Epidemiol. Infect. (1997), **119**, 101–103. Printed in the United Kingdom

SHORT PAPER

The stimulating effect of fosfomycin, an antibiotic in common use in Japan, on the production/release of verotoxin-1 from enterohaemorrhagic *Escherichia coli* O157:H7 *in vitro*

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(*Accepted 12 March 1997*)

SUMMARY

In 1996 Japan had a big outbreak of enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7, especially in Osaka prefecture where about 6000 patients (mainly schoolchildren) suffered from diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome (HUS) due to EHEC infection via school lunch. Our survey of doctors who took care of those patients showed that most patients had received antibiotics, especially fosfomycin which comprised 84% of the prescribed treatment. Since the administration of antibiotics for EHEC infection is under discussion, we analysed the effect of fosfomycin on production/release of verotoxins (VTs). Addition of fosfomycin into EHEC culture in CAYE broth at 2 and 5 h after start of incubation caused marked increase of VT1 release. This observation warns of the possibility of fosfomycin treatment making the disease worse.

Several risk factors for haemolytic uraemic syndrome (HUS) during infection with enterohaemorrhagic *Escherichia coli* (EHEC or STEC after Shiga-toxin producing *E. coli*) O157:H7 have been reported [1, 2]. These include extreme youth or old age, mental retardation, P antigen expression, and fever. The issue the risk involved in treatment with antibiotics, however, remains controversial. There is a clinical report that all 5 patients who received trimethoprim-sulphamethoxazole, but only 2 of 7 who received no antibiotics, developed HUS ($P = 0{\cdot}03$) [1]. If the antibiotic was a factor, it could be because antimicrobial agents killed *E. coli* O157:H7 releasing verotoxin (VT), or that the drug increases the production of VT. Walterspiel and colleagues have reported that subinhibitory concentrations of ciprofloxacin, co-trimoxazole, cefixime and tetracycline significantly increase extracellular VT in EHEC strains in this order [3].

Since EHEC is a bacterial infection, most clinicians are likely to prescribe an antimicrobial agent for this disease. In Osaka Prefecture this year there was an outbreak of EHEC O157:H7 involving over 6000 victims. We conducted a questionnaire survey of physicians who treated these patients. In their replies doctors indicated that 95·9% of patients received antimicrobial agents, of which fosfomycin (FOM) was the most frequently prescribed, comprising 84% of the prescribed treatments. Among 179 who received FOM 38 patients developed HUS. We were concerned if this most commonly prescribed antimicrobial agent in Japan has any effect on VT production or release in EHEC strains.

We first determined the minimum inhibitory concentration (MIC) of test organisms in our experimental conditions, which consisted of rotating (100 rpm) cultures in a small test tube at 37 °C in a medium of CAYE broth that was suitable for the production of VT [4]. The MIC for FOM and DOXY (doxycycline, a tetracycline compound) of an isolate (RIMD 0509890) of EHEC O157:H7 from the outbreak that occurred in Osaka prefecture was determined to be 1 mg/ml and 20 μg/ml, respectively

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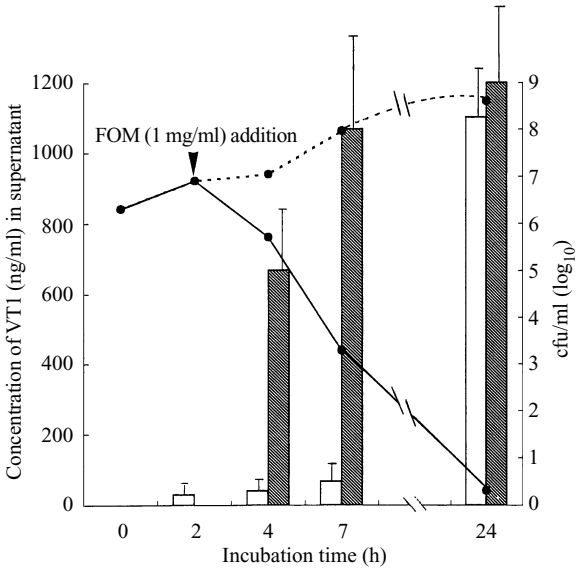

Fig. 1. Effect of fosfomycin (FOM) on the extracellular production of VT1. VT1 concentration (ng/ml): oblique shaded bars, with FOM; white bars, without FOM. Lines represent the number of living bacteria (cfu/ml) in cultures with, continuous line, and without FOM, broken line.

