Polish Journal of Microbiology
2005, Vol. 54, No 4, 301–304

Co-Infection of Hamsters with Toxin A or Toxin B-Deficient *Clostridium difficile* Strains

ADAM SZCZĘSNY[1,2], GAYANE MARTIROSIAN[1,3*], STUART COHEN[2] and JOSEPH SILVA Jr.[2]

[1] Department of Medical Microbiology Medical University of Silesia, Katowice, Poland
[2] Department of Internal Medicine, Division of Infectious and Immunologic Diseases University of California, Davis Medical Center, Sacramento, CA, USA
[3] Department of Histology and Embryology Center of Biostructure Research, Medical University of Warsaw, Warsaw, Poland

Received 22 June 2005, received in revised form 21 November 2005, accepted 24 November 2005

Abstract

Male Syrian hamsters (*Mesocricetus auratus*) were used to study interactions between different toxin deficient strains of *C. difficile*. After sensitization with clindamycin, hamsters were intragastrically co-infected with the appropriate dilutions corresponding to 100, 1000 and 10,000 cells of four (toxin A or B-deficient) *C. difficile* strains (8864, P-829, W-38 and W-74). In addition, a group of hamsters was infected with *C. difficile* VPI 10463, a reference toxigenic strain. Colonization and mortality was observed within 48 hours in the group of hamsters infected with the reference toxigenic strain. No clinical disease was observed in the groups of hamsters co-infected with the toxin A or B-deficient strains. Re-infection of these hamsters (co-infected with toxin deficient isolates) with *C. difficile* VPI 10463 resulted in clinical disease and death suggesting that these strains do not confer protection against infection with a toxigenic strain. Macroscopic and microscopic observations of the cecum of re-infected hamsters demonstrated uniformly multiple large hemorrhagic areas without pseudomembranes. Hamsters infected with as few as 100–500 cells of the toxigenic strain – VPI 10463 alone demonstrated pseudomembranes and multiple hemorrhages. These results suggest that even though the toxin deficient strains did not prevent re-infection with a toxigenic strain of *C. difficile*, they may play a role in the histopathologic changes after re-infections in the hamster model. Further studies with a larger number of hamsters and *C. difficile* strains of various molecular profiles are required to better understand the interaction between these strains.

Key words: *Clostridium difficile*, toxin deficiency, hamsters

Introduction

Clostridium difficile causes antibiotic-associated diarrhea (CDAD), colitis and pseudomembranous colitis (PMC) in humans through the actions of toxins (mainly A and B). *C. difficile*, a spore-forming anaerobic bacteria, is the predominant causative agent of nosocomial infectious diarrhea , and the most common cause of hospital acquired diarrhea in the USA (Martirosian *et al.*, 1995). CDAD has become a major clinical problem with the increased use of antibiotics such as clindamycin, penicillins, cephalosporins and others. Cases of *C. difficile* associated diarrhea have also been reported after cancer chemotherapy. Antibiotic treatment is problematic in patients with CDAD. Incidences of *C. difficile* – associated diseases are described even after treatment of patients with vancomycin or metronidazole (Szczęsny *et al.*, 2002). The later is preferred in cases of CDAD or PMC since vancomycin therapy is associated with colonization of patients with vancomycin-resistant enterococci.

Toxins A and B are considered the major virulence factors in *C. difficile*. Genes for toxins A and B (*tcdA* and *tcdB*) are located on a large 19.6 kb chromosomal fragment known as the pathogenicity locus (PaLoc) which is a prerequisite for virulence (Poxton *et al.*, 2001). The PaLoc also includes three other genes (*tcdC*,

* Corresponding author address: Department of Microbiology, Medical University of Silesia, 18 Medyków str., 40-572 Katowice, Poland, phone/fax: (48-32) 252 60 75, e-mail: gmartir@slam.katowice.pl; gmartir@ib.amwaw.edu.pl

tcdD, *tcdE*). Molecular changes in the PaLoc of *C. difficile* strains correlate with the virulence. Toxigenic strains of *C. difficile* produce both toxins and non-toxigenic strains lack both toxins and genes. Recently mutant strains producing only one toxin are described (toxin A-negative/toxin B-positive and toxin A-positive/toxin B-negative) (Rupnik *et al.*, 2005; Cohen *et al.*, 1998). One of these strains, 8864 (tcdA–/tcdB+) is known as a causative agent of diarrhea in humans and animals (Borriello *et al.*, 1992). Nevertheless limited work has been done on characterization of a possible virulence factors in such clinical isolates of *C. difficile*.

