Bioscience of Microbiota, Food and Health Vol. 42 (4), 229–235, 2023

Review

Microbial ecology between *Clostridioides difficile* and gut microbiota

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Received April 18, 2023; Accepted May 15, 2023; Published online in J-STAGE June 7, 2023

Clostridioides difficile **colonizes a polymicrobial environment in the intestine and is a causative agent for antibiotic-associated diarrhea (AAD) and pseudomembranous colitis (PMC). The most important virulence factors of *C. difficile* are bacterial toxins, and three toxins (toxin A, toxin B, and binary toxin) are produced by toxigenic strains. Other virulence factors include spores, flagella, capsules, biofilms, hydrolytic enzymes and adhesins. *C. difficile* infection (CDI) is specifically diagnosed by anaerobic culture and toxin detection by either nucleic acid amplification test (NAAT) or enzyme-linked immunosorbent assay (ELISA). For treatment of CDI, metronidazole, vancomycin and fidaxomicin are used based on the severity of CDI. Mutual interaction between *C. difficile* and gut microbiota is associated with pathogenesis of CDI, and decreased microbial diversity with altered gut microbiome was detected in CDI patients. Restoration of certain gut microbiota is considered to be potentially effective for the prevention and treatment of CDI, and an ideal goal for CDI patients is restoration of the gut microbiota to a healthy state. Fecal microbiota transplantation (FMT) is a highly successful method of microbiome restoration and has been reported to be effective for the prevention of recurrent CDI. In addition, approaches to restoring the gut microbiota by using probioitcs and live biotherapeutic products (LBPs) are currently being studied to examine the effect on CDI. Further microbial ecological research on *C. difficile* and gut microbiota could lead to a better understanding of the pathogenesis and treatment of CDI.**

Key words: ***Clostridioides difficile*****, gut microbiota, pathogenesis, microbial ecology, live therapeutic products**

INTRODUCTION

Microbial ecology is the study of relationships between microbes and their environments and how microbes interact with one another and their environments [1]. Microbial ecology includes interactions of microbes with humans, animals, plants, food, and surfaces which may serve as sources or reservoirs of microbes and is critically important, as microbes represent the majority of the genetic and metabolic diversity on the planet and drive most of the ecosystem processes.

Various microbes influence human microbiome, and disruption of the microbial ecosystem in humans may lead to diseases including infectious diseases [2]. It is evident that the diversity of microbes influences the composition of the human microbiota and consequently affects immune function and health outcomes. In humans, not only pathogens but also commensals modulate the gut microbiota and immune systems [2]. *Clostridioides difficile*, a causative pathogen for antibiotic-associated diarrhea (AAD) and pseudomembranous colitis (PMC), colonizes a polymicrobial environment in the intestine and responds to a dynamic microbial ecosystem [3, 4]. In this review article, the microbial-ecological significances of mutual interaction between *C. difficile* and gut microbiota are introduced and discussed.

CLOSTRIDIOIDES DIFFICILE

Microbiology

Clostridioides difficile was formerly called as *Clostridium difficile,* but according to its molecular classification, it was reclassified as *Clostridioides difficile* of the family of Peptococcaceae [5]. *C. difficile* is a Gram positive spore-forming obligately anaerobe bacterium, and it can colonize various hosts including humans and non-human mammals. Although it is not traditionally considered to be part of the human indigenous microbiota, it is estimated that *C. difficile* may comprise 1–3% of commensal bacteria in adult humans [6]. It is of note that it is detected in more than 80% of infants under 2 years of age without any gastrointestinal symptoms [7]. *C. difficile* ferments glucose, fructose, and mannitol but not arabinose, galactose, lactose, maltose, and sucrose [8]. It produces 3 different toxins, toxin A,

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doi: 10.12938/bmfh.2023-033

toxin B, and binary toxin, which are involved in its pathogenesis [9, 10]. *C. difficile* is isolated by anaerobic cultivation at 37°C for 2–3 days using CCFA (cycloserine-cefoxitin fructose agar) or CCMA (cycloserine-cefoxitin mannitol agar) medium. It is resistant to a broad spectrum of antibiotics, including beta-lactams and macrolides, but is sensitive to vancomycin, metronidazole, and fidaxomicin [11].