(the MIC of FOM to RIMD 0509890 was 6 μg/ml by the method recommended by NCCLS) [5]. The amount of VT in culture supernatants obtained after centrifuging (5000 g for 5 min) cultures in CAYE broth subsequent to adding either antibiotic was determined using a reversed passive latex agglutination test kit (*Escherichia coli* Verotoxin detection kit, Denka Seiken Co., Tokyo) in accordance with the procedure described in the manufacturer's manual. VT in the periplasmic space was assayed by the same method (*Escherichia coli* Verotoxin detection kit) with samples. After the above centrifugation, cell pellet was treated with polymyxin B (5000 IU/ml) at 37 °C for 30 min. Supernatant obtained by centrifugation of polymyxin B extract was used as periplasmic space sample. This kit can differentially detect two immunologically distinct, but biologically and structurally similar VTs (or Shiga toxins or Stxs), VT1 (Stx1) and VT2 (Stx2) [6].

A significant (eightfold) increase of VT1, but not VT2, was observed in supernatants of cultures containing a subinhibitory concentration (100 μg/ml) of FOM. On the other hand, with that (20 μg/ml) of DOXY VT levels decreased (VT1 2-fold, VT2 32-fold). The tendency for there to be increased levels of VT1 in conjunction with FOM was almost equally observed in all of the 10 EHEC O157:H7 strains tested. We furthermore examined the effect of FOM on the production/release of VT1 in culture supernatants with a concentration of MIC (1 mg/ml). This concentration of FOM killed test organisms when it was introduced 2 h after starting the culture. This experimental condition was designated to approximate the conditions in actual treatment. We found a marked increase of VT1, but not VT2, at 2 h (more than 13-fold) and 5 h (16-fold increase) after the addition of FOM (Fig. 1). During this period, the survival of the *E. coli* was decreased by hours and no detectable organism was present 22 h after FOM was introduced. In another three experiments, with conditions the same as those that yielded the results in Figure 1, we tested two separate samples obtained from culture supernatant and from periplasmic space 5 h after the time that FOM was added: concerning VT1 levels, supernatant samples without FOM contained 67 ng/ml and with FOM 1067 ng/ml; for periplasmic samples the results were 125 ng/ml without FOM and 31 ng/ml with FOM. These data suggest that the increase of VT1 in culture supernatants cultured in FOM-containing CAYE broth is mostly due to release of the VT1 toxin which is accumulated in periplasmic space in higher concentrations than VT2. This increased presence of VT1 in conjunction with FOM was not so marked when one MIC of FOM was added at 5 h after starting incubation, but when three MIC of FOM was added at the same time marked increase of VT1 in supernatant of 4 h culture after addition of antibiotics was observed that suggests the ratio of the number of bacteria to the concentration of FOM is critical. FOM did not significantly affect VT2 concentrations in either culture supernatant or periplasmic space.

FOM inhibits the cell wall synthesis of bacteria which results in the elongation of bacteria or bacterial lysis with abnormal divisions. This antibiotic is widely used, at least in Japan especially with children. In fact, in the recent mass outbreak of EHEC O157:H7 infection that occurred in Japan, according to our survey in Osaka area FOM was used in about 84% of the total cases. No precise data on HUS complication among EHEC O157:H7 infected cases experienced in the recent outbreak in Japan have yet been made available, but according to information in the mass media a few percent, at least, of EHEC O157:H7 infected cases developed HUS. The role of VT1 and VT2 in developing HUS is not clear, but a case report shows that EHEC producing only VT1 can cause HUS [7]. Although we think that further studies, including *in vitro* as well as *in vivo* experiments, are needed, we would like to call attention to the

https://doi.org/10.1017/S0950268897007541 Published online by Cambridge University Press

possibility that FOM treatment for EHEC patients may worsen their clinical course and in some cases result in HUS.

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