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Serum anti-toxin B antibody correlates with protection from recurrent *Clostridium difficile* infection (CDI)

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ABSTRACT

Background: Previous studies have demonstrated a correlation between *Clostridium difficile* anti-toxin A serum antibodies and protection against symptomatic disease and recurrence.
Methods: A neutralizing monoclonal antibody to *C. difficile* toxin A (CDA1) developed by MBL and Medarex, Inc. was studied in a phase II, randomized, double-blind, placebo-controlled trial in patients receiving standard of care treatment for *C. difficile* infection (CDI). Twenty-nine subjects received a single intravenous infusion of 10 mg/kg CDA1 and 17 subjects received placebo and were evaluated for recurrence of CDI during the 56-day study period. Serum antibodies against *C. difficile* toxin A and B were measured by ELISA and cytotoxicity assay at various time points before and after infusion.
Findings: CDI recurrence occurred in 5 of 29 (17%) in the CDA1 group and 3 of 17 (18%) (p = NS) in the placebo group with a trend toward delay in time to recurrence in the group treated with CDA1. The geometric mean concentration of antibody to an epitope of the receptor-binding domain of toxin B (0.300 and 1.20 μg/ml, respectively; $p = 0.02$) and geometric mean titer of neutralizing B antibody (8.00 and 100, respectively; $p = 0.02$) at study day 28 were lower for those subjects with recurrence compared to those who did not recur. In addition, a significantly greater proportion of subjects who recurred were infected with the epidemic BI/NAP1/027 strain compared with those that did not recur (88% vs. 22%; $p = 0.002$). Finally, in a multiple logistic regression analysis neutralizing anti-toxin B at day 14 ($p < 0.001$), anti-toxin A at day 28 ($p < 0.001$) and infection with the BI/NAP1/027 strain at enrollment ($p = 0.002$) were all predictive of CDI recurrence.
Interpretation: In this prospective study, lower concentrations of neutralizing anti-toxin B and anti-toxin A antibody and infection with the BI/NAP1/027 strain of *C. difficile* were significantly associated with recurrence of CDI.

1. Introduction

Clostridium difficile is a gram-positive, spore-forming, toxigenic bacterium and is the etiologic agent of pseudomembranous colitis in humans. Most strains of *C. difficile* produce two toxins, A and B, which are also virulence factors. Exposure to spores, commonly in the hospital environment, and disruption of the human gastrointestinal microbiota through the use of broad spectrum antibiotics are necessary for the initiation of disease. *C. difficile* is the most common cause of nosocomial infectious diarrhea in the United States and the incidence and attributable mortality of *C. difficile* associated disease in hospitalized patients is increasing [1,2]. A previously uncommon strain of *C. difficile* has been implicated in recent outbreaks in Canada and the United States [3–6]. This epidemic strain (BI/NAP1/027, Toxinotype III) is characterized by high level fluoroquinolone resistance and hyper-production of the pathogenic toxins A and B due possibly to deletions in the *tcdC* gene, a putative toxin repressor [3–5,7,8].

The importance of humoral immunity in resistance to infection and recurrence of CDI has been established in several clinical studies. Serologic responses to *C. difficile* toxins A and B are readily detectable in the general population and in patients with CDI [9,10].

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Furthermore, the magnitude of anti-toxin antibody response, particularly against toxin A, appears to correlate with resistance to symptomatic infection and protection against recurrence [11,12]. These data provided the rationale for the use of pooled intravenous immune globulin (IVIG) and the development of anti-toxin monoclonal antibodies as adjunctive therapy in severe, refractory or recurrent CDI [13–15].

MassBiologics (MBL), in partnership with Medarex, Inc. has developed fully human monoclonal antibodies directed against toxins A and B of *C. difficile*. We have shown that these antibodies can reduce mortality in acute and relapsing hamster models of *C. difficile* associated diarrhea [16]. In phase I clinical studies these antibodies are safe and well-tolerated when given as a single intravenous infusion to healthy adult volunteers [17,18]. A randomized, double-blind, placebo-controlled phase II study was undertaken in patients to examine the safety and efficacy of a human monoclonal antibody against toxin A of *C. difficile* (CDA1) with standard of care antibiotics to treat CDI. Serum samples were obtained from enrolled patients during the course of the study and the association of anti-toxin antibody concentrations with recurrent CDI was examined.

