MICROBIAL PATHOGENICITY

Development of systemic bacteraemia after oral inoculation of vancomycin-resistant enterococci in mice

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Bacteraemia caused by vancomycin-resistant enterococci (VRE) is an important clinical problem because there are only a few potent antimicrobial agents against such bacteria. Therefore, understanding the pathogenic mechanisms of VRE bacteraemia is important for prophylaxis. This study shows that treatment of mice with cyclophosphamide and a combination of metronidazole, kanamycin and vancomycin reduced normal intestinal flora and induced systemic VRE bacteraemia. Translocation of VRE and the normal intestinal flora to the mesenteric lymph nodes, liver, spleen and blood, and mortality rate were dependent on treatment with cyclophosphamide and each of the three antimicrobial drugs. Among the different strains studied, C57BL/6 mice were the most susceptible to VRE. The virulence of vancomycin-resistant *Enterococcus faecalis* was greater than that of vancomycin-resistant *Ent. faecium*. On the day after inoculation of VRE, *Escherichia coli* was also detected in many VRE-positive specimens including blood, liver and the mesenteric lymph nodes. Moreover, both VRE and *E. coli* were detected simultaneously in almost all blood samples obtained from dead and dying mice, and VRE organisms outnumbered *E. coli* in those samples by 100:1 or more. These results indicate that changes in normal intestinal flora by administration of antimicrobial drugs and severity of neutropenia induced by cyclophosphamide are important factors that contribute to the development of systemic VRE bacteraemia. *E. coli* may be intimately associated with the establishment of VRE translocation.

Introduction

Enterococci are normal inhabitants of the intestinal tract of man and animals, and 16 or more species of enterococci are currently recognised. Of these, two species, *Enterococcus faecalis* and *Ent. faecium*, are the most important causes of enterococcal infections in man. About 80–90% of enterococcal infections in man are caused by *Ent. faecalis* and 5–15% are caused by *Ent. faecium* [1, 2]. Singh *et al.* [3] indicated that the emergence of multidrug-resistant enterococci during the past decade has converted some enterococci from being difficult to treat to being impossible to treat. Enterococcal endocarditis has long been recognised as a therapeutic challenge, compared with endocarditis caused by other streptococci, and since vancomycin became the agent of ‘last resort’ for the treatment of

infections caused by multidrug-resistant, highly penicillin-resistant *Ent. faecium*, it was of great concern that the first reports of vancomycin-resistant enterococci in Europe began to appear in mid-1980s [4, 5]. Of nearly 11 000 enterococcal isolates examined in 1989 in the USA, 0.3% were resistant to vancomycin but 7.9% of isolates in 1993 were resistant, a 20-fold increase over the 4-year period [6]. Thus, vancomycin-resistant enterococci (VRE) are now the cause of serious problems in Europe and the USA [7, 8].

Two mechanisms might have played a role in the increased frequency of VRE: enterococci are part of normal gut flora, and the emergence of vancomycin resistance was associated with increased use of oral vancomycin for the treatment of documented and presumed *Clostridium difficile* infection [9–11]. Of note, resistance to vancomycin emerged in a strain highly resistant to ampicillin [12]. In a study reported by Jochimsen and co-workers [13], the main sites of infection were the bloodstream (31%), urinary tract (46%) and wounds (15%), while the main sites of

Received 27 Sept. 2000; revised version accepted 2 Feb. 2001.

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positive culture for colonised case-patients included the urinary tract (31%), wounds (9%) and stool or rectal swab (58%).

While the pathogenicity of the enterococci is solidly confirmed in cases of meningitis and endocarditis, it has been difficult to establish their role in bloodstream infections, because enterococci do not produce potent exotoxins [14].

Wells *et al.* [15, 16] described a murine model of enterococcal translocation across an intact intestinal epithelium in mice orally inoculated with *Ent. faecalis* and treated with broad-spectrum antibiotics. In their studies, 13% of infected mice died within 3 weeks but it was not clear whether death was due to overwhelming enterococcal infection or dissemination of other intestinal pathogens. The mechanisms of bacteraemia in enterococcal infections have not yet been established. Of particular interest is the fact that enterococci are part of the normal gut flora and do not possess any strong virulence factor. A study into possible mechanisms of enterococcal bacteraemia was initiated in order to understand the mechanism of entry of these problematic organisms into the bloodstream and identify factors associated with the development of enterococcal bacteraemia following oral inoculation of VRE organisms in mice.

