Protection Against *Clostridium difficile* Infection With Broadly Neutralizing Antitoxin Monoclonal Antibodies

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The spore-forming bacterium *Clostridium difficile* represents the principal cause of hospital-acquired diarrhea and pseudomembranous colitis worldwide. *C. difficile* infection (CDI) is mediated by 2 bacterial toxins, A and B; neutralizing these toxins with monoclonal antibodies (mAbs) provides a potential nonantibiotic strategy for combating the rising prevalence, severity, and recurrence of CDI. Novel antitoxin mAbs were generated in mice and were humanized. The humanized antitoxin A mAb PA-50 and antitoxin B mAb PA-41 have picomolar potencies in vitro and bind to novel regions of the respective toxins. In a hamster model for CDI, 95% of animals treated with a combination of humanized PA-50 and PA-41 showed long-term survival relative to 0% survival of animals treated with standard antibiotics or comparator mAbs. These humanized mAbs provide insight into *C. difficile* intoxication and hold promise as potential nonantibiotic agents for improving clinical management of CDI.

*Clostridium difficile* is a gram-positive, spore-forming, anaerobic bacterium that represents the leading cause of hospital-acquired diarrhea in developed countries [1, 2]. *C. difficile* infection (CDI) results in a spectrum of disease ranging from mild-to-severe diarrhea to fulminant colitis and death. The incidence and severity of CDI have increased markedly over the past decade, due in part to the emergence of unusually virulent, antibiotic-resistant strains. Chief amongst these are strains characterized as group BI by restriction endonuclease analysis, North American pulse-field type 1 (NAP1) by pulse-field gel electrophoresis, and ribotype 027 by polymerase chain reaction. CDI currently affects approximately 500 000 individuals and causes more than 20 000 deaths annually in the United States [1, 3].

CDI is typically precipitated when an individual is exposed to *C. difficile* spores while receiving antibiotics, which disrupt the normal colonic flora and provide an opportunity for *C. difficile* to flourish. Current practice for managing CDI involves discontinuing the culpable antibiotic and initiating treatment with metronidazole, vancomycin, or fidaxomicin [4]. Unfortunately, antibiotic therapy is associated with incomplete response or disease recurrence in approximately 30% of patients. The per-patient healthcare costs of CDI have been estimated to be approximately $4000 for primary cases and $16 000 for recurrent cases in the United States [5]. Consequently, the *C. difficile* bacterium places a significant burden on the healthcare systems of the United States and many other countries.

The main virulence factors of *C. difficile* are 2 large protein toxins, A and B. The toxins share similar size and domain organization composed of an aminoterminal glycosyltransferase domain followed by a proteolytic domain, a hydrophobic translocation domain, and a carboxy-terminal receptor-binding domain. Both toxins induce cell rounding and death by
glucosylating GTPases that are required for cytoskeletal integrity [6, 7]. These toxins have been reported to be overexpressed in hypervirulent strains [8], are absent from nontoxicogenic strains [9], and provide targets for novel therapies.

Neutralizing C. difficile toxins with monoclonal antibodies (mAbs) or vaccine-induced antibodies constitutes a nonantibiotic treatment strategy that has shown preclinical promise [10–16]. Initial clinical proof of principle was demonstrated recently with human anti-C. difficile toxin mAbs [17]. When used clinically in combination with antibiotic therapy, the mAbs significantly reduced the rate of CDI recurrence [17]. The results are consistent with prior findings that serum levels of endogenous antitoxin antibodies correlate with protection from primary and recurrent CDI [18, 19]. Although the toxin-encoding genes tcdA and tcdB are variable elements of the C. difficile genome [20, 21], little is known about how their genetic variation influences the activity of neutralizing antibodies.

We have generated novel humanized mAbs, PA-50 and PA-41, which define potent neutralization epitopes on toxins A and B, respectively. This report describes the mAbs’ binding properties and breadth of neutralizing activity. Additionally, combination therapy with PA-50/PA-41 in a well-established animal model of CDI resulted in long-lived protection from lethal disease beyond that observed with standard antibiotic therapy.

