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Binding of *Actinobacillus actinomycetemcomitans* lipopolysaccharides to *Peptostreptococcus micros* stimulates tumor necrosis factor α production by macrophage-like cells

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Peptostreptococcus micros is a gram-positive bacterium that has been associated with periodontitis and endodontic infections. In this study, we hypothesized that *P. micros* binds the immunomodulating component lipopolysaccharide derived from gram-negative bacteria to increase its capacity to stimulate cytokine production by host cells. The ability of *P. micros* to bind *Actinobacillus actinomycetemcomitans* lipopolysaccharide was demonstrated by an enzyme-linked immunosorbent assay and by immunoelectron microscopy. Pretreatment of *P. micros* cells with *A. actinomycetemcomitans* lipopolysaccharide was associated with a 49-fold increase in tumor necrosis factor alpha production by human monocytic cells U937 differentiated into adherent macrophages, compared to the stimulation with untreated *P. micros*. This effect was suppressed by incorporating polymyxin B, a lipid A-binding substance, during treatment of macrophage-like cells with lipopolysaccharide-coated *P. micros* cells. This is the first study reporting a binding interaction between lipopolysaccharide and a gram-positive bacterium. This interaction represents a new mechanism that could promote the inflammatory response during periodontitis.

Key words: *Actinobacillus actinomycetemcomitans*; lipopolysaccharide; macrophage; *Peptostreptococcus micros*; tumor necrosis factor α

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Peptostreptococcus micros is one of the few gram-positive bacterial species for which there is evidence supporting a role in periodontitis (1, 12, 14, 17). *P. micros* has also been associated with infected dental root canals (5) and with a number of extraoral infections including genital tract, intra-abdominal, pulmonary and brain infections (9). However, few studies have investigated the virulence determinants of *P. micros*. Kremer et al. (8) reported the ability of *P. micros*, more particularly the smooth morphotype, to adhere to oral epithelial cells. Gelatinase and hyaluronidase activities produced by *P. micros* have also been reported (10, 15).

Lipopolysaccharides, integral components of the outer membranes of periodontopathogenic gram-negative bacteria, are well known to be prominent inducers of inflammatory mediator production by

human cells (18). More particularly, Saglie et al. (13) reported that low concentrations of lipopolysaccharide from *Actinobacillus actinomycetemcomitans*, a bacterium strongly associated with several forms of periodontitis, stimulate human macrophages to dramatically increase the synthesis of messenger RNA coding for interleukin-1α (IL-1α), interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α). In this study, we hypothesized that this strongly immunomodulating lipopolysaccharide binds to the cell surface of *P. micros*, which is found in the same environment as *A. actinomycetemcomitans*, thus increasing the potential of this gram-positive bacterium to induce cytokine production by human macrophages.

Material and methods

Bacteria and growth conditions

P. micros HG1262, a rough morphotype strain (7), was kindly provided by Dr. J. J. M. van Steenbergen (Academic Centre for Dentistry, Amsterdam, the Netherlands). Lawns of bacteria were grown on Todd Hewitt agar plates (THB; BBL Microbiology Systems, Cockeysville, MD) for 3 days in an anaerobic chamber ($N_2/H_2/CO_2$, 80 : 10 : 10) at 37°C. Cells were harvested from the agar surface using a cotton swab and suspended in 50 mM phosphate-buffered saline (PBS) pH 7.2 to an optical density at 660 nm (OD_{660}) of 0.45 for the enzyme-linked immunosorbent assay (ELISA) or 0.25 for the immunoelectron microscopy analysis and the TNF-α induction assay.

Preparation of *A. actinomycetemcomitans* lipopolysaccharide

Lipopolysaccharides were isolated from *A. actinomycetemcomitans* ATCC 29522 (serotype b) by the method of Darveau & Hancock (3), which is based on protein digestion of a whole cell extract by proteinase K and successive solubilization and precipitation steps. The lipopolysaccharide preparation was freeze-dried and kept at −20°C. Using a protein assay kit (Bio-Rad Laboratories, Mississauga, Ontario, Canada), it was found that contaminant proteins, if present, were at a concentration of less than 1 μg/ml.

