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The non-toxigenic *Clostridium difficile* CD37 protects mice against infection with a BI/NAP1/027 type of *C. difficile* strain

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Abstract

C. difficile CD37, a clinical isolate from the USA, does not produce toxin A, B or binary toxin. The aim of this study was to determine whether strain CD37 can protect mice against infection from a challenge with a toxigenic *C. difficile* strain. Three groups of mice (n=10) were pretreated with a antibiotics cocktail for 5 days, switched to sterile water for 2 days, and given one dose of clindamycin (10 mg/kg) one day (day-1) before challenge (day 0) with a toxigenic *C. difficile* strain. Group 1 (CD37+UK6) was given 10^7 *C. difficile* CD37 vegetative cells by gavage twice a day on days -1 and -2, followed by challenge with 10^6 spores of the toxigenic *C. difficile* UK6 (BI/NAPI/027) on day 0; Group 2 (UK6) was infected with 10^6 *C. difficile* UK6 spores on day 0; Group 3 (CD37) was challenged with 10^6 CD37 vegetative cells on day 0. Our data show that pre-inoculation with strain CD37 provided significant protection (survival, $p < 0.001$ between groups CD37+UK6 and UK6) against subsequent infection with the strain UK6, while mice infected with CD37 only did not develop any symptoms of *C. difficile* infection (CDI). Our results highlight the potential use of CD37 as a therapeutic strain for the prevention of primary and recurrent CDI in humans.

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Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Introduction

Clostridium difficile is the leading cause of health care-associated infectious diarrhea [1]. Two toxins, TcdA and TcdB, are major virulence factors of *C. difficile* [2–4]. Both mortality and morbidity of *C. difficile* infection (CDI) have increased significantly in the last 15 years [5], partially due to the emergence of new virulent BI/NAP1/027 type *C. difficile* strains, some of which have increased toxin production and sporulation capacity, altered microbial resistance patterns (fluoroquilonone resistance) and produce an additional binary toxin [6, 7]. Interestingly, non-toxigenic *C. difficile* strains which lack the genes for TcdA, TcdB and the binary toxin exist in nature, and have been isolated from humans, although little is known about their biology [8].

Previous studies have shown that asymptomatic colonization by non-toxigenic *C. difficile* strains tend to decrease risk of CDI in humans [9]. Non-toxigenic *C. difficile* strains have been shown to prevent fatal CDI in hamsters and piglets [10] [11, 12]. *C. difficile* CD37 is a non-toxigenic clinical isolate from the USA [13]. In the strain CD37, the PaLoc (encoding toxin A and B) is absent, in its place is 115 bp of non-coding DNA [14].

CDI has been studied in a number of animal models, including hamsters, guinea pigs, rabbits, rats, germfree mice, conventional mice and germfree piglets [15–20]. The hamster model has been widely used. However, hamsters are extremely sensitive to *C. difficile*, rapidly develop clinical signs of CDI, and die within 2 to 3 days of infection dependent on strains used [21]. Therefore, this model does not represent the usual course and spectrum of CDI in human beings. The recently developed mouse and piglet CDI models are good alternatives for studying chronic CDI [19, 20], though large *C. difficile* challenging doses are required.

In the present study, we evaluated whether CD37 could be used as a therapeutic strain for the prevention of CDI in mice.

Materials and methods

Animals

C57BL/6 female mice (5 to 6 weeks old) were purchased from Charles River Laboratories, MA. All mice used in the experiments were housed in groups of 5 animals per cage under the same conditions. Food, water, bedding and cages were autoclaved. All studies followed the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved by the Tufts University Institutional Animal Care and Use Committee (IACUC) under the Protocol #G2012-70.

Spore preparation

C. difficile strains UK6 and CD37 were kindly provided by Drs. Abraham L. Sonenshein and Dale Gerding [22]. *C. difficile* strain UK6 belongs to the 027/BI21/NAP1 type, and produces TcdA, TcdB and binary toxin. Spores of *C. difficile* UK6 were prepared as described [23]. *C. difficile* CD37 is a non-toxigenic clinical isolate from the USA [13]. Strain CD37 is a poor sporulator, and vegetative cells were used to infect mice.

