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Receptor-mediated endocytosis of biofilm-forming *Enterococcus faecalis* by rat peritoneal macrophages

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***Background & objectives*: Enterococci are important nosocomial pathogens that are increasingly difficult to treat due to intrinsic and acquired resistance to antibiotics. Studies were taken up to identify virulence factors and to characterise pathogenic mechanisms of such infections to evaluate potential targets for treatments alternative to antibotic therapy. This study was carried out to evaluate the contribution of extracellular polysaccharide expressed by *Enterococcus faecalis* to resistance to phagocytosis and survival within rat peritoneal macrophages.**

***Methods*: Six *E. faecalis* clinical isolates were tested for their ability to survive within rat peritoneal macrophages. Cytochalasin D, colchicine and monodansylcadaverine were used to investigate the route of enterococcal entry inside macrophages.**

***Results*: Four of the isolates were able to produce extracellular polysaccharide and form biofilm after growth in glucose-supplemented medium, while no production could be detected in glucose deficient medium. Two isolates were polysaccharide-negative in both conditions. Isolates expressing extracellular polysaccharide were able to survive for more than 24 h compared to polysaccharide-negative bacterial cells of the same strain grown in glucose-deficient medium, which were readily cleared. Cytochalasin D virtually abolished the number of viable intracellular bacteria, after growth in either trypticase soy broth (TSB) or TSB supplemented with glucose; colchicine and monodansylcadaverine strongly affected survival of polysaccharide-positive bacteria, significantly more than that of polysaccharide-negative ones.**

***Interpretation & conclusion*: Biofilm-forming *E. faecalis* survived within rat peritoneal macrophages significantly better than polysaccharide-negative isolates. Perturbators of cytoskeleton and of surface receptors turnover, indicated receptors-mediated endocytosis as the most likely route for enterococcal entry into macrophages.**

Key words Biofilm - endocytosis - enterococci

Enterococci are important opportunistic pathogens, representing one of the leading causes of endocarditis and nosocomial bacteremia in the United States and northern Europe[1-3]. Almost all such infections are caused by the two species *Enterococcus faecalis* and *Enterococcus faecium*, responsible for 80 and 20 per cent of infections, respectively.

Virulence mechanism of this microorganisms is not well understood although some putative virulence factors have been described. Besides surface proteins and polysaccharide possibly involved in virulence, the ability of *E. faecalis* to survive inside polymorphonuclear leucocytes (PMN) and macrophages has been examined[4-6]. Translocation through the intestinal barrier

has long been indicated as one of the preferential portal of entry of enterococci for spreading to distant sites[7]; the ability of *E.faecalis* to survive within macrophages may contribute to pathogenesis by allowing translocation of bacteria through the intestinal mucosa and transport to different body parts. Further, resistance to killing by the PMN may represent a way to escape host defenses.

Aggregation substance (AS) has been indicated to be responsible for internalisation within PMN through a preferential route[4], allowing resistance to killing. We suggested that the ability to synthesise extracellular polysaccharides (slime) might mediate such alternate phagocytosis leading to enterococcal survival within peritoneal macrophages[6].

This study was done to examine the ability of slime-forming and non-slime forming *E. faecalis* isolates to survive within rat peritoneal macrophages, and to characterise the route of entry by using perturbators of cytoskeleton and inhibitors of surface-receptor turnover.

Material & Methods

Bacterial strains and media: Six *E. faecalis* isolates of different origin (endocarditis 2, catheter 2, biliary stent 1, environment 1) were examined in this study. The isolates were grown in plain trypticase soy broth (TSB, OXOID, Basingstoke, UK) or in TSB supplemented with 1 per cent glucose (TSBG).

Biofilm formation: To test for biofilm formation a quantitative adherence assay[6] was used. Briefly, 1:100 dilution of overnight cultures in TSB was used to inoculate wells in a microtiter polystyrene plate containing different media. After growth for 18 h at 37°C, plates were gently washed three times with phosphate buffered saline (PBS) *p*H 7.4, the adherent bacterial film was fixed by air drying at 60°C for 1h and then stained with Hucker's crystal violet; excess stain was washed off with tap water. The optical density of the biofilm was measured at 570 nm in an automatic spectrophotometer (Novapath™ Microplate Reader, BioRad Laboratories Inc., CA, USA).

Murine macrophages: Peritoneal macrophages were harvested from adult female rats as described[6]. Briefly, a peritoneal lavage was performed with 2 applications of 5 ml of Hank's balanced salt solution (HBSS). Recovered cell viability was determined by trypan blue dye exclusion. Cell suspensions, normalised to $2.5X10^5$ cells/ml in RPMI 1640 supplemented with 1 per cent foetal calf serum (FCS), were dispensed into 24 well plates and incubated for 30 min at 37°C under 5 per cent CO_2. Unattached cells were discarded by washing and macrophages further incubated for 24 h at 37°C with RPMI 1640, 1 per cent FCS, before infection. In experiments with inhibitors, all compounds were added to macrophages 30 min before infection and were present in the cell/bacteria suspensions throughout the experiments.

