Virulence Factors of *Clostridium difficile* and Its Pathogenesis in Intestinal Infection in Man

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Clostridium difficile was isolated in 1935 by Hall and O'Toole (29) from the feces of healthy neonate, and the microorganism was first designated *Bacillus difficilis*. The 'difficilis' was originated from the difficulty in cultivation of the microorganism. They showed that the microorganism had lethality to rabbit or guinea pig and edema-inducing activity. Later the microorganism was classified as *Clostridium difficile* in 1938 by Prévot (71). The pathogenicity of *C. difficile* to human had not been reported until when association of *C. difficile* with human pseudomembranous colitis (PMC) was demonstrated in 1978.

In 1893, Finney (19) first reported an occurrence of PMC in the patient with peptic ulcer after gastroenterostomy. The number of cases with PMC increased in parallel to an increase of use of antibiotics in the 1970s. Although *Staphylococcus aureus* was speculated to be a causative agent for PMC, there was no determinative evidence for the hypothesis. In 1977, Larson et al (48) reported that clostridial toxin was causative agent for PMC, and in 1978 toxigenic *C. difficile* was identified as a cause of PMC by Bartlett et al (3), George et al (23) and Larson et al (49). The isolation rate of *C. difficile* from feces of healthy adults was 0-12.8%, and that of the patients with PMC was 80-100% (59). It is considered that change of intestinal flora by administration of antimicrobial agent and following selection of toxin-producing *C. difficile* are background for the occurrence of PMC. Virulence factors of *C. difficile* and its pathogenesis in intestinal infection in man are discussed in this review by indicating not only previous reports in the literature but also our own experimental data.

VIRULENCE FACTORS OF *C. DIFFICILE*

Virulence factors of *C. difficile* reported so far are listed in Table 1. Toxins

Table 1. Virulence factors of *Clostridium difficile*

Virulence factor	Action	Reference
Toxins		1, 46, 53, 77
Toxin A (enterotoxin)	Enterotoxicity, cytotoxicity, mouse lethality	
Toxin B (cytotoxin)	Cytotoxicity, mouse lethality	
Mucosal association	Adherence to mucosa	6
Fimbriae	Adherence to mucosa	5
Capsule	Anti-phagocytosis	11, 76
Tissue degradative enzymes	Hydrolysis of mucus and destruction of intestinal cells	75
hyaluronidase		
chondroitin-4-sulfatase		
collagenase		

produced by *C. difficile* are considered to be the most important virulence factor. It is known that *C. difficile* produces two toxins: toxin A (enterotoxin) and toxin B (cytotoxin) (1, 77). Toxin A has an enterotoxicity inducing fluid accumulation in ileal loop response test. Toxin B has a strong cytotoxicity to cultured cells. Nakamura et al (65) found that strains with a particular serotype (serovar) are found in isolates from patients and are highly toxigenic, suggesting that surface characteristics of the bacteria are related with the pathogenesis. By animal experiments using hamsters administrated with clindamycin prior to *C. difficile* infection, Borriello et al (6) reported that *C. difficile* strains were classified into highly virulent strain causing death within two days after the inoculation, less virulent strain causing death between 3 and 14 days after the inoculation, and avirulent strain causing no death. *In vitro* toxin A production and association with intestinal mucosa were detected much more in highly virulent strains of *C. difficile* than in less virulent strains. On the other hand, there was no significant difference in *in vitro* toxin production between highly virulent and less virulent strains. They speculated that pathogenicity of *C. difficile* was determined by the intensity of association of the microorganism with intestinal epithelial cells. Fimbriae and capsule were reported as candidates for mucosal association factors (5, 11, 76). Their roles in adhesion, however, are not clear. As other factors associated with pathogenesis of *C. difficile*, tissue-degradative enzymes, hyaluronidase, chondroitin-4-sulfatase and collagenase, were reported (75). Further investigation on the tissue-degradative enzymes of *C. difficile* is needed.

