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ETIOPATHOGENESIS OF DISEASES CAUSED BY *CLOSTRIDIUM DIFFICILE*

Predrag Stojanović

University of Niš, Faculty of Medicine, Public Health Institute Niš, Serbia

Clostridium (C.) *difficile* is a typical representative of the genus Clostridium. After colonization of the intestinal tract, toxigenic C. *difficile* strains are capable to produce two exotoxins, enterotoxin (toxin A) and cytotoxin (toxin B), which cause diarrhea and colitis. Toxin A binds to specific carbohydrate receptors on the surface of intestinal cells and this is the beginning of damages in the intestinal tract which include destruction of the villi epithelium, limiting membrane, intercellular connections (zonula occludens) and surface of the mucosa. If only toxin B is injected into intestinal cells, it does not cause damage nor increased fluids secretion. Probably, the reason for this is the inability of the toxin to bind to the cell membrane receptor in the intestinal tract under normal physiological conditions. Toxigenic strains of C. *difficile* can be found in the intestines of healthy people, without any symptoms or clinical signs (asymptomatic colonization). However, in people with risk factors, they can cause diarrhea of varying severity and life-threatening pseudomembranous colitis. These diseases are known as C. *difficile* associated disease - CDAD. *Acta Medica Medianae 2015;54(1):60-65.*

***Key words**: Clostridium difficile, diarrhea, pathogenesis*

Contact: Predrag Stojanović
Faculty of Medicine, Bulevar dr Zorana Đinđića 81, Niš, Serbia
E-mail:pedjamicro@sezampro.rs

Introduction

C. *difficile* is a typical representative of the genus Clostridium, which includes a group of Gram-positive, moving, or aerotolerant anaerobic bacilli, widespread in soil and in the intestinal tract of animals. The spores form subterminally, rarely centrally. The spores are resistant to heat, drying and chemical agents, which allows them to survive in adverse environmental conditions. On electron microscopy, flagella and other growths on the bacterial cell can be seen. C. *difficile* was described in 1935, by the Hall and Toolea (1). C. *difficile* is considered as non-pathogenic bacteria, as it is cultivated from samples of healthy children. However, during the fifties of the last century this knowledge led to the discovery that C. *difficile* culture filtrate contains toxic products. In the course of 1977, it was proven that the C. *difficile* caused colitis in one patient, which ended in death. Further continuous research has shown that this bacterial species can produce two toxins (A and B) that are responsible for the onset of this disease (2).

In Serbia, the first isolates of C. *difficile* were obtained in IPH, Center for Microbiology in Niš, during 2005 (3). The first epidemic was observed in hospitalized patients accommodated in rooms at the Department of Intensive Care, Clinic of Neurology, Niš, followed by the hospital epidemic in Novi Sad and Požarevac (4). During the period 2005-10, the first study on the representation of non-toxigenic and toxigenic strains was conducted (5), as well as asymptomatic carriers (6) in the patients and people of Serbia. During 2014, the first isolates from samples of the environment were obtained (soil of the Clinical Center Niš and sludge from the territory of the municipality of Niška Banja) (7).

The aim of this paper is to present the latest findings on the pathogenic process at the level of the colon caused by C. *difficile*.

Virulence factors

Until After colonization of the gastro-intestinal tract, toxigenic strains of C. *difficile* secreted two exotoxins, enterotoxin (toxin A) cytotoxin (toxin B), which cause diarrhea and colitis (8).

Research conducted over the last 15 years has discovered other possible virulence factors of

C. *difficile* as well. Binary toxin (actin specific ADP-ribosylation toxin) is present in 1-8% toxigenic strains of C. *difficile* (9). Adhesins are considered important for the survival of the microorganism in the colon, which is still not confirmed. Several extracellular enzymes produced by C. *difficile* have different effects in vitro, but their role has not been defined yet. It is possible that these enzymes play a role in normal physiological processes of the gastrointestinal tract and may be important for the survival of the micro-organism (10). Recent studies have indicated that some pathogenic strains of C. *difficile* possess the S-layer on the cell surface. It is assumed to have a role in virulence (11).

