Clinical Studies of *Escherichia coli* O157:H7 Conjugate Vaccines in Adults and Young Children

SHOUSUN CHEN SZU¹ and AMINA AHMED²

¹Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; ²Levine Children’s Specialty Center—Pediatric Infectious Disease, Carolina Medical Centers, Charlotte, NC 28203

ABSTRACT Pediatric immunization has been the most effective measure to prevent and reduce the burden of infectious diseases in children. The recent inclusion of pneumococcal and meningococcal polysaccharide conjugates in infant immunization further reinforces their importance. Currently there is no human vaccine against enterohemorrhagic *Escherichia coli* (EHEC) infections. This review focuses on the human EHEC vaccine that has been studied clinically, in particular, the polysaccharide conjugate against *E. coli* O157. The surface polysaccharide antigen, O-specific polysaccharide, was linked to rEPA, recombinant exotoxin A of *Pseudomonas aeruginosa*. In adults and children 2 to 5 years old, O157-rEPA conjugates, shown to be safe, induced high levels of antilipopolysaccharide immunoglobulin G with bactericidal activities against *E. coli* O157, a functional bioassay that mimics the killing of inoculum in vivo. A similar construct using the B subunit of Shiga toxin (Stx) 1 as the carrier protein elicited both bactericidal and toxin-neutralizing antibodies in mice.

So far there is no clinical study of Stx-based human vaccine. Passive immunization of Stx-specific antibodies with humanized, chimeric, or human monoclonal antibodies, produced in transgenic mice, showed promising data in animal models and offered high prospects. Demonstrations of their safety and effectiveness in treating hemolytic-uremic syndrome or patients with EHEC infections are under way, and results are much anticipated.

For future development, other virulence factors such as the nontoxic Stx B subunit or intimin should be included, either as carrier protein in conjugates or as independent components. The additional antigens from O157 may provide broader coverage to non-O157 Stx-producing *E. coli* and facilitate both preventive and therapeutic treatment.

INTRODUCTION Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is a food-borne pathogen that can lead to complications such as hemorrhagic colitis and hemolytic-uremic syndrome (HUS), serious sequelae. In the United States, the most common *E. coli* serotype causing outbreaks is O157:H7, although non-O157 serotypes also cause the same disease, but in much fewer cases. The highest incidence rate is among children of preschool age (1, 2).

Prevention of *E. coli* O157 infection has been difficult because of the broad spectrum of contaminated sources, ranging from food such as beef, milk, produce, and fruits, to nonfood origins such as pool water and petting zoo animals (3, 4). Chemical or antimicrobial interventions are difficult to apply and have shown limited effectiveness (5). Since cattle are the major animal reservoir for *E. coli* O157, cattle vaccines to reduce carriage of *E. coli* O157 also were studied to a great extent and reached moderate success (6, 7).
An ideal *E. coli* O157 vaccine for humans should be safe and immunogenic in children and elicit bactericidal antibody that kills the inoculum upon contact. The infectious dose found in *E. coli* O157 outbreaks was usually low, around $10^2$ CFU (8). High levels of serum lipopolysaccharide (LPS) antibodies are detected following environmental exposure or symptomatic infection with *E. coli* O157 (9, 10). Although the protective role of these antibodies is unknown, evidence suggests that antibodies to the LPS of other enteric pathogens confer immunity by lysing the pathogens in the intestine (11). Convalescence from shigellosis, for example, provides LPS-specific resistance to infection, and vaccination with a *Shigella sonnei* LPS-based conjugate is efficacious in preventing infection (12). In clinical trials of a *Salmonella enterica* serovar Paratyphi A LPS-based conjugate vaccine, high levels of immunoglobulin G (IgG) anti-LPS with bactericidal activities were also induced in adults and preschool children (13). Because of the similarities between *E. coli* O157 and these gram-negative enteropathogens, vaccine-induced LPS antibodies to *E. coli* O157 may confer protection (14, 15).