C. DIFFICILE TOXINS

Some characteristics of toxins produced by *C. difficile* are listed in Table 2. Toxins A and B are eluted on anion-exchange column (Mono Q column) at 0.3–0.4 M and 0.6–0.7 M NaCl, respectively. Toxin A has an enterotoxicity which induces fluid accumulation with hemorrhage in ileal loop response test. In contrast, toxin B which induces strong cytotoxicity to cultured cells has no enterotoxicity

Table 2. Characteristics of *Clostridium difficile* toxins (37, 46, 53, 59, 77)

Characteristics	Toxin A	Toxin B
Enterotoxigenicity	+	—
Cytotoxicity	+	‡‡
Mouse lethality	+	+
Hemagglutination (HA) activity with rabbit erythrocytes		
4°C	+	—
37°C	—	—
Binding to hamster brush border membrane		
4°C	‡‡	N.T. ^{a)}
37°C	+	N.T.
Binding to bovine thyroglobulin		
4°C	+	—
37°C	—	—
Molecular weight		
non-denatured condition	400–600 kDa	300–400 kDa
denatured condition	200–300 kDa	200–300 kDa

^{a)} Not tested.

at all. The cytotoxicity of toxin B is much stronger by 100–1000 fold than that of toxin A. Both toxins have mouse lethality. Krivan et al (46) reported that toxin A binds temperature-dependently to hamster intestinal brush border membrane, rabbit erythrocytes and bovine thyroglobulin. Toxin B has not such binding activity. Krivan and Wilkins (47) reported a new purification procedure, thyroglobulin affinity chromatography (TGAC), for toxin A by taking advantage of temperature-dependent binding activity of the toxin to bovine thyroglobulin (binding at 4°C and releasing at 37°C). We have modified the purification procedure by applying the TGAC-thermal eluents into anion-exchange chromatography (Q Sepharose FF and Mono Q) (Fig. 1) (37). The highly purified toxin A was analyzed by polyacrylamide gel electrophoresis (PAGE) stained by silver. By native- and SDS-PAGE analysis, it was demonstrated that the molecular weight (MW) of toxin A in non-denatured and denatured conditions were 540 kDa and 240 kDa, respectively (Fig. 2). In SDS-PAGE, many minor bands ranging from 38 to 440 kDa were also detected by silver stain (Fig. 2B). Lyster et al (53) also reported a similar result in SDS-PAGE of toxin A, implying a possibility that toxin A is an aggregate of smaller subunits. The MW of toxin B is reported to be 300–400 kDa and 200–300 kDa in non-denatured and denatured conditions, respectively (1, 53, 77). Recently, DNA cloning of toxin gene has been performed and base sequence of the gene has been determined. The MW of toxins A and B were estimated to be 308 kDa (13) and 270 kDa (2), respectively.

As trisaccharide sequence, Gal α 1-3Gal β 1-4GlcNAc, is commonly detected on the surfaces of hamster brush border membrane, rabbit erythrocyte and bovine thyroglobulin to which toxin A binds temperature-dependently, the sequence was speculated to be a receptor for toxin A (46). However, it was reported that the

C. difficile VPI 10463 strain
 ↓
 Dialysis tubing-culture in modified
 BHI (anaerobically, 37°C, 5 days)
 ↓
 Culture filtrate 100 ml
 ↓
 Thyroglobulin affinity chromatography
 ↓
 Washing at 4°C
 ↓
 Thermal eluents at 37°C
 ↓
 FPLC using Q Sepharose FF column
 ↓
 Dialysis against 20 mM Tris-HCL (pH 7.5)
 ↓
 FPLC using Mono Q column

Fig. 1. Purification of toxin A by thyroglobulin affinity chromatography followed by anion-exchange chromatography.

Fig. 2. Polyacrylamide gel electrophoresis (PAGE) analysis of purified toxin A. (A) Native PAGE stained by silver: 1, molecular weight standards; 2, purified toxin A (5 μg). (B) SDS-PAGE stained by silver: 1, toxin A (5 μg) in reduced condition by β-mercaptoethanol; 2, toxin A (5 μg) in non-reduced condition.

trisaccharide is not detected on the surface of human intestinal epithelial cells (21). Recently, it was shown that a receptor for toxin A might be disaccharide of Galβ1-4GlcNAc detectable on the surface of human intestinal cell (80). We have shown that the peaks of hemagglutination (HA) activity and cytotoxicity of toxin A were dissociated by direct analysis of culture filtrate of *C. difficile* VPI 10463 strain in Mono Q-FPLC (fast protein liquid chromatography), suggesting a presence of non-hemagglutinating form (toxin A') of toxin A. Toxin A' was purified using TGAC-

