Colonization for the Prevention of *Clostridium difficile* Disease in Hamsters

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Studies suggest that asymptomatic colonization with *Clostridium difficile* (CD) decreases the risk of CD-associated disease (CDAD) in humans. A hamster model was used to test the efficacy of colonization with 3 nontoxigenic CD strains for preventing CDAD after exposure to toxigenic CD. Groups of 10 hamsters were given 10^6 nontoxigenic CD spores 2 days after receiving a single dose of clindamycin. Five days later, the hamsters were given 100 spores of 1 of 3 toxigenic CD strains previously shown to cause mortality within 48 h. Each nontoxigenic strain prevented disease in 87%–97% of hamsters that were challenged with toxigenic strains. Failure to prevent CDAD was associated with failure of colonization with nontoxigenic CD. Colonization with nontoxigenic CD strains is highly effective in preventing CDAD in hamsters challenged with toxigenic CD strains, which suggests that use of a probiotic strategy for CDAD prevention in humans receiving antibiotics might be beneficial.

Clostridium difficile (CD) is a common nosocomial pathogen that is the causative agent of pseudomembranous colitis and a major cause of antibiotic-associated diarrhea [1, 2]. Risk of CD-associated disease (CDAD) is primarily linked to antibiotic use; however, it is also associated with immunosuppression, chemotherapy, and gastrointestinal procedures [3]. A recent study reported that hospitalized patients who developed CDAD required additional time in the hospital, which contributed $3669 to the cost of a hospital admission [4]. Another study showed marked morbidity and mortality and an increasing incidence of CDAD [5]. Because of the high costs, morbidity, and mortality associated with CDAD, prevention is a continuing priority for health care providers.

Pathogenic strains of CD produce 2 large single-unit toxins, toxin A (enterotoxin) and toxin B (cytotoxin), which are the major known virulence factors for CDAD. Nontoxigenic strains of CD have also been found in surveillance studies of asymptomatic patients [6]. These nontoxigenic strains lack the genes to produce large clostridial toxins and are not known to be associated with human disease. Although toxigenic CD strains can induce symptomatic disease, they also have been detected in asymptomatic patients.

Previous studies in hospitalized patients indicate that prior asymptomatic colonization with CD (either toxigenic or nontoxigenic) reduces the risk of subsequent CDAD [6]. Presumably, if these patients acquire a toxigenic CD strain, they develop sufficient serum IgG antibody response to CD toxin A to prevent the clinical symptoms of CDAD, as was shown by Kyne et al. [7]. We hypothesize that colonization by a nontoxigenic CD strain will not cause harm to the host and will prevent the host from acquiring an infection by a toxigenic CD strain that could produce CDAD.

The Syrian Golden hamster model parallels human infection in the susceptibility of the hamster to CD infection after antimicrobial therapy [8–10]. The hamster model has been used to investigate the efficacy of prior colonization with nontoxigenic CD strains in preventing CDAD after toxigenic challenge [10, 11]. However, prior studies have demonstrated only partial or transient success in preventing CDAD in hamsters [11, 12].

In the present study, we selected 3 nontoxigenic CD strains and evaluated their ability to prevent CDAD in the hamster after challenge by 3 toxigenic CD strains. All CD strains were selected from our collection of >5000 isolates and were identified by the *Hin*dIII restriction endonuclease analysis (REA) method of molecular typing [13]. Three nontoxigenic strains with different REA types were selected on the basis of their high frequency of asymptomatic colonization of hospitalized patients (figure 1), and 3 toxigenic strains with different REA types were selected on the basis of their roles as outbreak strains in human epidemics [6, 14–16].

We used the hamster model to determine the colonization rates and CDAD prevention rates for each of the 3 nontoxigenic CD strains against early toxigenic challenge with each of the 3 epidemic strains. We also examined the usefulness of 2 of the nontoxigenic CD strains for long-term protection against toxi-

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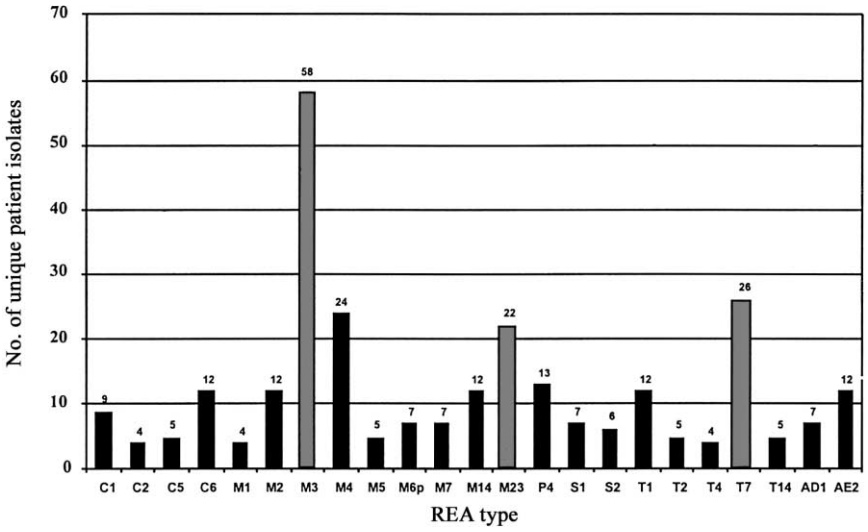

Figure 1. Frequency of isolation of *Clostridium difficile* of nontoxigenic restriction endonuclease analysis (REA) types from individual patients during 1982–1993. Gray bars indicate frequency of REA types used in hamster prevention trials.

genic challenge. Finally, we calculated the time from inoculation with the nontoxigenic strain to the onset of fecal colonization and compared the mean interval for each nontoxigenic CD strain with the efficacy of each strain in preventing CDAD in the hamster.

