Colistin inhibits E. coli O157:H7 Shiga-like toxin release, binds endotoxins and protects Vero cells

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SUMMARY

The role of antibiotics in the treatment of Shiga-like toxin (Stx)-producing E. coli infection is still controversial. This study investigated the effects of colistin on Vero cell cytotoxicity caused by the enterohemorrhagic EC O157:H7, and the effects of colistin on Stx and endotoxin release by EC O157:H7. Vero cells were incubated with supernatant collected from EC O157:H7 cultured for 18 h without (control) or with various concentrations of colistin. In the absence of colistin, Vero cell viability after 48 h was 29.1±6.3%. Under the same conditions, the overnight presence of colistin reduced cytotoxicity to Vero cells (viability: 97.3±5.0 to 56.5±14.4% for colistin concentrations ≥MIC). Sub-MIC concentrations of colistin also provided partial protection (viability: 38.8±12.5 to 36.6±14% for 0.125 and 0.06 mcg/ml colistin, respectively). Endotoxins contributed to the cytotoxic effects on Vero cells since lower but still significant protection was observed when colistin was added directly to the supernatant collected from cultures of untreated EC O157:H7.

Colistin reduced Stx release in a concentration-dependent manner, also at sub-MIC concentrations. Co-incubation of the supernatant from EC O157:H7 cultures with colistin markedly reduced the endotoxin concentration at all doses investigated. In conclusion, colistin protects Vero cells from EC O157:H7 at supra- and sub-MIC concentrations by inhibiting Stx release and binding endotoxins. Colistin might be a valuable treatment for clinically severe forms of EC O157:H7 infection.

INTRODUCTION

E. coli O157:H7 (EC O157:H7) is one of the causative pathogens of hemorrhagic colitis, which can be accompanied with life-threatening hemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura, and neurologic damage (Carter et al., 1987; Rangel et al., 2002; Steinborn et al., 2004; Gould et al., 2009; Mele et al., 2014). The morbidity and mortality from infection by this pathogen are still high (Carter et al., 1987; Rangel et al., 2002; Gould et al., 2009). EC O157:H7 is commonly part of the intestinal microbiota of cattle and is transmitted by contaminated food. Shiga-like toxins (Stx) released by infection which occurred in Germany in 2011 (Menne et al., 2010). In vitro studies have shown that some antibiotics (e.g., quinolones, trimethoprim, sulfamethoxazole, β-lactams) induce the release of Stx, while others do not (e.g., azithromycin, rokitamycin, doxycycline, clindamycin, fosfomycin) (Murakami et al., 2000; Hiramatsu et al., 2000; Dundas et al., 2005; Panos et al., 2006; Scheiring et al., 2010). However, a recent retrospective study showed that antibiotics were the most effective treatment during the outbreak of EC O104:H4 infection which occurred in Germany in 2011 (Menne et al., 2010). In vitro studies have shown that some antibiotics (e.g., quinolones, trimethoprim, sulfamethoxazole, β-lactams) induce the release of Stx, while others do not (e.g., azithromycin, rokitamycin, doxycycline, clindamycin, fosfomycin) (Murakami et al., 2000; Hiramatsu et al., 2003; McGannon et al., 2010). Despite the uncertainty on the efficacy and safety of antibiotic treatment, a recent article reported that 274 out of 474 (62%) patients with laboratory-confirmed EC O157:H7 infection took antimicrobial agents (Nelson et al., 2011). Colistin is a bactericidal polycationic antibiotic that targets the cell wall and does not interfere with DNA replication. It has also an anti-endotoxin effect. Several in vitro and in vivo studies have shown that colistin (like polymyxin B) binds to endotoxins in a stoichiometric fashion to form a stable complex with altered physico-chemical properties (Morrison et al., 1976; Jacobs et al., 1977), can reduce the release of inflammatory cytokines, and block some of the biological activity of these cytokines (Rifkind et al., 1966; Warren et al., 1985; Rogers and Cohen, 1986; Cirioni et al., 2007; Aoki et al., 2009; Nanjo et al., 2013).
Colistin is nephrotoxic, but when administered orally it is only slightly absorbed and is almost completely eliminated by the gastrointestinal tract and, therefore, in this circumstance there is no risk of nephrotoxicity. Colistin might, therefore, be a good candidate for the treatment of Shiga toxin-producing E. coli infection.

The aim of our study was to investigate the effects of colistin on Vero cell cytotoxicity caused by enterohemorrhagic E. coli O157:H7, and the effects of colistin on Stx and endotoxin release by EC O157:H7.

MATERIALS AND METHODS

Bacterial strain and antimicrobial agent

E. coli O157:H7 (vtx1+/vtx2+) [BG16413] and an E. coli Stx-negative strain [PV07004781] obtained from the Italian Institute of Health were grown in Mueller-Hinton broth (Oxoid, Basingstoke, Hampshire, UK) at 37°C. After 18 h the cultures were centrifuged at 3000 rpm for 15 minutes and the supernatants were filtered through 0.45-μm membrane filters (Millipore Corporation, Billerica, MA, USA) and stored at 4°C until assays were performed. Colistin sulfate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in water at 1000 mcg/ml and stored at -20°C.