Determination of lipopolysaccharide binding to *P. micros* cells

Binding of *A. actinomycetemcomitans* lipopolysaccharide to *P. micros* was first investigated by ELISA. *P. micros* cell suspension 110 μl ($OD_{660} = 0.45$) was added (100 μl) into wells of a microtiter plate (Maxisorp™ Nalg Nunc International, Maperville, IL), and allowed to attach by an overnight incubation at 4°C. Wells were washed three times with PBS, and *A. actinomycetemcomitans* lipopolysaccharide at 2 μg/ml (in PBS) was added. After incubation at 37°C for 1.5 h, unbound lipopolysaccharide was removed and wells were extensively washed three times with PBS containing 0.05% (v/v) Tween-20 (PBST). Unreacted sites were blocked by adding 100 μl of PBST containing 1% (w/v) gelatin to wells for 1 h at 37°C. After washing the wells three times with PBST, a rabbit antiserum directed to whole cells of *A. actinomycetemcomitans* ATCC 43718 (Y4, serotype b) (kindly provided by Dr. S. Asikainen, University of Helsinki), which reacts with lipopolysaccharide derived from the strain ATCC 29522 (data not shown), was diluted 1/500 in PBST containing 0.1% gelatin and added to wells. Following incubation at 37°C for 2 h, wells were washed three times with PBST, and a horseradish peroxidase-conjugated goat anti-rabbit IgG (1/3,000) was added prior to further incubation of the plate for 1 h at 37°C. Wells were washed three times with PBST and 100 μl of 3,3′,5,5′-tetramethyl benzidine at 0.1 mg/ml were added to each well, prior to incubating the plate at 37°C for 30 min in the dark. Following the addition of 1.8 N H_2SO_4, the absorbance at 450 nm (A_{450}) was measured with a microplate reader. Controls consisted in coating wells with gelatin instead of *P. micros* cells or omitting the incubation step with lipopolysaccharide. These A_{450} values, which correspond to the nonspecific binding of lipopolysaccharide to gelatin and of anti-*A. actinomycetemcomitans* rabbit antiserum to *P. micros* cells, respectively, were subtracted. The relative amount of lipopolysaccharide bound by the *P. micros* layer was estimated from a standard curve constructed by immobilizing into wells 500, 200, 100 or 50 ng of *A. actinomycetemcomitans* ATCC 29522 lipopolysaccharide.

Binding of *A. actinomycetemcomitans* lipopolysaccharide to *P. micros* was also evaluated by immunoelectron microscopy. The cell suspension of *P. micros* HG1262 was incubated with *A. actinomycetemcomitans* lipopolysaccharide (5 μg/ml) at 37°C for 1.5 h. *P. micros* cells were then washed with PBS three times to remove unbound lipopolysaccharide. After treatment (1 h) of cells with PBS containing 1% bovine serum albumin, *P. micros* cells were incubated (2 h) with anti-*A. actinomycetemcomitans* ATCC 43718 rabbit antiserum (1/50 dilution) absorbed with *P. micros* cells. A preimmune rabbit serum was used as control. Cells were then washed three times with PBS and incubated with a 1/10 dilution of immunogold conjugate EM goat anti-rabbit IgG (British Bio Cell International, Cardiff, UK) for 1 h prior to washing extensively. The cells were applied on Formvar-coated 200-mesh copper grids, stained with 2% uranyl acetate and examined by transmission electron microscopy (Hitachi H-800, Tokyo, Japan).

Cultivation and differentiation of U937 cells

The monocytic cell line U937 was purchased from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium supplemented with penicillin (50 μg/ml), streptomycin (50 μg/ml), and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. The cells were cultured at 2×10^5 cells/ml and split approximately three times per week to maintain the cell concentration between 1×10^5 and 2×10^6 viable cells/ml for optimal growth. Monocytes were differentiated into adherent macrophages by cultivation for 48 h in the presence of phorbol-12-myristate 13-acetate (PMA; Sigma Chemical Co., St. Louis, MO) at a concentration of 1 ng/ml. Thereafter, the cells were incubated in PMA-free supplemented RPMI 1640 medium for 24 h prior to use. Following this treatment, more than 90% of U937 cells are alive and differentiated into macrophages loosely attached to the plastic surface (4). The floating cells were then removed and adherent macrophage-like cells were collected using a cell scraper. Cells were washed three times by centrifugation ($200 \times g$ for 5 min) using RPMI 1640–10% FCS, and seeded into a 96-well plate at 10^6 cells/ml for the TNF-α induction assay.