Antibiotics administration and *C. difficile* challenge

Antibiotic administration was based on a previously published protocol [20]. The experimental design is illustrated in Fig. 1. Three groups of mice (n=10) were given a mixture of six antibiotics including ampicillin (200 mg/kg), kanamycin (40 mg/kg), gentamycin (3.5 mg/kg), colistin (4.2 mg/kg), metronidazole (21.5 mg/kg) and vancomycin (4.5 mg/kg) in the drinking water for 5 days. After 5 days of antibiotic treatment, all mice were given autoclaved water for 2 days, followed by a single dose of clindamycin (10 mg/kg) intraperitoneally 1 day before (day-1) challenge with *C. difficile* UK6 spores by gavage (day 0). One group (CD37+UK6) was given 10^7 *C. difficile* CD37 vegetative cells by gavage twice a day on days -1 and -2, followed by challenge with 10^6 *C. difficile* UK6 spores on day 0; the second group (UK6) was infected with 10^6 *C. difficile* UK6 spores on day 0; and the third group (CD37) was challenged with 10^6 CD37 vegetative cells on day 0.

Fecal cytotoxicity

Fecal cytoxicity was determined as described previously [15]. Fecal samples were collected, and dissolved at 0.1g/ml in sterile PBS containing protease inhibitor cocktail. After centrifugation the supernatants were recovered and stored at −80°C. To detect toxin-mediated cytotoxicity in fecal samples, the supernatants were filtered and serially diluted before adding to monolayers of Vero grown in 96-well plates. Each sample and dilution was duplicated. Toxin titers were defined as the highest dilution to cause 100% cytopathic effect (cell rounding) after overnight incubation. The specific activity caused by *C. difficile* toxins from fecal samples was confirmed with goat anti-TcdA and -TcdB polysera.

Statistical analysis

Mouse survivals were analyzed by Kaplan-Meier survival analysis with a log rank test of significance using Prism. Mean relative weight was analyzed by multiple t tests using the Prism. Results are expressed as means ± standard errors of means.

Results

Pre-inoculation of *C. difficile* CD37 protected mice against infection with a virulent strain *C. difficile* UK6

The experimental scheme is illustrated in Fig 1. After challenge with *C. difficile* UK6 spores (groups: UK6 and CD37+UK6) or *C. difficile* CD37 cells (group: CD37) on day 0, three groups of mice (n=10) were monitored for mortality (Fig 2A), weight loss (Fig 2B) and diarrhea (Fig 2C). While all mice in group UK6 developed diarrhea, only 30 % mice from the group CD37+UK6 developed diarrhea (Fig 2C). Eighty percent of group CD37+UK6 mice survived, which is significantly higher than survival of group UK6 mice (20%) ($p<0.01$) (Fig 2A). Mice in group UK6 also lost significantly more weight than those in group CD37+UK6 ($p<0.05$, on post-infection days 2 and 3). As expected, mice from the control group CD37 did not develop any signs of disease, neither diarrhea (Fig 2C) nor weight loss (Fig 2B), and all mice survived (Fig. 2A). Mice from the group CD37+UK6 (Fig. 2E) excreted much less toxins in the feces as compared with the group UK6 (Fig 2D). No toxins were detected in the feces from group CD37 (Fig 2F).

Discussion

The incidence of CDI is rising worldwide [24]. Standard therapy depends on treatment with vancomycin, fidaxomicin or metronidazole, none of which are fully effective [25]. Moreover, an estimated 15–35% of those infected with *C. difficile* relapse following treatment [26, 27]. In an effort to improve outcomes and reduce recurrences of CDI, interest has been renewed in the development of non-antibiotic and adjunct approaches to therapy. There has been considerable interest in the use of probiotics to prevent CDI.

