Development of a Vaccine Against Clostridium difficile Infection (CDI): Design, Purification, and Biological Activities of the Recombinant Toxin 1 Antigens

Jerzy Karczewski
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Development of a Vaccine Against *Clostridium difficile* Infection (CDI): Design, Purification, and Biological Activities of the Recombinant Toxin Antigens

Jerzy Karczewski, Ph.D.
Merck Research Laboratories, Vaccine Basic Research, West Point, PA, USA
Clostridium difficile Infection (CDI)

- *Clostridium difficile* infection (CDI) is a leading cause of nosocomial diarrhea
- CDI can lead to colitis, toxic megacolon, systemic inflammatory response syndrome, and death
- 500,000 cases/year in the US

• Substantial morbidity among patients:
  • Elderly (age >65 years)
  • Immunocompromised
  • Undergoing prolonged hospitalization
  • Receiving broad-spectrum antibiotics and/or proton pump inhibitors
• *Clostridium difficile* is an anaerobic spore-forming gram-positive bacterium

• The main virulence factors are two clostridial toxins: TcdA and TcdB

• Asymptomatic carriers of *C. difficile* have significantly higher serum anti-toxin IgG levels as compared with patients who develop primary or recurrent CDI (*J Med Microbiol. 2011 Aug;60(Pt 8):1070-9*)
Monoclonal Antibodies against TcdA and TcdB Reduce Recurrence of CDI in PII Clinical Study

- Treatment with monoclonal antibodies CDA1 and CDB1 reduces the rate of disease recurrence following SoC treatment (metronidazole or vancomycin)

• TcdA and TcdB are large (~300 kDa) proteins composed of N-terminal enzymatic domain (ED), glucosyl transferase, the middle region translocation domain (TD) and C-terminal, highly repetitive carbohydrate binding domain (CROPS).
• Native, formalin inactivated Toxoid A/B vaccine is safe and immunogenic, currently in Phase II (Sanofi-Aventis)
• Recombinant CROPS region, chimeric antigens, and DNA vaccines induced neutralizing antibodies are under evaluation as vaccine candidates
Objectives of fragmentation study:

1. Which CROPS segments induce protective antibodies?

2. Does TD play a role in the development of protective immunity?

3. Does ED contain additional neutralizing epitopes?
Expression and Purification of Recombinant Fragments of TcdB

SDS/PAGE

Purification yields

<table>
<thead>
<tr>
<th>Fragment name</th>
<th>Molecular weight kDa</th>
<th>Yield* mg/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>TransCROPS B</td>
<td>207</td>
<td>39</td>
</tr>
<tr>
<td>TMD B</td>
<td>142</td>
<td>50</td>
</tr>
<tr>
<td>B0</td>
<td>68</td>
<td>822</td>
</tr>
<tr>
<td>B1</td>
<td>62.4</td>
<td>582</td>
</tr>
<tr>
<td>B2</td>
<td>31.6</td>
<td>1836</td>
</tr>
<tr>
<td>B3</td>
<td>39.1</td>
<td>686</td>
</tr>
<tr>
<td>B4</td>
<td>32</td>
<td>800</td>
</tr>
<tr>
<td>ED B</td>
<td>63.9</td>
<td>505</td>
</tr>
</tbody>
</table>

* Assuming 20% biomass, after two-step purification

- All antigens were purified in the two-step process using Ni-affinity column followed by anion exchange chromatography.
- Highly purified antigens were obtained (SDS/PAGE) with purification yields 0.5-2 g/L for shorter fragments and 40-50 mg/L for longer antigens.
Antibody Titers in Hamsters Immunized With TcdB Antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>270 kDa</th>
<th>146 kDa</th>
<th>62 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>TD CROPS</td>
<td>TD CROPS</td>
<td>TD CROPS</td>
<td></td>
</tr>
</tbody>
</table>

Antibody titer

- Antibody titer was measured in plates coated with native TcdB, incubated with dilutions of sera, and detected with secondary antibody
- High-titer antibody response was observed following immunization with larger TcdB antigens
Neutralizing Activity of Antisera From Hamsters Immunized with TcdB Fragments

Assay Principle

<table>
<thead>
<tr>
<th>toxin + NAb</th>
<th>toxin</th>
<th>no toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Neutralizing Activity of Toxin B Antisera