Materials and methods

Organisms

Ent. faecalis V583 and Tx2486 were gifts from Dr B.E. Murray (University of Texas, Houston, TX, USA). The former strain has been described previously [17]. *Ent. faecalis* TMS2 and TMS5 were isolated from urine samples of Japanese patients. *Ent. faecium* TMS4 and TMS5 have been described previously [18].

Animals and treatment

CBA, C57BL/6, BALB/c, BALB/CNU, C3H, ICR, ICRNU/NU, BDF1, CDF1, DBA/2, DBA/1J, B6C3F1 and SCID strains of 5-week-old mice were a gift from Charles River Japan (Atsugi, Kanagawa, Japan). Mice were pretreated intraperitoneally with cyclophosphamide (Shionogi, Osaka, Japan) 250 mg/kg, 3 days before oral inoculation of VRE organisms. To reduce normal intestinal flora, a combination of metronidazole, kanamycin and vancomycin was administered orally once a day for 3 days, each at a dose of 100 mg/kg commencing on the same day as administration of cyclophosphamide, unless otherwise indicated. After treatment with antimicrobial agents and cyclophosphamide, mice were inoculated orally with $c. 1 \times 10^9$ VRE organisms from overnight cultures on blood agar plates. Mortality was assessed daily for 7 days after oral inoculation. Heart blood samples of deceased mice were obtained for detection of the pathogenic micro-organ-

isms. A portion of the sample (0.1 ml) was spread on a blood agar plate and incubated at 35°C for 24 h. All experiments were performed twice to confirm reproducibility. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Toho University School of Medicine.

Enumeration of viable bacteria in colon, mesenteric lymph nodes, spleen, liver and blood samples

Blood samples were obtained by heart puncture with a disposable heparinised syringe under ketamine-xylazine anaesthesia. The spleen, liver and mesenteric lymph nodes (MLN) were dissected out at the time of blood sampling but before the excision of the colon. Each organ was homogenised and diluted in pre-reduced Hanks's Balanced Salts Solution (Gibco Laboratories, Grand Island, NY, USA) supplemented with cysteine HCl (Sigma) 0.05% (HBSSs). In the next step, the colon was dissected out aseptically, immediately transferred to an anaerobic chamber (Forma Scientific, Marietta, OH, USA) and its contents were weighed, then suspended in HBSSs. The homogenate was serially diluted with the same solution and plated on to various plates: Sheep Blood Agar (Eiken, Tokyo, Japan) for total aerobes, EF Agar (Nissui, Tokyo, Japan) for enterococci, Enterococcosel Agar with vancomycin (BBL, MO, USA) for VRE, CPS-ID2 (bioMérieux, Paris, France) for *Escherichia coli*, GAM Agar (Nissui) for total anaerobes, BL Agar (Eiken) for *Bacteroides* spp. and LBS Agar (BBL) for *Lactobacillus* spp. Bacterial isolates were identified by standard methods and Vitek ID kits (bioMérieux).

Statistical analysis

Differences between groups in viable bacterial counts were examined for statistical significance by the Mann-Whitney U test.

Results

Changes in normal intestinal flora

The influence of the combinations of antimicrobial drugs on the normal intestinal flora was assessed first. For this purpose, mice ($n = 5$ /group) were pretreated with the antimicrobial drugs alone or in combination. Intraperitoneal administration of cyclophosphamide did not change the number or type of normal intestinal flora (data not shown). On the other hand, pretreatment with kanamycin reduced the colonic content of Enterobacteriaceae (mainly *E. coli*) by $1 \log_{10}$ cfu/g and *Lactobacillus* spp. by $4 \log_{10}$ cfu/g (Table 1). Administration of metronidazole reduced the number of total anaerobes and *Bacteroides* spp. by $c. 2 \log_{10}$ cfu/g, but increased the number of Enterobacteriaceae (mainly *E. coli*) to 10^8 cfu/g and that of *Enterococcus* spp. to 10^6 cfu/g. Simultaneous administration of