MATERIALS AND METHODS

Cell Lines, Purified C. difficile Toxins, and Supernatants

CHO-K1 and T-84 cells were obtained from American Type Culture Collection (ATCC, Rockville, Maryland). CHO-K1 cells were cultured in F-12K medium supplemented with 10% qualified fetal bovine serum (FBS) and l-glutamine, nonessential amino acids, and sodium pyruvate (Invitrogen). T-84 human colonic epithelial cells were cultured in a 1:1 mixture of F-12K and DMEM (Invitrogen) supplemented with 5% FBS, l-glutamine, nonessential amino acids, sodium pyruvate, and HEPES. Purified toxin and toxoid proteins from strain VPI 10463 were obtained from List Biological Laboratories (Campbell, California) or TechLab (Blacksburg, Virginia). C. difficile culture supernatants were produced at TechLab as described elsewhere [22].

Generation of Murine PA-50 and Murine PA-41

Female Balb/c mice (Charles River Labs, Wilmington, Massachusetts) were immunized subcutaneously with 2 or 3 doses of 10 μg of toxin A toxoid (inactivated with formaldehyde) with 10 μg Quil A adjuvant (Accurate Chemical, Westbury, New York) at 3-week intervals prior to boosting with increasing doses of active toxin A or B, also at 3-week intervals. The doses of toxin A were escalated from 20 ng to 2.5 μg, whereas doses of toxin B were escalated from 2 to 12.5 μg. Animals were boosted intraperitoneally with 10 μg toxin A or 20 μg toxin B 3 days before death. Hybridomas were generated by standard methods [23]. Hybridoma supernatants were tested for neutralization of toxin A or B on T-84 or CHO-K1 cells, respectively. Two potently inhibitory mAbs were designated murine PA-50 (mPA-50, antitoxin A) and murine PA-41 (mPA-41, antitoxin B). Hybridomas producing these mAbs were grown in cell culture or in mouse ascites, and murine mAbs were purified to >95% homogeneity by protein A chromatography, dialyzed into phosphate-buffered saline (PBS) and stored at −80°C.

Humanization of PA-50 and PA-41 and Preparation of Comparator mAbs

The variable regions of the heavy and light chain genes of mPA-50 and mPA-41 were cloned from the hybridomas using published methods [24]. Complementarity-determining regions and the relevant framework amino acids from the murine mAbs were grafted into human IgG1κ framework sequences. The humanized VH and VL regions were cloned into the expression vectors, pCON-gamma1 and pCON-kappa (Lonza Biologics, Berkshire, UK). Humanized PA-50 and PA-41 were produced as full-length IgG1κ antibodies in stably transfected CHO-K1SV cells (Lonza) and purified to >95% homogeneity by protein A chromatography. CDA1 and CDB1 [10, 17] were produced for use as comparator mAbs. Published DNA sequences encoding the heavy and light chain variable regions of CDA1/3D8 and CDB1/124 [25] were synthesized (DNA2.0, Menlo Park, California) and cloned into vectors pCON-gamma1 and pCON-kappa (Lonza). Full-length IgG1κ mAbs were expressed and purified as described above. CDA1 and CDB1 exhibited binding affinities, neutralization potencies, and hemagglutination activities in accordance with published data [25].

In Vitro Neutralization Assays

T-84 and CHO-K1 cells were used to evaluate neutralization activity of mAbs against purified toxin A and toxin B, respectively. T-84 or CHO-K1 cells were added to 96-well flat-bottom cell culture plates (Perkin Elmer) at 2 × 10^3 or 1.5 × 10^4 cells per well, respectively, and incubated for 4 hours at 37°C and 5% CO2. Toxin A (240 ng/mL) or toxin B (8 pg/mL) was combined with serially diluted mAbs (Falcon) for 1 hour at 37°C and then added to the cells. After incubation for 72 hours, 20 μL/well CellTiter-Blue (Promega) was added, and the plates were incubated for an additional 4 hours. Plates were read using a fluorescence excitation wavelength of 560 nm and an emission wavelength of 590 nm. Cell survival was compared in treated and untreated cultures, and the mAb concentration required for 50% neutralization (EC50) was calculated.
For *C. difficile* culture supernatants, toxin titer was determined by performing serial 2-fold dilutions of supernatants in the CHO-K1 and T-84 assays described above. The minimum dilution that caused >90% cytotoxicity was used in neutralization experiments.