<table>
<thead>
<tr>
<th>TcdB fragment</th>
<th>Neutralizing activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>++</td>
</tr>
<tr>
<td>B2</td>
<td>+</td>
</tr>
<tr>
<td>B3</td>
<td>+</td>
</tr>
<tr>
<td>B4</td>
<td>++</td>
</tr>
<tr>
<td>B0</td>
<td>+++</td>
</tr>
<tr>
<td>TMD</td>
<td>+++</td>
</tr>
<tr>
<td>TransCROPS</td>
<td>+++</td>
</tr>
<tr>
<td>Toxoid B</td>
<td>++++</td>
</tr>
</tbody>
</table>

- Native TcdB was incubated with tested antiserum for 1 hour and then added to IMR90 fibroblasts grown in 96-well microtiter plates. The extent of neutralization was assessed visually after 24-hour incubation.
- Antisera collected from hamsters immunized with recombinant TcdB fragments effectively neutralized cytotoxic effects of native TcdB.
C. difficile Challenge in Syrian Hamsters – Experimental Outline

- Immunization phase
- Disruption & colonization phase
  - “Opening of a colonization window” that allows C. difficile to germinate and establish an infection
  - Requires antibiotic disruption of the gut microbiota
- Pathological phase
  - Colitis, typhilitis, wet tail
  - Ultimately death
Hamsters (n=10) immunized with a combination of native Toxoid A and recombinant fragments of TcdB were challenged with *C. difficile* spores.

Kaplan-Meier analysis demonstrated prolonged survival of hamsters immunized with recombinant antigens as compared with adjuvant control.
Hamsters (n=10) were immunized with a combination of native TcdA and ED+TransCROPS TcdB fragments formalin treated (+F) or untreated (C).
Hamsters (n=10) were immunized with a combination of either native or recombinant fragments of TcdB and recombinant fragment of TcdA (A1) and challenged with *C. difficile* spores.
Development of Full-Length Recombinant mTcdA and mTcdB Vaccine

- Full-length mTcdA (310 kDa) and mTcdB (270 KDa) were expressed using recombinant DNA technology

- Several mutations were introduced to reduce toxicity (4-5 log reduction achieved)

- Both antigens were purified to homogeneity in a fully scalable process
# Biophysical Analysis of Full-Length Recombinant mTcdA and mTcdB

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Source</th>
<th>α-helix</th>
<th>β-sheet</th>
<th>Turns</th>
<th>Unordered</th>
<th>Tm (°C)</th>
<th>kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTcdA</td>
<td>Recombinant</td>
<td>23</td>
<td>26</td>
<td>21</td>
<td>30</td>
<td>65</td>
<td>286 (by AUC)</td>
</tr>
<tr>
<td>Toxin A</td>
<td><em>C. difficile</em></td>
<td>21</td>
<td>28</td>
<td>21</td>
<td>30</td>
<td></td>
<td>307 (Predicted)</td>
</tr>
<tr>
<td>Toxin A</td>
<td>Published*</td>
<td>32</td>
<td>23</td>
<td>22</td>
<td>22</td>
<td></td>
<td>307 (Predicted)</td>
</tr>
<tr>
<td>Toxoid A</td>
<td>Published*</td>
<td>32</td>
<td>25</td>
<td>19</td>
<td>24</td>
<td>59.8*</td>
<td>307 (Predicted)</td>
</tr>
<tr>
<td>mTcdB</td>
<td>Recombinant</td>
<td>23</td>
<td>27</td>
<td>20</td>
<td>30</td>
<td>&gt;85</td>
<td>240 (by AUC)</td>
</tr>
<tr>
<td>Toxin B</td>
<td><em>C. difficile</em></td>
<td>21</td>
<td>28</td>
<td>21</td>
<td>29</td>
<td></td>
<td>270 (Predicted)</td>
</tr>
<tr>
<td>Toxin B</td>
<td>Published*</td>
<td>36</td>
<td>21</td>
<td>18</td>
<td>25</td>
<td></td>
<td>270 (Predicted)</td>
</tr>
<tr>
<td>Toxoid B</td>
<td>Published*</td>
<td>36</td>
<td>18</td>
<td>18</td>
<td>28</td>
<td>55.8*</td>
<td>270 (Predicted)</td>
</tr>
</tbody>
</table>


- CD spectra and analytical centrifugation revealed secondary structure and overall size comparable to native toxins and in good agreement with published analysis
- Recombinant mTcdA and mTcdB induced high ELISA titers and neutralizing antibodies (not shown)
Efficacy of the Full-Length Recombinant mTcdA and mTcdB

- Recombinant TcdA/TcdB vaccine affords protection against *C. difficile* challenge (n=10)
- At the 100 µg dose (50 µg each) the level of protection is equivalent to benchmark toxoid vaccine
- Vaccine is also effective at 5-fold and 25-fold lower doses
Summary

- Fragment-based vaccine consisting of the recombinant CROPS fragments of Toxin A and B induced high ELISA titers, neutralizing antibodies, and good protection against *C. difficile* challenge. Larger fragments were performing considerably better than small fragments of the CROPS region.

