# *Porphyromonas gingivalis* LPS Lipid A Heterogeneity Significantly Affects Endothelial Cell Innate Host Responses

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**Purpose:** *Porphyromonas gingivalis* is an important gram-negative periopathogen strongly associated with adult type periodontitis. Lipopolysaccharide (LPS), a key component on the cell surface of gram-negative bacteria, is a potent immunomodulator that alerts the host of bacterial infection. The purpose of this study was to determine the contribution of P. gingivalis LPS heterogeneity to immunomodulation of innate host defenses.

**Materials and Methods:** *P. gingivalis* LPS was isolated and LPS heterogeneity was determined by a combination of matrix assisted laser desorption–time of flight (MALDI-TOF) mass spectroscopy coupled with gas chromatographic analysis of lipid A fatty acids. Endothelial cell E selectin activation was determined by semi-quantitative RT-PCR and IL-8 secretion was determined by ELISA.

**Results:** *P. gingivalis* lipid A heterogeneity was found to be due to culture medium conditions and extraction procedures and was not the result of degradation due to isolation procedures. Lipid A was identified as the component of LPS responsible for E selectin antagonism. Two different preparations of *P. gingivalis* LPS which differed in their lipid A species content were obtained. It was found that one was an agonist while the other was an antagonist for E selectin expression on human endothelial cells.

**Conclusions:** We suspect that the ability of *P. gingivalis* to synthesize and express multiple structurally different forms of lipid A represents a form of bacterially induced immunomodulation. This allows the bacterium to selectively evade and stimulate host cell responses in response to local environmental conditions contributing to its ability to occupy different niches in the host.

**Key words:** lipopolysaccharide, innate defense, immunomodulation

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## INTRODUCTION

### *The Microbial Oral Community is the most Completely Characterized Group of Bacteria that Persistently Colonize the Host*

The wealth of information available about the numbers and types of bacteria that persistently colonize the oral cavity is especially relevant to studies of bacterial / host interactions. Since van Leeuwenhoek's discovery of 'animacules' in gingival tooth scrapings in 1683 which began the science of bacteriology (Dobell, 1958) descriptive studies performed throughout the 20th century (Socransky and Haffajee, 1994) laid a foundation for current studies that utilize molecular techniques to identify non-cultivatable oral bacteria (Kroes et al, 1999), define oral transmission routes (Li and Caufield, 1998), and examine associations between species genotypes and disease (Griffen et al, 1999). The well-characterized shift from mostly gram-positive to gram-negative species (Socransky and Haffajee, 1994) that occurs in the transition from periodontal health to disease has been confirmed and further defined by DNA probe analysis of bacterial populations combined with multiple cluster and community ordination statistical methods (Socransky et al, 1998). For example, it is now known that periopathogenic bacteria including *Bacteroides forsythus, P. gingivalis, Treponema denticola*, and *Prevotella intermedia* (Socransky et al, 1998) group together in diseased sites and are also present in healthy sites (Ximenez-Fyvie et al, 2000). In addition, *in situ*

analysis has revealed that *P. gingivalis* and other oral pathogens occupy cheek epithelial cells as a reservoir apparently without deleterious effects to the host (Rudney et al, 2001). Clearly however, periopathogens have higher overall counts as well as greater proportions in diseased periodontal sites consistent with their association with destructive periodontitis (Ximenez-Fyvie et al, 2000). These extensive studies over the years provide certainty about the microbial flora and bacterial associations that persist in the oral cavity. In addition, the recent more sensitive and easier sampling techniques have revealed that *P. gingivalis* and other oral bacteria display a versatility in being able to persist in the oral cavity in a commensal relationship as well as act as pathogens associated with disease.

### *LPS is Eemployed by the Host to Detect Bacterial Infection*

Lipopolysaccharide, often called endotoxin as it is isolated from *Escherichia coli*, is a well known potent inflammatory immune activator (Ulevitch and Tobias, 1999). It has been termed a pattern recognition receptor ligand for the innate host defense system (Medzhitov and Janeway, 2000). LPS is present in all gram negative bacteria, is essential for bacterial viability, and contains a highly conserved lipid A structure consisting of a phosphorylated beta-(1,6)-glucosamine disaccharide substituted with hydroxylated and non-hydroxylated fatty acids (Takada and Kotani, 1992), fully filling the criteria for pattern recognition by host immune system.

