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Novel vaccine antigen combinations elicit protective immune responses against *Escherichia coli* sepsis

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ABSTRACT

Systemic infections caused by extraintestinal pathogenic *Escherichia coli* (ExPEC) have emerged as the most common community-onset bacterial infections and are major causes of nosocomial infections worldwide. The management of ExPEC infections has been complicated by the heterogeneity of ExPEC strains and the emergence of antibiotic resistance, thus their prevention through vaccination would be beneficial. The protective efficacy of four common ExPEC antigen candidates composed of common pilus antigens EcpA and EcpD and iron uptake proteins IutA and IroN, were tested by both active and passive immunization in lethal and non-lethal murine models of sepsis. Additionally, antibody raised to a synthetic form of a conserved surface polysaccharide, β-(1-6)-linked poly-*N*-acetylglucosamine (dPNAG) containing 9 monomers of (non-acetylated) glucosamine ($9GlcNH_2$) conjugated to tetanus toxoid TT ($9GlcNH_2$-TT) was tested in passive immunization protocols. Active immunization of mice with recombinant antigens EcpA, EcpD, IutA, or IroN elicited high levels of total IgG antibody of IgG1/IgG2a isotypes, and were determined to be highly protective against *E. coli* infection in lethal and non-lethal sepsis challenges. Moreover, passive immunization against these four antigens resulted in significant reductions of bacteria in internal organs and blood of the mice, especially when the challenge strain was grown in iron-restricted media. Inclusion of antibodies to PNAG increased the efficacy of the passive immunization under conditions where the challenge bacteria were grown in LB medium but not in iron-restricted media. The information and data presented are the first step toward the development of a broadly protective vaccine against sepsis-causing *E. coli* strains.

1. Introduction

Extraintestinal pathogenic *Escherichia coli* (ExPEC) normally reside in the human intestine but are capable of infecting extraintestinal sites like the blood, urinary tract, and meninges, using specific virulence attributes [1,2]. ExPEC are major causes of both community and nosocomial bacterial sepsis, with mortality ranging from 30% to 50% [3–5]. Clinical failure of antibiotic therapies, mainly due to multidrug resistance, increases the cost of care and results in prolonged morbidity for patients [6]. As a result, the prevention and control of these infections is a pressing concern.

A protective vaccine would be a useful strategy to prevent ExPEC infections. Efforts to develop vaccines against ExPEC have previously focused on specific virulence factors (O-antigens, OMP fractions, fimbriae, toxins, and iron-acquisition systems), or whole cells, but most of them were either not safe, poorly immunogenic, or did not provide cross-protection against ExPEC strains [7–12].

In order to develop a more effective vaccine against ExPEC sepsis, we tested siderophore receptors (IutA and IroN), which are highly prevalent among human ExPEC isolates [13]; and *E. coli* common pilus (ECP) [14] that plays a synergistic role in multiple steps of the infectious process [15–18]. Additionally chosen for passive vaccine studies were antibodies raised to a synthetic, deacetylated

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glycoform of the bacterial surface polysaccharide poly-β-(1-6)-*N*-acetyl-glucosamine (PNAG), a 9-mer of β-(1 → 6)-d-glucosamine conjugated to a tetanus-toxoid carrier protein (9GlcNH$_2$-TT), previously identified as a potential universal vaccine against pathogenic bacteria, including *E. coli* [19].

2. Methods

2.1. Ethics statement

New-Zealand-White rabbits and female BALB/c mice were obtained from Charles River Labs (Wilmington, MA). Vaccination and infection of animals were performed in accordance with protocols approved by the Arizona State University (ASU) Institutional Animal Care and Use Committee (IACUC) in dedicated facilities at the Biodesign Institute, ASU (Protocol number 1168R).

2.2. Antigens preparation

Genes encoding the selected antigens (EcpA, EcpD, IutA, IroN) (Table S1) were PCR amplified and cloned into pET-101/D-TOPO® vectors (Invitrogen). Recombinant proteins were expressed in *E. coli* BL21 and purified from inclusion bodies as His-tagged protein, using ProBond Ni-NTA resin columns (Invitrogen). The expressed proteins were 78 kDa (IroN), 74 kDa (IutA), 45 kDa (EcpD), and 21 kDa (EcpA), respectively.

2.3. Production of rabbit antibodies

Antisera to EcpA, EcpD, IutA, and IroN were raised by injecting subcutaneously (s.c.) rabbits with 250 μg of individual recombinant antigens (rAgs) in complete Freund's adjuvant, followed by two boosts at 3 weekly intervals with 250 μg of rAg in incomplete Freund's and two boosts in Montanide™ ISA 71 VG adjuvant. The concentration of antigen-specific rabbit IgG was measured by indirect ELISA using a goat-derived anti-rabbit IgG standard (Southern Biotech, Birmingham, AL). Rabbit antibodies raised to 9GlcNH$_2$-TT were prepared as previously described [19].