Materials and Methods

CD Strains

The nontoxigenic CD strains (REA types M3, M23, and T7) were selected on the basis of their high frequency among patient isolates in epidemiologic studies conducted from 1982 through 1993, primarily at the Minneapolis Veterans Affairs Medical Center but also at other US medical centers (figure 1). REA type M3 was the most frequently isolated nontoxigenic REA type, occurring in 58 of 403 nontoxigenic CD isolates from individual patients. REA types T7 and M23 also were found in isolates from individual patients at high frequencies (26 and 22 isolates, respectively). The toxigenic CD strains (REA types B1, J9, and K14) were responsible for epidemics in diverse US geographic regions from 1982 through 1995 [17]. The reference strain for each toxigenic REA type was isolated from a patient with CDAD [17].

REA

CD strains were grown anaerobically overnight in brain-heart infusion broth, and DNA was isolated, as described elsewhere [13], and then digested with *Hin*dIII. The fragments were separated on a 0.7% agarose gel, producing a characteristic banding pattern for each isolate that was visually compared with the patterns of the previously identified REA types in our library. Isolates are categorized into groups (letter designation) when they have ≤6 band differences (similarity index >90%); only isolates with identical patterns are assigned the same type (number designation) [13].

Phenotypic Assays of Toxin Activity in CD Culture Filtrates

Production of culture filtrates. For the toxin A immunoassay, bacterial strains were inoculated into 10 mL of reduced tryptic soy broth and incubated anaerobically at 37°C for 18–24 h. Cultures were centrifuged, and the supernatants were filtered through 0.45-mm syringe filters. For the human fibroblast cytotoxicity assay, bacterial strains were inoculated into 5 mL of chopped meat medium and incubated anaerobically at 37°C for 5 days. The supernatants were also filtered before assays were done, as described above.

Immunoassay for toxin A. We used the Tox-A test (TechLab) to assay CD culture filtrates for the presence of toxin A, but we modified the manufacturer's instructions to test culture supernatants instead of stool samples. In brief, culture supernatants were added to the test wells either neat or diluted 1:1 with the Tox-A test diluent. The plate was incubated at 35°C for 2 h with shaking. Washing and detection steps were as described in the manufacturer's instructions. The optical density of each well was read at 450 nm on a microplate ELISA reader. Samples with OD_{450} >200 were considered to be positive for toxin A, and samples with OD_{450} <100 were considered to be negative for toxin A.

Cell cytotoxicity assay of culture filtrates. CD culture filtrates were screened for cytotoxicity in a microtiter assay kit (*Clostridium difficile* Toxi-Titer cytotoxicity assay kit; Bartels) according to the manufacturer's instructions. This assay used

human fibroblasts as the target cells and specific anti-CD antiserum as the neutralizing antibody.

Antimicrobial Susceptibility

Each strain was tested for its susceptibility to various antimicrobial agents, including clindamycin (table 1). The susceptibility assay was a modification of the Etest strip method developed by AB Biodisk. In brief, CD isolates were grown overnight anaerobically at 36°C in 20 mL of reduced brain-heart infusion broth, washed in reduced PBS, and resuspended in 10 mL of PBS. Each isolate was diluted to an OD_{600} of 1.0, and 600 μL of the dilution was inoculated onto reduced taurocholate–fructose agar (TFA) plates (taurocholate–cycloserine–cefoxitin–fructose agar [TCCFA] plates [18] that contain no cycloserine or cefoxitin). After a brief drying period (~1–2 min), Etest strip gradients of each antibiotic were applied to each inoculated plate, and the plates were incubated anaerobically at 36°C overnight. MICs were measured after 24 h of incubation.

Hamster Assays

Preparation of spore inocula. Confluent cultures of each strain were grown anaerobically at 36°C on anaerobic blood agar plates (Columbia base; BBL) for 5–7 days to generate spore formation. The cultures were harvested with disposable loops into 10 mL of PBS with no added calcium or magnesium, washed in PBS, then heat-shocked at 56°C for 10 min to kill surviving vegetative cells. The spores were centrifuged, resuspended in Dulbecco's MEM, aliquoted, and frozen at −70°C. The frozen spores were quantitated 2 or 3 days before use by plating 100 mL of 10-fold serial dilutions of the spores onto TFA plates. After overnight incubation at 37°C in the anaerobic chamber, the plated colonies were counted and multiplied by the dilution factors to arrive at the average concentration of spores in the original freezer culture. Spores were diluted in Dulbecco's MEM for orogastric inoculation into hamsters.

Animals. Adult Syrian Golden hamsters weighing 100–130 g were obtained from the Sasco Division of Charles River Laboratories. Hamsters were housed individually in polypropylene isolator cages fitted with filter covers holding disposable polyester air filters. All food, water, bedding, cages, wire lids, and filter covers were autoclaved before use. Each group of 12 hamsters was housed in a separate small room to prevent cross-contamination with the different assay strains of CD.

