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Both leukotoxin and poly-*N*-acetylglucosamine surface polysaccharide protect *Aggregatibacter actinomycetemcomitans* cells from macrophage killing

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ABSTRACT

Two virulence factors produced by the periodontopathogen *Aggregatibacter actinomycetemcomitans* are leukotoxin, a secreted lipoprotein that kills human polymorphonuclear leukocytes and macrophages, and poly-*N*-acetylglucosamine (PGA), a surface polysaccharide that mediates intercellular adhesion, biofilm formation and detergent resistance. In this study we examined the roles of leukotoxin and PGA in protecting *A. actinomycetemcomitans* cells from killing by the human macrophage cell line THP-1. Monolayers of THP-1 cells were infected with single-cell suspensions of a wild-type *A. actinomycetemcomitans* strain, or of isogenic leukotoxin or PGA mutant strains. After 48 h, viable bacteria were enumerated by dilution plating, macrophage morphology was evaluated microscopically, and macrophage viability was measured by a Trypan blue dye exclusion assay. The number of *A. actinomycetemcomitans* CFUs increased approximately twofold in wells infected with the wild-type strain, but decreased by approximately 70–90% in wells infected with the leukotoxin and PGA mutant strains. Infection with the wild-type or leukotoxin mutant strain caused a significant decrease in THP-1 cell viability, whereas infection with the PGA mutant strain did not result in any detectable changes in THP-1 viability. Pre-treatment of wild-type *A. actinomycetemcomitans* cells with the PGA-hydrolyzing enzyme dispersin B rendered them sensitive to killing by THP-1 cells. We concluded that both leukotoxin and PGA are necessary for evasion of macrophage killing by *A. actinomycetemcomitans*.

1. Introduction

The Gram-negative bacterium *Aggregatibacter actinomycetemcomitans* (formerly *Actinobacillus actinomycetemcomitans*) has been implicated as a causative agent of several forms of severe periodontitis in humans [1]. In vitro, *A. actinomycetemcomitans* produces several factors that may contribute to its ability to colonize the oral cavity and cause disease. These include surface-exposed autotransporter proteins that bind to human buccal epithelial cells and type V collagen [2–4]; adhesive type IV pili and exopolysaccharides that mediate surface attachment, autoaggregation and biofilm formation [5,6]; and several exotoxins, including leukotoxin and cytolethal distending toxin, that kill host cells [7].

One of the best studied *A. actinomycetemcomitans* virulence factors is leukotoxin, a 114-kDa secreted lipoprotein that belongs to the RTX family of pore-forming bacterial toxins [8]. *A. actinomycetemcomitans* leukotoxin has been shown to kill polymorphonuclear leukocytes (PMNs) and macrophages isolated specifically from humans and Old World primates [9]. Human subjects harboring highly leukotoxic strains of *A. actinomycetemcomitans* are more likely to develop periodontitis than are subjects harboring minimally leukotoxic strains [10]. These findings suggest that leukotoxin may play a role in host cell killing and immune evasion in vivo. It has recently been shown that leukotoxin is also capable of lysing human erythrocytes in vitro [11]. Although numerous studies have confirmed that leukotoxin-producing strains of *A. actinomycetemcomitans* can kill host cells, only one study demonstrated that the leukotoxicity of *A. actinomycetemcomitans* strains correlates with their ability to avoid phagocytic killing [12]. In this study, a highly leukotoxic strain of *A. actinomycetemcomitans* (strain HK1519) was more resistant to killing by human PMNs than was a minimally leukotoxic strain

Abbreviations: PGA, poly-β-1,6-*N*-acetyl-D-glucosamine; PBS, phosphate buffered saline; TSA, Tryptic soy agar; TSB, Tryptic soy broth.

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(strain Y4). However, both *A. actinomycetemcomitans* strains used in this study were "smooth-colony" variants, which arise spontaneously upon repeated subculture of *A. actinomycetemcomitans* clinical isolates in broth [13]. Smooth-colony variants are usually deficient in intercellular aggregation and biofilm formation, phenotypes that are universally associated with fresh clinical isolates. The reason for using smooth-colony variants of *A. actinomycetemcomitans* in studies on phagocytic killing is that even small aggregates of cells formed by autoaggregating clinical strains are too large to be efficiently phagocytosed [14]. Therefore, a role for leukotoxin in immune evasion by biofilm-forming, clinical strains of *A. actinomycetemcomitans* has not yet been demonstrated.

A. actinomycetemcomitans also produces poly-*N*-acetylglucosamine (PGA), a surface polysaccharide that mediates intercellular adhesion, biofilm formation, and detergent resistance [6,15,16]. PGA is produced by several other pathogens including *Actinobacillus pleuropneumoniae*, *Escherichia coli*, *Yersinia pestis*, *Bordetella* spp., *Staphylococcus aureus* and *Staphylococcus epidermidis* [6,17–21]. Functions ascribed to PGA in other bacteria include abiotic surface attachment, intercellular adhesion, biofilm formation, epithelial cell attachment, and resistance to killing by antibiotics, antimicrobial peptides and human PMNs [21–28].