It has been reported that non-toxigenic *C. difficile* can protect hamsters against challenge with historic and epidemic toxigenic BI/NAP1/027 *C. difficile* strains [12]. Non-toxigenic *C. difficile* has been used to colonize and prevent CDI in two patients who experienced multiple relapses of CDI, and has been proven safe [11, 28]. More recently, non-toxigenic C. difficile strain M3 was evaluated for prevention of recurrent CDI in 129 patients in a randomized clinical trial; and it was shown that the strain M3 was well tolerated, colonized the gastrointestinal tract and significantly reduced CDI recurrence [29]. In this regard, non-toxigenic *C. difficile* could be considered as probiotics. *C. difficile* CD37 is the first non-toxigenic strain sequenced. More importantly, CD37 is a poor sporulator, making it an ideal strain for potential use in humans for treatment/prevention of primary and recurrent CDI. Therefore, in this project we evaluated its efficacy in protecting CDI in mice, and found oral inoculation of mice with *C. difficile* CD37 vegetative cells provided significant protection to mice against challenge with a clinical toxigenic BI/NAP1/027 type of *C. difficile* strain.

C. difficile CD37-mediated protection is not due to immune response induced by CD37 against the toxigenic strain, since anti-*C. difficile* antibodies cannot be significantly induced in mice within one or two days. The most logic interpretation of our observations would be that CD37 pre-colonization prevented the subsequent colonization of the toxigenic *C. difficile* as proposed previously [30]. However, due to the difficulties in differentially enumerating CD37 and UK6 from mouse feces, the hypothesis of colonization competition is difficult to test. Historically, live attenuated bacteria and viruses have been used as vaccines for human and animals [31] [32]. In this regard, *C. difficile* CD37 could be used as a potential vaccine candidate to prevent primary and recurrent CDI. An ideal vaccine should target both toxins and bacterial colonization with a goal to cure the toxin-mediated symptoms and reduce/prevent *C. difficile* transmission. Since strain CD37 is a poor sporulator, it would be an ideal vaccine candidate if engineered to carry immunodominant toxin epitopes. However, we should also be cautious in the possibility of non-toxigenic strains conversion into toxigenic strains in vivo though not reported so far [33].

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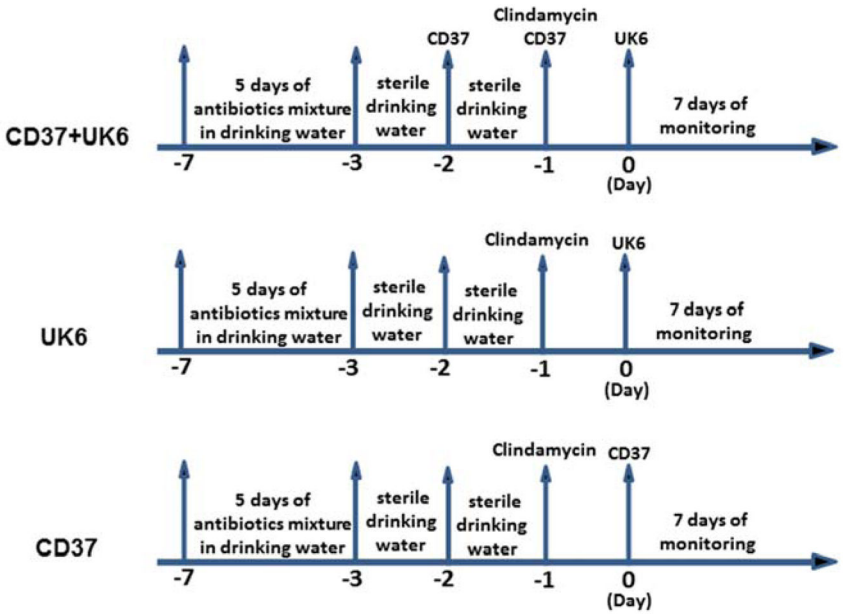