Antibiotic administration. Each hamster received a single dose of 30 mg/kg clindamycin orogastrically on day 0 of each study before challenge with CD strains.

Culture method. Three fecal pellets from each hamster were collected in 750 mL of sterile PBS. Fecal material was solubilized in the PBS with a sterile cotton swab and then swabbed onto 1 quadrant of prereduced TCCFA [17] and erythromycin (TCCFA-E) plates, which are TCCFA plates containing 5 μg/mL erythromycin. The inoculum was then streaked for isolation of individual colonies. Postmortem cecal contents were also inoculated on TCCFA and TCCFA-E plates. All cultures were incubated anaerobically at 36°C for 48 h. In all studies, nontoxigenic strain colonization (types M3, M23, and T7) was deduced from TCCFA plate–positive/TCCFA-E plate–negative cultures and confirmed by REA typing of the isolates. The presence of toxigenic strains (types B1, J9, and K14) was deduced from TCCFA plate–positive/TCCFA-E-plate–positive cultures and confirmed by REA typing of the isolates.

Colonization assessment. Cultures were assessed for growth and characteristic CD colony morphology on selective medium after 24–28 h of incubation. Hamsters for which cultures showed any growth of CD on the selective plates were referred to as being "colonized." Strain confirmation by REA typing was done on colonies isolated from ⩾3 hamsters from each study group.

Colonization of Hamsters with Nontoxigenic CD Strains

REA types M3, M23, and T7 were assayed in groups of 9 or 10 hamsters per study. Each hamster was treated with clindamycin on day 0, followed by orogastric inoculation on day 2 with 10^6 cfu of a nontoxigenic strain. Hamster fecal pellets were cultured before clindamycin was administered, daily on

Table 1. MICs after 24 h of a variety of antibiotics for *Clostridium difficile* strains used in a study of the protective value of colonization with nontoxigenic *C. difficile*.

Antibiotic	Assay range, μg/mL	MIC for strain, μg/mL					
		M3	M23	T7	B1	J9	K14
Ampicillin	0.016–256	2	3	1.5	1.5	0.75	3
Ciprofloxacin	0.002–32	>32	>32	>32	>32	>32	>32
Clindamycin	0.016–256	0.38	1	1.5	>256	>256	>256
Erythromycin	0.016–256	0.75	0.5	0.5	>256	>256	>256
Tetracycline	0.016-256	0.094	0.064	0.094	12	0.064	0.094
Trimethoprim-sulfamethoxazole	0.002–32	0.125	0.094	0.094	0.125	0.094	0.19
Metronidazole	0.002–32	0.38	0.38	0.5	0.25	0.038	0.5
Ceftriaxone							
Low dose	0.002–32	>32	>32	>32	>32	>32	>32
High dose	0.016–256	128	128	96	>256	>256	>256

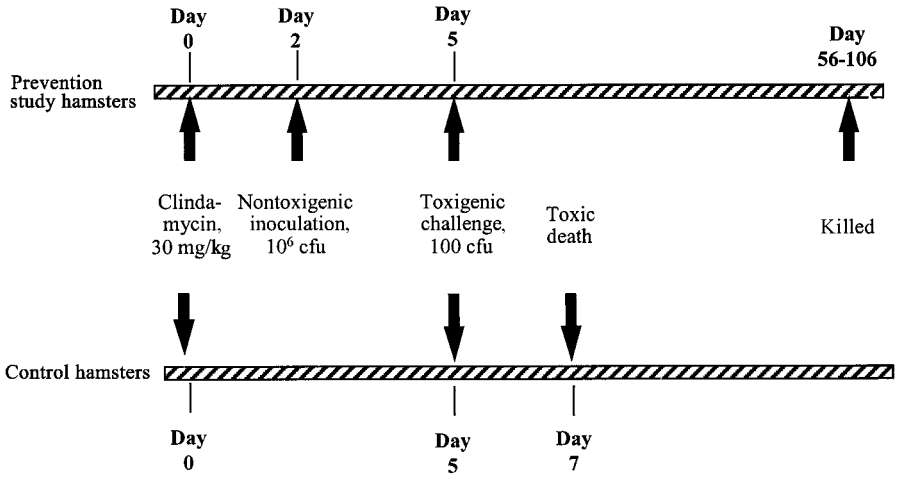

Figure 2. Time line for an early-challenge study of prevention of toxigenic *Clostridium difficile* infection involving 12 hamsters. All 12 hamsters were treated with clindamycin on day 0. Ten hamsters were orogastrically inoculated with 10^6 cfu of the nontoxigenic strain on day 2. All 12 hamsters were challenged with 100 cfu of the toxigenic strain on day 5.

days 0–8, on day 11, and once a week thereafter for a minimum of 30 days, to assess colonization onset and duration.