Genetic characteristics and structure of C. *difficile* toxins A and B

The gens that encode virulence of bacteria are organized into clusters. Such genetic "blocks" or "tapes" are called pathogenic islands. Hammond GA. et al. (12) described the boundaries of the island with the pathogenic genes of toxin A and B, which are called after the toxigenic element. Toxigenic C. *difficile* VPI element 10463 is 19.6kb in size and in addition to the genes of toxins A and B, contains three small ORF (eng. Open reading frame) segments, txe1 (also called Txeru), txe2 and txe3. Two smaller ORF segments, Txeru and txe2, are transcribed in the same direction as the genes of toxins.

The gene encoding toxin A is composed of 8130bp and the gene encoding the toxin B is smaller (7098bp). The final product of transcription of the coding toxin gene and translation of the mRNA is a major peptide of molecular weight 308kDa (2710 amino acids) and toxin A peptide of molecular weight 270kDa (2,366 amino acids) of toxin B (10).

Toxins are 50% identical in their structure and amino acids have a simple primary structure. Toxins have three functional parts (domains). F. Hoffman et al. (13) indicate that the enzymatic and toxic effects of toxins A and B are attached to its N-terminal domain. The central part of the toxin is a transmembrane domain. C-terminal (domain) of toxin receptor comprises a place and consists of repeating peptide elements.

The effects of C. *difficile* toxins A and B

Binding of C. *difficile* toxins A and B to the receptor structure

Specific binding of C. *difficile* toxin receptors located on the cell surface is the first step in cell intoxication. Previous research has shown that toxin A in cells of human origin can bind to Ga1β1-4G1cNAc and Ga1NAcβ1-3Ga1β1-4G1cNAc structure. Toxin A can also be associated with the carbohydrate antigens I, X, and Y, having a core structure of the type 2, similar carbohydrates Ga1α1-3Ga1ß1-4G1cNAc. All of these antigens, expressing humans cells of the intestinal epithelium. Antigen X is often found on the surface of neutrophils and of other leukocytes, indicating that toxin A can damage these cells (14). Receptor structures for toxin B has not been clarified yet (15).

The entry of C. *difficile* toxins A and B into a cell

Upon binding to cell surface receptors, both toxins enter the cell by endocytosis. This process of toxin A is visible on transmission electron microscopy. Toxins require passage through the acidic intracellular compartment and intoxicate cell. Intracellular route of toxin A is still unknown (16).

The mechanism of action of C. *difficile* toxins A and B

Catalytic fragments of toxins A and B spread to the cell cytosol and glycosylate the members of the family of GTP-binding proteins (GTPase, Rho proteins) that are involved in intracellular signal transmission (molecular switches). GTPase are characterized by low molecular weight (18-28kDa), C-terminal polyisoprenasations and property of binding and hydrolysis of guanine nucleotides. They are inactive in the GDP-bound form, whereas binding to GTP induces their activation, which leads to a variety of signals. The transition between inactive and active states is controlled by regulatory proteins: factors of exchange of guanin nucleotide (GEF), GTPase activating proteins (GAP) and inhibitors of guanin nucleotides dissociation (GDI). Rho proteins by binding to GTP induce changes in the structure and bind to the effector proteins (serine/threonine kinase possess Rho-binding domain), which leads to kinase activation (eg. ROKα/Rho kinase). In addition to the kinase, Rho effectors also include proteins without enzymatic activity (totekin and rofilin) that can serve as the core of multiprotein complexes, nodes of different signaling pathways (17).

The best characterized member of the family of GTP - binding proteins are Rho, Rac and Cdc42, which are involved in the regulation of the actin cytoskeleton. Rho protein controls the formation of stress fibers and focal adhesin, Rac is involved in the membrane ruffling, while Cdc42 is involved in the formation of filopodia (17,18).

Rho and Ras proteins are substrates for clostridial cytotoxins. Toxin B modifies Rho subfamily proteins, whereas toxin A glycosylate Rac and Cdc42 from the Rho subfamily, as weel as Ras, Rap and Ral from the Ras subfamily (19).