**Efficacy Studies**

Fifty-day-old Golden Syrian hamsters (Charles River Laboratories, Stone Ridge, New York) were pretreated (day −1) with a single subcutaneous dose of clindamycin at 50 mg/kg to disrupt the normal colonic flora. On the following day (day 0), hamsters received an oral dose (3.1 × 10⁶ colony-forming units in 0.5 mL) of a suspension of *C. difficile* (strain 545, ATCC 43596) [26]. Hamsters (8 or 10 per group) received either no treatment, vancomycin orally at 20 mg/kg twice daily (BID) × 5 days, starting on day 1, or mAbs administered intraperitoneally every other day (QOD) × 4, starting on day −1. Animals were weighed at least weekly and monitored daily for health and survival during the 39-day study. Necropsy was performed at study termination, and *C. difficile* cecal counts were determined following anaerobic culture at 37°C for 48 hours in selective medium. The limit of detection was 20 CFU/g of cecum contents.

Treatment of the animals was in accordance with regulations outlined in the USDA Animal Welfare Act (9 CFR Parts 1, 2, and 3) and/or the conditions specified in the “Guide for Care and Use of Laboratory Animals” (National Academy Press, Washington, DC, 1996).

**Statistical Analyses**

Neutralization data were fit to a 4-parameter logistic equation using GraphPad Prism software (v. 4.0; GraphPad, San Diego, California). Two-sided *t* tests or log-rank tests were used for comparison of means or survival data, respectively.

**RESULTS**

**Epitope Specificity**

Immunizations yielded a panel of murine mAbs with toxin-neutralizing activity. Included in this panel were mAbs mPA-50 and mPA-41 to toxins A and B, respectively. Biacore experiments indicated that the mPA-50 epitope is present in multiple copies on toxin A and does not overlap with the epitope for CDA1 (Supplementary Figure 1A). Immobilized mPA-50 specifically bound toxin A with an avidity of 0.16 nM. The mPA-41 was observed to bind a single site on toxin B with an affinity of 0.59 nM. Binding of mPA-41 to toxin B did not block subsequent binding of CDB1 (Supplementary Figure 1B). Neither mPA-50 nor mPA-41 showed measurable cross-reactivity with toxin B or toxin A, respectively, in Biacore or enzyme-linked immunosorbent assay (ELISA) studies (data not shown).

The mAbs’ binding sites were localized to specific regions of the respective toxins by limited proteolysis of the toxins followed by Western blotting (Supplementary Figure 1C and 1D). The mPA-50 bound the carboxy-terminal domain of toxin A, whereas mPA-41 bound the amino-terminal domain of toxin B. Collectively, the data indicate that mPA-50 binds multiple sites within the receptor-binding domain of toxin A, and mPA-41 binds a single site within the enzymatic domain of toxin B.

**In Vitro Neutralization Activity of Murine and Humanized mAbs**

Murine and humanized mAbs were analyzed for binding avidities and neutralization of purified toxins from strain VPI 10463 in vitro. Comparable binding avidities were observed for murine and humanized mAbs (data not shown). Murine and humanized forms of PA-50 neutralized the cytotoxicity of toxin A with EC₅₀ values of 90 pM (Figure 1A). The mPA-41 and humanized PA-41 neutralized toxin B with EC₅₀ values of 9 and 4 pM, respectively (Figure 1B). Essentially complete neutralization of toxins A and B was obtained at higher mAb concentrations. Humanized PA-50 and PA-41 thus recapitulate the toxin-neutralizing activities of the respective mouse mAbs.