Prevention of CDAD in Hamsters by Prior Colonization with Nontoxigenic Strains

Early toxigenic challenge. The effectiveness of nontoxigenic REA types M3, M23, and T7 in preventing CDAD in hamsters challenged with epidemic CD types B1, J9, and K14 was tested. Figure 2 shows the time course for all 9 studies of protection against early toxigenic challenge. Hamsters were assayed in groups of 12: 10 hamsters received a nontoxigenic strain before challenge with the toxigenic strain, and 2 hamsters received the toxigenic strain only and served as positive controls for susceptibility to infection and pathogenic dose. All 12 hamsters were treated with clindamycin on day 0 and were challenged with 100 cfu of the toxigenic strain on day 5; 10 of the 12 hamsters were orogastrically inoculated with 10^6 cfu of the nontoxigenic strain on day 2. Fecal pellets were collected and cultured for CD on days 0–8 and day 11 and then once a week until the conclusion of the study, to assess colonization onset and duration and prevention of CDAD.

Toxigenic challenge after day 40. Nontoxigenic REA types M3 and M23 were assayed for their long-term effectiveness in preventing CDAD in hamsters challenged with toxigenic strains B1 and J9 after day 40 of the experiment. Three separate assays were performed; in all assays, hamsters received clindamycin on day 0, followed by orogastric inoculation of 10^6 cfu of the nontoxigenic strain. Fecal pellets were collected and cultured for CD on days 0–8 and day 11 and then once a week until toxigenic challenge, after which pellets were cultured daily for 8 days and then once a week until the conclusion of the study.

In the first assay, 5 hamsters were inoculated with nontoxigenic strain M3 on day 2 and then challenged with 100 cfu of toxigenic strain B1 on day 62 of the study. In the second, 4 hamsters were inoculated with nontoxigenic strain M3 on day 9 and challenged with ~700 cfu of toxigenic strain J9 on day 63. In the third assay, 12 hamsters received clindamycin on day 0; 10 of those 12 hamsters were inoculated with nontoxigenic strain M23 on day 2, and all 12 hamsters were challenged with 100 cfu of toxigenic strain B1 on day 41. Hamsters were observed for onset and duration of colonization and prevention of CDAD.

Statistical Analysis

We used the χ^2 test to analyze differences in colonization and mortality rates among the 9 groups of hamsters that received nontoxigenic CD and subsequently were challenged on day 5 with toxigenic types of CD. The mean intervals between nontoxigenic administration and onset of intestinal colonization in the 9 early prevention assays were compared by use of 1-way analysis of variance.

Results

REA of CD strains. Each of the 6 CD strains in this study exhibited the pattern unique to its REA type designation, as is shown in figure 3. Nontoxigenic strains M3 and M23 showed 90% similarity in REA patterns, which placed them within the same group, group M. The REA patterns of the other strains were sufficiently dissimilar to place the strains in separate REA groups.

Antibiotic susceptibility. Nontoxigenic strains M3, M23, and T7 were all susceptible to ≤ 1.5 μg/mL clindamycin and were also susceptible to ampicillin, erythromycin, tetracycline,

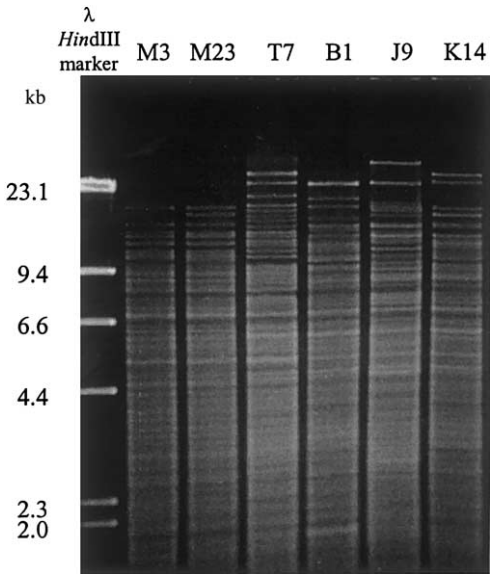

Figure 3. *Hin*dIII restriction endonuclease analysis (REA) patterns of nontoxigenic *Clostridium difficile* types (M3, M23, and T7) and toxigenic *C. difficile* types (B1, J9, and K14) used in a study of *C. difficile*–associated disease prevention. The *Hin*dIII-restricted λ-phage DNA ladder is shown in the first lane.

trimethoprim-sulfamethoxazole, and metronidazole, but they were resistant to ciprofloxacin and ceftriaxone (table 1). Toxigenic strains B1, J9, and K14 were resistant to both clindamycin and erythromycin at >256 μg/mL but were otherwise similar to the nontoxigenic strains in susceptibility to the other tested antibiotics; however, strain B1 was resistant to tetracycline (table 1).

Toxin activity of CD culture filtrates. Toxin A was detected by Tox-A immunoassay of culture filtrates from types B1, J9, and K14 (OD_{450} >1.2), whereas the culture filtrates from types M3, M23, and T7 had OD_{450} <0.05 and were reported to be negative for toxin A. The culture filtrates of the 3 toxigenic types (B1, J9, and K14) were shown to be cytotoxic and demonstrated cytopathic effects that were neutralized by specific anti-CD antiserum in the fibroblast cytotoxicity assay. The 3 nontoxigenic types (M3, M23, and T7) were not cytotoxic and demonstrated no cytopathic effects in this assay.