Table 3. Purification of non-hemagglutinating form (toxin A') of toxin A by Mono Q anion-exchange chromatography (33)

Fraction No.	Conc. of NaCl (M)	Toxin A ^{a)}		Toxin A' ^{a)}	
		Cytotoxicity ^{b)}	HA activity ^{c)}	Cytotoxicity	HA activity
37	0.29	<3	<1	6	<1
38	0.30	11	2	13	<1
39	0.31	13	5	14	<1
40	0.32	12	6	13	1
41	0.33	10	4	13	1
42	0.34	8	2	11	<1
43	0.35	6	<1	10	<1

^{a)} After thyroglobulin affinity chromatography (TGAC) of *C. difficile* culture filtrate, TGAC-thermal eluents and TGAC-unbound fractions were applied into Q Sepharose FF and Mono Q anion-exchange chromatography for purification of toxin A and toxin A', respectively.

^{b)} log₂CU/50 µl.

^{c)} log₂HAU/50 µl.

Table 4. Biological activity of toxin A and toxin A' (33, 34)

Toxin	Minimum dose (ng) causing each activity			Loop test ^{a)}
	Cytotoxicity	Mouse lethality	HA activity	
Toxin A	0.14	18	70	+(1.58) ^{b)}
Toxin A'	0.14	4.4	>8800	+(1.63)

^{a)} Ten µg of each toxin was injected into a rabbit ileal loop, and the rabbit was sacrificed at 10 hr after the injection.

^{b)} Ratio of the volume (ml) of the fluid accumulated in the loop to the length (cm) of the loop.

unbound fractions of culture filtrate by Q Sepharose FF- and Mono Q-FPLC (Table 3) (33). Toxin A' was demonstrated to have the same MWs (540 kDa in non-denatured and 240 kDa in denatured conditions) as those of toxin A, suggesting that toxin A' might be conformationally different from toxin A. Interestingly, toxin A' without HA activity has as much enterotoxicity as toxin A with HA activity (Table 4), implying a possibility that HA activity might be not necessary for induction of enteropathogenicity of toxin A (34).

Among many monoclonal antibodies (MAb) to *C. difficile* toxin A raised so far (14, 53, 73), PCG-4 produced by Lyster et al (53) was the only MAb to neutralize biological activity of toxin A. The MAb, PCG-4, neutralized both enterotoxicity and HA activity of toxin A, implying the presence of common epitope for enterotoxicity and HA activity. We have established 9 MAbs to *C. difficile* toxin A using highly purified toxin A for immunization of mice (38). Immunoblot analysis using 37B5, one of the 9 MAbs, showed that toxin A' reacted with the MAb as effectively as toxin A did (Fig. 3). It was also shown that 37B5 neutralized enterotoxicity of

Fig. 3. Immunoblot analysis of toxin A and toxin A' by using monoclonal antibody (37B5) to toxin A. (A) Immunoblot analysis after native PAGE of toxin A (5 μ g) (lane 1) and toxin A' (5 μ g) (lane 2). (B) Immunoblot analysis after SDS-PAGE of toxin A (5 μ g) (lane 1) and toxin A' (5 μ g) (lane 2).

toxin A, but not HA activity, sustaining the hypothesis that HA activity of toxin A is not necessary for induction of enterotoxicity.

Giuliano et al (28) reported the existence of the third toxin (toxin C) of *C. difficile*, which induces enterotoxicity without hemorrhage in ileal loop response test. The toxin was eluted near toxin B fractions in Mono Q-FPLC. However, due to a fragile characteristics of the toxin, further analysis has not been performed.

Although the mechanism of occurrence of diarrhea by *C. difficile* toxins has not been fully understood, pathogenic effect of toxins on intestinal cells is assumed as described below (20, 30, 59, 70). Toxin A binds to intestinal epithelial cells and is internalized into the cell. Consequently, infiltration of granulocytes into lamina propria of intestinal mucosa is followed by release of various chemical transmitters. Cytotoxic change of intestinal cells results in increased permeability inducing fluid secretion into intestinal lumen. By co-operation of cytotoxic effect of toxin A on intestinal cells, toxin B can enter the intestinal cell and exert its strong cytopathogenicity.