Determination of the minimal inhibitory concentration

The minimal inhibitory concentrations (MICs) of colistin were determined by the standard broth/tube macrodilution method (Clinical and Laboratory Standards Institute, 2015). After 18 h of incubation with different concentrations of colistin the bacterial growth was 8x10^6 CFU/ml at the MIC value, 6x10^6 CFU/ml at 1/2 MIC, 7x10^6 CFU/ml at 1/4 MIC, 7.2x10^5 CFU/ml at 1/8 MIC, while the growth in the control tube was 7.2x10^6 CFU/ml. The tubes were centrifuged at 3000 g for 15 min and the supernatants were collected to perform the toxicity test. The MIC for the EC O157:H7 strain used in our experiments was 0.25 mcg/ml (this value, albeit high, is in the range of those usually reported against E. coli strains).

Vero cell toxicity assay

The Vero (African green monkey kidney) cell line (ATCC, Rockville, MD, USA), which expresses high concentrations of globotriaosylceramides Glb3 and Glb4, the receptors for Stx in eukaryotic cells, were used for the cell culture toxicity assay. Vero cells were grown in Earle MEM (EuroClone S.p.A., Pero, Milan, Italy) supplemented with 10% fetal bovine serum (EuroClone). At confluence cells were trypsinized and seeded at 10,000 cells per well in Costar 96 well microplates for 24 h at 37°C in a 5% CO₂ humidified incubator. The medium was replaced and replaced with E. coli bacterial filtrate diluted in phenol red-free medium.

Stx release was assayed after treatment of E. coli cultures with colistin for 18 h at different concentrations ranging from 8 mcg/ml to 0.03 mcg/ml. In this case 100 μl of the supernatant from each colistin culture concentration were inoculated 1:10 into subconfluent Vero cell microplates in parallel with the untreated E. coli supernatant and incubated at 37°C in a 5% CO₂ humidified incubator. After 48 h cell viability was determined using a Vybrant MTT cell proliferation assay kit (Invitrogen, Eugene, Oregon, USA) according to the manufacturer’s instructions. Briefly the supernatant was replaced with 100 μl of fresh phenol red-free medium and cells were incubated with 10 μl stock solution at 37°C for 4 h. Next, 100 μl of the second reagent were added and incubated at 37°C for another 4 h. After mixing each sample the absorbance was read at 570 nm.

For each experiment a Stx-negative E. coli was tested in parallel with the Stx O157:H7-positive strain.

Shiga toxin detection

Stx was detected by enzymatic immunoassay (EIA) using the Premier HECH EIA rapid test (Meridian, Bioscience Europe, Inc., Cincinnati, OH, USA) according to the procedure described by the manufacturer. Briefly 100 μl of each centrifuged supernatant from EC O157:H7 cultures treated with colistin from 8 mcg/ml to 0.03 mcg/ml were tested diluted 1:10 in the HEAC EIA test for 1 h at room temperature. Polyclonal anti-Stx was added for the detection after washing for 1 h at room temperature. After washing, substrate was added and incubated for 10 min at room temperature. Stop solution was added and the optical density (OD) of each well was measured spectrophotometrically at 450 nm. Positive and negative controls supplied by the manufacturer of the EIA kit were used with each assay run to provide quality assurance of the reagents. Results were considered positive when the OD was ≥0.180. The same EIA kit was also used to evaluate the reduction of toxins in the supernatant treated for 3 h at 37°C with the same colistin concentration and then diluted before being added to the EIA microplate. This method does not differentiate between Stx1 and Stx2.

Endotoxin determination

Endotoxin concentrations were measured by a commercially available LAL kinetic turbidimetric assay (Charles River Laboratories, USA) using the microplate reader Elx808 (Biotek, USA) controlled by LAL-specific software Endoscan V (Charles River Laboratories, USA). Supernatant samples were filtered with 0.2 μl endotoxin-free filters and diluted in endotoxin-free water to reach dilution factors from 100,000 to 1,000,000.

The endotoxin concentration was determined as described by the manufacturer using also a B-glucan blocker buffer (code BG120, Charles Rivers Laboratories, USA). Endotoxin standards (from 50 EU/ml to 0.05 EU/ml) were tested in each run, and the endotoxin concentration in the test samples was calculated by comparison with the standard curve. The spike value was 0.5 EU/ml. All sample results satisfied the requirements of the current European Pharmacopoeia (spike recovery, R value and negative controls) (European Pharmacopoeia, 2014).

Statistical analysis

All data are presented as mean ± standard deviation and were analyzed by the unpaired t-test for single comparisons. A p value <0.05 was considered to be statistically significant.