The hamster provides the most useful model to study the pathophysiology of CDAD (Sambol *et al.*, 2001). It is the closest model of CDAD to that observed in humans. Fatal colitis in the hamster model occurs 3–5 days after administration of one dose of an antibiotic and toxigenic *C. difficile* strain. In this preliminary study, we used hamster model to investigate interactions between different mutant strains of *C. difficile* given intragastrically as a mixture.

Experimental

Materials and Methods

Strains of *Clostridium difficile*. Table I, lists the strains of *C. difficile* and their phenotypic characteristics. All strains were cultured on Brucella Blood agar (Anaerobic Systems, San Jose, CA). Plates were incubated anaerobically at 37°C for 48 hours. Strains were identified by routine laboratory methods (Martirosian *et al.*, 2004). One colony of each strains was inoculated into Brain Heart Infusion broth – BHI (Difco) and incubated anaerobically at 37°C overnight. Cultures were centrifuged at 3000 rpm for 10 min to pellet the cells. Cells were re-suspended in sterile Phosphate Buffer Saline (PBS). Dilutions of each *C. difficile* strain corresponding to 100, 1000 and 10,000 cells were prepared in PBS. The appropriate dilutions of four *C. difficile* strains (8864, P-829, W-38 and W-74) were mixed and used as the inoculum. *C. difficile* VPI 10463, a reference toxigenic strain was used as the control for clinical disease. Dilutions of the reference strains were done as described above. Each strain was tested for susceptibility to clindamycin and erythromycin by the E-test method (AB Biodisk, Sweden). For detection of both toxins (A and B) of *C. difficile* strains, Tox A/B ELISA test (TechLab, USA) and PCR for toxin A and B genes (primer pairs YT-28 & YT-29 and YT-17 & YT-18) was performed as previously described. Additionally TCD toxin A test (Becton Dickinson, USA) for detection of toxin A was also performed (Kuhl *et al.*, 1993; Martirosian *et al.*, 2004). Toxin B was detected *in vitro* by cytotoxicity and neutralization assays on McCoy cell line. PCR ribotyping was performed to compare all five *C. difficile* strains. For PCR – ribotyping primers for amplification of 16S-23S rRNA intergenic spacer were used as previously described (Martirosian *et al.*, 1995).

Table I
Molecular profile of *Clostridium difficile* strains

Strain	Characteristics	Source
VPI 10463	tcdA+/tcdB+	ATCC
8864	tcdA–/tcdB+	ATCC
P-829	tcdA+/tcdB–	UCDMC[1]
W-38	tcdA+/tcdB–	UCDMC[1]
W-74	tcdA–/tcdB+	UCDMC[1]

[1] These strains are part of the *C. difficile* collection at the University of California, Davis Medical Center, and were isolates from cases of CDAD in Seattle, WA (Cohen *et al.*, 1998).

Hamsters. Twenty four male Syrian hamsters 6–7 weeks old (*Mesocricetus auratus*) were obtained from Charles River. Hamsters were housed in groups of 4 in isolator cages with air filters fitted in their lids. They were observed during a one week period and fecal pellets were screened for the presence of *C. difficile* by direct application of a small amount of material on a selective medium Cycloserine Cefoxitine Fructose Agar (CCFA) supplemented with sodium taurocholate. Twenty hamsters were sensitized on day 0 with 3 mg/ml (subcutaneously – SC) of clindamycin (Cleocin phosphate, Pharmacia). Two days after receiving the antibiotic, hamsters were infected respectively with: the mixture of *C. difficile* toxin deficient strains (groups 3, 4, 5), VPI 10463 (group 6). Hamsters were infected by intragastric intubation (gavage) with the appropriate feeding needles. Gavage was performed following light anesthesia with Sevoflurane. As controls, a group of hamsters (group 1) received saline (SC) instead of clindamycin and 0.5 ml of saline was administered intragastrically. In addition, the clindamycin control group (group 2) received clindamycin (SC) and 0.5 ml of saline intragastrically. All hamsters were weighed on day 0 and every day there after. Hamsters were observed and scored twice daily for the following symptoms: lethargy, weight loss, onset to wet tail and time to death, for a two-week period. Every second day during this two weeks period fecal pellets from each hamster was cultured for the presence of *C. difficile* on selective CCFA plates with sodium taurocholate, as described above. After a two-weeks period, all animals in groups 3, 4 and 5 were re-infected with *C. difficile* VPI 10463.