Table 1. Effects of drug administration on the normal intestinal flora

Bacterial flora	Mean (SD) viable counts in colon (log ₁₀ cfu/g of content) in mice given				
	Control	Kanamycin	Metronidazole	Kanamycin + metronidazole	Kanamycin + metronidazole + vancomycin
Total aerobes	5.6 (0.2)	6.5 (1.2)	8.2 (0.2)*	7.2 (0.4)*	6.1 (1.2)
Enterobacteriaceae	5.4 (0.3)	4.1 (0.3)*	8.1 (1.2)*	6.5 (0.5)*	1.8 (2.3)*
<i>E. coli</i>	5.3 (0.2)	4.0 (0.4)*	8.1 (0.1)*	ND	1.8 (2.3)*
<i>Enterococcus</i> spp.	5.1 (0.3)	5.1 (3.3)	6.0 (0.4) [†]	4.8 (0.2)	1.8 (2.3)*
Total anaerobes	9.0 (1.1)	9.6 (1.2)	8.1 (0.1) [†]	6.1 (2.5)*	5.2 (1.0)*
<i>Bacteroides</i> spp.	8.4 (1.6)	9.6 (1.2)	7.0 (0.1) [†]	6.0 (2.1)*	ND
<i>Lactobacillus</i> spp.	7.7 (0.1)	3.2 (2.2)*	7.4 (0.2)	3.4 (2.4)*	3.6 (0.8)*
<i>Clostridium</i> spp.	ND	ND	ND	ND	ND

*p < 0.01 compared with control.

[†]p < 0.05 compared with control.

kanamycin and metronidazole markedly reduced the numbers of viable *E. coli* to below the detection limit and the total anaerobes, *Bacteroides* spp. and *Lactobacilli* spp. to 1–0.01%, compared with control mice. In mice pretreated with all three antimicrobial agents, the bacterial counts of Enterobacteriaceae, *E. coli*, *Enterococcus* spp., total anaerobes, *Bacteroides* spp. and *Lactobacillus* spp. diminished significantly to <0.1%, relative to control mice.

Translocation of normal intestinal organisms by treatment with antimicrobial agents and cyclophosphamide

Bacterial counts in blood, liver, spleen and MLN tissues were below detection limits in control and cyclophosphamide-treated mice (n = 5/group, Table 2). However, in two of five mice treated with all three antimicrobial agents, both *E. coli* and enterococci or *E. coli* alone were detected in MLN, but not in other tissues. In mice treated with cyclophosphamide and all three antimicrobial agents, *E. coli* was detected in all MLN samples as well as in 20% of spleen samples (one of five), while *E. coli* alone was detected in 80% of liver samples (four of five), but no organisms were detected in any of the blood samples. The viable counts of *E. coli* detected in various tissues were 10–200 cfu/tissue.

Based on these results and for the purpose of establishing VRE bacteraemia in mice, kanamycin, metronidazole and vancomycin were administered orally together with intraperitoneal injection of cyclophosphamide.

Effects of kanamycin, metronidazole, vancomycin and cyclophosphamide on mortality rate due to oral administration of VRE

Administration of oral kanamycin, metronidazole or vancomycin alone did not influence mortality rate in infected mice (n = 10) pretreated with cyclophosphamide. On the other hand, the mortality rate of mice (n = 10) pretreated with cyclophosphamide and with two antimicrobial agents was 10% and mortality increased further to 30% when the three antimicrobial agents were used simultaneously. In repeat experiments with 30 animals under treatment with cyclophosphamide and the three antimicrobial drugs, the mortality rate of 30% was confirmed. The effect of pretreatment with cyclophosphamide on mortality rate was also examined. There were no deaths in mice (n = 10) not treated with cyclophosphamide but treated with kanamycin, metronidazole and vancomycin at doses of 100 mg/kg/day for 3 days.