**Breadth of In Vitro Neutralization of Humanized mAbs**

Because *C. difficile* exhibits intrastain heterogeneity in the genes encoding toxins A and B, studies were undertaken to examine the breadth of toxin neutralization by PA-50 and PA-41. These studies employed toxin-containing supernatants generated from diverse *C. difficile* isolates from North America and Europe (Table 1). The panel included ribotypes 001, 002, 003, 012, 014, 017, 027, and 078 in approximate frequency proportion to that observed clinically [27, 28], with the exception of ribotype 017 tcdA−tcdB+ strains, which are overrepresented in the panel. Supernatants from tcdA−tcdB+ strains were used as tools to identify cells that were refractory to killing by supernatants containing toxin B alone and thus would be suitable for examining cytotoxicity mediated by toxin A. Strain VPI 10463 was included in the panel and allowed a comparison of results obtained with purified and unpurified toxins. CDA1 and CDB1 were tested as comparator mAbs.

PA-50 neutralized toxin A in a strain-independent manner. The median EC₅₀ was 32 pM (range, 20–127 pM; Table 1), and Hill slopes typically exceeded 2 (Figure 2A). PA-50 was more active than CDA1 against each of the test isolates. The greatest potency differences were observed for hypervirulent ribotype 027 strains; PA-50 was approximately 1000-fold more potent than CDA1 (*P* = .0002) against these strains, as well as against a ribotype 078 strain included in the panel.
PA-41 inhibited each of the \( \text{tcdA}^- \text{tcdB}^+ \) strains with a median EC\(_{50} \) of 23 pM (range, 7.7–129 pM; Table 1) (Figure 2B). PA-41 was generally more effective than CDB1 against \( \text{tcdA}^- \text{tcdB}^+ \) strains and was approximately 500-fold more potent against the ribotype 027 strains \((P = .003)\). CDB1 was more effective than PA-41 against ribotype 017 \( \text{tcdA}^- \text{tcdB}^+ \) strains; however, the reverse was true for a ribotype 036 \( \text{tcdA}^- \text{tcdB}^+ \) strain. Finally, PA-41 and PA-50 exhibited comparable activities against crude and purified forms of VPI 10463 toxins (Table 1 and Figure 1).

**Efficacy of PA-50 and PA-41 in a Hamster Model of CDI**

In vivo efficacy was examined in hamsters. The study examined 20 and 50 mg/kg/mAb doses of PA-50 and PA-41 used in combination, that is, “PA-50/PA-41”, in animals challenged with the ribotype 012 strain 545. Strain 545 represents a heterologous challenge strain that is unrelated to the ribotype 003 strain (VPI 10463) used in generating these mAbs. Strain 545 is similar to VPI 10463 in terms of the susceptibility of its toxins to neutralization by PA-50, PA-41, and the comparator mAbs (Table 1). Control groups in the study received no treatment, vancomycin, or the CDA1/CDB1 combination (Table 2).

Untreated animals had a median survival of 2 days, with the longest survival being 3 days. In contrast, 19 of 20 animals treated with either 20 or 50 mg/kg/mAb of PA-50/PA-41 survived through the 39-day study (Table 2 and Figure 3A). One PA-50/PA-41 animal perished on day 8 with symptoms consistent with CDI.

Animals treated with the CDA1/CDB1 combination, or with vancomycin, displayed intermediate survival. Treatment with CDA1/CDB1 at 20 or 50 mg/kg/mAb resulted in median survival times of 11 and 14 days, respectively. Maximum survival was 30 days. The results observed for CDA1/CDB1 in this study are consistent with the 55% survival at 11 days post-infection observed in a prior hamster study [10]. Vancomycin treatment improved median survival to 20 days, but all vancomycin-treated animals experienced a fatal relapse of disease typical of this model. Given the similar outcomes observed for the 20 and 50 mg/kg/mAb doses in mAb-treated animals, the data for these dose groups were pooled in plotting survival curves (Figure 3A). The survival benefit for treatment with PA-50/PA-41 was significant \((P < .0001)\) relative to each of the other treatment groups.