Fig. 1. Experimental scheme of pretreatment of mice with *C. difficile* CD37 in a mouse model of *C. difficile* infection

After 5 days of antibiotic treatment, mice were given autoclaved water for 2 days, followed by a single dose of clindamycin (10 mg/kg) intraperitoneally 1 day before (day-1) challenge with *C. difficile* UK6 spores by gavage (day 0). The first group (CD37+UK6) was given 10^7 *C. difficile* CD37 vegetative cells by gavage twice a day on days -1 and -2, followed by challenge with 10^6 *C. difficile* UK6 spores on day 0; the second group (UK6) was infected with 10^6 *C. difficile* UK6 spores on day 0; the third group (CD37) was challenged with 10^6 CD37 vegetative cells on day 0. Mice were monitored for diseases for 7 days.

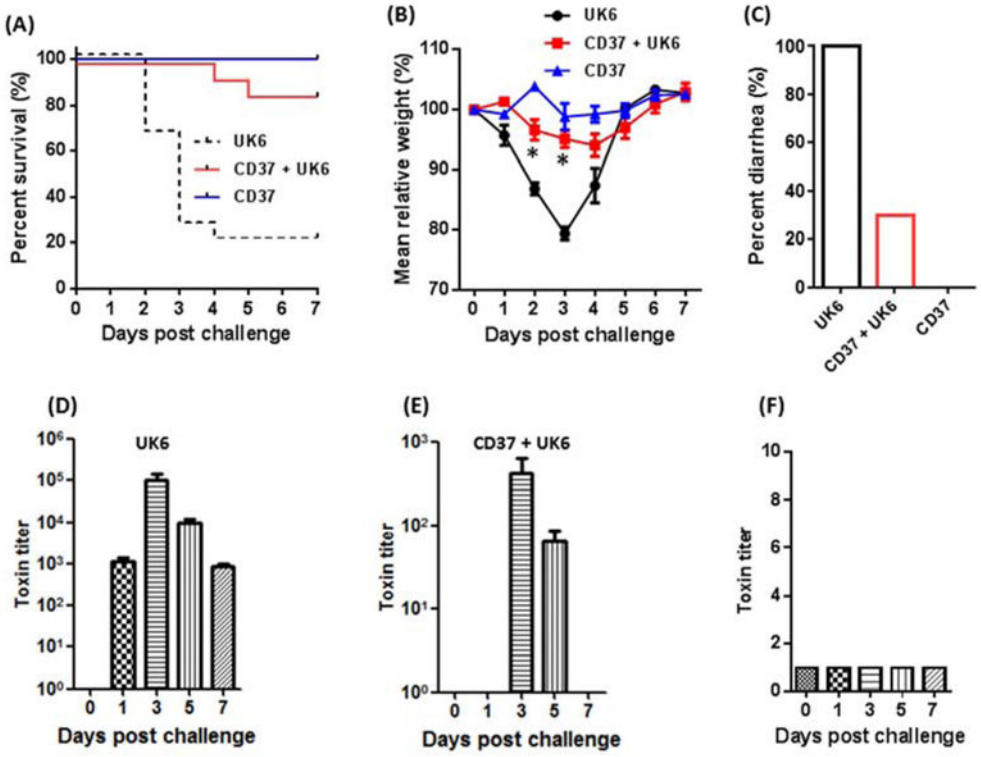

Fig. 2. Pre-inoculation of *C. difficile* CD37 protected mice against infection with a virulent strain *C. difficile* UK6

After challenge with UK6 or CD37, mice were monitored for survival ($P < 0.001$ between groups UK6 and CD37+UK6) **(A)**, weight loss (* $p<0.05$ on post-infection days 2 and 3 between groups UK6 and CD37+UK6) **(B),** and occurrence of diarrhea **(C)**. *C. difficile* toxin levels in feces from group UK6 **(D)**, CD37+UK6 **(E) and CD37(F)**.