Table 2. Effects of drug administration on translocation of normal intestinal flora

Groups of mice	Number positive/number tested	Identity of translocating species (cfu/tissue sample) in			
		Blood	Liver	Spleen	MLN
Normal	0/5	NG	NG	NG	NG
CY-treated	0/5	NG	NG	NG	NG
Drug-treated	2/5	NG	NG	NG	<i>E. coli</i> (80) Enterococci (60)
Drug- and CY-treated	5/5	NG	NG	NG	<i>E. coli</i> (30)
		NG	<i>E. coli</i> (10)	NG	<i>E. coli</i> (10)
		NG	<i>E. coli</i> (200)	NG	<i>E. coli</i> (10)
		NG	<i>E. coli</i> (200)	NG	<i>E. coli</i> (30)
		NG	NG	<i>E. coli</i> (160)	<i>E. coli</i> (80)
		NG	<i>E. coli</i> (10)	NG	<i>E. coli</i> (10)

CY, cyclophosphamide; NG, no growth; MLN, mesenteric lymph nodes.

Comparison of virulence between species

Oral inoculation of vancomycin-resistant *Ent. faecalis* VS83 organisms in mice ($n = 10$) pretreated with cyclophosphamide and three antimicrobial agents resulted in 30% mortality rate with the majority of deaths occurring 2 days after inoculation (range 1–4 days) and was 20% in those inoculated with other vancomycin-resistant *Ent. faecalis* Tx2386, TMS2 or TMS5. In comparison, the mortality rate was 10% in mice ($n = 10$) inoculated with vancomycin-resistant *Ent. faecium*.

Comparison of virulence between mouse strains

Differences in mortality rates in different strains of mice were assessed. For this purpose, each strain of mice ($n = 10$ each) pretreated with cyclophosphamide and three antimicrobial agents was inoculated orally with *Ent. faecalis* V583. The mortality rate in C57BL/6, DBA/2 and B6C3F1 strains was 30%, 20% and 10%, respectively, and death was observed 1–3 days after oral inoculation of the organisms. There were no deaths in other CBA, BALB/c, BALB/CNU, C3H, ICR, ICRNU/NU, BDF1, CDF1, DBA/1J and SCID strains

of mice. Based on the above results, *Ent. faecalis* V583 was used as the infecting organism and C57BL/6 strain mice as the experimental animal in the remaining experiments.

Translocation of VRE in cyclophosphamide- and drug-treated mice

On the day after oral inoculation of VRE, normal mice and mice pretreated with cyclophosphamide or all three antimicrobial drugs, or both (10 animals/group) were killed to determine the extent of VRE translocation. In the case of oral administration of VRE in normal mice, VRE were detected in 50% of MLN samples at 10–470 cfu/tissue sample (Table 3). Furthermore, VRE alone and both VRE and *E. coli* were detected in each blood sample of those VRE-positive samples. Micro-organisms were not detected in any liver and spleen samples. In the case of neutropenic mice, only 10 cfu of VRE/tissue sample were detected in two MLN samples, and VRE and staphylococci were detected in each liver sample (Table 4). On the other hand, VRE were detected in 60% of blood samples, and *E. coli* was simultaneously present in c. 70% of these samples. In contrast, in the case of mice treated with three

Table 3. Translocation of intestinal bacteria into the MLN, liver, spleen and blood of normal mice

Mouse no.	Identity of translocating species (cfu/tissue sample) in			
	Blood	Liver	Spleen	MLN
1	NG	NG	NG	NG
2	VRE (30) <i>E. coli</i> (20)	NG	NG	VRE (10)
3	NG	NG	NG	NG
4	NG	NG	NG	VRE (470)
5	NG	NG	NG	NG
6	VRE (10)	NG	NG	VRE (10)
7	NG	NG	NG	NG
8	NG	NG	NG	NG
9	NG	NG	NG	VRE (40)
10	NG	NG	NG	VRE (90)

NG, no growth; MLN, mesenteric lymph nodes.

Table 4. Translocation of intestinal bacteria into the MLN, liver, spleen and blood of neutropenic mice without administration of antimicrobial drugs

Mouse no.	Identity of translocating species (cfu/tissue sample) in			
	Blood	Liver	Spleen	MLN
1	VRE (10) <i>E. coli</i> (10)	NG	NG	NG
2	VRE (200) <i>E. coli</i> (20)	NG	NG	NG
3	NG	Staphylococci (300)	NG	NG
4	NG	NG	NG	NG
5	VRE (200) <i>E. coli</i> (70)	NG	NG	NG
6	VRE (270)	NG	NG	VRE (10)
7	VRE (50)	NG	NG	NG
8	NG	NG	NG	VRE (10)
9	VRE (80) <i>E. coli</i> (130)	VRE (30)	NG	NG
10	NG	NG	NG	NG