- Full-length recombinant mTcdA and mTcdB vaccine induced neutralizing antibodies and induced long-lasting protection equivalent to benchmark toxoid vaccine.

- The final vaccine image including formulation, stability, and dosing is under development.
# C. difficile Vaccine Team

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- Carolee Welebob (Clinical)
- Swati Gupta (Epidemiology)
- Tim Herring (Epidemiology)
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- Lois Lockledge (Commercial)
- Stella Reed (Regulatory)
- Lisa Plitnick (Safety Assessment)
- Beth Arnold (Clinical Assays)
- Rocio Marchese (Clinical Assays)
Thank you!
Muito Obrigado!
Cytotoxic Effects of *C. difficile* Toxins

Legend

- **E**: Holotoxin A or B
- **E**: Enzymatic domain
- **:** Translocation domain
- **:** Binding domain
- **:** Endosomal H⁺ ATPase

- **Rho GTPases (RhoA, Rac1, cdc42)**
  - **Rho**: active
  - **Rho⁻**: inactive
- **Inhibition**
- **Activation**

Barth et al, JBC 276(2001):10670-10676
Rupnik et al, Microbiology 151(2005):199-208
Pfeifer et al, JBC 278(2003):44535-44541
Abstract

*Clostridium difficile* is the major cause of antibiotic-associated diarrhea and pseudomembranous colitis, a disease associated with significant morbidity and mortality. The disease is mostly of nosocomial origin, with elder patients undergoing anti-microbial therapy being particularly at risk. A new, hypervirulent strain of *C. difficile* called NAP1 (027) has been implicated in outbreaks associated with increased morbidity and mortality since the early 2000s. This epidemic strain is responsible for increased incidence of *C. difficile*-associated diarrhea related not only to antibiotic exposure, but infection is also associated with GI surgery, prolonged hospitalization, and immune-compromising conditions. *C. difficile* produces 2 large toxins: Toxin A and Toxin B. These 2 toxins act synergistically to damage and impair the colonic epithelium and are primarily responsible for the pathogenesis associated with *C. difficile* infection (CDI). Testing the feasibility of toxin-based vaccination against *C. difficile* is being investigated. A native Toxoid A- and B-based vaccine was reported to be safe and immunogenic in healthy volunteers.

We generated a toolbox of *E. coli* expressed Toxin B fragments covering the entire molecule and systematically explored these fragments as components of an experimental vaccine. We observed a robust immune response in hamsters vaccinated with the recombinant toxin fragments. The antiserum obtained from immunized hamsters was shown to neutralize cytotoxic effects of Toxin B *in vitro* (in cell-based neutralization assay). Hamsters immunized with the combination of full-length Toxoid A and fragments of Toxin B were protected against lethal challenge with *C. difficile* spores.

We also evaluated that recombinant full-length mTcdA and mTcdB vaccine and demonstrated excellent performance in a hamster challenge model, equivalent to benchmark toxoid vaccine.

The use of recombinant *C. difficile* toxins could afford improved protection, vaccine thermal stability, and facilitate manufacturability of the vaccine. The additional studies, including thermal stability, formulation, safety, and dosing, will be needed to establish the final vaccine image.
Western Blotting Analysis of Recombinant Toxin B Fragments

A. Monoclonal antiTcdB (Novus)

B. Polyclonal antiTcdB/TcdA

• Recombinant Toxin B fragments were separated on SDS/PAGE, transferred onto nitrocellulose, and probed with monoclonal antibody (5A8-E11) developed against full-length native Toxin B (Novus Biologicals) (A) or polyclonal antisera from rhesus monkeys immunized with Toxin A/Toxin B (B)
C. difficile Infection Prevention: Biotherapeutics, Immunologics, and Vaccines