### *Gram-Negative Periopathogenic Bacteria Contain Low Biological Reactivity Lipopolysaccharide (LPS)*

Early studies indicated (Mansheim et al, 1978; Fujiwara et al, 1990) and more recent studies have confirmed (Darveau et al, 1995; Pulendran et al, 2001) that LPS and its isolated lipid A component obtained from gram negative oral anaerobic bacteria do not elicit host responses in a manner similar to the classic *E. coli* type endotoxin. Early studies examining endotoxin lethality in mice demonstrated that gram negative oral anaerobic LPS was not nearly as potent as enterobacterial LPS (Takada and Galanos, 1987; Isogai et al, 1988) and did not induce a Schwartzman reaction (Mansheim et al, 1978; Fujiwara et al, 1990), a systemic innate immune inflammatory response typically associated *E. coli* type endotoxin (Takada and Kotani, 1992). These observations resulted in this type of LPS being designated as having low biological activity. However, LPS obtained from *P. gingivalis* induces inflammatory responses in non-responder LPS mice (Kirikae et al, 1999), subsequently shown to have a mutation in TLR 4 (Poltorak et al, 1998; Qureshi et al, 1999), suggesting that their LPS contains a TLR 4 independent mechanism for cell activation. Subsequently, numerous *in vitro* studies by our laboratory (Darveau et al, 1995; Cunningham et al, 1996, 1999) and others (Fujiwara et al, 1990; Ogawa et al, 1994; Ogawa and Uchida, 1996) have confirmed that *P. gingivalis* LPS is both less potent and it elicits a different pattern of inflammatory mediators (Ogawa and Uchida, 1996; Tanamoto et al, 1997) when compared to *E. coli* LPS. Indeed, it has recently been reported that the dendritic cell response to *P. gingivalis* bacteria and its isolated LPS is significantly different than *E. coli* (Pulendran et al, 2001).

### *Host Responses to LPS Vary Significantly Depending upon Lipid A Structure*

The biological activity of LPS is due to the lipid A portion of the molecule (Loppnow et al, 1989). Consistent with this, numerous LPS structural studies have demonstrated that the number, chain length, and position of lipid A fatty acids as well as the number of phosphate groups attached to glucosamine disaccharide backbone can significantly affect biological activity (Takada and Kotani, 1992; Loppnow et al, 1989; Schumann et al, 1996). The first definitive structure activity relationship studies with lipid A employed a series of chemically synthesized *E. coli* lipid A analogues (Takada and Kotani, 1992). These studies demonstrated that lipid A can affect all the classic endotoxic activities and have given considerable structure-function information. These studies also demonstrated that although the canonical lipid A structure is necessary for the full range and potent activity of lipid A, slight alterations in lipid A structure are still recognized by the innate host defense system. These structural alterations significantly affect the host response both in its potency and range of responses.

### *Bacteria can Regulate their Lipid A Structure in Response to Environmental Conditions*

One component of bacterial pathogenesis is the ability to coordinately express virulence genes in response to host microenvironments (Mekalanos, 1992). *Salmonellae typhimurium* contains a PhoP-PhoQ sensor kinase and transcriptional activator system which regulates genes required for intracellular survival and cationic peptide resistance (Miller et al, 1989). It has been demonstrated that a constitutive *S. typhimurium* mutant designated PhoPc, which mimics in part the intracellular regulation state of the bacteria, contains a modified lipid A species composition (Guo et al, 1997). These modifications resulted in *S. typhimurium* LPS that contained two additional lipid A species, one modified with an aminoarabinose moiety and the other containing a 2-OH myristic fatty acid instead of myristic acid (Guo et al, 1997). These additional lipid A species rendered both whole bacteria and isolated LPS less potent activators of E selectin and TNFα when compared to

wild type or another PhoP- minus mutant (Guo et al, 1997). This was the first description of genetic regulation that resulted in lipid A alterations which modified the innate host response. In addition, *Yersinia pestis*, the causative agent of the bubonic plague, displays lipid A heterogeneity that is dependent upon incubation temperature, a key component in the flea to human transmission of this pathogen (Kawahara et al, 2002). These studies demonstrate that bacteria can modify their lipid A structure to elicit either more or less potent innate host responses depending upon local host microenvironmental conditions.