The purpose of the present study was to determine whether leukotoxin and PGA protect cells of a biofilm-forming, clinical strain of *A. actinomycetemcomitans* against macrophage killing. In this report we describe the antimicrobial activity of the human macrophage cell line THP-1 against *A. actinomycetemcomitans* clinical strain IDH781, and isogenic, biofilm-forming leukotoxin- and PGA-deficient mutant strains. To overcome the problem of intercellular autoaggregation, we utilized a filtration protocol to prepare single-cell suspensions from tightly adherent *A. actinomycetemcomitans* biofilms. Our results suggest that both leukotoxin and PGA play a key role in immune cell evasion by clinical strains of *A. actinomycetemcomitans*.

2. Results

2.1. Phenotypes of A. actinomycetemcomitans strains

In the present study we utilized a wild-type *A. actinomycetemcomitans* clinical isolate (strain IDH781 [29]), and isogenic leukotoxin and PGA mutant strains. The leukotoxin mutant strain contained a transposon insertion in the leukotoxin structural gene *ltxA* [30] and the PGA mutant strain contained a transposon insertion in *pgaC*, which encodes the integral membrane glycosyltransferase responsible for the polymerization and secretion of PGA [6]. All three strains produced biofilms in 96-well microtiter plates as determined by using a crystal violet binding assay (Fig. 1A). In all cultures, the broth remained optically clear and contained $<1\%$ of the CFUs in the well. Biofilm colonies produced by the leukotoxin and PGA mutant strains exhibited a morphology that was very similar to the morphology exhibited by wild-type biofilm colonies (data not shown). These data are consistent with the results of previous studies demonstrating that PGA is not essential for biofilm formation by *A. actinomycetemcomitans* in the 96-well microplate assay [6,15].

The amount of PGA in wild-type and mutant *A. actinomycetemcomitans* biofilms was measured by staining the biofilms with Congo red dye, which binds to PGA [6]. As expected, biofilms produced by the PGA mutant strain bound significantly less Congo red dye than did wild-type biofilms (Fig. 1B). The amount of Congo red bound by biofilms produced by the leukotoxin mutant was the same as the amount bound by wild-type biofilms, indicating that the leukotoxin mutant strain was not deficient in PGA production.

The amount of leukotoxin produced by all three *A. actinomycetemcomitans* strains was measured by treating the leukotoxin-sensitive cell line HL-60 for 6 h with bacterial culture supernatants, and then measuring HL-60 cell viability by Trypan blue exclusion (Fig. 2A). As expected, supernatants from the *A. actinomycetemcomitans* wild-type strain killed significantly more HL-60

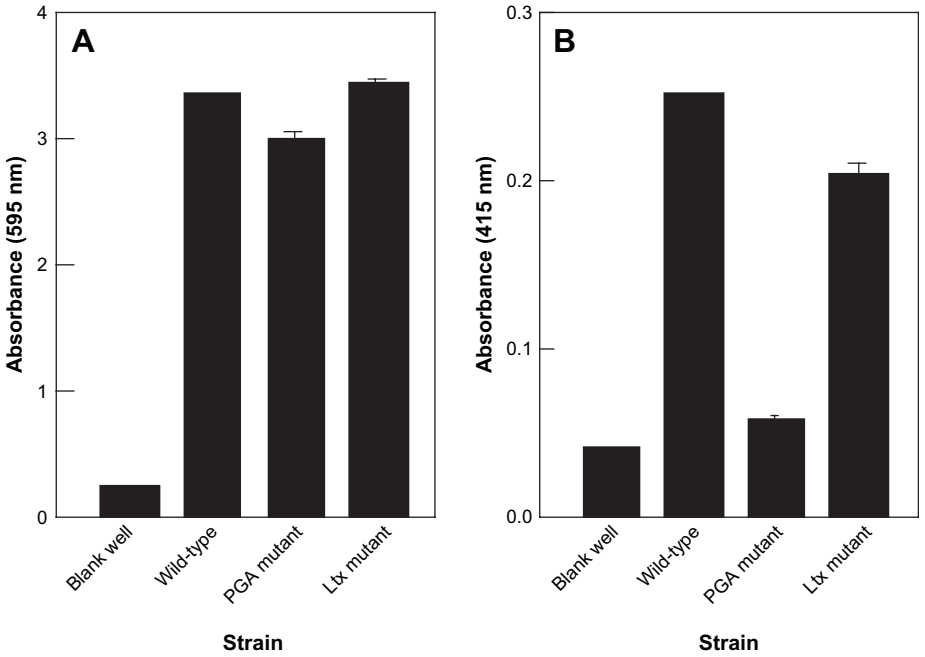

Fig. 1. Phenotypes of wild-type and isogenic leukotoxin (Ltx) and PGA mutant *A. actinomycetemcomitans* strains. (A) Biofilm formation in 96-well microtiter plates. Biofilms were stained with crystal violet dye. The amount of bound crystal violet (absorbance at 595 nm) is proportional to biofilm biomass. (B) Quantitation of PGA production in wild-type and mutant biofilms. Biofilms were stained with Congo red dye. The amount of bound Congo red (absorbance at 415 nm) is proportional to PGA production. All values in panels (A) and (B) indicate the mean and standard deviation for triplicate wells.