Comparison of Enterotoxin Production, Cytotoxin Production, Serogrouping, and Antimicrobial Susceptibilities of Clostridium difficile Strains Isolated from AIDS and Human Immunodeficiency Virus-Negative Patients

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Received 9 July 1992/Accepted 7 December 1992

We analyzed and compared Clostridium difficile strains isolated from diarrheic stools of 49 human immunodeficiency virus (HIV)-negative and 50 AIDS patients. Our results suggest that distribution patterns of serogroups are different in these two populations. Serogroup C (which has been previously reported to be very resistant to antimicrobial agents) represents 66.0 and 18.4% of the isolates from AIDS and HIV-negative patients, respectively (P < 0.001); the selection of serogroup C could be explained by multiple antibiotic pressure to which AIDS patients have been subjected.

Clostridium difficile is the major etiological agent of pseudomembranous colitis, antibiotic-associated diarrhea (AAD), and antibiotic-associated colitis (AAC), especially in elderly and immunocompromised patients (patients with hematologic malignancies or patients undergoing chemotherapy) (7). Pathophysiological mechanisms of C. difficile-associated intestinal infections lead to release of toxins A (cytotoxin) and B (cytotoxin) (7).

The purpose of this study was to analyze and compare parameters such as in vitro toxin production, serotypes determined by the technique of Delmée et al. (3), and antimicrobial susceptibility related to C. difficile strains isolated from patients with AIDS and human immunodeficiency virus (HIV)-negative patients.

Study cohort. The study cohort consisted of 50 patients with AIDS and 49 HIV-negative patients with AAD or AAC whose stools harbored C. difficile but no other enteric pathogen. They were selected at random from all of the cases of C. difficile-associated diarrhea or colitis diagnosed in our laboratory from January to December 1991. Clinical information about diarrhea history, underlying diseases, and associated antimicrobial therapy was obtained by reviews of patients’ charts.

Bacterial isolates. Stool specimens were inoculated into a medium selective for C. difficile (TCCA) consisting of brain heart infusion supplemented by 5% defibrinated horse blood, 0.1% sodium taurocholate, 250 mg of cycloserine per liter, and 10 mg of cefoxitin per liter (11) and were incubated at 37°C for 48 h in an anaerobic atmosphere. The identification of suspicious colonies was confirmed by biochemical techniques (Rapid 32A; BioMérieux).

Stool cytotoxicity assay. Fresh stool specimens were diluted 1:10 (wt/vol) in phosphate-buffered saline and centrifuged (GT 422; Jouan) at 3,500 rpm for 30 min. The supernatant was filtered on a Millipore filter (0.45-μm pore size); the sterile filtrate was inoculated on confluent monolayers of MRC-5 cells prepared in microtiter plates and incubated at 37°C in a CO2 (6.5%) atmosphere for 48 h. The specificity of the cytopathic effect (rounding off of the cells) was confirmed by its neutralization with C. sordelli antisera (Anaerobes Unit, Institut Pasteur, Paris, France).

In vitro cytotoxin production assay. In vitro cytotoxin (toxin B) production was tested on spun and filtered supernatants of a 5-day brain heart infusion broth culture (in order to obtain maximum cytotoxin production) inoculated on confluent monolayers of CHO-K1 cells. The titer was defined as the logarithm of the reciprocal dilution giving a cytopathic effect for at least 50% of the cells.

Enterotoxin assay. Detection of enterotoxin (toxin A) was performed on a 5-day brain heart infusion broth culture supernatant by a double sandwich enzyme-linked immunosorbent assay technique with a monoclonal antibody (anti-toxin A) coated on microtiter wells; an anti-rabbit alkaline phosphatase conjugate and substrate were added to reveal the enzymatic activity (1). The amount of toxin A was determined on a standard curve drawn with a reference toxin A. The titer was expressed as the logarithm of the quantity of toxin A (in nanograms) per milliliter of supernatant.

Serogrouping. Serogrouping was performed by slide agglutination with different antisera by the method of Delmée et al. (3). Antisera were prepared by immunizing rabbits with Formol-treated reference strains. Serogrouping was done by mixing 1 drop of each antiserum with 1 drop of C. difficile culture. A positive reaction was recorded when complete clumping of the bacterial cells against a clear background was observed.

Antimicrobial susceptibility. Antimicrobial susceptibility was determined by the ATB-ANA system (BioMérieux). In summary, C. difficile strains were suspended in distilled water, transferred into a semisolid growth medium, and inoculated onto strips. After 24 h of anaerobic incubation, growth was read by an automatic system (ATB Expression; API, BioMérieux). The results were expressed as susceptible or resistant. Results were obtained for five antibiotics (tetracycline, chloramphenicol, erythromycin, rifampin, and

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clindamycin) to which *C. difficile* has shown a wide variation in susceptibility (2).

**Statistical methods.** Differences with group proportions were assessed by the chi-square test adapted to large-scale samples, and comparison of means was performed by Student’s *t* test. Two-tailed tests of significance at *P* < 0.05 were used to determine statistical significance.

The isolates from AIDS and HIV-negative patients came from 23 and 26 different wards, respectively (corresponding to 9 and 17 different hospitals, respectively), thus excluding a possible nosocomial spread of the organism and an epidemic situation. Patients with AIDS were mostly hospitalized in infectious care units, whereas HIV-negative patients came from intensive care units or gastroenterology and oncology wards.