### *P. gingivalis LPS may also Contain Lipid A Heterogeneity*

Two separate studies have structurally examined the lipid A found in *P. gingivalis* LPS preparations (Kumada et al, 1995; Ogawa, 1993). In one study the major lipid A species found was a tri-acylated monophosphorylated form with a negative ion mass of 1195 (Ogawa, 1993). In the other study, multiple *P. gingivalis* lipid A structural isoforms were observed. However, two forms predominated which were tetra-acylated monophosphorylated forms with molecular mass ions of 1435 and 1450 respectively (Kumada et al, 1995).

These structures are depicted in Fig 1 and differ from the canonical *E. coli* lipid A structure in the number of phosphates, and the number, type, and position of the fatty acid chains. A study from our laboratory has demonstrated that all three of these *P. gingivalis* lipid A species (1195, 1435, and 1450) are present in our purified LPS preparations (Bainbridge et al, 2002). In addition, another study employing tri-reagent to extract LPS has found that *P. gingivalis* contains multiple lipid A species (Yi and Hackett, 2000). The possible reasons for the apparent discrepancies in the predominant type of *P. gingivalis* lipid A found between these studies include variations in the strain of *P. gingivalis* employed, growth conditions, and isolation procedures.

## RESULTS

### *P. gingivalis LPS Displays Lipid A Heterogeneity*

In the following series of experiments with the use of a new crude LPS extraction procedure which employs commercially available tri-reagent (Yi and Hackett, 2000), it is shown that *P. gingivalis* LPS contains more major lipid A mass ions than previously described and that both the $MgCL_2$/ETOH and phenol/water LPS purification procedures failed to extract all of the different lipid A mass ions equally.

Tri-reagent extraction of LPS from *P. gingivalis* 33277 and subsequent lipid A cleavage (Caroff et al, 1988) revealed numerous major lipid A mass ions that clustered around m/z 1450, 1690, and 1770 (Fig 2a - see Fig 1 for structures of the major lipid A mass ions). The lipid A species found clustered around each of these mass ions differ by single methylene units smaller (m/z 1420 and 1435 adjacent to 1450; 1675 adjacent to 1690; and 1705 and 1755 adjacent to 1770) and single methylene units larger (m/z 1465 and 1480 adjacent to 1450; 1705 adjacent to 1690; and 1785 and 1800 adjacent to 1770). This pattern of different lipid A mass ions is indicative of fatty acid chain length heterogeneity accounting for some of the different lipid A species. In contrast, LPS extracted from whole cells by either the $MgCl_2$ / ETOH (Fig 2b) or phenol / water (Fig. 2c) were missing both clusters of lipid A mass ions centered at 1690 and 1770. The tri-acylated monophosphorylated lipid A at m/z 1195 was not present in the LPS preparations obtained by the three different extraction procedures. These data demonstrate that *P. gingivalis* LPS displays significant lipid A heterogeneity and that different

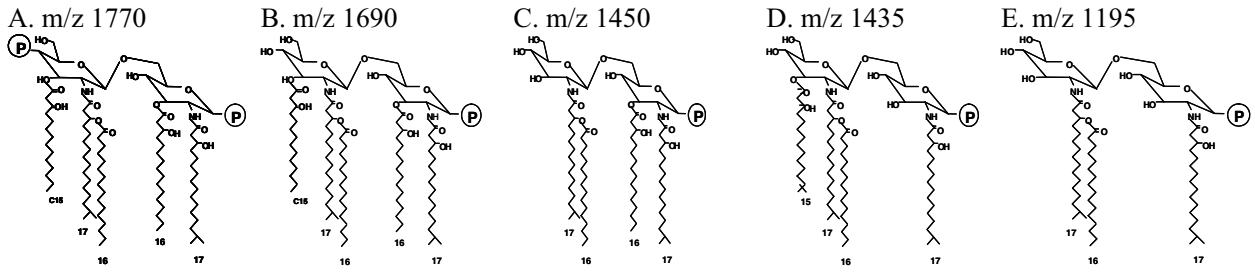


**Fig 1** Major characterized *P. gingivalis* lipid A mass ions. *P. gingivalis* displays lipid A heterogeneity in that LPS isolated from a single species contains multiple lipid A species. Kumada (Li and Caufield, 1998) has elucidated the structures of several of the major lipid A mass ions. These lipid A structures differ in the number of phosphates and fatty acids attached to the glucosamine backbone.