Both toxins induce a change of microfilament system which is composed of dissociation of actin filament and intracellular accumulation of globular actin (69, 79). This seems to be a cause of the cytotoxicity of cultured cells by *C. difficile* toxins. It was reported that both toxins did not inhibit phagocytic activity of neutrophils (9). On the other hand, Däubener et al (10) showed that both toxins inhibited monocyte-dependent T cell proliferation. Toxin A has been reported to have promotive effect on proliferation of spleen cells in the presence of calcium ionophore (58) and enhancing activity of cell-mediated cytotoxicity by peripheral mononuclear cells (56). A detailed relationship between *C. difficile* toxins and immune system should be determined in the future.

Table 5. Antimicrobial susceptibility of *Clostridium difficile* strains (64)

Antimicrobial agent	Number of strains showing MIC ($\mu\text{g/ml}$) of									
	≤ 0.39	0.78	1.56	3.13	6.25	12.5	25	50	≥ 100	
Rifampicin	78	1								
Metronidazole	79									
Fusidic acid	70	9								
Vancomycin		10	69							
Penicillin G		59	9	6	5					
Ampicillin		28	37	9	5					
Cephalothin		1	36	25	13	4				
Cephaloridine		1	8	32	31	7				
Carbenicillin					48	14	14	3		
Neomycin							1	12	66	
Gentamicin								4	75	
Cefoxitin								3	76	
Cefalexin, sulfamethoxazole, trimethoprim, kanamycin, streptomycin, nalidixic acid, cycloserine									79	
Erythromycin	29	13							37	
Clindamycin		3	8	30	1				37	
Lincomycin			2	12	17	10	1		37	
Chloramphenicol			46	10	9			14		
Tetracycline	11	40	8	1				19		

ANTIMICROBIAL SUSCEPTIBILITY OF *C. DIFFICILE* AND INCREASED TOXIN PRODUCTION OF *C. DIFFICILE* BY ANTIMICROBIAL AGENTS

It is known that *C. difficile* is more resistant against various antimicrobial agents than any other clostridia (18, 27, 64, 67). Our experimental data on antimicrobial susceptibility of *C. difficile* are shown in Table 5. *C. difficile* is highly resistant against aminoglycosides, trimethoprim, sulfamethoxazole, nalidixic acid, cycloserine, neomycin, cefoxitin and cephalixin. On the other hand, *C. difficile* has sensitivity to rifampicin, metronidazole, fusidic acid, vancomycin, ampicillin and penicillin G. With respect to susceptibility to erythromycin, clindamycin, lincomycin, chloramphenicol and tetracycline, *C. difficile* strains are divided into two groups, resistant and sensitive strains to these agents. The fact that *C. difficile* has high resistance against many antimicrobial agents seems to be closely related with selective proliferation of *C. difficile* in the patients with PMC or AAD after administration of antimicrobial agents.

It has been reported that toxin production of *C. difficile* is increased in the presence of various antimicrobial agents, particularly clindamycin (22). In contrast, when *C. difficile* was incubated in continuous flow culture, toxin production was enhanced by addition of vancomycin or penicillin, but not clindamycin (68). Nakamura et al (62) examined the effect of clindamycin on cytotoxin (toxin B) production using 80 *C. difficile* strains (56 toxigenic and 24 nontoxigenic). It was

noted that cytotoxin production was markedly increased in 6 toxigenic strains. Toxin A production was also reported to be increased in the presence of clindamycin (31).

SPORULATION OF *C. DIFFICILE*

C. difficile spores can survive for longer than 5 months under atmosphere (44). It is difficult to recover *C. difficile* spores by ordinary growth medium like brain heart infusion (BHI). The recovery rate of most strains was lower than 0.01% in BHI medium. However, the recovery is distinctly enhanced when either the spores are treated with 0.5 M sodium thioglycolate at 50°C for 30 min and inoculated in lysozyme (10 µg/ml)-containing medium (thioglycolate-lysozyme method) (32, 66), or a medium supplemented with sodium taurocholate (0.1%) is used for spore recovery medium (taurocholate method) (83). The mean value of recovery rates of 103 *C. difficile* strains in the taurocholate method (89%) was significantly higher than that in thioglycolate-lysozyme method (64%) (39). The determinative conditions of heating or alkali treatment that alter *C. difficile* spores were 80°C for 10 min or 0.1 N NaOH for 15 min, respectively (40). For recovery of such altered spores, thioglycolate-lysozyme method was much more effective than taurocholate method (Table 6) (40). It was reported that toxin production was detected after the exponential growth phase, and maximum yield was found in culture filtrate at 4–5 days after inoculation when bacterial growth was already arrested (42, 50), suggesting