**Table 1. Neutralization of Toxins From Diverse *Clostridium difficile* Strains In Vitro**

<table>
<thead>
<tr>
<th>Ribotype</th>
<th>Strain</th>
<th>Antitoxin A mAbs</th>
<th>Antitoxin B mAbs</th>
<th>EC(_{50}), pM</th>
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<tr>
<td>001</td>
<td>CCL14137</td>
<td>39</td>
<td>611</td>
<td>9.7</td>
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<tr>
<td>001</td>
<td>MH5</td>
<td>127</td>
<td>284</td>
<td>18</td>
</tr>
<tr>
<td>001</td>
<td>Pitt 2</td>
<td>27</td>
<td>247</td>
<td>13</td>
</tr>
<tr>
<td>002</td>
<td>UVA17</td>
<td>26</td>
<td>825</td>
<td>129</td>
</tr>
<tr>
<td>003</td>
<td>VPI 10463</td>
<td>20</td>
<td>1271</td>
<td>7.7</td>
</tr>
<tr>
<td>012</td>
<td>545</td>
<td>38</td>
<td>4552</td>
<td>15</td>
</tr>
<tr>
<td>012</td>
<td>630</td>
<td>54</td>
<td>1019</td>
<td>111</td>
</tr>
<tr>
<td>014</td>
<td>UVA30/TL42</td>
<td>51</td>
<td>625</td>
<td>21</td>
</tr>
<tr>
<td>017</td>
<td>CCL13820</td>
<td>N/A</td>
<td>N/A</td>
<td>&gt;10(^5)</td>
</tr>
<tr>
<td>017</td>
<td>F1470</td>
<td>N/A</td>
<td>N/A</td>
<td>&gt;10(^5)</td>
</tr>
<tr>
<td>017</td>
<td>Pitt 102</td>
<td>N/A</td>
<td>N/A</td>
<td>&gt;10(^5)</td>
</tr>
<tr>
<td>027</td>
<td>CCL678</td>
<td>29</td>
<td>58950</td>
<td>77</td>
</tr>
<tr>
<td>027</td>
<td>CCL14402</td>
<td>ND</td>
<td>ND</td>
<td>19</td>
</tr>
<tr>
<td>027</td>
<td>CD196</td>
<td>61</td>
<td>132600</td>
<td>16</td>
</tr>
<tr>
<td>027</td>
<td>HMC553</td>
<td>29</td>
<td>109000</td>
<td>24</td>
</tr>
<tr>
<td>027</td>
<td>Montreal 5</td>
<td>29</td>
<td>87090</td>
<td>36</td>
</tr>
<tr>
<td>027</td>
<td>Montreal 7.1</td>
<td>31</td>
<td>109400</td>
<td>29</td>
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<tr>
<td>027</td>
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<td>43</td>
<td>108100</td>
<td>29</td>
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<tr>
<td>036</td>
<td>8864</td>
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<td>N/A</td>
<td>370</td>
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<tr>
<td>078</td>
<td>Pitt 07</td>
<td>32</td>
<td>&gt;10(^5)</td>
<td>59</td>
</tr>
</tbody>
</table>

Data are representative of 2–3 replicates for each mAb/strain combination. Abbreviations: N/A, not applicable; ND, not done.
Additional evaluations included weight measurements, gross necropsy, and cecal titer of *C. difficile* at termination. Mean body weights of animals treated with vancomycin or PA-50/PA-41 decreased during the first week postinfection and then rebounded (Figure 3B). By day 39, the mean body weights of animals treated with PA-50/PA-41 were similar to those of healthy, uninfected animals that were housed in parallel (*P* > 0.05). The mean body weights of animals treated with CDA1/CDB1 declined steadily (Figure 3B).