NG, no growth; MLN, mesenteric lymph nodes.

antimicrobial drugs, VRE alone and both VRE and *E. coli* were detected in MLN samples of each of four mice (Table 5). VRE were detected in liver and spleen samples of five and four mice, respectively, and *E. coli* was simultaneously detected in 60% of liver samples and 25% of spleen samples. VRE alone and both VRE and *E. coli* were detected in blood samples of two and one mouse, respectively. In the case of neutropenic mice treated with three antimicrobial drugs, VRE were detected in 90% of MLN samples at 10–3000 cfu/tissue sample (Table 6). Furthermore, *E. coli* or staphylococci were simultaneously detected in 50% of these samples. VRE alone, both VRE and *E. coli* or staphylococci and *E. coli* alone were detected in liver samples of three, three, one and one mouse, respectively. VRE alone, both VRE and *E. coli* or staphylococci and *E. coli* alone were detected in spleen samples of one, two, one and three mice, respectively. Both VRE and *E. coli* were detected in three blood samples, and bacterial counts of VRE and *E. coli* were 10–1000 cfu/ml and 10–80 cfu/ml, respectively. VRE alone were detected in two blood samples. In mice harbouring VRE at

1000 cfu/ml, VRE and *E. coli* were also detected in the liver, spleen and MLN.

When 24 mice were examined under the same conditions except for oral administration of the three antimicrobial agents at a higher dose (each 200 mg/kg), the mortality rate increased to 46% and death was observed 1–4 days after oral inoculation of the organisms. The viable counts of intestinal flora were assayed to determine the type of bacteria responsible for this high mortality (Table 7). At day 3 before the final dose of the agents, $3.9 \log_{10}$ cfu of VRE/g were detected, and the causative bacteria was identified as vancomycin-resistant *Ent. gallinarum*. On day 1 after the final dose of antimicrobial agents, the viable counts of all tested microbes were below the detection limits except for total aerobes (average $2.1 \log_{10}$ cfu/g). Viable counts in the intestinal flora recovered to almost basal levels at day 28 after inoculation of organisms. However, viable counts of inoculated vancomycin-resistant *Ent. faecalis* were still $8.1 \log_{10}$ cfu/g at day 28 after inoculation.

Table 5. Translocation of intestinal bacteria into the MLN, liver, spleen and blood of normal mice treated with antimicrobial drugs

Mouse no.	Identity of translocating species (cfu/tissue sample) in			
	Blood	Liver	Spleen	MLN
1	VRE (30)	VRE (310)	VRE (220)	VRE (600)
	...	<i>E. coli</i> (90)	<i>E. coli</i> (40)	<i>E. coli</i> (410)
2	<i>E. coli</i> (370)	<i>E. coli</i> (520)	NG	<i>E. coli</i> (50)
	VRE (100)	VRE (250)	...	VRE (710)
3	NG	VRE (2500)	NG	VRE (270)
	...	<i>E. coli</i> (160)	...	<i>E. coli</i> (10)
4	NG	VRE (30)	NG	NG
5	NG	NG	VRE (10)	NG
6	NG	NG	NG	VRE (10)
7	NG	NG	NG	VRE (20)
8	NG	VRE (40)	VRE (150)	VRE (200)
	...	...	...	<i>E. coli</i> (420)
9	VRE (10)	NG	NG	VRE (80)
10	NG	NG	VRE (20)	VRE (20)

N, no growth; MLN, mesenteric lymph nodes.

Table 6. Translocation of intestinal bacteria into the MLN, liver, spleen and blood of neutropenic mice treated with antimicrobial drugs