Colonization of hamsters with nontoxigenic CD strains. To test the onset and duration of colonization with nontoxigenic CD strains, REA type M3 was administered to 9 hamsters; all 9 hamsters were colonized by the strain within 3 days after inoculation (day 5 of the assay). The mean time to onset of colonization was 2.2 days after inoculation. M3 colonization persisted in the hamsters for a mean of 31.8 days after inoculation, and the hamsters remained free of disease until the end of the study, at day 62. REA type M23 was administered to 10 hamsters, and all 10 were colonized within 4 days after inoculation (mean time to onset of colonization, 2.5 days after inoculation). Colonization with M23 persisted in all 10 hamsters until the end of the study, at day 41. REA type T7 was administered to 10 hamsters, and all 10 hamsters were colonized by day 3 of the assay (mean time to onset of colonization, 1.0 days after inoculation). T7 colonization persisted in all 10 hamsters until the end of the study, at day 197. In all 3 colonization studies, hamsters remained disease free until the conclusion of each study.

Prevention of CDAD by prior colonization with nontoxigenic strains: early toxigenic challenge. In all studies, the 2 control animals that received only the toxigenic strain died within 48 h of toxigenic challenge, which confirmed that they were susceptible to infection and that an adequate toxic dose was used. The presence of the toxigenic strains was confirmed by culture of postmortem cecal contents on TCCFA and TCCFA-E plates.

Figure 4*A* shows colonization and prevention rates among hamsters inoculated with the 6 CD strains. All 10 hamsters that were inoculated with M3 and then challenged with B1 were colonized with M3 by day 5 and remained disease free after toxigenic challenge with B1 until the end of the study, at day 70. M3 colonization, determined by detection of the strain in fecal pellets, lasted a mean of 50 days after the onset of colonization with M3. Type B1 was not detected in any of the 10 hamsters. The rate of disease prevention after challenge with type B1 was significantly higher among hamsters that received M3 than among control hamsters that were not inoculated with M3 ($P < .001$).

Nine of 10 hamsters that were inoculated with M3 and then challenged with J9 were colonized with M3 by day 5 after inoculation. All 9 of the M3-colonized animals remained disease free after toxigenic challenge with type J9 until the end of the study, at day 58. The tenth hamster, which did not become colonized with M3, died within 48 h after J9 challenge, at the same time as the 2 control hamsters, which were inoculated with J9 only. Cultures from the tenth hamster on TCCFA and TCCFA-E plates were positive, and the isolates subsequently were identified as J9 by REA typing. The rate of disease prevention after challenge with J9 was significantly higher among hamsters that were inoculated with M3 than among control hamsters ($P = .007$).

All 10 hamsters that were inoculated with M3 and then challenged with K14 became colonized with M3 by day 4 after inoculation and remained disease free after toxigenic challenge with K14 until the end of the study, at day 85. Type K14 was not found at any time point in any of these 10 hamsters. The rate of disease prevention after challenge with K14 was significantly higher among hamsters that were inoculated with type M3 than among control hamsters ($P < .001$). The mean colonization rate for M3 in these 3 studies was 97%, and disease was prevented in all animals that became colonized with M3 after inoculation.

Figure 4*B* shows results of the M23 prevention studies. Nine of 10 hamsters that were inoculated with M23 before challenge with B1 at day 5 became colonized with M23 by day 6 after

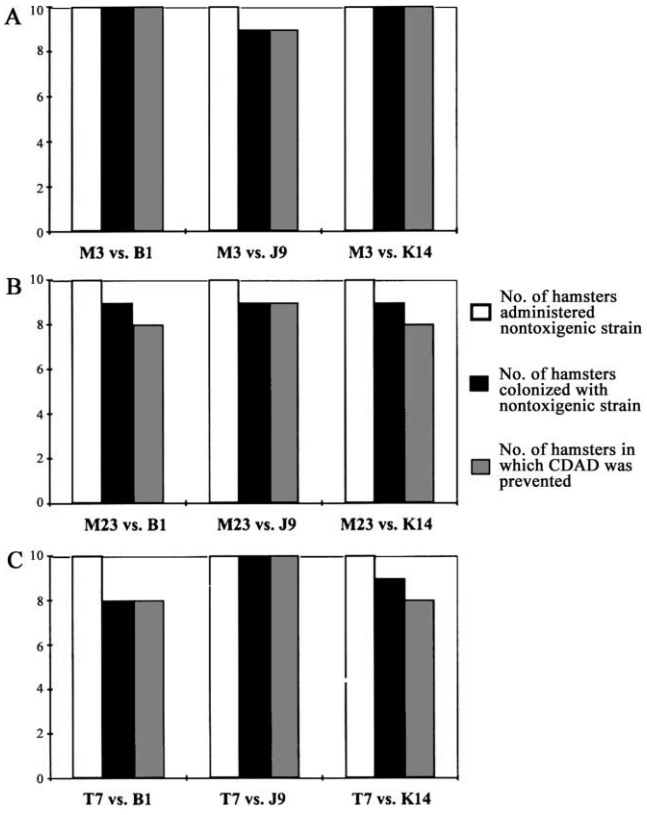

Figure 4. Colonization by nontoxigenic restriction endonuclease analysis (REA) types and prevention of *Clostridium difficile*–associated disease (CDAD) in hamsters. Bars indicate no. of animals colonized by nontoxigenic *C. difficile* REA type M3 *(A),* type M23 *(B),* and type T7 *(C)* and challenged with 3 different toxigenic *C. difficile* REA types (B1, J9, and K14).