Table 2. Survival Outcomes by Treatment Group in the Hamster Efficacy Study

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (route)</th>
<th>Treatment days</th>
<th>No. of animals</th>
<th>Median survival, d</th>
<th>Day 39 survival, %</th>
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<tbody>
<tr>
<td>No treatment</td>
<td>None</td>
<td>None</td>
<td>8</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>20 mg/kg BID (PO)</td>
<td>1–5</td>
<td>10</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>CDA1/CDB1 combination</td>
<td>20 mg/kg each mAb QOD (IP)</td>
<td>–1, 1, 3, 5</td>
<td>10</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>CDA1/CDB1 combination</td>
<td>50 mg/kg each mAb QOD (IP)</td>
<td>–1, 1, 3, 5</td>
<td>10</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>PA-50/PA-41 combination</td>
<td>20 mg/kg each mAb QOD (IP)</td>
<td>–1, 1, 3, 5</td>
<td>10</td>
<td>N/A</td>
<td>100</td>
</tr>
<tr>
<td>PA-50/PA-41 combination</td>
<td>50 mg/kg each mAb QOD (IP)</td>
<td>–1, 1, 3, 5</td>
<td>10</td>
<td>N/A</td>
<td>90</td>
</tr>
</tbody>
</table>

Abbreviations: BID, twice daily; IP, intraperitoneally; mAb, monoclonal antibodies; N/A, not applicable; survival exceeded 50% at the end of the study; QOD, every other day.
At day 39, the gastrointestinal tracts of the 19 surviving PA-50/PA-41 animals appeared similar to those of uninfected animals (data not shown). Cecal titers of C. difficile were either undetectable (<1.3 log₁₀ CFU, n = 11) or low (4.15 ± 0.76 log₁₀ CFU, n = 8) in the PA-50/PA-41-treated animals. In contrast, inflamed gastrointestinal tracts were observed in some or all of the animals in the other treatment groups at time of death. C. difficile was detected in 4 of 4 untreated animals (mean CFU = 8.96 ± 0.59 log₁₀, P < .0001 relative to PA-50/PA-41) and in 4 of 4 vancomycin-treated animals (mean CFU = 6.01 ± 0.93 log₁₀, P < .017 relative to PA-50/PA-41) for which cecal analyses were performed. Most hamsters treated with CDA1/CDB1 had little to no cecum contents at the time of analysis, which precluded quantitation of C. difficile titers. The empty ceca were an unexpected finding that may relate to prolonged disruption of normal gastrointestinal function in these animals.

A second study compared the efficacies of PA-50 and PA-41 used alone or in combination (see Supplementary Data). Treatment groups included vehicle, vancomycin, 40 mg/kg PA-50 alone, 40 mg/kg PA-41 alone, and PA-50/PA-41 at 20 mg/kg/mAb or 40 mg/kg/mAb. All animals that received either dose of the PA-50/PA-41 combination survived until the end of the study (42 days, Supplementary Figure 2). Median survival times for the other treatment groups were 2 days for vehicle, 2 days for PA-41, 3 days for PA-50, and 16 days for vancomycin.

**DISCUSSION**

This study describes novel humanized mAbs, PA-50 and PA-41, that broadly neutralize toxins from conventional and hypervirulent strains of C. difficile in vitro and define important neutralization epitopes on toxins A and B. When used in combination in a stringent hamster model of CDI, the mAbs provided long-lived protection against lethal disease beyond that observed for standard antibiotic therapy. The gastrointestinal tracts of the animals treated with the PA-50/PA-41 combination were characterized by a healthy appearance and low or undetectable levels of C. difficile, indicating that therapy led to normalization of the colon. PA-50 and PA-41 potently neutralized toxins from genetically diverse C. difficile strains representative of the current epidemic.