Pathogenesis

Virulence factors of *C. difficile* are listed in Table 1 [3, 12]. Toxins are the most important virulence factors, and toxin-producing strains (toxigenic strains) induce AAD and PMC after disruption of indigenous gut microbiota due to antibiotic treatment. Toxin A, formerly called as enterotoxin, is encoded by *tcdA* in PaLoc (pathogenicity locus) and inactivates Rho protein via its glucosyltransferase activity [13]. Similarly, toxin B, formerly called as cytotoxin, is encoded by *tcdB* in PaLoc and inactivates Rho protein via its glucosyltransferase activity. Encoded by *cdtA/cdtB* in CdtLoc (Cdt locus), binary toxin (CDT) is a two-component toxin and is composed of CDTa and CDTb, which are responsible for toxin activity and binding to the receptor of LSR (lipolysis- stimulated lipoprotein receptor), respectively [14]. CDTa is an ADP-ribosylating toxin and inhibits polymerization of actin molecules. Then, cell structure is changed with formation of microtubule protrusion which increases the adhesion of extracellular *C. difficile.* Bacterial strains without the production of toxins are called non-toxigenic ones and do not induce any diseases. Other virulence factors include spores, flagella, capsules, biofilms, hydrolytic enzymes and adhesins [15]. In addition to bacterial virulence factors, cytokines, reactive oxygen species, and nitric oxide are host-derived virulence factors and are associated with the induction of inflammation and cell injury.

C. difficile *infection (CDI)*

C. difficile is detected by anaerobic culture and nucleic acid amplification test (NAAT) targeting mainly toxin A [16–19]. Enzyme-linked immunosorbent assays (ELISAs) for toxin A and B are used for the detection of toxigenic strains. The glutamate dehydrogenase (GDH) test is also used for detection of both toxigenic and non-toxigenic *C. difficile,* but it should be noted that other clostridial species including proteolytic *Clostridium botulinum* and *Clostridium sporogenes* produce GDH [20, 21]. For diagnosis of CDI, in addition to isolation of *C. difficile* by anaerobic culture, the combination of the NAAT and GDH is clinically used. Risk factors of CDI are listed in Table 2, and they include antibiotic exposure, older age, hospitalization, nursing home stay, and use of proton pump inhibitors (PPIs) or histamine 2 receptor blockers (H2RAs) [22–25]. Broad spectrum penicillins and cephalosporins, clindamycin, and fluoroquinolones possess a higher risk of CDI induction than other antibiotics. Patient age of 65 years or older increases the risk of CDI 5- to 10-fold, compared with patents <65 years of age. Most cases of CDI are linked to hospitalization or nursing home stays. Gastric acid suppressants including PPIs and H2RAs may disrupt the intestinal indigenous microbiota, allowing for *C. difficile* colonization. Other risk factors include immunosuppressants, inflammatory bowel diseases, gastrointestinal surgeries, transplantations and chronic renal diseases.

Table 1. Virulence factors of *Clostridioides difficile* infection

Virulence factor	Main effect
Spore-forming ability	Survival activity against stresses
Flagella	Motility
Capsule	Anti-phagocytosis
Biofilm formation	Resistance to antibiotics/oxygen stress
Hydrolytic enzymes (hyaluronidase, gelatinase, collagenase)	Destruction of tissue
Adhesins (Cwp66, Fbp68, SLP)	Adherence to epithelial cells
Cwp84	Proteolytic activity
CD2831	Collagen binding protein
Toxin A	Enterotoxigenic activity
Toxin B	Cytotoxic activity
Binary toxin (CDT)	Disruption of cytoskelton
Cytokines	Induction of inflammation, chemotaxis
Reactive oxygen species	Injury of intestinal epithelial cells
Nitric oxide	Injury of DNA