inoculation. One hamster never became colonized with M23; that hamster died of B1 CDAD on day 7. Of the 9 M23-colonized hamsters, 1 hamster was found have types M23 and B1 in fecal pellets on day 6. Type B1 eventually prevailed, and the hamster died of CDAD on day 8. Colonization with M23 persisted in the remaining 8 hamsters, and these 8 had negative results of testing for type B1 and were disease free until the end of the study, at day 77. The difference in the rate of disease among control hamsters that were not inoculated with M23 and hamsters that were inoculated with M23 before B1 challenge was significant ($P = .028$). In the M23 prevention study in which B1 challenge was delayed until day 6, 10 of 10 hamsters were colonized with M23 by day 5 of the assay. All 10 had negative results of testing for type B1 and were disease free until the end of the study, at day 35.

Nine of 10 hamsters that were inoculated with M23 before challenge with J9 became colonized with M23 by day 6; the average onset of colonization occurred at day 4. The tenth hamster did not become colonized with M23 and subsequently died, on day 6, of J9 CDAD. The 9 M23-colonized hamsters remained free of J9 and were disease free until the end of the study, at day 56. The rate of disease prevention after challenge with type J9 was significantly higher among hamsters inoculated with M23 than among control hamsters ($P = .007$).

Nine of 10 hamsters that were inoculated with M23 and then challenged with K14 became colonized with M23 by day 6 (mean onset of colonization, day 4.8). The tenth hamster became infected with type K14 and died of CDAD within 24 h of toxigenic challenge. The 9 M23-colonized hamsters lived until the end of the study, at day 106. However, 6 of the 9 hamsters became cocolonized with type K14 transiently by day 7, and 1 hamster had K14 infection at day 13. This hamster developed symptoms of mild wet-tail disease at day 20 (but recovered), was free of K14 colonization by day 22, and remained disease free until the end of the study, at day 106. The other 5 cocolonized hamsters had only transient K14 colonization that lasted from onset at day 7 until after day 9, when K14 was no longer found in fecal pellet cultures. The rate of disease prevention after challenge with type K14 was significantly higher among hamsters that were inoculated with type M23 than among control hamsters ($P = .007$). The mean rate of colonization with M23 in these 3 studies, in which toxigenic challenge was administered at day 5, was 90%, and the rate of prevention of disease was 93% among colonized animals.

Figure 4*C* shows the results of T7 prevention studies. Eight of the 10 hamsters that were inoculated with T7 became colonized between days 5 and 6 and remained colonized with T7 and disease free after toxigenic challenge with type B1 until the end of the study, at day 84. Both T7 and B1 were detected in 1 of the 8 hamsters on day 6, but B1 colonization disappeared by day 7, and the hamster never developed symptoms. The remaining 2 hamsters never became colonized with T7. These 2 hamsters died within 48 h of toxigenic challenge with type B1. The rate of disease prevention after challenge with type B1 was significantly higher among T7-inoculated hamsters than among control hamsters that were not given type T7 ($P = .028$).

All 10 of the hamsters that were inoculated with T7 and then challenged with J9 became colonized with T7 by day 4 (within 24 h after T7 inoculation) and remained disease free after toxigenic challenge with J9 until the end of the study, at day 55. Nine of the 10 hamsters remained colonized with T7 until the end of the study, at day 55. One of the 10 hamsters became T7 negative at day 41. The rate of disease prevention after challenge with J9 was significantly higher among T7-inoculated hamsters than among control hamsters ($P < .001$).

Nine of 10 hamsters that were inoculated with T7 before challenge with K14 became colonized with type T7 by day 5 after inoculation. One of the 9 colonized hamsters subsequently died, on day 12, 7 days after toxigenic challenge, but never exhibited symptoms of CDAD or K14 colonization in either fecal pellets or cecal contents. The cause of death was not determined. The tenth hamster failed to become colonized with T7 and died 48 h after toxigenic challenge with type K14. The