Based on this postulation, our goal is to develop vaccines that elicit serum bactericidal IgG in young children. Several investigational polysaccharide conjugate vaccines were prepared at the National Institutes of Health. The vaccines were composed of detoxified LPS for *E. coli* O157, covalently linked to the carrier protein rEPA, a recombinant exoprotein of *Pseudomonas aeruginosa*. Here we review the clinical studies of O157-rEPA investigational vaccines conducted in adults and children 2 to 5 years old (16–19).

One of the major virulence factors in enterohemorrhagic *E. coli* (EHEC) is the Stx secreted by both the O157 and non-O157 serotypes. Therefore, an ideal human vaccine is one that elicits neutralizing antibody against Stx. Passive immunization with neutralizing antibody remains the only effective therapy for many other toxin-mediated diseases (20). However, up until the present, the only data supporting the development of Stx-based vaccines for both active and passive immunization are in preclinical stage. The Stx-based vaccine is reviewed briefly in this article.

**METHODS AND RESULTS**

**Investigational LPS-Based Vaccines**

The O-antigen of *E. coli* O157 consists of a linear copolymer of the tetrasaccharide repeating unit, $\alpha-D$-GalpNAc(1-2)$-\alpha-D$-PerpNAc-(1-3)$-\alpha-L$-Fucp-(1-4)$-\beta-D$-Glcp-(1-3), where PerpNac represents perosamine, 4-aminobutyryl-6-deoxy-d-mannose (17). The O-antigen can be extracted from LPS and detoxified by acetic acid (yielding O-specific polysaccharide [OSP]) or by anhydrous hydrazine to remove the O-acyl-linked lipid chains (yielding DeALPS) (13). Both OSP and DeALPS are not immunogenic without conjugation to a carrier protein. Three investigational vaccines had been prepared by covalently linking to the carrier protein rEPA and designated as O157 OSP-rEPA1, OSP-rEPA2, and DeALPS-rEPA. The carrier protein rEPA was chosen because it has been demonstrated to be clinically safe and served effectively in several polysaccharide conjugates (21). It also has the advantage of not overloading the already crowded routine vaccines with additional doses of diphtheria or tetanus toxoids.

The conjugates passed preclinical immunogenicity tests in mice and followed the safety requirements of the Code of Federal Regulations and were approved by the U.S. Food and Drug Administration as investigational vaccines. Each 0.5-ml dose contained 25 μg of polysaccharide and an approximately equal amount of rEPA. This dosage followed established studies on conjugate vaccines for noncapsular polysaccharides and is slightly higher than the licensed *Haemophilus influenzae* type b, pneumococcal, or meningococcal conjugate vaccines (12, 13).

**Clinical Studies**

Both the phase I and phase II clinical studies of O157 conjugate vaccines were conducted at the Carolina Medical Centers, Charlotte, NC. Briefly, in phase I, 87 healthy adults were injected once with one of the three conjugates (18). After safety and immunogenicity were demonstrated, the conjugate that elicited the highest antibody was chosen for the phase II study where 49 children, 2 to 5 years old, were recruited and divided into two groups receiving one or two doses of the vaccine (19).

The *E. coli* O157 conjugate vaccines were safe for all ages. There were no fever cases except for one child who had a temperature of 38.2°C 72 h after the second dose was administered. The local reactions were all mild and subsided within 24 h. In phase I, serum assays, including lactate dehydrogenase or alkaline phosphatase, serum bilirubin, and indirect bilirubin, were performed 1 week after injection to evaluate liver function. Six (7%) had asymptomatic elevations (up to 35% above the normal range) in one or more serum assays that returned to normal within 5 weeks. There were no significant differences in serum transaminase levels between pre- and postvaccination in children in the trial.
Antibody Responses
The serum anti-LPS IgG responses and bactericidal titers in vaccinees were used as the end-point markers for vaccine evaluation. The responses were examined before and 1, 4, and 26 weeks after immunization for adults and 1, 6, 10, and 26 weeks after the first injection for children.

All adults had low levels of preimmune anti-LPS IgG (measured in enzyme-linked immunosorbent assay [ELISA] units), and this level was approximately two times higher than those detected in young children (vide infra) (10, 18). The higher background level in adults could be a result of longer environmental exposure to cross-reactive organisms containing perosamine residue in their LPS, such as *Citrobacter* species, *Yersinia enterocolitica, Salmonella urbana, Pseudomonas maltophilia, and Brucella melitensis* (22–28).