Table 2. Risk factors for *Clostridioides difficile* infection

Risk factor	Remarks
Antibiotic exposure	Greater risk with penicillin, cephalosporins, clindamycin, and fluoroquinolones
Older age	Increased risk in patients >65 years old
Hospitalization	Increased risk with longer hospitalization
Nursing home stay	Comorbidities and frequent antibiotic therapy are observed in residents
Use of proton pump inhibitors (PPIs) or histamine 2 receptor blockers (H2RA)	Induction of disruption of gut microbiota by gastric acid suppressants
Others	Immunosuppressants, inflammatory bowel diseases, gastrointestinal surgeries, transplantations, chronic renal diseases

doi: 10.12938/bmfh.2023-033

For the non-serious CDI patients, metronidazole is recommendable as the first-choice drug, and vancomycin and fidaxomicin are recommendable for serious cases of CDI or cases of CDI with a high risk of recurrence [16–19, 26, 27]. Even after effective chemotherapy for the primary CDI, recurrent CDI occurs in 10–25% of patients due to either relapse of infection with the original strain or re-infection with different strain [28]. For prevention of recurrent CDI, several prophylactic interventions have been using passive immunization with a monoclonal antibody (bezlotoxumab) to toxin B, antibiotic prophylaxis, and microbiota-targeted therapy, including faecal microbiota transplantation (FMT), probiotics, and live biotherapeutic products (LBPs), have been reported [29, 30]. Bezlotoxumab has been reported to be effective for the prevention of recurrent CDI in combination with antibiotics [31]. A recent meta-analysis showed that oral vancomycin prophylaxis, one of the antibiotic prophylaxis interventions, was associated with reduced rates for both primary and secondary CDI in the patient with high risk factors [32]. The effects of FMT, probiotics, and LBPs on CDI are introduced later in this review. For the prevention of CDI in hospitals and nursing homes, use of gloves and disposable gowns by medical staff, healthcare personnel, and visitors is recommended throughout entire diarrheal episodes. It is important to recognize that alcohol-based hand hygiene is not effective due to spore formation by *C. difficile,* and that mechanical hand washing with running water and soap prevents the spread of *C. difficile.* It has been reported that more than 1,000 ppm of chlorine is effective for environmental cleaning [33].

INTERACTION BETWEEN *C. DIFFICILE* AND THE GUT MICROBIOTA

It has been reported that a toxigenic *C. difficile* strain induced lethal colitis in germfree (GF) mice but that no serious colitis was induced by the toxigenic strain in conventional (CV) mice harboring healthy commensal microbiota, indicating that healthy gut microbiota suppresses the outgrowth of *C. difficile* [34]. We showed that the growth of *C. difficile* was inhibited by a mix of intestinal microbiota (*Enterococcus avium, Klebsiella pneumoniae, Parabacteroides distasonis, Eubacterium lentum, Clostridium ramosum* and *Clostridium perfringens*) isolated from infant feces in continuous flow culture, suggesting that the inhibition may be due to the consumption of amino acids by gut microbiota [35].

Antibiotic therapy disrupts the intestinal commensal microbiome, reducing microbiota diversity and colonization resistance, which may lead to CDI, including AAD and PMC [36]. Gut dysbiosis is generally defined as “any change to the composition of resident commensal communities relative to the community found in healthy individuals”, and it can lead to deficient education of the host immune system and subsequent development of immune-mediated diseases [37].

Correlations between CDI and the gut microbiota have been analyzed by culture-independent genomic techniques, including next-generation sequencing (NGS). Pyrosequencing analysis of fecal specimens of the patients with CDI and *C. difficile*-negative nosocomial diarrhea (CDN) showed decreases in microbial diversity and species richness compared with those of healthy controls (HCs), and the relative abundances of Ruminococcaceae and Lachnospiraceae were significantly decreased in CDI and CDN patients [38]. Goldberg *et al.* [39] reported that higher abundances of both clostridial species and Bacteroidetes were observed in their control and non-*C. difficile* diarrhea groups compared with their CDI and *C. difficile* carrier groups, indicating an inverse association between CDI and the abundances of Bacteroidetes and other clostridial species in human intestine. Recently, Berkell *et al.* [40] reported that CDI patients demonstrated decreased microbial diversity and altered gut microbiome composition with decreases in Lachnospiraceae and Ruminococcaceae, and increase in *Enterococcus.* Gut microbiota compositions associated with *C. difficile* colonization and infection have been recently reviewed [41]. In the people with asymptomatic colonization of *C. difficile* (AC), the relative abundances of *Prevotella, Alistipes, Bacteroides, Bifidobacterium, Dorea, Coprococcus* and *Roseburia* were decreased, while in CDI patients the relative abundances of Lachnospiraceae, Ruminococcaceae, *Blautia, Prevotella, Dialister, Bifidobacterium, Roseburia, Anaerostipes, Faecalibacterium* and *Coprococcus* were decreased. It was also indicated that increases in the abundances of Enterococcaceae and *Enterococcus* were associated with CDI.