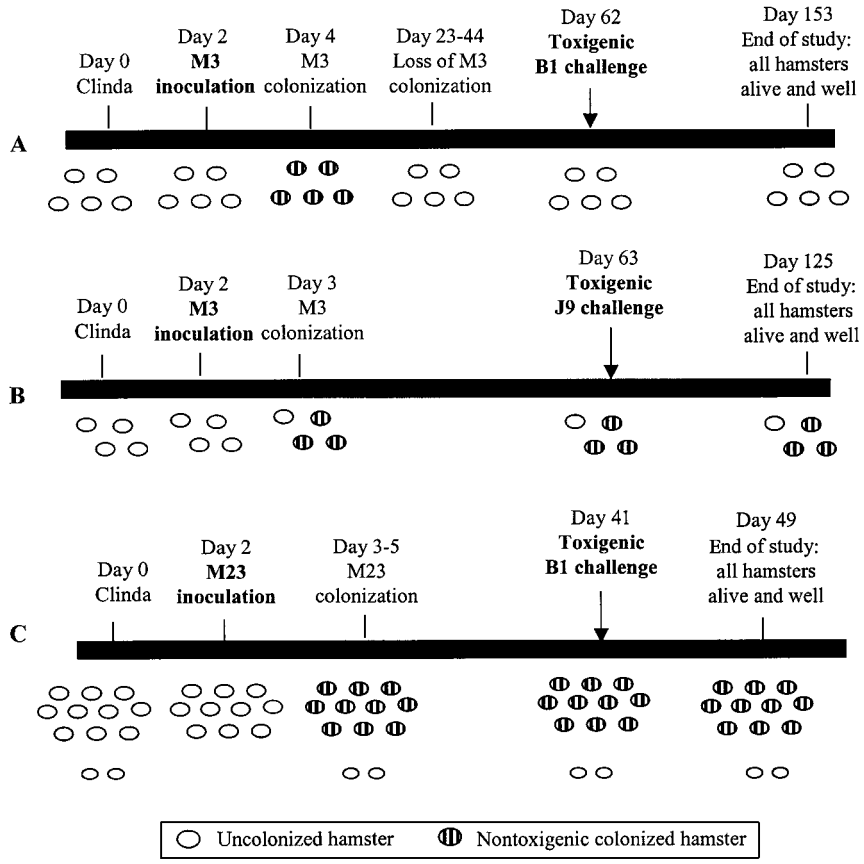

Figure 5. Long-term prevention in hamsters colonized with nontoxigenic *Clostridium difficile* (CD) strains against "late" (after day 40) toxigenic challenge. Colonization efficiency and CD-associated disease prevention results are shown for hamsters colonized with nontoxigenic CD restriction endonuclease analysis (REA) type M3 and challenged with toxigenic type B1 *(A),* colonized with nontoxigenic type M3 and challenged with toxigenic type J9 *(B),* and colonized with nontoxigenic type M23 and challenged with type B1 *(C).* Clinda, clindamycin.

rate of disease prevention after K14 challenge was significantly higher among hamsters that were inoculated with type T7 before challenge than among control hamsters ($P = .028$). The mean rate of colonization with T7 for these 3 studies was 90%, and the rate of prevention of toxigenic CDAD was 100% among colonized animals.

Time to colonization. For each of the 3 nontoxigenic CD strains, the mean times to onset of colonization in all 3 early toxigenic challenge studies were as follows: for REA type M3, 2.2 days; for type M23, 2.7 days; and for type T7, 2.8 days ($P = .002$, for REA type M3 vs. types M23 and T7).

Prevention of CDAD by prior colonization with nontoxigenic strains: toxigenic challenge after day 40. Five hamsters that were inoculated with nontoxigenic strain M3 on day 2 became colonized within 48 h of the nontoxigenic inoculation and maintained M3 colonization until day 23–44. All 5 hamsters survived challenge with toxigenic strain B1 on day 62 of the study and remained free of disease until the conclusion of the study, at day 153 (figure 5*A*).

Three of 4 hamsters that were inoculated with nontoxigenic strain M3 on day 9 became colonized within 24 h and maintained M3 colonization until the end of the study, at day 125. One hamster never became colonized with M3. All 4 hamsters survived toxigenic challenge with strain J9 on day 63 (figure 5*B*).

Twelve hamsters received clindamycin on day 0. Of the 10 hamsters inoculated with nontoxigenic strain M23 on day 2, all 10 became colonized 24–72 h later with M23 and remained colonized with M23 until the end of the study, at day 49. All 12 hamsters were challenged with toxigenic strain B1 on day 41, and all 12 survived until the end of the study, at day 49 (figure 5*C*).

Discussion

Nosocomial infection with toxigenic strains of CD and resultant CDAD remain common problems in hospitals and extended-care facilities. Current studies show an average increase in hospital stay of 3.6 days for patients with CDAD. The annual cost of this disease in the United States is conservatively estimated to be $1.1 billion [4]. The prevention of diarrhea in

patients undergoing antibiotic therapy has been attempted previously by incorporation of biologic or probiotic agents, including the yeast *Saccharomyces boulardii* [19], *Lactobacillus* species, and *Bifidobacterium* species [20]. The incidence of diarrhea has been reduced by these strategies, but CDAD rates have not been significantly reduced [19, 20].

Hospitalized patients who are asymptomatically colonized with CD strains have a decreased incidence of CDAD [6], which suggests that prior colonization with CD exerts a preventive effect against subsequent CDAD. This observation has been supported by findings in studies involving a hamster model in which prior colonization with nontoxigenic CD strains was used to protect against toxigenic CD challenge [11, 12]. However, initial protection was only partial and was not durable over the long term in the study by Borriello and Barclay [12], in which the toxigenic CD strain eventually overtook the nontoxigenic colonization in most of the previously protected animals, causing fatal CDAD 10–48 days after challenge with the toxigenic strain.

In this study, we modified the hamster protocol to model our current hypothesis of transmission of CD in the hospital setting, specifically in the use of spores, the most likely infectious form of CD in the aerobic environment, and in the use of low but very effective doses of highly infectious toxigenic strains from human epidemics in hospitals. We also selected as the prevention strains nontoxigenic REA types that were among the most frequent colonizers of patients in the hospital environment. In our 9 prevention studies (90 hamsters), which ranged in length from 35 to 106 days of observation, we observed only 2 instances of late-onset disease, in which 1 animal died of CDAD due to strain B1 at day 8 and 1 animal developed nonfatal wet-tail disease due to strain K14 on day 20. Both incidents occurred during colonization with preventive strain M23. However, we may have reduced the level of toxigenic CD in the environment by frequent cage changes in the first week of the study, although we did not take any measures to prevent coprophagic behavior in the test animals.

