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Short communication

Opsonophagocytic assay as a potentially useful tool for assessing safety of enterococcal preparations

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Abstract

Enterococci are commonly used in foods and food additives. A number of putative virulence factors are usually evaluated to assure that the strains used are not harmful. We propose an additional test to assess the safety of these bacteria by testing the susceptibility to opsonophagocytic killing. One probiotic *Enterococcus faecalis* strain was compared to a collection of 27 clinical isolates and our results indicate that 89% of the clinical strains were less susceptible to killing mediated by normal rabbit sera. Opsonophagocytic killing is the best in vitro surrogate for a protective immune response against bacteria, and the susceptibility of bacteria against normal rabbit sera indicates that these strains may not be able to survive in the bloodstream of the host. Further studies comparing a larger collection of pathogenic strains with commensal isolates are necessary to confirm these findings.

Keywords: *Enterococcus*; Opsonophagocytosis; Safety; Probiotics

1. Introduction

Enterococci are Gram-positive bacteria found in a number of foods. These bacteria are also used in pre- or probiotic preparations (Aarestrup et al., 2002). Enterococci are common pathogens in hospitalised patients, especially those with immune deficiencies such as patients with cancer, recipients of solid organ transplants, and neonates. Although the intrinsic virulence of enterococci is generally low, they can cause serious and life-threatening infections in immunocompromised patients (Murray, 2000).

Little is known about the virulence factors of enterococci (Mundy et al., 2000). A cytolysin/bacteriocin in enterococci has been described and shown to increase virulence in a rabbit corneal infection model (Jett et al., 1994). A surface protein associated with clumping of enterococci, called aggregation substance, has been associated with increased virulence in a rat model of enterococcal endocarditis (Schlievert et al., 1998). While both factors show virulence traits in appropriate infection models, the clinical role of these factors in causing or sustaining human infection is not clear (Mundy et al., 2000). Numerous studies could not find a correlation between clinical strains and the presence of these two putative virulence factors (Jett et al., 1994; Mundy et al., 2000). Clinical isolates that do not possess cytolysin or aggregation substance have been shown to be capable of causing

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serious and life-threatening infections; on the other hand, the presence of these factors is not necessarily predictive of a strain's pathogenicity, since commensal bacteria that possess these factors have been isolated from both patients and healthy individuals.

The use of potentially pathogenic bacteria in food or probiotic preparations is problematic, and various tests have been proposed for assessing the potential risk associated with the use of bacterial strains for these purposes (Franz et al., 2001). It is obvious that strains used as food additives or probiotics should not possess antibiotic-resistance traits that could be transferred to bacteria in the physiologic flora. Another potential risk is the replacement of commensal strains with more pathogenic bacteria from exogenous sources.

Opsonophagocytic killing is the best in vitro surrogate for a protective immune response against bacterial pathogens (Pier et al., 1994a,b). Not all opsonic antibodies are protective, but all antibodies that provide antibacterial immunity are opsonic. For Gram-positive organisms, there is no direct bactericidal activity from antibodies in concert with complement because of the absence of an outer membrane, and opsonic killing requires the interplay of antibodies, complement, and phagocytes (Salyers and Whitt, 2002).

We believe that using an opsonophagocytic assay to test the susceptibility of enterococcal isolates used in the food industry or as probiotic preparations might offer an additional, clinically relevant way to assess the safety of these potential pathogens.

2. Materials and methods

Bacterial strains tested in opsonophagocytic assays were 21 clinical isolates from Japan and England (Maekawa et al., 1992) as well as strains from the United States commonly used for research purposes, such as OG1RF (Xu et al., 1998), V583 (Sahm et al., 1989), and FA2-2 (Chow et al., 1993). The probiotic *Enterococcus faecalis* strain S1 tested was kindly provided by SymbioPharm (Herborn, Germany).

The opsonophagocytic assay was performed as described (Huebner et al., 1999). In brief, PMNs were freshly prepared from human blood collected from healthy adult volunteers; 20–30 ml was mixed with an equal volume of dextran/heparin-buffer and incubated at 37 °C for 1 h. The upper layer containing the leukocytes was collected, and hypotonic lysis of the remaining erythrocytes was accomplished by resuspending them in 1% NH_4Cl and by subsequent washing steps with RPMI (Gibco, Grand Island, NY) plus 15% fetal bovine serum. Trypan-blue exclusion was used to determine the number of viable cells, and the final PMN count was adjusted to 2×10^7 neutrophils/ml. Serum used as the complement source was obtained from normal healthy volunteers, and adsorbed with homologous *E. faecalis* at 4 °C for 1 h before use.