Mouse no.	Identity of translocating species (cfu/tissue sample) in			
	Blood	Liver	Spleen	MLN
1	NG	NG	NG	VRE (20)
2	NG	NG	NG	NG
3	VRE (10)	VRE (20)	VRE (200)	VRE (100)
	<i>E. coli</i> (10)	<i>E. coli</i> (10)	<i>E. coli</i> (100)	<i>E. coli</i> (30)
4	NG	<i>E. coli</i> (180)	<i>E. coli</i> (20)	<i>E. coli</i> (30)
5	VRE (400)	VRE (70)	VRE (30)	VRE (3000)
	...	Staphylococci (300)	Staphylococci (150)	Staphylococci (3000)
6	VRE (500)	VRE (10)	...	VRE (3000)
	<i>E. coli</i> (80)	...	<i>E. coli</i> (20)	<i>E. coli</i> (2000)
7	VRE (1000)	VRE (200)	VRE (250)	VRE (70)
	...	<i>E. coli</i> (100)	<i>E. coli</i> (100)	<i>E. coli</i> (250)
8	NG	VRE (50)	NG	VRE (10)
9	VRE (10)	VRE (5000)	...	VRE (80)
	<i>E. coli</i> (20)	<i>E. coli</i> (500)	<i>E. coli</i> (20)	<i>E. coli</i> (10)
10	VRE (50)	VRE (130)	VRE (20)	VRE (20)

NG, no growth; MLN, mesenteric lymph nodes.

Table 7. Characterisation of colonic bacteria after administration of metronidazole, kanamycin and vancomycin, each at 200 mg/kg

Bacterial flora	Mean (SEM) viable counts in colon (log ₁₀ cfu/g of content) on [†]				
	-3	1	3	14	28
Total aerobes	5.9 (0.5)	2.1 (1.1)*	7.0 (1.0)*	9.4 (0.3)*	7.1 (1.2)*
Enterobacteriaceae	5.1 (0.4)	ND	4.7 (3.2)	7.0 (1.2)*	5.8 (1.3)
<i>E. coli</i>	4.7 (0.7)	ND	4.7 (3.2)	7.0 (1.2)*	5.8 (1.3)
<i>Enterococcus</i> spp.	4.9 (0.5)	ND	6.8 (0.9)*	9.3 (0.3)*	8.1 (2.1)*
VRE	3.9 (1.5)	ND	6.8 (0.9)*	9.2 (0.5)*	8.1 (2.1)*
Total anaerobes	8.1 (0.6)	ND	4.3 (2.9)*	2.4 (2.6)*	7.2 (1.0)
<i>Bacteroides</i> spp.	8.1 (0.7)	ND	4.3 (2.9)*	2.4 (2.6)*	6.9 (1.6)
<i>Lactobacillus</i> spp.	6.6 (0.7)	ND	ND	ND	5.6 (0.8)
<i>Clostridium</i> spp.	ND	ND	ND	ND	ND

*Days numbered in relation to time of final administration of antimicrobial drugs.

[†]p < 0.01 compared with data at -3 days.

In the present study, inoculated VRE were detected in all blood samples obtained from dead and dying mice. Simultaneously, *E. coli* was detected in almost all these mice, and the number of *E. coli* organisms was ≤10% of the number of VRE.

Discussion

Intestinal colonisation and overgrowth of orally inoculated microbes cannot occur unless the stability of the normal flora is upset by prior treatment with antimicrobial agents, use of purgatives, fasting or other factors. The normal intestinal flora resists colonisation by extraneous organisms. Previous studies have suggested that broad-spectrum antimicrobial agents such as the third-generation cephalosporins increase the risk of acquiring enterococcal-associated systemic infection by creating a selective milieu for enterococcal superinfection [13, 14, 19–21]. Moreover, the use of third-generation cephalosporins has been used as a marker for enterococcal superinfection that can be fatal in some cases [14, 22]. Furthermore, when the suitability of antimicrobial agents for prophylactic administration was investigated, the combination of kanamycin and metronidazole induced a good outcome except for the overgrowth of enterococci [23]. For this reason, these agents were selected to disturb the normal intestinal flora of mice in the present study.

Studies in gnotobiotic mice colonised only with anaerobic bacteria indirectly confirmed that anaerobic bacteria acted to prevent intestinal colonisation of orally administered bacterial pathogens [24]. Hentges *et al.* [25] indirectly indicated that other groups of intestinal bacteria might also function to prevent intestinal colonisation by potential pathogens. In the present study, a decrease of one log₁₀ of anaerobic or aerobic bacteria by administration of metronidazole or kanamycin was not lethal in mice orally inoculated with VRE. Moreover, none of the mice died 6 days after oral inoculation of VRE, although at 14 days after final administration of the three agents, each at a dose of 200 mg/kg, counts of viable total anaerobes and *Bacteroides* spp. were