All three conjugates elicited a significant rise of anti-LPS IgG in just 1 week after the injection, with 82% having greater than a 4-fold rise (Fig. 1). The escalation of antibodies shortly after immunization is important since the vaccine could be considered as a useful control measure during outbreaks before the source of contamination is identified and to block primary or secondary transmissions (29). The antibody levels continued to rise 4 weeks after the injection, and the geometric mean (GM) in the recipients of OSP-rEPA was slightly higher than that induced by the conjugate prepared with hydrazine-treated LPS, DeALPS-rEPA. At 6 months, the levels of anti-LPS IgG waned to ~33 ELISA units (EU) for all three conjugates, with 97% of volunteers continuing to have greater than a 10-fold rise than the preinjection levels.

To a lesser degree than the IgG response, O157-rEPA conjugates also induced increases in serum anti-LPS IgM and IgA levels. Interestingly, there is no correlation between the serum IgG and IgM or IgA antibody titers.

The highest incidence of HUS caused by *E. coli* O157 infection occurred in children under 6 years of age (30–32). We chose this target age group, children 2 to 5 years old, to study the safety and immunogenicity of our O157-rEPA conjugate. Children had very low anti-LPS IgG preinjection levels; however, within 1 week of injection, 81% responded with a greater than 4-fold rise in their serum anti-LPS IgG levels (Fig. 2). The antibody levels continued to rise; 6 weeks after the first injection, the GM reached 11.36 EU, with 98% having >10-fold increase compared with the preinjection levels (one child had a 6-fold rise). At all time intervals, the postinjection GM of anti-LPS IgG is significantly higher than the GM of the preinjection level.

Children who received a second injection of O157-rEPA at week 6 had an increase of antibodies measured 4 weeks later. However, at 26 weeks there was no difference in anti-LPS IgG levels between the groups receiving one or two injections. The lack of a booster response in this age group was also observed in other polysaccharide conjugate vaccines, such as *Salmonella* serovar Paratyphi A and *Shigella flexneri* type 2a (12, 13). At 26 weeks, all children in the study except one continued to have >4-fold higher anti-LPS IgG than their preinjection levels (Fig. 2).

The serum bactericidal assay has been a reliable and reproducible functional bioassay for gram-negative organisms such as *Salmonella* serovar Typhimurium and *Neisseria meningitidis* group C and serves as a good surrogate for protection (33, 34). The assay is mediated by antibody- and complement-induced lysis of the bacterial cells, mimicking the killing of the inoculums in vivo. In Table 1 we list the bactericidal titers and corresponding levels of anti-LPS IgG and IgM in representative serum samples from children before and 26 weeks after the first injection. After the sera were treated with 2-mercaptoethanol to inactivate IgM function, we observed that there was a direct correlation between the level of IgG anti-LPS and the bactericidal titers ($R^2 = 0.78$).

There is a possibility for the O157 LPS-based vaccines to protect against other pathogens that have cross-reactive LPS (22, 24). For instance, the LPS of *Vibrio cholerae* O:1 constitutes a monosaccharide repeat of perosamine, coinciding with one of the four sugars in *E. coli* O157 O-antigen. It has been reported that LPS antibodies against *V. cholerae* O:1 cross-react with...
E. coli O157 (22). We also observed some low-level cross-reaction in sera from children injected with O157-rEPA with V. cholerae O:1 serotype Inaba, but not with serotype Ogawa. Interestingly, there is no correlation between the anti-LPS titers to E. coli O157 and those to V. cholerae (R²<0.2) (19).

The most essential virulence factor of STEC is Stx, in particular type 2. Its role as both a prophylactic and a therapeutic antigen has also been observed in animal models and in epidemiology findings (9, 35). The major hindrance in development of an Stx2-based vaccine is the difficulty in producing a sufficient amount for vaccine preparation. In a proof-of-principle test, we conjugated OSP with the nontoxic recombinant B subunit of Stx1 (Stx1B) by two methods, by direct attachment or by linker adipic dihydrazide. Weaning mice injected with either conjugate elicited bactericidal antibodies to E. coli O157 and neutralizing antibodies to holotoxin Stx1 in vitro (Table 2). However, there was no observed cross-neutralization against Stx2 (36). Mutants with various promoters for Stx2B fragments have been constructed and offered future potential in this approach (unpublished data).