For the opsonophagocytic assay, 100 μl of PMNs, 100 μl of bacteria (2×10^7/ml, confirmed by viability counts), 100 μl of pooled normal rabbit serum (1:500 dilution) (Huebner et al., 1999), and 100 μl of human serum at a dilution of 1:20 as complement source were mixed. The mixture was incubated on a rotor rack at 37 °C for 90 min, and samples were plated at time 0 and after 90 min. Percent killing was calculated by comparing the colony counts in the inoculum (i.e., viable counts at time $0 - T_0$) to the colony counts after a 90-min incubation at 37 °C (T_{90}), using the following formula: (mean cfu at T_0) − (mean cfu at T_{90})/(mean cfu at T_0)*100. Values less than zero indicated growth during the incubation period and are subsequently presented as 0% killing.

3. Results and discussion

Fig. 1 shows the opsonophagocytic killing of 27 clinical enterococcal isolates. The range of killing

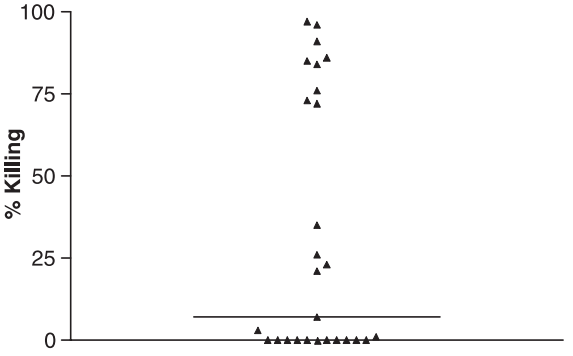

Fig. 1. Opsonophagocytic killing of 27 clinical *E. faecalis* isolates.

values observed was between 0% and 97%. The median value was 7% for these clinical strains. One third of the collection (i.e., 9/27) showed killing values of 72% and higher, while more than half of the strains (14/28) had killing values of less than 8%. In the probiotic strain tested, 90% was killed by pooled normal rabbit serum indicating that this strain was highly susceptible to opsonophagocytic killing as compared to the collection of clinical isolates. Only 11% (3/28) of the strains tested were more susceptible than the probiotic strain to opsonophagocytic killing.

Enterococci are major pathogens in many hospitals, especially in intensive care units and in immunocompromised patients (Murray, 2000). The use of enterococci as food additives and as probiotic preparations raises concerns about the possibility of colonizing susceptible hosts with potentially dangerous pathogens.

We recently identified a capsular polysaccharide in *E. faecalis* and *Enterococcus faecium* (Huebner et al., 1999) that was able to induce protective antibodies against enterococcal infections in a mouse sepsis model (Huebner et al., 2000). The antigen was identified in an opsonophagocytic assay as an in vitro surrogate for a protective immune response (Huebner et al., 1999). Structural analysis showed the antigen to be a teichoic acid (Wang et al., 1999).

The protective immune response against bacterial infections involves three major components: (a) phagocytes such as granulocytes, (b) complement components, and (c) antibodies (see Fig. 2). Complement alone (i.e., complement component C3b), and complement in concert with antibodies (i.e., complement component C1), are responsible for killing by the alternative or classical pathway, respectively (Salyers and Whitt, 2002). However, these mechanisms might not be relevant for most Gram-positive organisms because of the presence of a protective capsule. Opsonisation of bacteria by complement (i.e., C3b) or by specific antibodies is an important mechanism