The most significant effect modification of GTPase is the collapse of the actin cytoskeleton (20). Intoxicated cells slow down all physiological processes, the whole cell is becoming smaller and rounded, which was initially followed by formation of refractive fibers which extend radially. In the further process of intoxication, retraction fibers

disappear and cells completely acquire a spherical shape, while in the terminal phase the cells are partially separated. The morphological changes are accompanied by layering of filaments F-actin of the actin cytoskeleton. Stress fibers of cells disappear and the rest of actin filaments accumulate in perinuclear area. Microtubules and intermediate filaments are secondarily affected. It takes only a few molecules of toxins to damage cells, because the toxins have enzymatic action inside the cell (20).

Consequntial effects of C. *difficile* toxins A and B on the cell and differences in the cytotoxic potential of toxins A and B

Toxin A (enterotoxin) in microgram doses damage the bowel epithelium. It firstly destroys the epithelium of the villi, then the limiting membrane, which leads to the damage of the surface mucosa. Toxin A also destroys the tight junctions (lat. zonula occludens). This effect is due to the destruction of actin fibers and inactivation of Rho function (actin fibers regulate the complex of solid connection). Toxin A also induces the production of various cytokines and neurokina, which has been found to play a significant role in the pathogenesis. All of these toxic damages lead to increased permeability of the colon, secretion of fluids, as a basis for watery stools (20,21).

Variations in the production of some C. *difficile* toxins

It was long thought that toxigenic strains of C. *difficile* produce two toxins (A+/B+ strain). However, in 1991, a strain of C. *difficile* 8864 was identified, which produces only toxin B (A-/B + strain) (22). Strain 8864 had a major deletion (5.6KB) in the gene encoding the synthesis of toxin A. The second variation is cultured from samples of children and belongs to serotype F (eg, strain 1470). PCR analysis indicates that the A-/B + strains have a small deletion areas (approximately 1.7KB) with gene encoding the synthesis of toxin A (in the repeating units).

The immune response

Established characteristic of colitis caused by C. *difficile* is a massive influx of neutrophils from the circulation in the mucosa of the colon. It occurs as a consequence of production IL-1, IL-8, TNF and leukotrienes B4 by activated phagocytes tissue - mast cells. Samples of patients' colonic mucosa with colitis caused by C. *difficile* showed vascular congestion, neutrophilic infiltration of the lamina propria and signs of inflammation (23). Pothoulakis C. et al. (24), in the experiments on mice, demonstrated the importance of the fat cells in the recruitment of neutrophils and fluid secretion induced by toxin A (in vivo). Isolated mast cells also showed a response to toxin A, liberating TNFa and substance P. Administration of toxin A in the intestine of rats leads to the release of substance P from sensory and dorsal lumbal ganglia and parts of the mucosa, which precedes the appearance of fluid secretion and colonic mucosal necrosis. Substance P is a peptide found in the bowel tissue and the CNS, which has the role of neurotransmitters. This indicates that the administration of substance P antagonists can inhibit TNF alpha response, as evidenced in rats treated by substance P antagonist prior to administration of toxin A (24). Toxin A also affects the phospholipase A2, and therefore the production of prostaglandins and leukotrienes. Toxin A can cause separation and apoptosis of enterocytes. It diffuses through the damaged epithelium and interacts with inflammatory cells in the lamina propria (25).

Pathogenesis

The primary condition for the manifestation of the pathogenicity of C. *difficile* is a change in the normal relations of bacteria colonizing the digestive tract, which is a barrier to the proliferation of pathogenic microorganisms. More than 90% of infections caused by C. *difficile* occur after prolonged use of antibacterial drugs. The effect of antibacterial drugs disturbs the normal flora of the colon, allowing endogenous and exogenous microorganisms (C. *difficile*) to proliferate and colonize the colon. The disturbance of the intestinal motility is also a prerequisite for the occurrence of infections (22,26).