Table 6. Recovery of spores of *C. difficile* altered by heat or alkali (39, 40)

Treatment	Strain	Relative recovery rate ^{a)} (%)	
		Taurocholate method	Thioglycolate-lysozyme method
80°C, 10 min	KZ 1628	2.9	80
	KZ 1648	2.1	41
	KZ 1610	0.73	58
	KZ 1660	0.050	28
90°C, 10 min	KZ 1628	<0.00001	33
	KZ 1648	<0.00001	10
	KZ 1610	<0.00001	18
	KZ 1660	<0.00001	17
0.1 N NaOH 15 min	KZ 1628	91	32
	KZ 1648	60	46
	KZ 1610	1.3	50
	KZ 1660	5.9	34
0.2 N NaOH 15 min	KZ 1628	0.074	41
	KZ 1648	0.049	40
	KZ 1610	0.0034	48
	KZ 1660	0.017	34

^{a)} Ratio of the number of recovered spores after various treatments to that of spores heated at 70°C for 10 min recovered by taurocholate methods.

Table 7. Relationship between sporulation and cytotoxin production of *Clostridium difficile* KZ 1630 strain (36)

Time (hr) after inoculation ^{a)}	Untreated			Acridine orange-treated ^{b)}		
	Vegetative cells ^{c)}	Spores ^{c)}	Cyto- toxicity ^{d)}	Vegetative cells	Spores	Cytotoxicity
12	7.3	ND ^{e)}	ND	7.6	ND	ND
24	8.1	3.2	6	8.1	ND	2
36	7.9	5.9	10	8.2	1.2	3
48	7.9	6.8	12	8.2	2.0	5
60	7.9	7.1	12	8.0	3.9	7
72	7.5	7.0	12	7.8	4.1	7

^{a)} Three-tenth ml of 10⁶-diluted culture of the strain incubated at 37°C for 5–6 hr was inoculated into 15 ml m-BHI medium.

^{b)} Acridine orange (30 µg/ml) was added 12 hr after the inoculation.

^{c)} log₁₀CFU/ml.

^{d)} Extracellular cytotoxicity (log₂ CU/50 µl).

^{e)} Not detected.

a relationship between toxin production and sporulation of *C. difficile*. However, Onderdonk et al (68) showed no correlation between cytotoxin production and sporulation in studies with a continuous flow culture system. Ketley et al (43) also reported that the kinetics of spore production by *C. difficile* were not paralleled by release of toxin A *in vitro* and that toxin A was not found to be associated with either purified whole spores or spore coats. In their experiments, however, considerable large number of spores in the inoculum was applied, which made the analysis on the relationship between sporulation and toxin production more difficult. In contrast, by inoculating of 10⁶-diluted KZ 1630 culture which had been incubated anaerobically for 5–6 hr to give an initial count of less than 10 vegetative cells/ml with no spores, the relationship between cytotoxin production and sporulation was reinvestigated (36). Cytotoxin production was coincidental to the phase of spore formation of *C. difficile* (Table 7). It was also shown that addition of a sporulation inhibitor, acridine orange (30 µg/ml), decreased significantly both sporulation and cytotoxin production in spite of no effect on the growth of vegetative cells (Table 7). These results suggest closer relationship between sporulation and cytotoxin production.