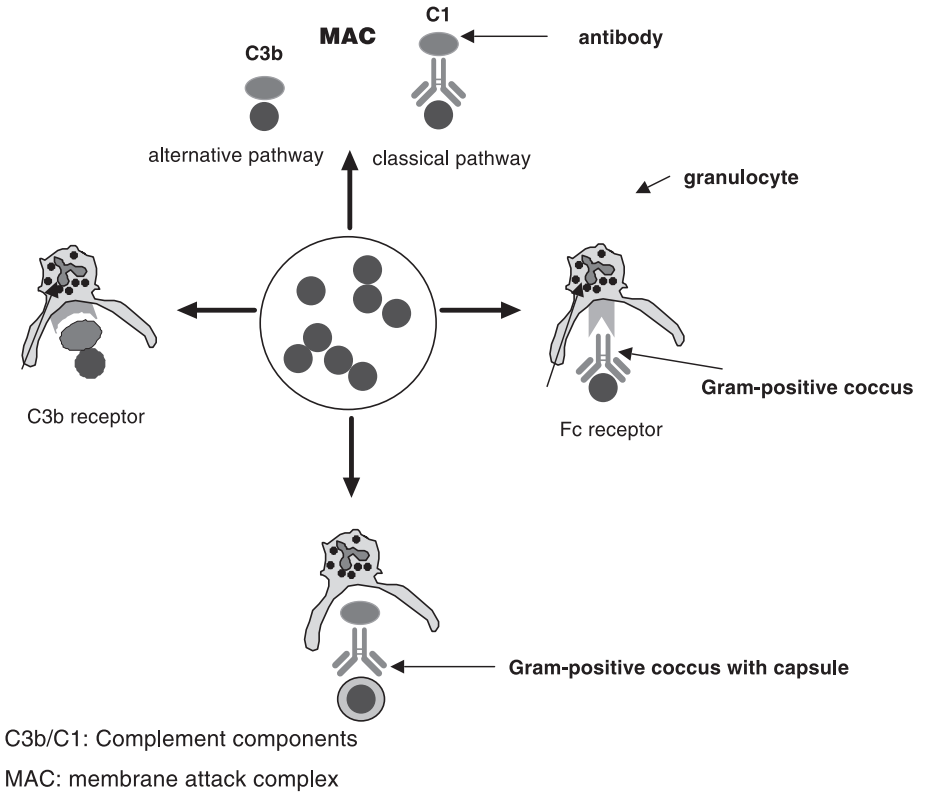

Fig. 2. Protective immune response against bacterial infections.

allowing phagocytosis of the bacteria by professional phagocytes such as granulocytes. However, for encapsulated organisms, all factors- i.e., complement, phagocytes, and specific antibodies directed at the capsular antigen- might be necessary for killing.

In the experiments presented, we showed that pooled normal rabbit serum contributed to opsonic killing, raising the question of how specific antibodies against enterococcal polysaccharides develop in normal rabbits and in humans. We and others could demonstrate that low titers of specific antibodies to enterococci are present in the sera of healthy humans and of normal rabbits (Arduino et al., 1994; Huebner et al., 1999). These antibodies are probably induced by constant translocation of low numbers of enterococci in the gastrointestinal tract of healthy individuals (Wells et al., 1990, 1991). Enterococci are present in high numbers (up to 10^7 cfu/g faeces) in the normal flora of the gut of the majority of humans, and only the intestinal epithelial layer prevents systemic spread of the bacteria in the host. Wells et al. (1991) have shown that specific factors such as systemic treatment with antibiotics or mucosal damage due to cytotoxic drugs may promote bacterial translocation. They have also speculated that bacteria can readily translocate from the intact intestinal lumen into mesenteric lymph nodes (Wells et al., 1990, 1991). This exposure to enterococcal antigens might result in the low levels of antibodies against enterococci observed in several studies.

One possibility for the susceptibility of specific strains, such as the probiotic strain used in the present study, may be due to the absence of a polysaccharide capsule. However, this cannot be the case because we were able to visualise a capsule in the probiotic strain using immune electron microscopy together with rabbit sera raised against purified capsular polysaccharide of *E. faecalis* 12030 (data not shown). It is not known what proportion of enterococcal strains have a capsule, but preliminary results indicate that at least 60% of a collection of 159 clinical isolates possesses one of four different polysaccharides identified so far (unpublished results).

The data presented indicate that enterococcal strains differ in their susceptibility to opsonic killing. The resistance to opsonic killing may be a factor allowing specific strains to persist in the bloodstream. However, the clinical importance of this phenomenon needs to be assessed in a comparison of pathogenic versus commensal isolates. Our data support a possible role of the opsonophagocytic assay in assessing the safety of enterococcal preparations as food additives or probiotics.

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