Table II shows the animal groups and treatment.

Table II
Groups of hamsters and treatment

Group 1	Saline s.c. (0.2 ml) + 0,1 ml saline by gavage (Control group)
Group 2	Clindamycin (3 mg s.c. in 0.2 ml) + 0.1 ml saline by gavage (Clindamycin control)
Group 3	Clindamycin s.c. + 0.1 ml mixture of *Clostridium difficile* strain (10,000 cells)
Group 4	Clindamycin s.c. + 0.1 ml mixture of *Clostridium difficile* strain (1000 cells)
Group 5	Clindamycin s.c. + 0.1 ml mixture of *Clostridium difficile* strain (100 cells)
Group 6	Clindamycin s.c. + 0.1 ml VPI 10643 *Clostridium difficile* strain (100–500 cells)

Results and Discussion

Characterization of *C. difficile* strains. All 5 *C. difficile* strains demonstrated similar colony morphology and characteristic *C. difficile* biochemical reactions. Results of antibiotic susceptibility testing by E-tests are presented in Table III. E-tests demonstrated susceptibility to clindamycin and erythromycin in all *C. difficile* strains. Only one strain W-74 demonstrated higher MIC (2.0 μg/ml) of clindamycin and erythromycin, compared with 4 other *C. difficile* strains (range 0.094–1.0 μg/ml).

Toxigenicity and PCR-ribotyping of *C. difficile* strains. All five *C. difficile* strains used in this experiment demonstrated positive results in Tox A/B ELISA designed for detection of both toxins A and B. The culture filtrates of each *C. difficile* strains were analyzed for cytotoxicity using fibroblasts cell line. Strains VPI 10463 and 8864 demonstrated cytopathic effect on tissue culture, and this effect was neutralized by the specific anti-*C. difficile* serum (TechLab, USA). The culture filtrates of strains P-829, W-38, W-74 showed no cytopathic activity on cells. Toxin A activity using the TOX A ELISA kit was positive for all strains except strain 8864.

The results for PCR of toxin A and B gene and PCR ribotyping of *C. difficile* are shown in Table III.

Table III
Characterization of *C. difficile* strains

C. difficile strains	Clindamycin/Erithromycin MICs (μg/ml)		Tox A/B ELISA	TCD Tox A	Cytotoxicity and neutralization	PCR for toxin genes (A/B)	PCR ribotyping
VPI 10643	0.38	0.50	+	+	+	+/+	C
8864	0.50	0.75	+	–	+	–/+	A
P-829	0.094	0.38	+	+	–	+/–	B
W-38	0.60	1.0	+	+	–	+/–	D
W-74	2.0	2.0	+	+	–	+/–	E

Hamsters. Clinical disease was not observed in the groups of hamsters co-infected with the toxin deficient strains (groups 3, 4, 5) or in the control groups (1 and 2). No *C. difficile* growth was obtained from fecal samples taken from hamsters of groups 1, 2, 3, 4 and 5. In the case of group 6 (strain VPI 10463) we observed colonization and mortality of all animals within 48 hours after infection. All the hamsters showed pseudomembranes and hemorrhages in the colonic mucosa, both, macroscopically and microscopically (histology slides). *C. difficile* was cultured from fecal contents of all hamsters in group 6. Toxigenicity, antibiotic susceptibility testing and PCR-ribotyping were consistent with the characteristics of the reference strain VPI 10463 (Table III). After the two weeks period we re-infected (by intragastric intubation – gavage) 12 hamsters (group 3, 4 and 5) which had previously received the mixture of toxin deficient *C. difficile* strains, with strain VPI 10463 (100–500 cells). Within two days, all hamsters showed clinical symptoms of CDAD and died. *C. difficile* was cultured on selective CCFA plates from the fecal contents of all animals. All characteristics of the strain isolated were identical to strain VPI 10463. Macroscopic and microscopic studies of colons demonstrated multiple large hemorrhagic areas without pseudomembranes.