2. Subjects, materials and methods

2.1. Study design

The trial was conducted at fourteen hospitals in California and one hospital in Massachusetts from July 2005 through August 2007. Hospitalized patients ≥18 years of age with diarrhea (≥3 bowel movements/day for 2 days or >6 bowel movements/day for 1 day) and a positive stool test for *C. difficile* toxin were eligible for the study. Patients were also required to be receiving standard of care treatment (SoC) for CDI, defined as either metronidazole or oral vancomycin at the time of enrollment. Patients with severe or fulminant *C. difficile* colitis, history of chronic diarrheal illness, underlying illness(es) with a score of 4 on a modified Horn's index at enrollment [19], or previous receipt of a monoclonal antibody product or any investigational study agent within 30 days of enrollment were excluded. Enrolled subjects were randomized to either a single intravenous infusion of 10 mg/kg of CDA1 or placebo (0.9% sodium chloride) by a 2:1 assignment. Recruitment began after approval from the Institutional Review Boards (IRB) from each clinical site and written informed consent was obtained.

Subjects were followed for 56 days after study infusion for safety and clinical outcomes. Recurrent CDI was defined as a new episode of diarrhea associated with a positive *C. difficile* toxin stool test after discontinuation of SoC and resolution of prior CDI episode. Serum samples for the measurement of anti-toxin antibodies were obtained before and multiple times after infusion through day 56. The study was terminated early to evaluate anti-toxin A and anti-toxin B human monoclonal antibodies as combination adjunctive treatment for CDI.

2.2. Serum antibody assays

All available serum samples were analyzed by a standardized ELISA and a cell-based toxin neutralization assay. These assays were performed on coded samples by laboratory personnel who had no knowledge of treatment assignment. Unless otherwise specified, a recombinant fragment of the receptor-binding domain of *C. difficile* toxin A or toxin B was used to coat ELISA plates. This fragment, designated fragment 4, is the region of toxin A recognized by CDA1 [16]. Subjects' serum samples and control sera were added to plates, incubated for 90 min, followed by incubation with alkaline phosphatase-conjugated anti-human IgG $F(ab')_2$. After exposure to substrate, the absorbance was determined at 405 nm in a standard plate reader. The IgG antibody concentration of each sample was determined by interpolation from a standard curve using the four parameter curve fitting program of SoftMax Pro software (Molecular Devices, Sunnyvale, CA). The lower limit of detection for this assay was 0.1 and 0.6 μg/ml for anti-toxin A and B antibodies, respectively. Anti-toxin A and anti-toxin B antibody concentrations below the limit of detection were assigned values 1/2 the lower limit for statistical analyses. In addition to measuring antibodies specifically to a fragment of the receptor-binding domain (fragment 4), a whole toxin ELISA, using holotoxin A or B to coat ELISA plates was performed as previously described [11,20].

A cell-based cytotoxicity assay was used to determine the neutralizing antibody titers present in the serum samples. *C. difficile* toxin A or B were incubated with either subjects' sera or control monoclonal antibody. The toxin-antibody mixtures were then added to confluent IMR-90 cells. After incubation, the cytopathic effect was determined by microscopic observation. The titer of neutralizing antibody capable of inhibiting 50% of cytopathic effect was then derived from comparison with the standard curve.

2.3. Determination of C. difficile strain type

Isolation of *C. difficile* from patient stools obtained at study enrollment and isolate typing was performed in blinded fashion using selective cycloserine-cefoxitin-fructose agar with taurocholate added to enhance vegetation of spores, followed by typing of isolates by restriction endonuclease analysis (REA) to determine the *C. difficile* typing group as previously described [21]. The current epidemic strain circulating in North America and Europe is identified as group BI by REA and correlates with PCR-ribotype 027 and North American pulse field type NAP1 [22].

2.4. Statistical analysis

Statistical comparisons of enrollment characteristics, serum concentrations and titers of anti-toxin antibodies and clinical outcomes between CDA1 and placebo treatment groups were made using parametric and non-parametric tests. Logistic regression models were used to determine the significance of these variables in predicting recurrence. JMP (version 7.0 SAS institute) was used for statistical analyses.

3. Results

3.1. Study population

A total of 47 patients were enrolled in the study. One subject was followed only for safety analysis due to a randomization error. Of the 46 evaluable patients, 29 subjects received CDA1 and 17 subjects received placebo. The demographics and clinical characteristics of the enrolled patients were similar. There were no significant differences with respect to age or mean duration of SoC prior to enrollment. Fifteen of 29 (51.7%) of subjects in the CDA1 group and 12 of 17 (70.6%) of the placebo subjects received metronidazole only ($p = 0.24$) and 12 of 29 (41.4%) and 4 of 17 (23.5%) received both metronidazole and vancomycin, respectively ($p = 0.34$) during the 56-day study period. The mean duration (days) of SoC treatment of the initial episode was similar between the two groups (16.0 vs. 17.9, $p = 0.57$). Stool samples were available from 41 of the 46 evaluable subjects, *C. difficile* was cultured from 36, and the strain was determined in 35 of these samples. Thirteen samples (37%) had the BI/NAP1/027 epidemic strain and the percentage of subjects infected with this strain was similar in the two groups (Table 1).