Electron microscopy: Infected macrophages were fixed with 2.5 per cent glutaraldehyde in 0.1 *M* sodium cacodylate buffer (*p*H 7.4) for 1 h and postfixed with 1 per cent OsO_4 in 0.1 *M* sodium cacodylate buffer for 1 h at room temperature[8]. Fixed specimens were washed, dehydrated through a graded series of ethanol solutions (30 to 100% ethanol) and embedded in Agar 100 (Agar Scientific Ltd., UK). Ultrathin sections obtained by a MT-2B Ultramicrotome (LKB Pharmacia, Sweden) were stained with uranyl acetate and lead citrate and examined by an EM 208 Philips electron microscope (The Netherlands).

Chemicals: Cytochalasin D and colchicine, diluted in phosphate buffered saline (*p*H 7.4), and monodansylcadaverine (MDC), dissolved in dimethyl sulphoxide (DMSO), were prepared as stock solutions and added to the cell culture medium at 1 and 5, 5 and 100 µg/ml, respectively.

All inhibitors were tested for possible effects on cell and bacterial viability by trypan blue exclusion test and cfu counting, respectively, of treated and untreated samples.

Results

Biofilm formation: Ability to form biofilm was tested by the quantitative plate test after growth in plain TSB or in TSBG medium. Four strains were slime producers when grown in TSB supplemented with 1per cent glucose (TSBG), but slime-negative in absence of additional glucose; isolates EFS 38 and EFS 90 were biofilm negative in all conditions (Table I).

Table I. Source and virulence factors pattern of the six *E. faecalis* isolates used in this study

Isolates	Source	pAD1	pAM373	*Cyl*A	*esp*	*gel*E	Slime	
							TSB	TSBG
EFS 9	Endocarditis	+	-	-	+	-	NP	FP
EFS 38	Biliary stent	-	-	-	+	-	NP	NP
EFS 44	Catheter	-	+	-	-	-	NP	FP
EFS 57	Environment	-	-	-	+	-	NP	FP
EFS 85	Catheter	+	-	+	-	+	NP	FP
EFS 90	Endocarditis	-	-	-	-	+	NP	NP

pAD1, pAM 373-aggregation factor; *CylA*, cytolisin; *esp*, enterococcal surface protein; *gel*E, gelatinase; TSB, tripticase soy broth; TSBG, TSB supplemented with 1% glucose; NP, non-biofilm producing; FP, biofilm producing

Survival of E. faecalis inside rat peritoneal macrophages: The isolates examined belonged to our laboratory collection, which had been characterised by PCR for the presence of genes coding putative virulence factors including the aggregation factor (AS) pAD1 and pAM373, cytolisin (*cyl*A), gelatinase (*gel*E) and enterococcal surface protein (*esp*) (Table I). Survival ability, expressed as percentage of the inoculum recovered from infected macrophages at 24 h, varied between isolates. In all cases, however, survival was enhanced for glucose-grown bacteria compared to those grown in glucose-free medium (Table II). We could not find correlations with any of the genetic traits considered.

Well preserved bacterial cells were observed inside macrophage vacuoles at 24 h after infection, either in single-cell or in multiple cells-containing vacuoles (Fig.1).

Route of enterococcal entry into macrophages: Different inhibitors of cellular structures were used to examine the mechanism of enterococcal entry into the cells. Isolate EFS 85, which showed the highest survival levels, was used; EFS 85 was grown in TSB and/or TSBG to determine the effect of the extracellular polysaccharides on phagocytosis. Cytochalasin D, at concentration of 5 µg/ml, virtually abolished the number of viable intracellular bacteria, after growth in either TSB or TSBG (97.5 and 99%, respectively, Fig.2). Colchicine reduced TSBG grown-EFS 85 entry into macrophages by 95 per cent, while the effect on polysaccharide-lacking cells was weaker (27%). MDC also dramatically redued the number of viable intracellular polysaccharide-positive

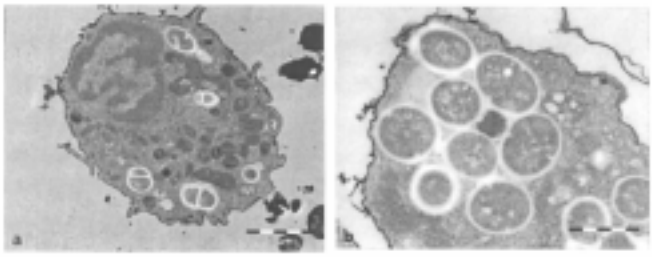

Fig.1. Well preserved enterococcal cells visible inside macrophages after 24 h infection, either in single-cell tight vacuoles (**a**) or in larger vacuoles containing numerous bacteria (**b**).