Lesniak *et al.* [42] recently investigated the correlation between gut microbiota and the severity of colitis caused by *C. difficile* inoculation in a mouse model (Table 3). GF mice were inoculated with human fecal samples, and mice were challenged 2 weeks later with 10^3 *C. difficile* RT027 spores. Serious epithelial damages, tissue edema, and inflammation were detected in the intestine of the moribund mice, and they died due to lethal colitis and toxin production following infection. In the moribund mice, *Akkermansia, Bacteroides, Clostridium sensu stricto,* and *Turicibacter* were detected at higher relative abundances. In non-moribund mice following *C. difficile* inoculation, *Anaerotignum, Enterocloster,* and *Murimonas* were more abundant. *Bacteroides* OTU7 was associated with toxin production and moribundity, and *Enterocloster* and *Murimonas* were associated with no detection of toxins and a low histopathologic score. These results revealed groups of microbiota associated with both severe and mild CDI outcomes, suggesting the possibility of identifying patients at high or low risk of developing more severe disease.

RESTORATION OF THE GUT MICROBIOTA IN THE TREATMENT OF CDI

The role of the gut microbiota is prevalent throughout the entire life cycle of *C. difficile,* from spore formation and germination to the development of infection, and it is possible that restoration of certain intestinal microbiota might be effective for prevention and treatment of CDI [43, 44]. An ideal goal for CDI patients is restoration of the gut microbiota to a healthy state [45]. It has been reported that the reduced presence of *Bacteroides* is associated with negative consequences for gastrointestinal disorders including CDI [46]. It is possible that *Bacteroides* may activate the host immune system to limit entry and proliferation of potential pathogens including *C. difficile* [47].

FMT

FMT is a highly successful method of microbiome restoration for the prevention of recurrent CDI [36]. van Nood *et al.* [48] examined the effect of FMT on the patients with recurrent CDI. The percentages cured without relapse were 81%, 93%, 23% and 31% in standard vancomycin treatments with a single FMT, multiple

doi: 10.12938/bmfh.2023-033

Table 3. Relationship between gut microbiota and severity of *Clostridioides difficile* infection* (modified from Lesniak *et al.* [42])

	Moribund mice (M1–M6)	Non-moribund mice (N1–N9)
C. difficile titer (CFU/g stool)		
1 dpi**	10^3–10^8	10^2–10^7
10 dpi	Deceased	10^2–10^8
Toxin titer (log10) 2 dpi	1–4	1–3
10 dpi	Deceased	1–3
Pathological score	7–10	0–8***
Microbiota	Increased	Increased
	Akkermansia	*Anaerotignum*
	Bacteroides	*Enterocloster*
	Clostridium sensu stricto	*Murimonas*
	Turicibacter	
Association with toxin production	Positive	Negative
	Bacteroides OTU7	*Enterocloster*
		Murimonas

*Germfree mice were inoculated with human fecal samples, and the mice were challenged 2 weeks later with 10^3 *C. difficile* RT027 spores.
**dpi; days post infection
***Pathological scores were determined at the end of the experiment.