INTESTINAL INFECTION BY *C. DIFFICILE*

C. difficile is a causative microorganism of PMC and some cases (20%) of AAD (24). PMC is a severe inflammatory disease in colon mucosa with formation of pseudomembrane composed of fibrin, mucus, leukocytes and necrotic intestinal epithelium. The more antimicrobial agent was used for patients in the 1970s, the more the number of the cases of PMC increased. Major clinical symptoms are diarrhea, abdominal pain and fever occurring after antimicrobial agent administration. The change of intestinal flora caused by the administration of the drug

results in selective proliferation of *C. difficile* with production of various virulence factors including toxins A and B. Although clindamycin and lincomycin were first considered to be the main causative antimicrobial agents for PMC and AAD, it is now known that all antimicrobial agents except for non-oral administration of aminoglycosides are associated with the occurrence of PMC or AAD. It is considered that antimicrobial agents are closely related with both selective proliferation of *C. difficile* and increased toxin production by *C. difficile*. Isolation rates of *C. difficile* were 0–12.8%, 0–50% and 80–100% from the feces of healthy adults, patients administrated with antimicrobial agent, and patients with PMC, respectively (25, 59, 61, 81). In contrast, high isolation rate (30–80%) was observed in the feces of healthy babies, particularly neonates, and approximately 30% of the feces showed positive-cytotoxicity (59). Despite that toxigenic *C. difficile* was isolated and high cytotoxicity (10^2 – 10^6 CU/50 μ l) was detected in the fecal extract, no diarrhea was induced in the intestine of neonates. The reason why no symptom of enterocolitis was induced in the babies remains to be determined. Krivan et al (46) supposed that the receptor for *C. difficile* toxins might not be expressed on the surface of neonate intestinal cells. However, Rolfe (72) recently reported that there was no significant difference in the binding activity of toxin A between adult and neonate hamster brush border membranes.

LABORATORY TESTS FOR *C. DIFFICILE*

Laboratory tests for *C. difficile* are listed in Table 8. For isolation of *C. difficile*, anaerobic incubation using CCFA (cycloserine-cefoxitin-fructose-agar) (26) is performed. As mentioned above, thioglycolate-lysozyme and taurocholate methods are effective for the recovery of *C. difficile* spores. Cytotoxicity assay is a sensitive method for detection of *C. difficile* toxins. Cultured cells treated with *C. difficile* toxins exhibited cytopathic effect of cell rounding with refractile appearance (Fig. 4).

Table 8. Laboratory tests for *C. difficile* (35, 41, 54, 59, 60, 63, 84)

Test	Target	Remarks
Anaerobic culture	<i>C. difficile</i>	<i>C. difficile</i> can be isolated Two to three days are needed for incubation
Cytotoxicity assay	<i>C. difficile</i> toxins	The most sensitive method (detectable for 1 pg of toxin B) Cultured cells are needed
Enzyme-linked immunosorbent assay (ELISA)	<i>C. difficile</i> toxins	High specificity Special apparatus is needed Rapid screening assay
Latex agglutination assay	<i>C. difficile</i> antigen (glu- tamate dehydrogenase)	Nonspecific reaction is observed
Polymerase chain reaction (PCR)	Toxin genes	Detectable for small number of toxigenic <i>C. difficile</i> Existence of inhibitors in fecal extract

Fig. 4. Cytotoxicity assay for *C. difficile* toxins. (A) Untreated BHK-21/W1-2 (baby hamster kidney) cells. (B) BHK-21/W1-2 cells treated for 24 hr with *C. difficile* culture filtrate containing both toxin A and toxin B.

Fig. 5. DEAE-Sephacel anion-exchange chromatography profile of toxigenic strain (KZ 616) of *C. difficile*. ●, cytotoxicity; —, A₂₈₀; ---, Molar concentration of NaCl; LA, latex agglutination-positive fractions.

As it is possible to detect pg level of toxin B by the cytotoxicity assay, this test is considered to be a 'gold standard' for detection of the toxins. As another detection method for *C. difficile* toxins, enzyme-linked immunosorbent assay (ELISA) was developed and has been recently clinically available (12, 54). Reversed passive latex agglutination test kit (CD check•D-1 kit) of Mitsubishi Chemical Industries, Tokyo, Japan, was developed as a rapid screening test for toxin A (D-1 toxin) (1, 45). It was reported that not only toxigenic *C. difficile* strains but also nontoxigenic strains gave positive reaction in the latex agglutination test (35). In addition, other microorganisms of *C. sporogenes*, proteolytic *C. botulinum*, *Bacteroides asaccharolyticus*, *Peptostreptococcus anaerobius*, showed positive reaction in the test (4, 35, 57), indicating nonspecific cross-reaction of the latex test kit. We have checked latex agglutination (LA) activity and cytotoxicity with all fractions after anion-exchange chromatog-