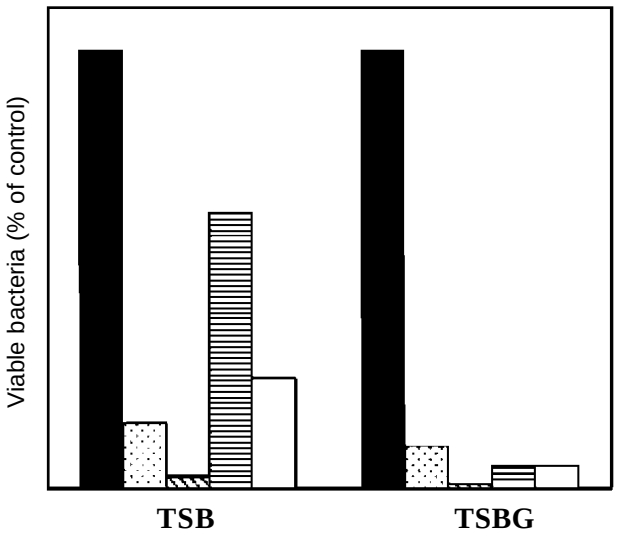

Fig.2. Effect of surface receptors turnover and cytoskeleton pretubators on EFS 85 survival after growth in TSB (polysaccharide-negative cells) and TSBG (polysaccharide-expressing cells). Cytocalasin D at 1 µg/ml ▨; cytocalasin D at 5 µg/ml ▧; colchicine 5 µg/ml ▤; monodansylcadaverine (MDC) 100 µg/ml □; control bar ■. Control is normalised to 100% and the effect of chemicals indicates the percentage of inhibition. Results shown are the mean of at least three experiments performed in duplicate.

Table II. Survival of different *E. faecalis* isolates inside rat peritoneal macrophages

Isolate	Medium	T_0	T_3	T_{24}
		(% of the inoculum)		
EFS 9	TSB	1.2	0.002	-
	TSBG	16.2	0.0125	0.002
EFS 38	TSB	0.33	0.009	-
	TSBG	0.40	0.014	0.012
EFS 44	TSB	0.53	0.006	-
	TSBG	0.60	0.09	0.02
EFS 57	TSB	1.3	0.006	-
	TSBG	12.2	0.022	0.004
EFS 85	TSB	5.0	0.011	0.003
	TSBG	18.5	0.65	0.076
EFS 90	TSB	0.13	0.0056	-
	TSBG	0.2	0.006	-

TSB, tripticase soy broth; TSBG, TSB with 1% glucose

E. faecalis by 95 per cent (Fig.2). Again, TSB grown cells were affected at a lower extent (75%).

Discussion

Enterococci are opportunistic pathogens which can cause severe infections in different body systems, particularly among immunocompromised patients. Translocation through the intestinal barrier has been indicated as one of the preferred portals of entry of enterococci for spreading to distant sites; Gentry-Weeks *et al*[5] showed that *E. faecalis* was able to survive within peritoneal macrophages, which might contribute to pathogenesis by allowing translocation of bacteria through the intestinal mucosa and hindering antimicrobial therapy. We reported[6] on the ability of *E. faecalis* to survive within rat peritoneal macrophages up to 48 h, and suggested extracellular polysaccharide could be involved in extended survival. Rakita and colleagues[4] showed that strains bearing AS stimulated their own phagocytosis by PMNs into larger phagosomes allowing survival, compared to AS-negative cells. The present findings confirmed that the survival of *E. faecalis* seemed to be related to the ability of bacteria to synthesise extracellular polysaccharide induced by the presence of an additional carbohydrate source in the medium.

To determine the route of entry of enterococci, inhibitors of the surface-receptors turnover and cytoskeleton perturbators were used. As known, polymerized filamentous actin contributes to the process of phagocytosis, while microtubules take part in the transportation of endosomes and lysosomes within the cells[9,10]. MDC is a frequently used inhibitor of receptor-mediated endocytosis, which acts through the increase of lysosomal *p*H and the decrease of receptor turnover rate[9]. As expected, cytochalasin D, which inhibits the polymerisation of filamentous actin[11], dramatically affected the entry of enterococci within the macrophages. Interestingly, colchicine, which blocks the elongation of microtubules[11], and MDC[9], which slows down the surface receptors turnover, strongly inhibited entry/ survival of polysaccharide-positive bacterial cells. The effect on cells of the same strain not expressing the extracellular polysaccharide was much weaker. These data suggested that a receptor-mediated endocytosis might be involved in macrophage entry of polysaccharide-positive enterococcal cells leading to bacterial survival; interaction of specific macrophage receptors with polysaccharide moieties might mediate phagocytosis without inducing bactericidal functions.

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