RESULTS

Cytotoxicity of the supernatant obtained from EC O157:H7 incubated with different concentrations of colistin

Vero cells were incubated with the supernatant collected from EC O157:H7 cultured for 18 h without (control) or with supra- and sub-MIC concentrations of colistin. In the absence of colistin, Vero cell viability after 48 h was 29.1 ± 6.5%. Under the same conditions, the overnight pres-
Colistin and Shiga-like producing E. coli

**Materials and Methods.** The amount of Stx released was significantly evaluated in the supernatant diluted 1:10 as described in the Materials and Methods. The amount of Stx released was significantly lower in the presence of all concentrations of colistin than in the absence of the antibiotic (control). *p<0.05.

**Effect of colistin on Stx concentration.** EC O157:H7 cultures were treated overnight with colistin and the release of Stx was collected after 18 h, was added to cultures of Vero cells: cell viability is represented by the black bars. White bars represent the viability when colistin was added directly to the supernatant. An asterisk denotes a statistically significant difference compared with control (no colistin). EC = EC O157:H7; CST = colistin.

**DISCUSSION**

Although the use of antibiotics for the treatment of Stx-producing E. coli infections is controversial, there is a general consensus that if an antibiotic is used, it should not elicit the SOS response - which can increase Stx release. Some antibiotics (e.g., ciprofloxacin, norfloxacin, ampicillin, kanamycin) (Thi et al., 2011; Blazquez et al., 2012; Brochmann et al., 2014), regardless of the drug-target interaction, also exert their bactericidal activity through the formation of hydroxyl radicals (OH•) that cause DNA damage and promote the SOS response (Kohanski et al., 2007). Nagnmouchi et al. (2013) reported that colistin can cause DNA damage, but other two studies showed that the bactericidal activity of colistin is not associated with OH• formation (Brochmann et al., 2014) and that colistin does not induce the SOS response in E. coli (Thi et al., 2011). Moreover, Uemura et al. (2004) reported that cultures of enterotoxemic E. coli O139 (a pathogen causing the edema disease in pigs) were treated with x1 or x5 MIC concentrations of colistin, the release and production of Stx, evaluated by Vero cell cytotoxicity, was equal or less than in untreated controls.

E. coli infections may result in an increase in free endotoxin and enhancement of inflammation either when infections are untreated or when antibiotics are administered, irrespective of the agent used (Friedland, 1993). The role of endotoxins in the pathophysiology of Stx-releasing E. coli disease is not known, but elevated levels of lipopolysaccharide-binding protein have been observed in children with EC O157:H7 infection who developed HUS (Proulx et al., 1999) and it has been hypothesized that endotoxins may contribute to the thrombotic process of HUS (Karpman et al., 1997; Stahl et al., 2009; Karpman, 2012). To evaluate the role of endotoxins in Vero cell cytotoxicity we
added colistin directly to the supernatant which was then added to Vero cells. The presence of colistin, which markedly reduced endotoxin concentration (Figure 3), protected Vero cells from the lethal injury otherwise caused by the supernatant (Figure 1), suggesting that endotoxins released by EC O157:H7 might contribute to the intestinal damage that can occur in patients infected by this bacterium. The ideal antibiotic for the treatment of Stx-producing E. coli infection should reduce the bacterial burden, avoid the release of Stx and be safe. Data from the literature and our results suggest that colistin could be a valuable candidate. Since there is no evidence of bacteremia during infection with Stx-producing E. coli (Karpman, 2012), an oral antibiotic with activity limited to the gastrointestinal tract is sufficient and probably advisable. Colistin can be safely administered orally at high doses as colistin sulfate, which is only slightly absorbed by the gastrointestinal tract, thus reducing the carriage of EC O157:H7. Our results show that colistin not only does not favor Stx release, but that it actually inhibits Stx release, even at sub-MIC concentrations (Figure 2). Finally, colistin binds EC O157:H7 endotoxins (Figure 3) which might contribute to tissue damage, as shown by Vero toxicity experiments (Figure 1).

A limitation of our study is that our assay did not discriminate between Stx1 and Stx2, which are both produced by human Stx-producing E. coli. Several observations suggest that Stx2 may be more virulent in human disease than Stx1 (Louise and Obrig, 1995; Jacewicz et al., 1999; Mele et al., 2014). We cannot, therefore, state whether colistin affected the release of Stx1, Stx2 or both. Another limitation was that we tested only one E. coli Stx-producing strain. Molecular studies have demonstrated the existence of distinct subpopulations of Stx-producing EC O157:H7 which have different virulence (Griff et al., 1998; Manning et al., 2008; Tozzoli et al., 2014) and, therefore, our results might not apply to all Stx-producing strains.

In conclusion, colistin has properties that could make it a valuable treatment for Stx-producing E. coli infection and it might be worth investigating its oral use in severely ill patients.

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**Compliance with Ethical Standards**

The authors declare that they have no conflicts of interest (financial or non-financial).

**References**


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