The mAbs’ breadth of activity is notable in light of the considerable genotypic and phenotypic variation within the toxins [20, 29, 30]. PA-50 and PA-41 neutralized toxins produced by the hypervirulent ribotype 027 strains with picomolar activity, whereas comparator mAbs exhibited nanomolar activity. This result is consistent with reduced binding of CDA1 to toxin A from ribotype 027 strains [31]. Toxin B from ribotype 027 strains exhibits marked sequence variation associated with increased cytotoxicity in vitro [21, 29]. However, this sequence divergence did not affect the activity of PA-41, which binds an epitope within the amino-terminal domain. Overall, the findings indicate that the epitopes for PA-50 and PA-41 are broadly conserved through the ribotype 027 lineage. Further delineation of the epitopes for PA-50 and PA-41 may provide insight into structure-function relationships of C. difficile toxins.

CDI is typically caused by C. difficile strains that produce both toxins A and B. However, tcdA⁻tcdB⁺ strains, predominantly ribotype 017, can cause disease [32, 33]. Ribotype 017
strains exhibit reduced pathogenicity in hamsters [34] and encode an atypical tcdB whose amino-terminal region bears 70%–80% sequence identity with both tcdB from VPI 10463 and lethal toxin (tcsL) from C. sordellii [35]. Phenotypically, ribotype 017 tcdB has hybrid characteristics and exhibits the receptor-binding properties and glucosylating specificities of typical tcdB and tcsL toxins, respectively [35]. The atypical amino-terminal region of ribotype 017 tcdB provides a likely explanation for why it was not neutralized by PA-41. Although ribotype 017 strains can be regionally prevalent [36–39], overall they comprise <2% of the strains encountered in recent international phase 3 clinical studies [27, 28]. In contrast to the findings for ribotype 017 strains, PA-41 effectively neutralized toxin from a ribotype 036 tcdA−tcdB− strain.

PA-50 exhibited a steep dose-response neutralization curve with Hill coefficients >2, indicating cooperative inhibition. Cooperative interactions are common in nature and are characterized by Hill coefficients of >1 [40]. PA-50 binds toxin A in a multivalent fashion, a condition that is often necessary, but not sufficient for cooperativity. We are not aware of another mAb that shows cooperative neutralization of C. difficile toxin. Studies to ascertain if cooperativity occurs at the level of PA-50 binding to toxin A and to determine the valency of the PA-50 epitope on toxin A are ongoing.

Combination treatment with PA-50/PA-41 was highly efficacious in the well-established hamster model of CDI. A short course of treatment with PA-50/PA-41 resulted in 95% survival of animals at 39 days postinfection, compared with 0% survival of animals that received no treatment, standard antibiotic therapy, or comparator mAbs. At 39 days postinfection, animals treated with PA-50/PA-41 had normal weights and showed no obvious gastrointestinal lesions. C. difficile bacterial counts could not be recovered from most animals, reflecting a >6-log10 clearance relative to untreated animals. One likely explanation for these findings is that mAb neutralization of toxins in the absence of antibiotics enabled reestablishment of protective microfloral flora in the gastrointestinal tracts of the animals.

Either toxin A or toxin B alone can cause fatal disease in hamsters [34, 41, 42], and mAbs to both toxins are generally required for maximum efficacy [10, 43]. Consistent with these findings, treatment with PA-50 or PA-41 alone exhibited minimal activity (Supplementary Data), underscoring the requirement for combination treatment. The QOD mAb treatment regimen used here was selected on the basis of a prior study of antitoxin mAbs [10]. Given the high-level efficacy observed for PA-50/PA-41, further exploration of dose and schedule is warranted. Examination of additional mAb combinations (eg, PA-50/CDB1, CDA1/PA-41, and 3 or 4 mAb combinations) is also of future interest.

In summary, humanized anti-C. difficile toxin mAbs PA-50 and PA-41 define novel neutralization epitopes that are broadly conserved across contemporary and historical strains of C. difficile. Combination treatment of animals with PA-50/PA-41 provided durable protection against lethal disease in hamsters. The properties of these mAbs make them attractive development candidates as a potential nonantibiotic therapy for CDI.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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