2.4. Bacterial challenge strain

Mice were challenged with urosepsis *E. coli* CFT073 [20] (Table S1) grown in either Lysogeny Broth (LB) [21] at 37 °C with or without 2,2′-bipyridyl (100 μM) with aeration until an OD$_{600}$ of ∼0.85 or in Dulbecco's Modified Eagle Medium (DMEM) + 0.5% Mannose + 2,2′-bipyridyl (100 μM), at 28 °C for 48 h standing and the OD$_{600}$ value of culture was adjusted to ∼0.85. The strain was stored at −80 °C in peptone–glycerol medium.

According to NCBI's BLASTn, the genome of CFT073 (AE014075.1) contains the sequences for *ecpA*(*matB*) (NP_752341.1), *ecpD*(yagW) (NP_7523338.1), *iutA* (NP_755498.1), *iroN* (AAN79707.1), and *pgaABCD* locus encoding [22].

2.5. Vaccination and challenge

2.5.1. Active immunization

As shown in Fig. S1, mice were s.c. injected with rAgs, either alone or in combinations [two or four antigens (high or low doses)] (Table S2), in phosphate buffered saline (PBS), emulsified in ISA 71 VG adjuvant, and boosted on day 23 (Table S2). On days 21 and 40, blood was collected by submandibular bleeding and sera were stored at −80 °C. On day 42, mice were challenged intraperitoneally (i.p.) with CFT073. In the non-lethal challenge (∼3.5×10^7 CFU), necropsies were performed after 24 or 48 h of challenge. In the lethal challenge (∼1.8×10^8 CFU), death was recorded for 48 h after inoculation; survived mice were euthanized and necropsied. Bacterial loads were determined in blood and organs.

2.5.2. Passive immunization

Mice were i.p. immunized with 200 μl of either pre-immune rabbit serum (control) or antigen-specific rabbit IgG antiserum (50 μg). Twenty-hour later, mice were boosted with the same serum samples. After a further 4 h, mice were i.p. challenged with ∼1.8×10^8 CFU of CFT073 grown under different conditions as described above. Necropsies were performed 24 h post-challenge with enumeration of bacteria in blood and organs.

2.5.3. Serum antibody titers

Antigen-specific IgG and IgG1/IgG2a isotypes titers in serum against each antigen were determined by ELISA [23], in 96-well plates, using a rAg coating concentration of 2 μg/ml. The endpoint titer was defined as the reciprocal of the highest dilution that gave an OD$_{405}$ twice that of the control.

2.6. Statistical analysis

Comparison analyses of data were performed using Kruskal–Wallis test followed by Dunn's multiple comparisons (bacterial clearance and ELISA data) and Fisher's exact and the Log-rank (Mantel–Cox) tests (Survival and mean death time data). $P < 0.05$ were considered statistically significant. All statistical tests were performed using GraphPad Prism 6.0 software.

3. Results

3.1. Expression of selected antigens

As determined by immunoblotting [24], CFT073 expressed PNAG in all conditions tested; when grown in LB at 37 °C [25], it expressed IutA and IroN at low levels, but not Ecp. In iron-restricted LB, a condition known to induce over-expression of iron-regulated outer-membrane proteins (IROMPs) [26], CFT073 over-expressed IutA and IroN, but did not express Ecp. Finally, in iron-restricted DMEM at 28 °C, it expressed Ecp and iron-uptake antigens at high levels.

3.2. Antigen-specific IgG elicited in vaccinated mice

Evaluation of antigen-specific (EcpA, EcpD, IutA, and IroN) total IgG in pooled-sera from 3 mice (equal volumes) in each group at days 21 and 40 post-immunization determined that IgG responses against the antigens varied depending on the vaccine composition (Fig. 1A). While anti-EcpA IgG were detected as early as day 21 post-vaccination in all EcpA-vaccine groups and persisted or even increased at day 40 post-vaccination, anti-IutA and anti-IroN IgG were only detected at day 40 post-vaccination in all IutA- and IroN-groups. In the EcpD groups, with the exception of the group vaccinated with all antigens at a low dose, in which anti-EcpD IgG were detected only at 40 day post-vaccinations, anti-EcpD IgG were detected at day 21 and persisted and even increased at day 40 post-vaccination in other groups (Fig. 1A). Our data have also shown that antibodies against specific antigens are cross-reactive with other antigens tested (Fig. 1).

3.3. Level of IgG titers elicited in mice

In a repeat experiment with a similar design, but in which serum samples tested were collected at day 40 and were not pooled, we determined that the levels of IgG elicited in different groups varied (Fig. 1B).