The pathogenic process begins by binding of toxin A to receptors on the mucosal side of intestinal cells, which causes the early transepithelial signals of unknown nature. The signal induces the release of mediators on the basolateral side of the epithelium, activates neurons and starts the secretion of fluids. Some mediators also activate tissue macrophages to produce proinflammatory cytokines. All this leads to relaxing of intracellular hard colon connection, wherein the inflammatory cell infiltrate in the epithelium and damage the mucosa. Toxin A damages the villi of enterocytes, epithelial membrane and can cause a complete erosion of the mucosa. In response to the damage, a viscous bloody exudate occurs. All of these events allow toxin B to pass through the epithelium, and came to the basolateral surface of cells, where it binds to receptors causing necrosis and mucosal inflammation. Now toxins can also directly activate immune cells, which can cause significant pathological changes, which sometimes result in formation of focal pseudomembranes (Figure 1) (10,22,27).

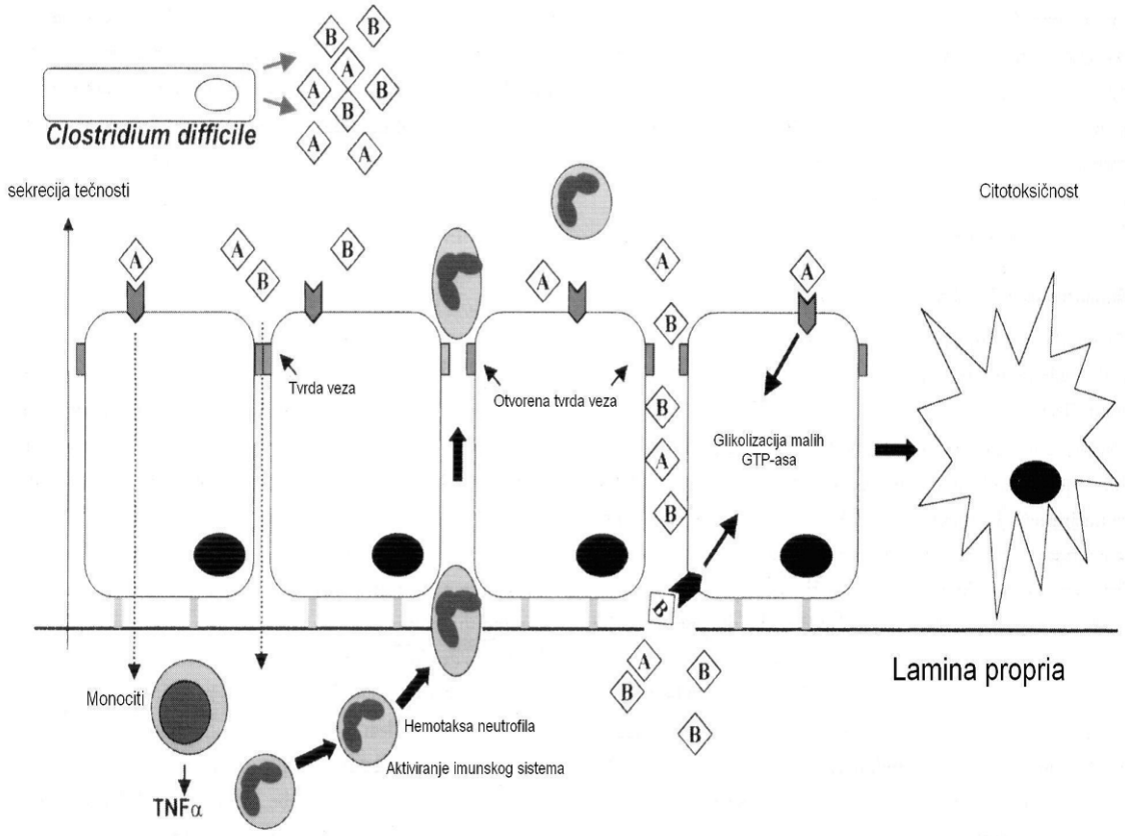

Figure 1. The effect of toxin A and B Clostridium difficile on the intestine epithelium

Pathological changes

The toxins of C. *difficile*, by acting on human colon cells, lead to the formation of shallow ulcers on the surface of the mucosa. Serum proteins, mucus and inflammatory cells come to the surface of a shallow ulcer and form the characteristic pseudomembranes on the colon. Rise of the inflammatory exudate in the mucosa creates a typical "volcanic" lesions in colitis caused by C. *difficile*.

