Effects of subinhibitory concentrations of clindamycin on the morphological, biochemical and genetic characteristics of *Bacteroides fragilis*

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Abstract

The effects of subinhibitory concentrations of clindamycin on the morphological, biochemical and genetic characteristics of species of the *Bacteroides fragilis* group isolated from children with diarrhea were determined. The minimal inhibitory and subinhibitory concentrations for clindamycin were determined. Minimal inhibitory concentration values ranging from 0.25 to 512 $\mu g\,mL^{-1}$ were observed. Cultures grown with clindamycin were used to determine the macroscopic morphological characteristics, cellular viability, ultrastructural characteristics and DNA integrity. Clindamycin did not alter colonial morphology, but after 6 h elongated cells were observed. Also, extracellular vesicles and electron-lucent areas inside the cytoplasm were observed. Bacteria treated with clindamycin also showed fragmentation of DNA as determined by electrophoresis. The alterations produced by clindamycin might be indicative of a possible modification of the structures involved in bacterial pathogenesis.

Introduction

Bacteroides fragilis is considered an important clinical pathogen and it is the most common anaerobe isolated from human clinical specimens producing endogenous infections (Duerden, 1994). Species of the *B. fragilis* group have shown an increase in resistance to most of the antimicrobial agents traditionally used for treating anaerobic infections.

It is well known that antimicrobial drugs can produce alterations in the host's indigenous microbiota, selecting resistant organisms which can appear as opportunistic pathogens (Bezirtzoglou, 1997). Subinhibitory concentrations of antimicrobial agents cause multiple effects on a variety of organisms, which include morphological and ultrastructural changes, alteration in the ability of the bacteria to be killed by phagocytic cells, biochemical behavior and changes in attachment capabilities (Lorian, 1986).

Clindamycin, one of the antibiotics frequently used in subinhibitory studies, is clinically useful in infections involving *B. fragilis* (Gorbach & Bartlett, 1974). This antibiotic affects bacterial protein synthesis producing lysis, but also induces morphological alterations such as elongation and, sometimes, fusiform morphology in Gram-negative aerobic organisms, whereas Gram-positive cocci enlarge. Although the effects of subinhibitory doses of an antibiotic on aerobic organisms have been evaluated extensively, less attention has been directed towards anaerobic bacteria, including species of the *B. fragilis* group. Moreover, there have been reports of filament formation in *Bacteroides* spp. exposed to several antibiotics (Guss & Bawdon, 1984).

Lewis (2000) has proposed that the well-described phenomena of bacterial autolysis after the exposure to antibiotics or other harsh environmental conditions may actually represent situations where bacterial programmed cell death is working to eliminate damaged cells from the bacterial population.

In this study, the effects of clindamycin on the morphological, biochemical and genetic characteristics of *B. fragilis* were determined.

Materials and methods

Organism, culture conditions and antimicrobial susceptibility

Nine species of the *Bacteroides fragilis* group isolated from children with diarrhea were used as follows: five *B. fragilis* (AUS-290, AUS-296, AUS-300, AUS-342 and AUS-343), one *B. distasonis* AUS-318, one *B. vulgatus* AUS-356, one *B. uniformis* AUS-391 and one *B. ovatus* AUS-352, and as a control, *B. fragilis* ATCC 43858. The organisms were stored in skim-milk (10%) at −80 °C, until use, at the Anaerobe Laboratory, Department of Microbiology, ICB, USP.

The organisms were grown in 5 mL of brain heart infusion broth (BHI), in an anaerobic atmosphere (90% N_2/10%CO_2) at 37 °C for 48 h. The minimal inhibitory concentration (MIC) for clindamycin (Hydrochloride clindamycin, Upjohn) was determined by a macrodilution method (National Committee for Clinical Laboratory Standard, 1997). The lowest drug concentration that still allowed bacterial growth but generated morphological alterations when observed with a light microscope was defined as the subinhibitory concentration.