raphy using DEAE-Sephacel of *C. difficile* culture filtrates (Fig. 5). The LA-positive activity was detected in the fractions between toxins A and B, indicating that LA-positive antigen is not toxin A. The nonspecific reaction in the LA test has been also reported by Lyerly and Wilkins (55) and Borriello et al (4). Recently, it was shown that the reactive antigen in the LA test is glutamate dehydrogenase produced by *C. difficile* (51). According to the data on base sequences of the toxin genes (13, 84), PCR (polymerase chain reaction) method using two primers synthesized has been developed for rapid test of *C. difficile* toxins (41, 84). As it was reported that there was an inhibitor for PCR in the fecal extract, clinical application of the PCR method has not been performed.

THERAPY FOR *C. DIFFICILE* INFECTION

Therapy for *C. difficile*-induced PMC or AAD is listed in Table 9. The first step for the therapy is to suspect *C. difficile* infection and stop the administration of the causative antimicrobial agent. Next, administration of either vancomycin (0.5–2.0 g/day) or metronidazole (1.0–1.5 g/day), effective drugs to *C. difficile*, is recommended (17, 78). Cholestyramine to bind *C. difficile* toxins has also been used, especially in difficult cases. Bacteriotherapy using either complex undefined combinations of fecal flora in the form of a fecal enema (7, 15) or a nontoxigenic avirulent strain of *C. difficile* (74) was employed for the therapy of PMC. Bowden et al (7) reported that 13 of 16 patients with PMC responded dramatically following administration of fecal enema. A different approach using nontoxigenic *C. difficile* strains by Seal et al (74) gave a successful effect to treat a case of relapse and to prevent further relapse in another patient.

Corthier et al (8) reported that prior administration of *Saccharomyces boulardii* decreased mortality of the gnotobiotic mouse monoassociated with *C. difficile*. It was shown that this inhibitory effect was induced by viable yeast but not by heated or irradiated yeast, and that higher doses, at least 1.0×10^9 cells, were needed for the protection in the mice from lethal ileocectitis by *C. difficile* (16). Recently, the sequencing of an internal fragment of the toxin A gene revealed the presence of repeat nucleotide sequences, consisting of nearly a third of the gene (8133 base pairs) at the 3' end (13, 84). Lyerly et al (52) examined the effect of vaccination

Table 9. Therapy for antimicrobial agent-associated pseudomembranous colitis or diarrhea by *Clostridium difficile* (7, 15, 17, 59, 74, 78)

1. Stop administration of the antimicrobial agent responsible for the colitis or diarrhea
2. Administration of effective antimicrobial agent
Vancomycin (0.5–2.0 g/day) 1–2 wk
Metronidazole (1.0–1.5 g/day) 1–2 wk
3. Binding substance to <i>C. difficile</i> toxins: cholestyramine
4. Bacteriotherapy/bacterioprophyllaxis
Fecal enema
Nontoxigenic <i>C. difficile</i> strain

with a nontoxic recombinant peptide comprising 33 of the 38 repeating units of toxin A gene on protection of ileocectitis in hamster by *C. difficile*. It was noted that the antiserum against the recombinant peptide neutralized the enterotoxic and cytotoxic activity of the toxin and that hamsters vaccinated with the peptide were partially protected (4 out of 8 hamsters vaccinated) against *C. difficile* ileocectitis. The two latter methods are not now applied as a clinical therapy, but it is possible that they will be new effective therapeutic method for *C. difficile* diseases.

CONCLUDING REMARKS

Willis (82) stated that "Among all the many advances that have been made in our understanding of anaerobic bacteria and anaerobic bacterial disease during the last 40 years, elucidation of the syndrome of antibiotic-associated pseudomembranous colitis ranks as a major triumph". However, the mechanism by which *C. difficile* colitis occurs has not been fully understood. *C. difficile* spores can survive for longer period under an aerobic condition. Many cases of PMC and AAD have been reported to be induced by nosocomial transmission (85). Outbreaks of diarrhea due to this organism have been documented in a number of settings including cancer wards, chronic care facilities and post surgical wards. It is stated that *C. difficile* is an important nosocomial pathogen for the 1990s (85). Further analysis on both virulence factors of *C. difficile* and the mechanism of the occurrence of PMC or AAD remains to be done.

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