markedly less than in control mice with abundant growth of VRE. Stroud *et al.* [14] reported that the use of metronidazole, a therapeutic agent found to be a risk factor for increased morbidity and mortality, served as a potential marker for gastrointestinal tract inflammatory process; there was a positive correlation between metronidazole use and the development of *C. difficile* colitis in only 3 of 17 patients exposed to metronidazole. However, the remaining 14 patients in their study were diagnosed with some form of gastrointestinal tract inflammation (e.g., peritonitis, colitis, abdominal abscess, or cholecystitis) before the onset of enterococcal primary bloodstream infection [14]. These results indicate that other factor(s) (e.g., gastrointestinal tract inflammation) are probably involved in the translocation of intestinal microbes – with the exception of *Bacteroides* spp. Although intestinal anaerobic bacteria outnumber aerobic bacteria in the intestine by 100:1 or even 1000:1, anaerobes rarely translocate to extra-intestinal regions. For example, Hill [26] reported that of 74 indigenous strains of rabbit caecal anaerobes, none significantly adhered *in vitro* to the caecal epithelium. One hypothesis argues that anaerobes preferentially colonise the intestinal mucus layer and cannot attach to the epithelium. There is evidence that elimination of the mucus layer results not only in a noticeable increase in the populations of bacteria directly adherent to the enteric surface but also in the dissemination of normal intestinal bacteria into extra-intestinal tissues such as the liver and spleen [27]. The present study showed translocation of *E. coli* following changes in the normal intestinal flora induced by the administration of three drugs. This finding indirectly suggested that administration of three drugs might eliminate the mucus layer. Of particular interest was the fact that the appearance of VRE in the blood was intimately associated with translocation of the organism from the intestine to mesenteric lymph nodes. Colonisation of VRE in the intestine is a primary factor inducing systemic VRE bacteraemia.

The present data demonstrated that bacterial counts in the liver, spleen and MLN were higher and the ratio of organisms translocated into these tissues was higher

than in mice not treated with cyclophosphamide. This finding strongly indicates that one of the risk factors enhancing systemic bacteraemia is neutropenia [14, 20, 21]. Furthermore, preliminary experiments showed that intraperitoneal administration of cyclophosphamide at a dose of 250 mg/kg resulted in severe leucopenia (~900 cells/ml) on day 4 after administration, although the number of leucocytes rapidly increased and recovered to ~6000 cells/ml at day 7 after cyclophosphamide administration (data not shown). Following these changes, VRE organisms were administered orally on day 3 after cyclophosphamide treatment to enhance their translocation and migration to the bloodstream and subsequent growth in various tissues evading phagocytosis and killing.

Cytolytic (haemolytic) and protease (or gelatinase) activities, aggregation substance, lipoteichoic acid and hyaluronidase are known virulence factors for *Ent. faecalis* [27]. However, few reports have demonstrated similar virulence factors for *Ent. faecium*. Thus, the frequency of *Ent. faecalis* infection is higher than that caused by *Ent. faecium* among vancomycin-susceptible enterococcal bacteraemia [19]. In the present study, similar results were obtained with respect to pathogenicity between vancomycin-resistant *Ent. faecalis* and *Ent. faecium*. Of course, the frequency of bacteraemia with vancomycin-resistant *Ent. faecium* is higher than with vancomycin-resistant *Ent. faecalis* because the frequency of acquiring vancomycin resistance in the former is higher than in the latter [28, 29]. When mice were killed the day after oral inoculation of VRE, many VRE-positive specimens contained *E. coli*, but VRE organisms outnumbered *E. coli* in those samples by $\geq 100:1$. Only VRE are detected in blood samples of patients with bacteraemia and as these patients often receive antibacterial agents before admission [20–22], this clinical phenomenon might be explained by the presence of VRE and other co-existing organisms (mostly *E. coli*) that are susceptible to antibiotics given to these patients. Lipopolysaccharide is capable of inducing cytokines and chemokines that are known as key mediators and orchestrate the host immuno-inflammatory response to bacterial infection [30]. Taken together, these data show that *E. coli* may be closely associated with the induction of VRE bacteraemia. The role of *E. coli* in VRE bacteraemia is presently under investigation.

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