DISCUSSION AND FUTURE DEVELOPMENT

The simple thesis of this review is to demonstrate the possibility that, similar to polio, typhoid fever, and cholera, parenterally administered vaccines can protect against orally transmitted diseases such as infections with E. coli O157. The outermost carbohydrate moiety of E. coli O157 is the O-antigen of LPS, and conjugates synthesized with O-antigen were shown to be safe and immunogenic and elicited bactericidal antibodies in young children. Shigella sp. and E. coli are closely related in genetics and pathogenicity (14, 15). Evidence from S. sonnei and S. flexneri type 2b efficacy trials showed that OSP conjugate vaccines, based on the same construct, are efficacious against similar disease even during an outbreak (29).

We have noticed an age-dependent anti-LPS IgG response between pre- and postinjection sera. The higher background level of anti-LPS in adults compared to that in children was also noticed by others (10). These preexisting LPS antibodies could come from prolonged exposure to other gram-negative organisms containing cross-reactive LPS, and may in turn explain the

![Image](https://www.asmscience.org/MicrobiolSpectrum)

**Figure 2** Serum anti-Vi IgG response in children 2 to 5 years old receiving one injection (●) or a booster dose 6 weeks later (■); n = 25 in each group. No statistical difference between the groups at all periods. doi:10.1128/microbiolspec.EHEC-0016-2013.f2

### Table 1. Reciprocal bactericidal activity of serum LPS antibodies elicited in 2- to 5-year-old children injected with E. coli O157-rEPA conjugates

<table>
<thead>
<tr>
<th>Volunteer no.</th>
<th>IgG titer (IgG ELISA units)</th>
<th>IgM titer (IgM ELISA units)</th>
<th>Bactericidal titer&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole serum</td>
<td>Serum treated with 2-ME&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Whole serum</td>
</tr>
<tr>
<td>ECO 161</td>
<td>100</td>
<td>1:1280</td>
<td>1:640</td>
</tr>
<tr>
<td>023</td>
<td>33.63</td>
<td>1:2560</td>
<td>1:1280</td>
</tr>
<tr>
<td>033</td>
<td>24.44</td>
<td>1:320</td>
<td>1:160</td>
</tr>
<tr>
<td>035</td>
<td>17.21</td>
<td>1:320</td>
<td>1:320</td>
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<tr>
<td>041</td>
<td>14.37</td>
<td>1:320</td>
<td>1:160</td>
</tr>
<tr>
<td>043</td>
<td>11.28</td>
<td>1:320</td>
<td>1:160</td>
</tr>
<tr>
<td>047</td>
<td>12.98</td>
<td>1:320</td>
<td>1:160</td>
</tr>
<tr>
<td>048</td>
<td>21.87</td>
<td>1:320</td>
<td>1:160</td>
</tr>
<tr>
<td>054</td>
<td>18.55</td>
<td>1:320</td>
<td>1:160</td>
</tr>
<tr>
<td>055</td>
<td>21.60</td>
<td>1:320</td>
<td>1:160</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sera collected from children 42 days after the first injection of the conjugate vaccine.

<sup>b</sup>Titers are expressed as the inverse of dilution giving 50% killing. The anti-LPS IgG titer is calculated using a reference serum randomly assigned 100 EU for IgG (ECO 161). Similarly, a separate reference serum assigned 100 EU for IgM (ECO 110). Correlation coefficient for IgG vs. serum, R² = 0.75; for IgM vs. serum treated with 2-mercaptoethanol (2-ME), R² = 0.78. All bactericidal titers compared with IgM, no correlation (R²<0.10).