TNF-α induction assay

Equal volumes (500 μl) of the bacterial cell suspension ($OD_{660} = 0.25$) and *A. actinomycetemcomitans* lipopolysaccharide (5 μg/ml in PBS) were mixed and incubated at 37°C for 1.5 h to allow interactions between bacteria and lipopolysaccharide. PBS was used as a blank control for replacing either *P. micros* cells or *A. actinomycetemcomitans* lipopolysaccharide. Following the incubation, unbound and loosely attached lipopolysaccharide were

removed by three consecutive centrifugations (10,000 ×*g* for 10 min). Finally, *P. micros* cells were suspended in the original volume of RPMI 1640–10% FBS, and 100 µl of the suspension was added to PMA-differentiated U937 cells cultivated into a 96-well plate. *A. actinomycetemcomitans* lipopolysaccharide (final concentration of 5 or 1 ng/ml) were also directly added to macrophage-like cells as positive controls. After a 24-h incubation, the TNF-α concentration in the culture supernatants was measured using a commercial ELISA kit (Human TNF-α CytoSets™, Biosource, Camarillo, CA) according to the manufacturer's instructions. To investigate the effect of polymyxin B (Sigma Chemical Co.), a lipid A-binding substance, PMA-differentiated U937 cells were stimulated by lipopolysaccharide-coated *P. micros* in the presence of the compound at 10 µg/ml.

Results

The binding of lipopolysaccharide isolated from *A. actinomycetemcomitans* ATCC 29522 on the surface of *P. micros* HG1262 cells was first demonstrated by ELISA using bacteria immobilized onto wells of a microtiter plate (Table 1). From the standard curve and assuming that all lipopolysaccharide applied attached on the bottom of wells, the amount of lipopolysaccharide bound by the *P. micros* layer was estimated to be in the range of 100–200 ng. In order to confirm the binding of *A. actinomycetemcomitans* lipopolysaccharide to the cell surface of *P. micros*, an immunoelectron microscopy analysis was performed. As shown in Fig. 1B, numerous gold beads, not uniformly distributed, were found to be associated with the cell surface of *P. micros* HG1262, indicating the presence of bound lipopolysaccharide. Very few beads attached to the cells when using a preimmune rabbit serum (Fig. 1A) instead of anti-*A. actinomycetemcomitans* rabbit antiserum, or when omitting the treatment with *A. actinomycetemcomitans* lipopolysaccharide (data not shown).

Production of TNF-α by PMA-differentiated U937 monocytic cells following stimulation with *P. micros* treated or not with *A. actinomycetemcomitans* lipopolysaccharide is shown in Fig. 2. The amount of TNF-α secreted following stimulation with *P. micros* alone was 13.1 ± 4.2 pg/ml. Pretreatment of *P. micros* with *A. actinomycetemcomitans* lipopolysaccharide was associated with a 49-fold increase in TNF-α production, compared to the stimulation with untreated *P. micros*. This effect was completely suppressed by including polymyxin B during stimulation. Stimulation of macrophage-like cells directly with *A. actinomycetemcomitans* lipopolysaccharide at

Table 1. Immunological detection of *A. actinomycetemcomitans* lipopolysaccharide bound to *P. micros* HG1262 or immobilized onto microtiter wells

Assay condition	Immunological detection of LPS (A_{450})*
P. micros cells immobilized onto well and treated with 200 ng LPS	0.120 ± 0.012**
500 ng LPS immobilized onto well	0.253 ± 0.009†
200 ng LPS immobilized onto well	0.182 ± 0.024†
100 ng LPS immobilized onto well	0.118 ± 0.021†
50 ng LPS immobilized onto well	0.045 ± 0.014†

LPS, lipopolysaccharide.
*The means ± standard deviations of three assays are presented.
**The values corresponding to nonspecific binding to lipopolysaccharide to gelatin-coated wells and to nonspecific binding of anti-*A. actinomycetemcomitans* antiserum to *P. micros* cells were subtracted.
†The value corresponding to nonspecific binding of anti-*A. actinomycetemcomitans* antiserum to gelatin-coated wells was subtracted.

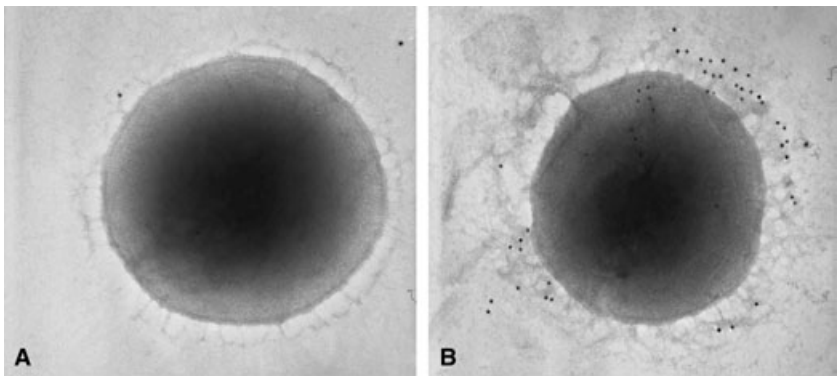

Fig. 1. Immunolocalization of *A. actinomycetemcomitans* lipopolysaccharide bound to *P. micros* HG1262 cells by transmission electron microscopy. Bacteria were successively treated with *A. actinomycetemcomitans* lipopolysaccharide, preimmune rabbit serum (A) or anti-*A. actinomycetemcomitans* rabbit antiserum (B), and colloidal gold-conjugated goat anti-rabbit immunoglobulin G.