The mean ages were estimated to be 36 ± 9 and 61 ± 25 years for patients with AIDS and HIV-negative patients, respectively. These two populations were not similar in regard to sex ratio; males were predominant among patients with AIDS (43 males and 7 females) but not among HIV-negative patients (24 males and 25 females). These differences are mainly due to the epidemiological characteristics of AIDS.

We also determined prior antibiotic therapy received during the 6 weeks preceding the onset of diarrhea or colitis; the mean number of antibiotics received was estimated to be 1.3 per patient in the HIV-negative group versus 2.9 per patient for the AIDS group.

The results of stool cytotoxicity and in vitro toxin production tests are summarized in Table 1. All toxigenic strains isolated from patients with AIDS and HIV-negative patients released both toxins A and B in vitro. We also found that the stool cytotoxin assay was negative for 11 patients with AIDS and 3 HIV-negative patients, although *C. difficile* strains isolated from their stools had been shown to be toxigenic in both the in vitro cytotoxin and enterotoxin production tests.

Serotyping of *C. difficile* isolates from patients with AIDS showed that serogroup C was predominant (66.0% of all strains); this was not the case for isolates from HIV-negative patients (18.4%; *P* < 0.001). Among isolates from the HIV-negative group, a wide distribution pattern of serotypes A, C, D, G, H, and K was observed (Table 1). Resistance of *C. difficile* strains to the five antibiotics tested was significantly higher among isolates from patients with AIDS than among isolates from HIV-negative patients (Table 1).

The aim of this study was to determine whether *C. difficile* strains isolated from patients with AIDS could be distinguished from strains isolated from HIV-negative patients with regard to toxin production, serogrouping, and antimicrobial susceptibility.

Most *C. difficile* isolates are toxigenic in both HIV-negative and AIDS patients (89.8 and 88.0%, respectively) and can account for cases of diarrhea or colitis in these patients. Nontoxigenic strains belong to serogroups D (five strains), K (one strain) and A (one strain); their significance remains unclear. Patients with nontoxigenic *C. difficile* strains can be defined merely as carriers, and their diarrhea or colitis should be explained by other mechanisms. For 14 patients (11 patients with AIDS and 3 HIV-negative patients), we found a discrepancy between a negative stool cytotoxin assay and a positive in vitro toxin production assay. We hypothesize that the in vivo toxin level might be too weak to account for the negative stool cytotoxicity assay but sufficient to induce diarrhea or colitis in patients with previous intestinal mucosal damage (such as AIDS patients with HIV enteropathy or a history of repetitive gastrointestinal infections or patients under chemotherapy). In this case, we surmise that the stool cytotoxicity assay, which is considered the “gold standard” method for detecting *C. difficile*-associated intestinal diseases, might lack reliability for diagnosis. We believe that the bacterial culture of toxigenic *C. difficile* in stools may indicate a potential risk factor for development of a *C. difficile* AAC or AAD, particularly in immunocompromised patients such as those with AIDS, even if the stool cytotoxicity assay is negative.

Several methods are reliable for demonstrating that *C.
difficile-associated gastrointestinal infections can be nosocomially acquired and can be responsible for outbreaks of diarrhea among immunocompromised patients (6, 8, 9). Among these, the serogrouping technique is a rapid, simple, and practical method for typing C. difficile. Delmée et al. (3, 10) have previously shown that different C. difficile serogroups vary in toxin production. For example, strains from serogroups A and C are often toxigenic, whereas strains from serogroup D or B are usually nontoxigenic. Delmée et al. also pointed out that different serogroups could be associated with different clinical pictures (5, 10). For example, serogroups A, G, H, and K are isolated mainly from adult patients with pseudomembranous colitis or AAD, whereas serogroup D is isolated mainly from asymptomatic neonates and small children. Typing of C. difficile also revealed that serogroup C isolates are often responsible for outbreaks (4). We demonstrated that the distribution patterns of C. difficile serogroups in patients with AIDS are different from those in HIV-negative patients. Serogroup C represents the majority of the isolates in patients with AIDS, whereas a wide distribution pattern of different serogroups is observed in HIV-negative patients. We excluded an epidemic situation because of methodical patient selection. Patients came from different and distant French hospitals over a period of 12 months. In agreement with a previous report by Delmée and Avesani (2), we showed that serogroup C is associated with a pattern of strong resistance to antimicrobial agents. This could account for the predominance of serogroup C in patients with AIDS who have been treated with several antimicrobial agents for opportunistic infections. We hypothesize that repetitive antibiotic pressures may eliminate susceptible strains of C. difficile and could finally select resistant strains of exogenous (environmental) or endogenous origin.

C. difficile is the major cause of AAD and AAC. Because patients with AIDS spend considerable time in hospitals and are often given antimicrobial therapy, clinicians should keep this pathogen in mind when searching for the cause of diarrhea in this patient group. We showed that serogroup C represents the majority of isolates in AIDS patients with AAD or AAC and that this finding could reflect the multiple antibiotic therapy which patients with AIDS often receive.

We are deeply indebted to M. Sebold (Anaerobes Unit, Institut Pasteur) for her generous gift of C. sordelli antiserum.

REFERENCES