Bacterial growth curve

Initially, all the cultures grown in anaerobiosis with or without the respective subinhibitory concentrations of clindamycin (Table 1) were used to determine the growth curves. Bacterial growth without the drug was used as control. Every 3 h of a total of 74 h of incubation an aliquot was removed to observe the morphological characteristics of the bacteria; optical density (OD) was measured using a spectrophotometer ($A_{550\,nm}$) (Jenway, Model 6300). The cellular viability was also determined every 3 h during the 74 h growth by determining the viable cell counts after serial dilution of the samples, transferring 10 μL onto blood agar and incubating in anaerobiosis. All the experiments were performed in duplicate.

Table 1. The minimal inhibitory and subinhibitory concentrations of clindamycin used to determine morphological alterations produced during bacterial growth

Strains	Minimal inhibitory concentration (μg mL^{-1})	Subinhibitory concentration (μg mL^{-1})
Bacteroides fragilis AUS-290	256	0.125
Bacteroides fragilis AUS-296	512	0.5
Bacteroides fragilis AUS-300	0.25	0.0078
Bacteroides fragilis AUS-342	32	1
Bacteroides fragilis AUS-343	16	0.06
Bacteroides uniformis AUS-391	0.25	0.0078
Bacteroides distasonis AUS-318	0.25	0.016
Bacteroides ovatus AUS-352	256	0.125
Bacteroides vulgatus AUS-356	256	0.125
Bacteroides fragilis ATCC 43858	0.5	0.015

Morphological and physiological analyses

Analyses of bacterial colonial and cellular morphology

The bacteria were observed every 3 h of growth in BHI with or without clindamycin, Gram stained and viewed under a light microscope (Leica). Images were taken using a CCD camera (Hitachi model KP-D 581U). Aliquots were streaked onto blood agar and, after incubation in anaerobiose at 37 °C, colonial morphology was observed.

Biochemical assays

Bacteroides fragilis grown with and without clindamycin were examined for the production of catalase H_2S, indole, gelatinase and bacteriocin, hydrolysis of esculin, hydrophobicity and sugar fermentation (Holdeman *et al.*, 1977; Summanen *et al.*, 1983).

Transmission electron microscopy (TEM)

Ten milliliter samples of each broth culture with or without clindamycin were centrifuged for 20 min, at 3000 ***g***, to obtain bacterial pellets, which were rapidly fixed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2% volume in volume (v/v) glutaraldehyde and 2.5% v/v formaldehyde, for 4 h at 4 °C in the dark. After several washings in the same buffer, pellets were postfixed in 1% weight in volume (w/v) osmium tetroxide, then dehydrated in a graded series of ethanol and embedded in Spurr resin (Electron Microscopy Sciences, Fort Washington). Ultrathin sections collected on 200-mesh copper grids were stained with uranyl acetate and lead citrate before examination in a JEOL 1010 TEM.

Observation of the capsule of strain AUS-290 was performed using ruthenium red staining. Samples were prepared as described above, except that ruthenium red stain 0.2% w/v was included at both fixation steps.

DNA isolation and electrophoresis

After 30 h growth in BHI with and without clindamycin, aliquots were obtained for DNA extraction as follows: bacteria were centrifuged (10 000 ***g***, 10 min) and the pellet was resuspended in 300 μL of lysis buffer [40 mM Tris-acetate, pH 7.8; 20 mM sodium acetate; 1 mM ethylenediamine tetra-acetic acid (EDTA), pH 8.0; 1% w/v sodium dodecyl sulfate] and 50 μL of 10 mg mL^{-1} proteinase K, and the tubes were incubated at 37 °C, for 30 min. The extracts were treated with 300 μL of chloroform/isoamyl alcohol

(CIA – 24 : 1) and centrifuged (12 000 ***g***, 4 °C). The aqueous phase was transferred into a new tube and 200 μL of Tris-EDTA buffer (pH 8.0) were added. The extraction was repeated, and the DNA was precipitated with 500 μL of isopropyl alcohol. DNA was resuspended in 50 μL of Tris-EDTA buffer (pH 8.0). Aliquots of 20 μL of DNA were analyzed by electrophoresis in 2% w/v agarose gel, at 90 V, for 90 min.