FMTs, bowel lavage, and no additional treatment, respectively, indicating the significant efficiency of FMT in the prevention of recurrent CDI. Pomares Bascuñana *et al.* [49] reported the results of a meta-analysis that evaluated the effectiveness of FMT for the treatment of *C. difficile* diarrhea based on 15 studies selected from a total 5,266 studies. It was shown that the effectiveness of FMT was 82% (95% confidence interval [CI]: 75–89%) and that the included studies were highly homogenous (80%). It was also indicated that the efficacy of FMT increases with the number of doses and that it is equivalent or superior to the gold standard antibiotic regimes (vancomycin and fidaxomicin). Song *et al.* [50] recently reported the results of a meta-analysis concerning the effectiveness of FMT for severe or fulminant CDI (SFCDI) based on 10 studies including a total of 240 patients. They indicated that FMT resulted in the resolution of SFCDI within 4 weeks in 211/240 patients, giving a pooled estimate of 88% (95% CI: 83–91%) and that the mean number of FMT required was 1.6 for severe CDI resolution and 2.0 for fulminant CDI resolution. In the Clinical Practice Guidelines for CDI in adults and children of the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA), FMT is recommended for patients with multiple recurrences of CDI who have failed appropriate antibiotic treatments [16]. Similarly, the Clinical Guidelines for Prevention, Diagnosis, and Treatment of CDI of the American College of Gastroenterology (ACG) suggest FMT be considered for patients with severe and fulminant CDI refractory to antibiotic therapy and recommend FMT be delivered through colonoscopy or capsules for treatment of recurrent CDI [17]. The Treatment Guidelines for CDI of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) indicated that FMT may be a rescue therapy for patients with severe complicated CDI that have deteriorated despite CDI antibiotic treatment and for whom surgery is not feasible [18]. The Clinical Practice Guidelines for Management of CDI of the Japanese Society of Chemotherapy and Japanese Association of Infectious Diseases (JSC/JAID) indicated that FMT cannot be recommended solely based on its efficacy (weak recommendation against use) [19]. Recently, Juul *et al.* [51] reported that FMT was also effective in a treatment for primary CDI. However, further studies on the screening and standardization of methods used to harvest stool and process it for FMT need to be performed. In addition, the two main concerns for FMT are the risk of transferring infectious pathogens from the donor to the recipient, and the development of autoimmunological disorders [52].

Probiotics

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [53]. An alternative for CDI therapy comprises the delivery of beneficial probiotic microorganisms into the intestinal tract to restore the microbial balance [44]. Many probiotic microorganisms including *Bifidobacterium, Lactobacillus, Bacteroides, Clostridium,* and *Saccharomyces,* have been reported to have an inhibitory effects on the growth of *C. difficile* [54, 55]. Specific species such as *Lacticaseibacillus rhamnosus* or *Saccharomyces boulardii* have been studied for the prophylaxis or treatment of CDI with moderate certainty evidence in the meta-analysis [54]. We have previously reported that the *Clostridium butyricum* MIYAIRI588 strain was effective for the prevention of CDI in not only basic but also clinical studies [56–58]. A meta-analysis of probiotic efficacy for gastrointestinal diseases showed that the effects of probiotics on gastrointestinal diseases, including CDI and AAD, were different among probiotic species and that VSL#3 (including 8 bacterial strains of *Lacticaseibacillus casei* subsp. *paracasei, Lactiplantibacillus plantarum, Lactobacillus acidophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium breve,* and *Streptococcus thermophilus*) and *C. butyricum* MIYAIRI 588 had strong inhibitory effects [59]. The effects of bacteriocin produced by commensal microbiota on *C. difficile* were also reported. Commensal *Bacillus thuringensis* was shown to produce thuricin CD, which is a bacteriocin with activity against *C. difficile* [60], while *Limosilactobacillus reuteri* was shown to compete with other indigenous microbiota to produce reuterin from glycerol, in order to inhibit the growth of *C. difficile* [61].

Although various probiotics have been reported to be effective to prevent AAD and cure CDI, various recent clinical guidelines for the treatment of CDI do not strongly recommend the use of probiotics. The IDSA/SHEA and ACG guidelines recommend against probiotics for the prevention of primary CDI and CDI recurrence due to insufficient evidence of efficacy [16, 17]. Similarly, the ESCMID guidelines indicated that routine administration of probiotics to prevent CDI when on antibiotic treatment is not recommended during antibiotic treatment [18]. In contrast, American Gastroenterology Association (AGA) Clinical Practice Guidelines recommend the use of specific probiotics in adults and children on antibiotic treatment, in adults and children with pouchitis, and in preterm low-birth-weight infants, with conditional recommendations [62]. The Japanese guidelines of the JSC/JAID indicated a weak recommendation for probiotics to prevent CDI in patients at risk of CDI, but probiotics are not recommended for the prevention of recurrence of CDI [18].