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>n</th>
<th>Titer to Stx1&lt;sup&gt;a&lt;/sup&gt; GM (25-75%)</th>
<th>Titer to Stx2</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli O157 LPS</td>
<td>5</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>OSP-AH-Stx1B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>8,040 (6,400–15,250)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>OSP-Stx1B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>14,040 (12,250–18,600)</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

<sup>a</sup>Neutralization of Stx1 or Stx2 was measured by using HeLa cell monolayers incubated with dilutions of 100 pg of toxin/ml of serum. Sera from mice immunized with saline or LPS alone showed titers of <10. The titers are the highest serum dilutions to yield 50% neutralization. 14,440 vs. 8,040, P<0.03.

<sup>b</sup>O-polysaccharide conjugated with Shiga toxin 1B with a linker.

<sup>c</sup>O-polysaccharide conjugate with Shiga toxin 1B without a linker.
age-related incidence rate of _E. coli_ O157 (1, 2, 30, 31, 32). Adults also responded with about 4-fold higher anti-LPS IgG than children at all time intervals. However, at 26 weeks, the level of antibodies in children remained about 12 times higher than the adults’ preimmune level, implying that the children after vaccination had elevated immunity to _E. coli_ O157.

There are advantages to using LPS as the vaccine source: the O-specific antigen is stable, its purity and chemical composition can be identified unambiguously, the polysaccharide can be produced in large quantity and is suitable for vaccine production, the detoxification procedures are well established, the residual endotoxicity level can be validated to meet the requirements of the regulatory guidelines, and, most of all, serum LPS antibodies elicited by the conjugates demonstrated bactericidal activity against _E. coli_ O157 and can be adopted as a functional bioassay for potency test.

However, there are limitations of LPS-based vaccines. For example, the induced LPS antibodies do not neutralize Stx, the major virulence factor of EHEC. This shortcoming limits its usefulness in prophylaxis and treatment during an outbreak, especially for non-O157 outbreaks. In one attempt to compensate for this shortcoming, we conjugated OSP with the B subunit of Stx1. Mice injected with OSP-Stx1B elicited bactericidal antibodies with high neutralization titers against Stx1 (36). Since Stx type 2 is the most common type found in EHEC outbreaks, an ideal vaccine would include Stx2 B-subunit or nontoxic recombinant Stx2 mutants as part of the vaccine component (37–39). Another obstacle that human vaccine development faces, either LPS- or Stx-based vaccine, is the planning of an efficacy trial to demonstrate the effectiveness. Most EHEC cases occur in outbreaks at no particular locations or regions, and because of the unpredictable nature of the disease epidemiology, designing an efficacy trial for future human vaccines bears inherent difficulty.

LPS that enables gram-negative organisms to escape complement fixation was considered as one of the virulence factors for _E. coli_ O157 (40). There are other virulence and attachment factors such as adhesin intimin, Tir, and EspA proteins, and some showed various degrees of protection against _E. coli_ O157 in animal models (41–44). Attempts to include these protein antigens as chimeric recombinant proteins in transgenic plants have also reached some preclinical success (43, 44). In the future development of _E. coli_ O157 human vaccine, including such virulence factors either as the carrier protein for an OSP conjugate or as separate components in a combined formulation, could be beneficial. Concurrent immunization with multiple antigens may generate synergistic protection, broaden the coverage to the non-O157 STEC serotypes, and facilitate both preventive and therapeutic treatments (45).

Although plasmapheresis is a common emergency intervention for patients with HUS, the plasma used was not enriched with specific Stx neutralization antibodies. Other nonspecific measures aimed at lowering systemic Stx levels in patients include immunoadsorption, IgG replacement activated charcoal absorption, or kidney dialysis, and their effectiveness remains controversial (46, 47). Several reviews showed monoclonal antitoxin with humanized, chimeric, or human monoclonal antibodies produced in transgenic mice was successful in animal models and offered high prospect (48–50). A recent Stx challenge study showed that administration of Stx2A or Stx2B human monoclonal antibodies could significantly reduce the Stx accumulation in kidney, accompanied by a short-term elevation of Stx-antibody complex in liver during clearance (51). With these plausible results, clinical demonstration of passive immunization with these or similar Stx-neutralizing antibodies is much anticipated.

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