Endoscopic examination of the colon mucosa or rectum shows the yellow or dirtily-white beaches, sized 0.2-2cm. Edema and hypothermia were also present, as the x-rays give a typical picture of "thumbprints". Irregular schedule beach is probably related to the amount of secreted toxins. If the concentration of toxins increases, damage fields can be joined and thus form pseudomembranous colitis (PMC) (28,29).

Clinical manifestations

Toxigenic strains of C. *difficile* can be found in the intestines of healthy individuals without any irritation (asymptomatic colonization); however, in people with risk factors they can cause diarrhea of varying severity to life-threatening PMC (C. *difficile* associated disease - CDAD) (30).

Frequent clinical manifestation of CDAD is colitis with absence of pseudomembranes. Nausea, vomiting, loss of appetite, abdominal pain, cramps and watery diarrhea (slimy, dirty-green, smelly liquid stool) are present. Possible events include dehydration, fever and leukocytosis (31).

Pseudomembranous colitis is a classic manifestation of fully expressed colitis caused by C. *difficile* (32).

Fulminant colitis as a manifestation of CDAD occurs in 3% of patients but is often accompanied by complications such as perforation, ileus, megacolon and lethality (33).

One of the most serious manifestations of CDAD is toxic megacolon which is diagnosed on the basis of colon dilation (greater than 7 cm) and present signs and symptoms of severe intoxication (fever, chills, dehydration, leukocytosis) (33).

CDAD may complicate ulcerative colitis or Crohn's disease. Pseudomembrane is rarely observed because the colon is already damaged by inflammatory process (34).

Conclusion

Data from reference literature, as well as the results obtained in our own investigations indicate that the formation of a broad spectrum of diseases caused by C. *difficile* species depend on the different pathogenic mechanisms whose knowledge and understanding facilitates diagnosis and interpretation of the proven presence of these bacteria in the intestine.

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ETIOPATOGENEZA OBOLJENJA IZAZVANIH *CLOSTRIDUM DIFFICILE*

Predrag Stojanović

Univerzitet u Nišu, Medicinski fakultet, Institut za javno zdravlje Niš, Srbija

Clostridium (C.) difficile je tipični predstavnik roda Clostridium. Nakon kolonizacije intestinalnog trakta, toksigeni sojevi *C. difficile* mogu lučiti dva egzotoksina, enterotoksin (toksin A) i citotoksin (toksin B), koji prouzrokuju dijareju i kolitis. Toksin A se vezuje za specifične ugljenohidratne receptore na površini crevnih ćelija i tako započinje oštećenje intestinalnog trakta. Prvo razara epitel vrhova crevnih resica, a zatim graničnu membranu, što dovodi do ogoljenja površine mukoze. Toksin A, takođe, razara međućelijske čvste spojeve (zonula occludens). Toksin B ne pravi oštećenja i ne izaziva povećano lučenje tečnosti ukoliko se samo on injektuje u kolon. Razlog za to je verovatno nemogućnost ovog toksina da se veže za receptor granične membrane ćelije intestinalnog trakta u normalnim fiziološkim uslovima. Toksigeni sojevi C. *difficile* se mogu naći i u intestinumu zdravih osoba, ne izazivajući nikakve smetnje (asimptomatska kolonizacija), ali kod osoba sa prisutnim faktorima rizika mogu izazvati dijareje različite težine i po život opasan pseudomembranozni kolitis. Oboljenja koja izaziva poznata su kao bolesti povezane sa prisustvom *C. difficile* (*Clostridium difficile* associated disease - CDAD). *Acta Medica Medianae 2015; 54(1):60-65.*

***Ključne reči**: Clostridium difficile, dijareja, patogeneza*