LPS extraction procedures yield different ratios of these lipid A species.

### *A P. gingivalis LPS Fraction which is Highly Enriched for the Lipid A Mass Ion at m/z 1690 has been Obtained*

P. gingivalis lipid A species need to be isolated and characterized to more fully understand their individual contribution to the role of this bacterium in host colonization. It was found that the culture medium can significantly affect P. gingivalis lipid A species content in that when P. gingivalis is incubated in a medium different than that shown in Fig 2, a P. gingivalis lipid A fraction that is highly enriched for the lipid A mass ion at m/z 1690 is obtained (Fig 3a). This LPS preparation is designated PgLPS1690.

This provided good evidence that P. gingivalis was able to regulate the number and types of lipid A species found in LPS. However, it also provided the opportunity to determine if the lipid A species found at m/z 1435 and 1450 were degradation products of the mass ion found at m/z 1690. It is possible that the LPS extraction and analysis procedures were contributing to the lipid A heterogeneity. Specifically, the lipid A hydrolysis procedure which involves 100°C heat treatment under mild acid conditions for 60 minutes may not only release lipid A from intact LPS, but may also partially degrade the lipid A during hydrolysis resulting in smaller partial lipid A species. This was a particular concern because the 1435 and 1450 lipid A species could easily be generated from the 1690 peak by the release of C16:OH and C15:OH respectively (see Fig 2 and lipid A structures Fig 1). The LPS preparation that contained one major lipid A mass ion cluster at m/z 1690 and a single peak at m/z 1440 was subjected to lipid A hydrolysis for varying times at 100 C (Fig 3). It was reasoned that if the LPS hydrolysis procedure was contributing to the lipid A heterogeneity by artificially creating partial lipid A structures that their generation would be subject to lipid A hydrolysis times. For example, if the standard 60-minute hydrolysis resulted in the generation of lipid A mass ions of 1435 and 1450, longer hydrolysis times may result in the total conversion of the lipid A species at 1690 to smaller lipid A species and less hydrolysis time might generate less of the smaller lipid A species. However, this was not found. In two separate experiments prolonged LPS hydrolysis (Fig 3, panel c) did not result in the generation of lipid A species with lower mass. In fact, in one experiment hydrolysis overnight at 100 C resulted in the same pattern of lipid A mass ions peaks that was observed at two hours (data not shown). This data demonstrates that lipid A mass ion at m/z 1690 is stable and lipid A hydrolysis does not generate the lipid A mass ions at m/z 1435 or 1450. Consistent with this, hydrolysis of E. coli lipid A for two hours also did not result in the generation of partial lipid A species (data not shown). Furthermore, the experiment revealed that the