To determine whether the prevention conferred by nontoxigenic CD colonization became attenuated over time, we undertook late challenge studies in which M3- and M23-colonized hamsters were challenged with toxigenic CD strains 39–61 days after nontoxigenic inoculation. These studies showed that prevention of disease was persistent in hamsters colonized with nontoxigenic CD. However, prevention of CDAD after late toxigenic challenge was also seen in 3 hamsters that were initially treated with clindamycin and did not become colonized with nontoxigenic strains, which suggests that the hamsters were no longer susceptible to toxigenic CD infection at the time of the late challenge, presumably as a result of recovery of normal gastrointestinal flora.

We performed a pilot study to determine the period of CDAD susceptibility after oral administration of clindamycin in our model. Hamsters were challenged with 2×10^4 cfu of type J9 spores at varying time points after clindamycin administration. We found that the hamsters were no longer susceptible to infection by days 14–21 after clindamycin treatment (data not shown). Larson and Borriello [21] found that, in hamsters given a single oral dose of 3 mg of clindamycin, clindamycin remained detectable in the cecal contents for up to 11 days at a level (4–6 μg/g) sufficient to inhibit growth of a clindamycin-sensitive strain of CD, H-1. This level of clindamycin is also likely to inhibit growth of normal intestinal flora. After 11 days, if the normal flora begins to reestablish itself in the hamster gut, it could again form a protective barrier against CD infection, thus correlating with our results that show loss of CDAD susceptibility beginning at days 14–21. Larson and Borriello [21] found hamsters to be susceptible to toxigenic challenge with 10^4 spores as late as 74 days after administration of oral clindamycin, and, therefore, there may be considerable variation in the time to recovery of flora in hamsters after clindamycin dosing.

Our study results show a strong positive correlation between successful colonization of hamsters with nontoxigenic CD types and subsequent prevention against toxigenic challenge ($P < .0005$). In our experimental design, the time to onset of colonization with nontoxigenic CD strains is an important parameter in successful prevention. In the 9 prevention studies, type M3 exhibited the shortest time to onset of colonization (mean, 2.3 days), in contrast to the other 2 nontoxigenic strains, M23 (mean, 2.8 days) and T7 (mean, 2.9 days) ($P = .002$). Type M3 also was associated with the highest survival rate; 29 of 30 hamsters that were inoculated with M3 survived toxigenic challenge, compared with 26 of 30 for each of the other strains (P was not significant).

Two hamsters did not exhibit colonization with the nontoxigenic strain before toxigenic challenge and subsequently became colonized with both the nontoxigenic and the toxigenic type simultaneously (on the same day). The toxigenic type eventually overtook the nontoxigenic type, within 1–2 days, in 1 of these hamsters; that animal died. The other hamster survived. In general, cocolonization with nontoxigenic and toxigenic types failed to cause disease in hamsters when the nontoxigenic strain first appeared in fecal pellets $\geq$24 h before the nontoxigenic strain was detected. In 6 of the 7 instances in which a hamster with cocolonization survived, the nontoxigenic strain was detected $\geq$1 day before the nontoxigenic strain was detected. Toxigenic CD detection was transient in these instances, lasting only 2–3 days in fecal pellets before disappearing permanently, whereas the nontoxigenic strain persisted in the long term.

These results suggest that nontoxigenic CD colonization before toxigenic CD challenge is required for effective prevention of CDAD. Nontoxigenic CD colonization may be difficult to achieve or maintain under conditions of continuous administration of certain antibiotics, as occurs in patients undergoing antibiotic therapy. Colonization may be dependent on the antibiotic-resistance characteristics of the nontoxigenic strain. In

these studies, all 3 nontoxigenic strains (M3, M23, and T7) were susceptible to clindamycin in vitro at concentrations of ≤ 1.5 μg/mL. Nevertheless, all 3 strains demonstrated successful colonization (mean rate of colonization, 92%) of assay animals when the nontoxigenic strain was administered 48 h after clindamycin, which suggests that antibiotic-susceptible strains of CD can successfully colonize animals treated with an antibiotic to which the organism is susceptible but that there may be some delay in colonization as a result of the presence of antibiotic in the gastrointestinal tract. Further studies are required to examine the relationship between colonization with nontoxigenic CD and subsequent preventive effects during continuous antibiotic treatment.

In conclusion, we found that colonization with nontoxigenic strains of CD before challenge with toxigenic CD strains is highly effective in preventing CDAD in the hamster model, both after early challenge and after late challenge. To date, only 2 cases have been reported in the literature in which a nontoxigenic CD was administered to patients, and in both cases, it was given as an adjunct to CDAD treatment to prevent recurrence of CDAD [22]. Neither patient experienced any side effects from the nontoxigenic CD, but the efficacy of the approach was not clear. Our findings support the possible use of nontoxigenic CD strains as probiotics for primary prevention (and as possible treatment for recurrence) of CDAD in patients undergoing antibiotic therapy, particularly in environments where the nosocomial incidence of CDAD is high.

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