Several reports indicate that previous colonization with nontoxigenic *C. difficile* may be protective against colonization with a toxigenic strain (Wilson and Sheagren, 1983). Attempts have been made to treat patients with a nontoxigenic strain to prevent further relapses with a toxigenic strain. Even though some

results have been promising, it is not clear whether this approach will be useful in clinical practice. This preliminary study was undertaken to determine if previous infection with toxin A or B-deficient strains confers protection in hamsters against infection with a toxigenic strain. No colonization or clinical symptoms were observed when hamsters were co-infected with *C. difficile* toxin deficient strains (3 toxin B-deficient and one toxin A-deficient strain). Co-infection with as high as 10,000 cfu of these isolates did not result in clinical symptoms. Re-infection with *C. difficile* VPI 10463 resulted in death within 48 hours. These results suggest that previous infection with toxin deficient isolates does not result in protection against colonization and disease development by a toxigenic strain. Why a mixture of these toxin deficient isolates does not cause clinical symptoms in the hamster remains to be investigated. It is possible that adherence plays a major role in this case, and that other genetic characteristics of the deficient strains related to adherence mechanisms and colonization may play a role. In summary, one implication derived from this study is that there may be other pathogenic factors that are co-linked to the pathogenicity locus which are important in colonization and development of clinical colitis. Thus, strains having the ability to produce only one toxin may have other pathogenic factors that are eliminated or not expressed. These can include production of anti-chemotactic factors, proteinases, alterations in adhesion, *etc.* The studies of these factors are very important because biotherapy still holds promise for either preventing *C. difficile* colitis or treatment of patients with active disease and/or relapses. The mutant isolates studied do not appear to offer any significant protection against toxigenic *C. difficile* strains in the hamster model. However, a larger study with more animals and combination of strains fully characterized may be provide useful information.

Acknowledgements. We would like to thank Dr Yajarayma Tang-Fredman for critical comments and advices.

Literature

Borriello S.P., B.W. Wren, S. Hyde, S.V. Seddon, P. Sibbons, M.M. Krishna, S. Tabaqchali, S. Manek and A.B. Price. 1992. Molecular, immunological, and biological characterization of a toxin A-negative, toxin B-positive strain of Clostridium difficile. *Infect. Immun.* **60**: 4192–4199.

Cohen S., Y. Tang, B. Hansen and J.Jr. Silva. 1998. Isolation of a toxin B-deficient mutant strain of *C. difficile* in a case of recurrent *C. difficile* – associated diarrhea. *Clin. Infect. Dis*. **26**: 410–412.

Kuhl S.L., Y.J. Tang, L. Nawarro, P.H. Gumerlock and J.Jr. Silva. 1993. Diagnosis and monitoring of *C. difficile* infections with the polymerase chain reaction. *Clin. Infect. Dis.* **16**: 234–238.

Martirosian G., S. Kuipers, A. van Belkum, H. Verbrough and F. Meisel-Mikolajczyk. 1995. PCR ribotyping and arbitrarily primed PCR for typing of *C. difficile* from Polish maternity hospital. *J. Clin. Microbiol.* **33**: 2016–2021.

Martirosian G., A. Szczęsny, S.H. Cohen and J.Jr. Silva. 2004. Isolation of non-toxigenic strains of *C. difficile* from cases of diarrhea among patients hospitalized in hematology/oncology ward. *Pol. J. Microbiol*. **53**: 197–200.

Poxton I.R., J. McCoubrey and G. Blair. 2001 The pathogenicity of *C. difficile*. *Clin. Microbiol. Infect*. **7**: 421–427.

Rupnik M., B. Dupuy, N.F. Fairweather, D.N. Gerding, S. Johnson, I. Just, D.M. Lyerly, M.R. Popoff, J.I. Rood, A.L. Sonenshein, M. Thelestam, B.W. Wren, T.D. Wilkins and C. von Eichel-Streiber. 2005. Revised nomenclature of *C. difficile* toxins and associated genes. *J. Med. Microbiol.* **54**: 113–117.

Sambol S.P., J.K. Tang, M.M. Merrigan, S. Johnson and D.N. Gerding. 2001. Infection of hamsters with epidemiologically important strains of *C. difficile*. *J. Infect. Dis.* **183**: 1760–1766.

Szczęsny A., A. Kański and G. Martirosian. 2002. Incidence of pseumembranous colitis after vancomycin-treated MRSA infection. *Clin. Microbiol. Infect.* **8**: 58–59.

Wilson K.H. and J.N. Sheagren. 1983. Antagonism of toxigenic *C. difficile* by nontoxigenic *C. difficile*. *J. Infect. Dis.* **147**: 733–736.