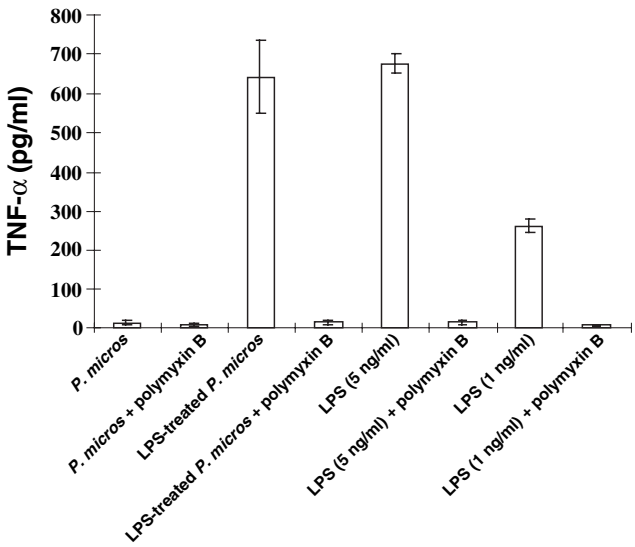

Fig. 2. TNF-α production by PMA-differentiated U937 monocytic cells stimulated by cells of *P. micros* HG1262 treated or not with *A. actinomycetemcomitans* lipopolysaccharide (LPS) or by *A. actinomycetemcomitans* lipopolysaccharide alone. The effect of adding polymyxin B is also reported. Each value represents the mean ± standard deviation of three assays.

5 ng/ml showed a TNF-α induction comparable to that obtained with lipopolysaccharide-coated *P. micros*, suggesting that a very small amount of lipopolysaccharide is required to stimulate cytokine induction.

Discussion

In this study, we clearly demonstrated the capacity of *P. micros* cells to bind *A. actinomycetemcomitans* lipopolysaccharide on their surface. To our knowledge, this is the first report demonstrating the binding of lipopolysaccharide to the cell surface of a gram-positive bacterium. Whether the binding of *A. actinomycetemcomitans* lipopolysaccharide to *P. micros* observed in this study is specific is not clear. Using a different method, we showed that ^{3}H-radiolabeled lipopolysaccharide from *Porphyromonas gingivalis* and *Fusobacterium nucleatum* attached poorly to *P. micros* cells, whereas that from *Treponema denticola* bound significantly (data not shown). It thus appears that the binding capacity of lipopolysaccharide is not unique to *A. actinomycetemcomitans* but that there are variations in lipopolysaccharide from different bacteria. The binding properties of *A. actinomycetemcomitans* lipopolysaccharide were also investigated by Okuda et al. (11). They showed that this particular lipopolysaccharide can bind to saliva- or serum-coated hydroxyapatite and can agglutinate human erythrocytes.

Our results indicated that *A. actinomycetemcomitans* lipopolysaccharide bound to *P. micros* cells conferred to this gram-positive bacterial species the capacity to induce a strong TNF-α response in macrophage-like cells. Polymyxin B, a substance binding to the lipid A moiety of lipopolysaccharide, inhibited this phenomenon, thus strongly suggesting that the increased production of TNF-α is specifically caused by *A. actinomycetemcomitans* lipopolysaccharide rather than by minor contaminants that may have been present in our lipopolysaccharide preparation.

Results from this study suggest that lipopolysaccharide released from *A. actinomycetemcomitans* can attach to *P. micros*, resulting in a population of bacteria that could be a strong stimulant for human macrophages. Lipopolysaccharide is released *in vivo* from the bacterial cells during growth but also when bacteria die or lyse. Serum components and antibiotics may also induce the release of lipopolysaccharide (2, 16). The phenomenon reported in this study represents a new mechanism by which gram-positive bacteria such as *P. micros* might contribute to cytokine induction in a periodontal lesion, thus favoring inflammation. Interestingly, the poor treatment response individuals showed significantly elevated proportions of *P. micros* in both their active and inactive sites compared to the good treatment response group (6). Through the binding of gram-negative lipopolysaccharide, this bacterial species may contribute to the maintenance of the inflammatory response.

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