Results

Ten species of the *Bacteroides fragilis* group showed MIC values ranging from 0.25 to 512 μg mL^{-1} clindamycin (Table 1). All the strains tested were analyzed for their characteristics after treatment with subinhibitory concentrations of clindamycin, as shown in Table 1. The strains displayed similar growth curves and showed a subpopulation of cells with filamentous morphology after drug treatment as assessed by light microscopy (data not shown). Strain *B. fragilis* AUS-290 was chosen from the resistant strains for further characterization.

Bacteroides fragilis AUS-290 was affected by clindamycin after 6 h of growth, displaying a significant decrease in the growth rate (Fig. 1a). In order to determine the viability of the cultures, the effect of clindamycin on *B. fragilis* AUS-290 at different time points was determined (Fig. 1b). The results showed that the viability correlated well with the growth curve, indicating that this concentration of the drug had an effect upon growth, but was not killing the cells.

There was no evidence of alterations in the morphology of the colony of *B. fragilis* AUS-290 growing in 0.125 μg mL^{-1} clindamycin. However, after 6 h, *B. fragilis* AUS-290 showed elongated cells and filaments as observed by light microscopy (Fig. 2). The capsule was also observed

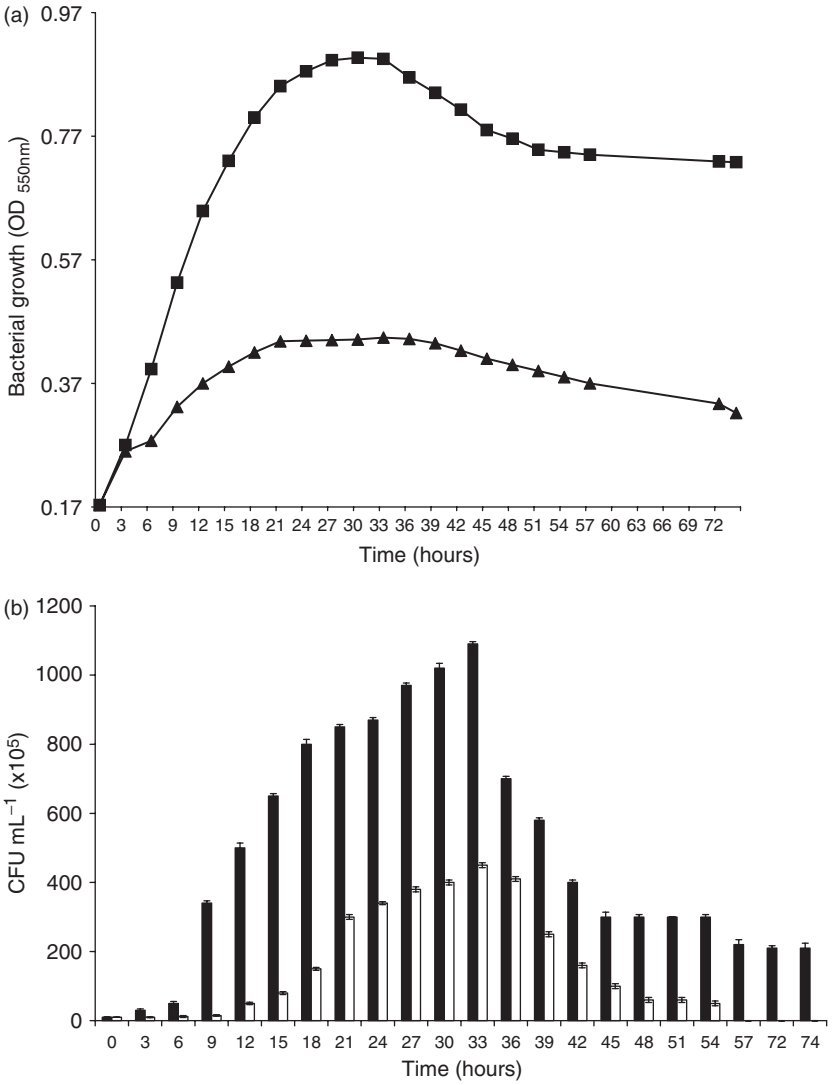

Fig. 1. (a) *Bacteroides fragilis* AUS-290 grown in brain heart infusion (BHI) broth in the presence and absence of clindamycin. Growth was measured by determining absorbance at 550 nm. Cultures: ■, without antibiotic; ▲, 0.125 μg mL^{-1} clindamycin. (b) Cellular viability of *B. fragilis* grown in BHI broth. Viability was determined by counting colonies after serial dilution of each culture. Cultures: ■, without antibiotic; □, 0.125 μg mL^{-1} clindamycin.