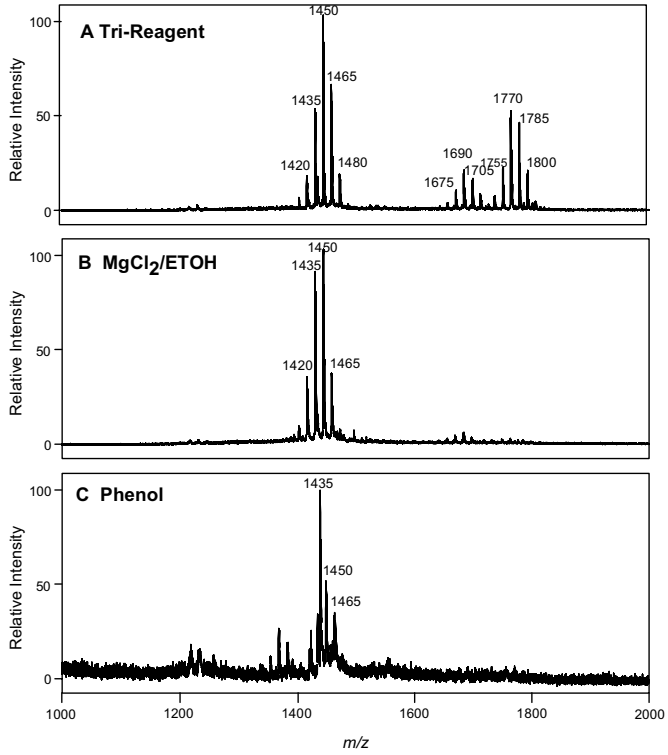


**Fig 2** Characterization of *P. gingivalis* lipid A species by negative ion mass spectrometry. *P. gingivalis* LPS was obtained by either **(a)** tri-reagent procedure (Yi and Hackett, 2000); **(b)** cold / $MgCL_2$ ETOH procedure (Darveau and Hancock, 1983); or **(c)** phenol / $H_20$ procedure (Westphal and Jann, 1965). Lipid A was cleaved and separated from the LPS as described by Caroff et al (1988). Matrix assisted laser desorption time of flight (MALDI-TOF) was performed as previously described (Guo et al, 1997). All values given are average mass rounded to the nearest whole number for singly charged deprotonated molecules. Tri-reagent extracted LPS yields two major lipid A mass ions at m/z 1690 and m/z 1770 which are missing or significantly reduced when either $MgCL_2$ / ETOH **(b)** or phenol/water **(c)** procedures are used to purify the LPS.

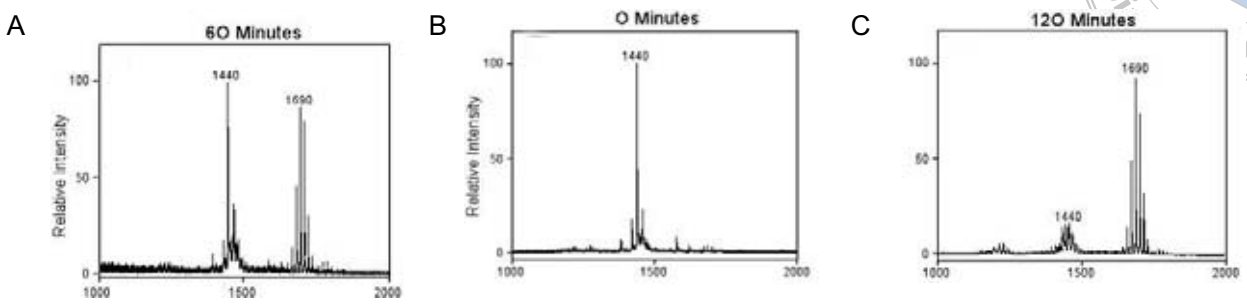


**Fig 3** Negative ion mass spectrum of *P. gingivalis* LPS preparations. *P. gingivalis* LPS was extracted with tri-reagent (Yi and Hackett, 2000) and subjected to lipid A hydrolysis for varying times (indicated for each panel). MALDI-TOF analysis was performed (Guo et al, 1997) and the fragmentation pattern of lipid A generated from *P. gingivalis* LPS is shown.

mass ion at m/z 1440 did not require heating to be released from the LPS (Fig 3, panel b) and was subject to degradation after 2 hours (Fig 3, panel c). The observation that the mass ion at m/z 1440 did not require hydrolysis to be observed in MALDI-TOF experiments demonstrates that this mass ion is a non-lipid A component that appears in some of our crude LPS preparations. We isolated this component by a chloroform/methanol extraction, consistent with it being a non-lipid A component, and found that it did not exhibit any TLR2 or TLR4, nor endothelial cell E selectin activating or antagonistic activity (data not shown). Therefore, culture medium and not lipid A degradation was responsible for the difference in the lipid A species content observed between Figs 2a and 3a.

## *P. gingivalis Lipid A is Sufficient for Endothelial Cell Antagonism*

We have previously described that the human endothelial cell innate host response to *P. gingivalis* LPS is markedly different than that observed with *E. coli* LPS in that it fails to significantly elicit expression of E selectin or IL-8 and was an antagonist for E selectin and IL-8 secretion response to *E. coli* LPS (Darveau et al, 1995). We have subsequently performed MALDI-TOF analysis of the different LPS preparations employed in these previous studies and found that these preparations were similar in lipid A content to that shown in Fig 2b (this LPS preparation is designated $PgLPS_{1435/1450}$) in that they were severely reduced in the amount of the lipid A mass ion at m/z 1690 and highly enriched lipid A species with molecular masses of 1435 and 1450 (see Bainbridge et al, 2002; Darveau et al, 2004). Next, it was determined if the lipid A component of these *P. gingivalis* LPS preparations was sufficient to obtain endothelial cell antagonism.