<table>
<thead>
<tr>
<th>Prevention Intervention</th>
<th>Effectiveness in Humans</th>
<th>Rapidity of Prevention</th>
<th>Duration of Prevention</th>
<th>Primary Prevention</th>
<th>Recurrence Prevention</th>
<th>Projected Cost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fecal transplants</td>
<td>Excellent</td>
<td>Rapid (1-2 days)</td>
<td>Likely to be effective until further antibiotics</td>
<td>Unknown</td>
<td>Yes</td>
<td>Low</td>
</tr>
<tr>
<td>Non-toxigenic C. difficile</td>
<td>Unknown but expected to be excellent from natural colonization</td>
<td>Rapid (1-2 days)</td>
<td>Likely to be effective until microbiota recovers or further antibiotics</td>
<td>Yes</td>
<td>Yes</td>
<td>Low</td>
</tr>
<tr>
<td>Injectable vaccine</td>
<td>Unknown</td>
<td>Slow (weeks to months)</td>
<td>Unknown but should be long</td>
<td>Yes</td>
<td>Yes</td>
<td>Low</td>
</tr>
<tr>
<td>Mucosal vaccine</td>
<td>Unknown</td>
<td>Slow (weeks to months)</td>
<td>Unknown but should be long</td>
<td>Yes</td>
<td>Yes</td>
<td>Low</td>
</tr>
<tr>
<td>Monoclonal antibodies</td>
<td>Excellent</td>
<td>Rapid (immediate)</td>
<td>Unknown but expected to be transient</td>
<td>Probable</td>
<td>Yes</td>
<td>Very high</td>
</tr>
</tbody>
</table>

Gerding DN
Points of Treatment of CDI

- C. difficile acquisition
- Antimicrobial(s)
- Asymptomatic C. difficile colonization
- CDI

- Hospitalization
- Give non-toxigenic C. difficile, other biotherapeutics, or monoclonal antibodies
- Vaccinate or give monoclonal antibodies to prevent CDI

Antibiotic Rx plus monoclonal antibodies or vaccine or fecal transplant or non-toxigenic C. difficile

Gerding DN
Sequence Similarity Between TcdA and TcdB

ENZYMATIC DOMAIN (ED) (Rho Glucosyl Transferase)
TRANSLOCATION DOMAIN (TD) (???)
BINDING DOMAIN (BD) (Carbohydrate-Binding Domain CBD)

TcdA
8133 bp

TcdB
7101 bp

Similarity

1 300 600 900 1200 1500 1800 2100 2400 2700
C. difficile Infection

• 500,000 hospitalizations in US due to CDI – up to 5% mortality
• Anaerobic spore-forming gram-positive bacillus
• Causes C. difficile-associated infection (CDI or CDAD)
• Damage to gut lining → diarrhea → pseudomembranous colitis → toxic megacolon → sepsis → death
• Main mode of transmission is fecal-oral in hospital setting
Expression and Purification of Recombinant Fragments of TcdB

(Coomassie Blue)

Western Blotting (anti6His)

- Synthetic genes encoding TcdB fragments were cloned into pET30a vector, expressed in (BL21/DE3) *E. coli*, and purified by two-step chromatography using Ni-affinity column followed by anion exchange chromatography.
### Expression of TcdB Fragments in *E. coli* – Purification Yields

<table>
<thead>
<tr>
<th>Fragment Name</th>
<th>Amino Acids</th>
<th>Mw</th>
<th># of Residues</th>
<th>pl</th>
<th># of Cys</th>
<th>Purity, %</th>
<th>Yield* mg/Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>TransCROPS B</td>
<td>545-2367</td>
<td>207</td>
<td>1832</td>
<td>4.37</td>
<td>8</td>
<td>77</td>
<td>39</td>
</tr>
<tr>
<td>TMD B</td>
<td>1129-2367</td>
<td>142</td>
<td>1248</td>
<td>4.3</td>
<td>4</td>
<td>~50</td>
<td>50</td>
</tr>
<tr>
<td>B0</td>
<td>1786-2367</td>
<td>68</td>
<td>592</td>
<td>4.11</td>
<td>1</td>
<td>95</td>
<td>822</td>
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<tr>
<td>B1</td>
<td>1836-2367</td>
<td>62.4</td>
<td>542</td>
<td>4.13</td>
<td>1</td>
<td>99.3</td>
<td>582</td>
</tr>
<tr>
<td>B2</td>
<td>1836-2101</td>
<td>31.6</td>
<td>275</td>
<td>4.4</td>
<td>0</td>
<td>99.3</td>
<td>1836</td>
</tr>
<tr>
<td>B3</td>
<td>1949-2275</td>
<td>39.1</td>
<td>336</td>
<td>4.29</td>
<td>1</td>
<td>99.6</td>
<td>686</td>
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<tr>
<td>B4</td>
<td>2102-2367</td>
<td>32</td>
<td>276</td>
<td>4.07</td>
<td>1</td>
<td>99.6</td>
<td>800</td>
</tr>
<tr>
<td>ED B</td>
<td>63.9</td>
<td>559</td>
<td>4.85</td>
<td>1</td>
<td>91</td>
<td>505</td>
<td></td>
</tr>
</tbody>
</table>

* Assuming 20% biomass, after two-step purification