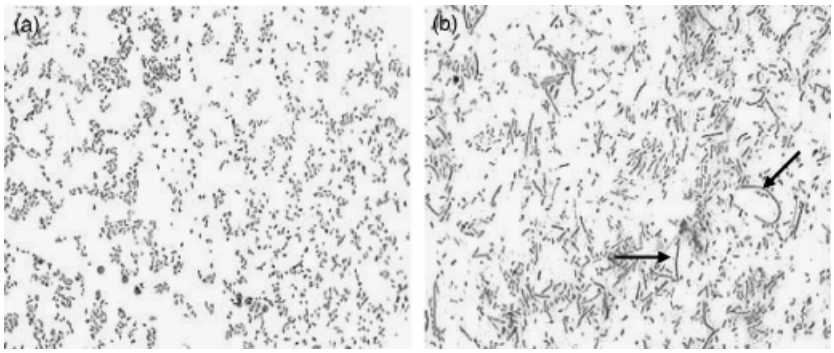

Fig. 2. Light microscope (oil-immersion) of *Bacteroides fragilis* AUS-290 grown for 6 h in brain heart infusion (BHI) broth with or without clindamycin. (a) Control and (b) with 0.125 μg mL^{-1} clindamycin. Arrows show the elongated and the filamentous cells. Magnification × 100.