LBPs

Compared with conventional probiotics, novel beneficial effects on human health have been reported for a wide range of microbial probiotics, and such probiotics are called next-generation probiotics (NGPs). NGPs include various bacterial species such as *Faecalibacterium prausnitzii, Akkermansia muciniphila, Bacteriodes fragilis*/*Bacteroides uniformis, Eubacterium hallii,* and cocktails of *Clostridium* clusters IV and XIVa [63]. According to the pharmaceutical application, NGPs with novel therapeutic functions are termed LBPs [64]. An LBP is defined as "a biological product that contains live organisms such as bacteria and that is applicable to the prevention, treatment, or cure of a disease or condition of human beings". O'Toole *et al.* [64] listed several candidates for LBPs and showed the biological functions targeting various diseases (infectious diseases, inflammatory diseases, malignancies) for 11 strains of the following 8 bacterial species: *Bacteroides xylanisolvens, Bacteroides ovatus, Bacteroides dorei, B. fragilis, Bacteroides acidifaciens, C. butyricum, F. prausnitzii,* and *Lactococcus lactis*.

Approaches to restoring the gut microbiota by using novel LBPs are currently being studied to examine the effect on CDI. It has been recently reported that SER-109 spores containing approximately 50 specific species of only Firmicutes, which were isolated from healthy donors, were effective for recurrent CDI [65]. The percentage of patients with recurrence of CDI was 12% in the SER-109 group and 40% in the placebo group, and the observed safety profile of SER-109 was similar to that of a placebo. Khanna *et al.* [66] recently reported the efficacy and safety of RBX2660, which consists of a broad consortium of microbes prepared from human stool in a phase III randomized double-blind, placebo-controlled trial for prevention of recurrent CDI. Both a microbiota suspension of RBX2660 and a placebo, normal saline, were administered rectally. The model-estimated treatment success rate was 70.6% with RBX2660 versus 57.5% with the placebo, and the incidence of treatment-emergent adverse events was higher in the RBX2660 group than that in the placebo group. In November 2022, the US Food and Drug Administration (FDA) approved RBX2660 (REBYOTA^{TM}) for the prevention of recurrence of CDI following antibiotic treatment for recurrent CDI in individuals 18 years of age and older. Dsouza *et al.* [67] recently reported the results of a phase 1 trial to examine colonization and modulation of the gut microbiota in healthy volunteers by VE303, which is comprised of 8 commensal clostridial strains and was developed for the treatment of recurrent CDI. It was shown that VE303 strains optimally colonized healthy volunteers if dosed over multiple days after vancomycin pretreatment and that VE303 promoted the establishment of a healthy state of the microbiota community.

Bacteriophage

Scientific research on bacteriophage has become a hot topic because antimicrobial resistance (AMR) of pathogenic bacteria is now a serious problem around the world. Fujimoto *et al.* [68] identified several novel endolysin sequences from the prophage sequence of *C. difficile,* and showed that synthesized endolysins exhibited bacteriolytic activity *in vitro* and were effective in a mouse model of CDI. Theoretical and practical findings from pre-clinical and clinical evaluations of the safety and effectiveness of bacteriophage therapy need to be clarified for therapeutic application of this novel therapy for CDI [69]. A metagenome data-based next-generation phage therapy for CDI will be developed in the future [70].

CONCLUSION

In 2017, the US Centers for Disease Control and Prevention (CDC) considered CDI to be a major health threat, with 223,900 national cases among hospitalized patients, eventually leading to 12,800 deaths [71]. In its 2019 AR (antibiotics resistance) Threats Report, in addition to carbapenem-resistant *Acinetobacter, Candida auris,* carbapenem-resistant Enterobacterales, and drug-resistant *Neisseria gonorrhea, C. difficile* is listed in the category of urgent threats microorganisms. Therefore, methods of rapid diagnosis and effective treatment and prevention of CDI are expected to be established soon. Further microbial ecological research on *C. difficile* and the gut microbiota could lead to a better understanding of the mechanisms of pathogenesis following *C. difficile* infection and to further development of microbiome-restoring methods, including FMT, probiotics, LBPs, and phage therapy.

CONFLICT OF INTEREST

The author is employed by Miyarisan Pharmaceutical Co. LTD.

ACKNOWLEDGEMENTS

The author is very grateful to the following scientists for kind supervision and outstanding collaborations; Emeritus Professor Shinichi Nakamura, Kanazawa University, Emeritus Professor Atsushi Ozawa, Tokai University, Professor Haruhiko Taguchi and Professor Takako Osaki, Kyorin University, and Dr. Motomichi Takahashi and Dr. Kentaro Oka, Miyarisan Pharmaceutical Co. Ltd.

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