA fragments L1—3, L2—3, and L2—4 were $(5.4 \pm 0.4) \times 10^5$, $(3.2 \pm 0.1) \times 10^5$, and $(5.5 \pm 0.4) \times 10^5$ at 24 hours after infection, respectively, and were undetectable at 60 hours after infection (Table 1). This indicates that these specific recombinant OmpA fragments protected the mice against *E. coli* infection and that this protection correlated with bacterial clearance.

Discussion

In Taiwan, maternal Group B *streptococcus* (GBS) screening during pregnancy and intrapartum antibiotic prophylaxis has been promoted since 1996. Like early-onset neonatal sepsis, although this strategy decreases the incidence of GBS meningitis, a concurrent increase in the incidence of *E. coli* infection has been observed. A 29-year-long assessment of the evolving trends of neonatal and childhood bacterial meningitis in northern Taiwan revealed GBS and *E. coli* as the two most common pathogens of neonatal meningitis; *E. coli*, however, was the most common pathogen of neonatal meningitis in 2008—2012.[3]

Table 1 Remnant bacteria in the brain after *Escherichia coli* infection.[a]

Time after infection (h)	Mean colony-forming unit (±SD)/brain				
	12	24	36	48	60
RS218	$(2.3 \pm 0.3) \times 10^6$	$(2.4 \pm 0.2) \times 10^7$	$(6.0 \pm 0.3) \times 10^7$	NT	NT
RS218 + L1−2	$(8.5 \pm 0.2) \times 10^5$	$(4.0 \pm 0.2) \times 10^6$	$(1.5 \pm 0.3) \times 10^7$	$(3.8 \pm 0.1) \times 10^7$	$(8.6 \pm 0.1) \times 10^7$
RS218 + L1−3	$(6.0 \pm 0.2) \times 10^5$	$(5.4 \pm 0.4) \times 10^5$	$(2.6 \pm 0.5) \times 10^5$	$(1.0 \pm 0.2) \times 10^5$	ND
RS218 + L1−4	$(1.1 \pm 0.2) \times 10^6$	$(4.5 \pm 0.4) \times 10^6$	$(1.8 \pm 0.2) \times 10^7$	$(4.3 \pm 0.1) \times 10^7$	$(9.2 \pm 0.0) \times 10^7$
RS218 + L2−3	$(5.6 \pm 0.1) \times 10^5$	$(3.2 \pm 0.1) \times 10^5$	$(1.2 \pm 0.2) \times 10^5$	ND	ND
RS218 + L2−4	$(5.6 \pm 0.2) \times 10^5$	$(5.5 \pm 0.4) \times 10^5$	$(2.5 \pm 0.3) \times 10^5$	$(1.0 \pm 0.2) \times 10^5$	ND
RS218 + L3−4	$(1.3 \pm 0.3) \times 10^6$	$(5.0 \pm 0.3) \times 10^6$	$(1.8 \pm 0.2) \times 10^7$	$(4.3 \pm 0.2) \times 10^7$	$(8.8 \pm 0.1) \times 10^7$

[a] Groups of three mice were inoculated intracerebrally with 5×10^5 *E. coli* RS218. Brain was collected at various time points after treatment. Remnant bacteria in brain were quantified.
L = loop; ND = not detectable; NT = not tested.

The OmpA protein is the major determinant enabling *E. coli* to adhere to and invade into BMECs and astrocytes, and this binding is the most critical step for *E. coli* K1 infection.[21,22] OmpA consists of 325 amino acids, and its N-terminal β-barrel domain is essential for effective folding and function. OmpA proteins lacking C-terminal residues 228−325 or 194−325 are effectively incorporated into the outer membrane and confer all known OmpA phenotypes.[23,24] Moreover, purified N-terminal amino acids 1−171 of OmpA bind directly to BMECs, whereas a derivative lacking all four extracellular loops cannot.[10] Reportedly, preincubation of BMECs with OmpA, solubilized from the outer membrane of OmpA$^+$ *E. coli*, inhibited *E. coli* invasion.[20] Similarly, the purified N-terminal OmpA also decreased the association of *E. coli* K1 with BMECs dose-dependently.[10] We previously reported that the recombinant, full-length OmpA protein can protect astrocytes by avoiding their activation after *E. coli* infection.[21] In addition, bacterial entry into BMECs is governed by L1, L2, and L3.[25] Notably, the short peptides corresponding to L1 and L2 can block the OmpA$^+$ *E. coli* invasion of BMECs.[20] In addition, the OmpA regions in L1, L2, and L4 resist serum bactericidal activity through increased binding to a complement regulator protein, C4b-binding protein. Moreover, the OmpA regions in L1 and L2 are responsible for survival in tissues and/or blood, resulting in high-grade bacteremia, which is a prerequisite for the onset of meningitis.[26]

Based on our research, this is the first report demonstrating that recombinant OmpA fragments can protect mice from infection caused by the intracerebral injection of *E. coli* RS218. The remnant bacteria in brains infected with *E. coli* RS218 along with recombinant OmpA fragments L1−3, L2−3, and L2−4 were almost undetectable at 60 hours after infection. These data therefore indicate that the OmpA fragments L1−3, L2−3, and L2−4 can protect mice from severe neuronal damage and death. In bacterial meningitis, although antibiotic therapy is the first choice for management, neurological complications can seldom be averted. Thus, the possible protective effects and routes of administration of recombinant OmpA fragments for patients with bacteremia require further investigation.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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