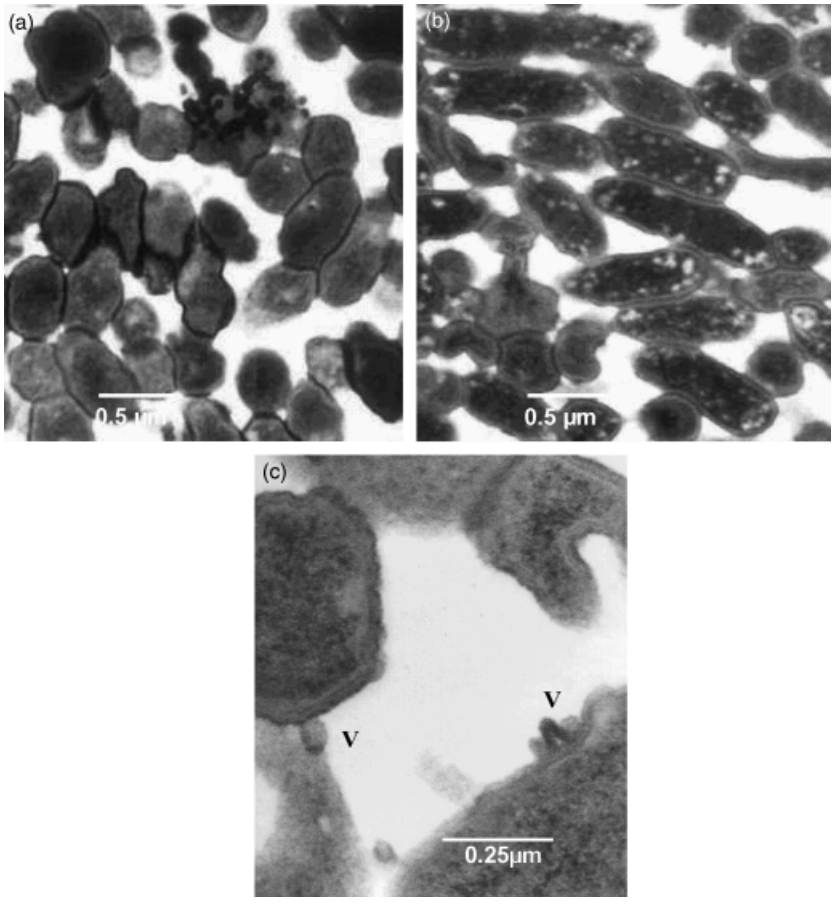

Fig. 3. Transmission electron micrograph of *Bacteroides fragilis* AUS-290 grown for 27 h without (a) and with (b) 0.125 μg mL^{-1} clindamycin. Note the numerous electron-lucent areas inside the clindamycin-treated cells. Magnification × 25 000. (c) *Bacteroides fragilis* AUS-290 grown for 27 h in brain heart infusion broth with 0.125 μg mL^{-1} of clindamycin. V, extracellular vesicles. Magnification × 66 500.

in the culture without clindamycin, and in the culture with an antibiotic, a decrease in the number of cells displaying this structure was observed (data not shown), agreeing with previous results (Zaleznik *et al*., 1986). Ultrastructural examination of *B. fragilis* AUS-290 incubated with 0.125 μg mL^{-1} clindamycin for 27 h revealed the presence of elongated cells, extracellular vesicles and electron-lucent (clear) areas inside their cytoplasm, suggesting a possible structural degradation (Figs 3b and c).

All the strains were tested for metabolic characteristics and were shown to produce catalase, ferment sugars and hydrolyze esculin, and displayed a hydrophilic characteristic. These strains did not produce H_2S, indole, gelatinase and bacteriocin, and these characteristics were not altered when growing in subinhibitory concentrations of clindamycin (data not shown).

In order to determine whether the antibiotic was affecting the chromosome, DNA samples extracted from *B. fragilis*

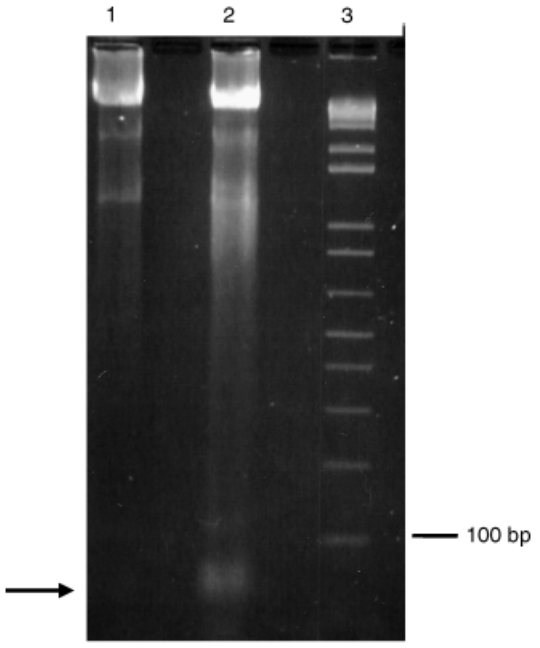

Fig. 4. Agarose gel electrophoresis of DNA isolated from *Bacteroides fragilis* AUS-290. Lane 1: *B. fragilis* AUS-290 grown without clindamycin; lanes 2: *B. fragilis* AUS-290 grown in brain heart infusion broth with 0.125 μg mL^{-1} clindamycin for 30 h; lane 3: DNA molecular weight marker (1 kb Plus DNA ladder, Invitrogen, San Paulo, Brazil). The arrow indicates the low molecular weight DNA fragments, in lane 2.

AUS-290 grown in 0.125 μg mL^{-1} clindamycin (30 h) were analyzed by electrophoresis. It was observed that the DNA from treated cells showed low molecular weight fragments, while the DNA from untreated cells was intact (Fig. 4).

Discussion

Species of the *Bacteroides fragilis* group are the anaerobic bacteria most commonly associated with a wide variety of endogenous clinical infections, especially *B. fragilis* species.

It has been mentioned that the bacteria with antimicrobial resistance mechanisms are able to grow and to multiply in gradually higher concentrations of antibiotics. It was also suggested that both genetic and physiological bacterial factors could selectively suppress the susceptibility to the antibiotic killing action (Tuomanen *et al.*, 1986). As a bacterial population consists of different subpopulations of cells, there is much to be elucidated about these phenomena among bacteria of the *B. fragilis* group (Avila-Campos *et al.*, 1991; Nakano & Avila-Campos, 2004).

Although the cellular targets of most antibiotics have been well characterized, it is still unknown how the interaction between an antibiotic and its target actually triggers cellular death. Subinhibitory concentrations of a variety of antibiotics ranging from β-lactam antibiotics to streptomycin have been found to alter the morphology and the ultrastructure in several bacterial species, including alteration in the number and location of ribosomes, elongation and enlargement of the bacteria and changes in the appearance of the cell wall (Svanborg-Edén *et al.*, 1978; Lorian, 1986).

Conversely, it is known that antibiotics that directly or indirectly produce an alteration in protein synthesis induce Gram-negative bacilli to elongate and sometimes to assume fusiform morphology. Most of the studies on the effect of subinhibitory doses of antibiotics on bacteria have been concentrated on aerobic organisms, although there have been reports of filament formation in *Bacteroides* spp. exposed to several antibiotics (Guss & Bawdon, 1984).

Studies have shown that exposure of anaerobically grown *B. fragilis* to clindamycin and to erythromycin resulted in a reduction in the size of the capsule, without alterations in the morphology of the bacteria, and that exposure to cefoxitin resulted in filamentation (Guss & Bawdon, 1984). In our study, 0.125 μg mL^{-1} clindamycin produced a decrease in the number of cells of *B. fragilis* AUS-290 with capsule, as well as an increase in elongated and filamentous cells. It is known that a bacterial filament results when bacilli grow but do not separate into new individual bacterial cells. Filament formation in *B. fragilis* group species upon exposure to cefoxitin has been demonstrated in some previous studies, while other studies could not demonstrate filamentation (Guss & Bawdon, 1984; Fang *et al.*, 2002).

Moreover, it appears that the treatment with clindamycin induced extracellular vesicle formation (Fig. 3c); in control cultures, no vesicle was observed (data not shown). The release of extracellular vesicles may prove to have important implications for the pathogenesis of *B. fragilis* as suggested by Patrick *et al.* (1996). Here, we also observed that when exposed to 0.125 μg mL^{-1} clindamycin, cultures of *B. fragilis* AUS-290 showed a severe decrease in both viable count and absorbance relative to the control, suggesting that the drug is inhibiting cell growth.

Namavar *et al.* (1986) observed no effect on the cellular morphology of *B. fragilis* grown in subinhibitory concentrations of clindamycin by optical electron microscope and TEM. However, in our study, organisms developed filamentation and elongated cells after exposure to 0.125 μg mL^{-1} clindamycin, and the morphological changes occurred rapidly and were pronounced (Figs 2 and 3). This fact indicates that clindamycin has a lower bactericidal effect on the cell, which may be due to the lower growth of this organism.

Conversely, subinhibitory concentrations may induce alterations in the morphology, ultrastructure, biochemistry and multiplication rate of microorganisms and they certainly elicit serious consequences on their virulence as well as their susceptibility to the host immune defenses (Lorian,

1986; Zaleznik *et al.*, 1986). Moreover, in this study, clindamycin did not induce changes in the biochemical characteristics tested.

Our data showed DNA fragmentation, producing low molecular weight fragments in the cells treated with $0.125\,\mu g\,mL^{-1}$ clindamycin (Fig. 4). This pattern is frequently observed in apoptotic eukaryotic cells, and it was also observed in *Helicobacter pylori* cells, which had undergone programmed cell death (Cellini *et al.*, 2001).

In conclusion, all the alterations of the cellular morphology in *B. fragilis* AUS-290 observed in this study may occur as an effort towards adaptation to adverse environmental conditions, as proposed by Ferreira *et al.* (1991). The growth pattern, the morphological changes and the DNA fragmentation induced by clindamycin in *B. fragilis* species might be indicative of a possible modification of the structures involved in bacterial pathogenesis.

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