Initially, *P. gingivalis* LPS, which is similar to other LPS species in that it contains a heterogeneous mix of LPS molecules that differ in the number of O antigen repeats attached to the lipid A (Vasel et al, 1996), was fractionated on a Sepharose gel exclusion column which separates LPS molecules by size. *P. gingivalis* LPS column fractions were collected, subjected to SDS-PAGE and stained with silver (Fig 4a). Separation of LPS molecules containing a large number O antigen subunits attached to the core and lipid A (fractions 22, 23, 24) from those that only contain lipid A and lipid A attached to the core (lanes 26, 27, 28) were found. Next increasing concentrations of the different *P. gingivalis* LPS column fractions were added to human endothelial cells exposed to 10 ng *E. coli* LPS and the amount of IL-8 in the culture supernatant was measured after 24 hr as previously described (Darveau et al, 1998). It is shown that both fractions that contained O antigen (22 and 23) and those that did not (26 and 28) blocked IL-8 secretion equally well. This demonstrated that the core and attached lipid A components of LPS were sufficient for IL-8 antagonism and that O antigen was not required (Fig 4b). In a separate experiment, *P. gingivalis* LPS was treated as described by Caroff et al (1988) to cleave and separate lipid A from core and O antigen LPS components (isolated lipid A was confirmed by MALDI-TOF and gas chromatographic analysis, data not shown) and examined for its ability to block IL-8 secretion. It was found that both isolated LPS and purified lipid A blocked IL-8 secretion in response to *E. coli* LPS equally well (Fig 4c). These experiments demonstrated that *P. gingivalis* lipid A, specifically lipid A mass ions at m/z 1435 and 1450 were sufficient for human endothelial antagonism.

## *$PgLPS_{1435/1450}$ Antagonizes E Selectin in Response to $Pg_{1690}$*

Next the endothelial cell E selectin response to the *P. gingivalis* LPS preparation that contains predominantly the cluster of lipid A mass ions at m/z 1690 ($Pg_{1690}$, Fig 3a) was examined (Fig 5).

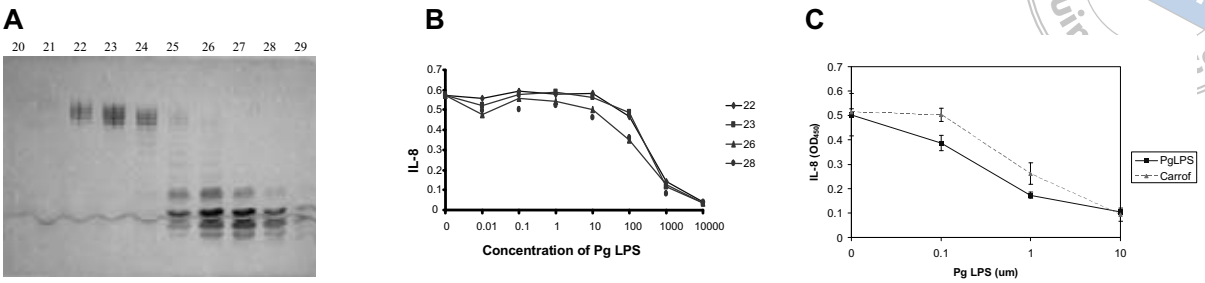


**Fig 4** *P. gingivalis* lipid A is sufficient for endothelial cell antagonism. In **(a)** *P. gingivalis* LPS was subjected to Sepharose gel exclusion column (Pugin et al, 1993) that separates LPS molecules by size. In **(a)** fractions were collected, subjected to SDS-PAGE and stained with silver. The numbers at the top of each lane correspond to the fraction number obtained after column chromatography. In **(b)** increasing concentrations of select *P. gingivalis* LPS column fractions (indicated in the legend) were added to human endothelial cells exposed to 10 ng *E. coli* LPS and the amount of IL-8 in the culture supernatant was measured after 24 hours as previously described (Darveau and Hancock, 1983). In Fig **(c)** purified *P. gingivalis* LPS was treated as described by Caroff et al (1988) to cleave and separate lipid A from core and O antigen LPS components and examined for its ability to block IL-8 secretion.

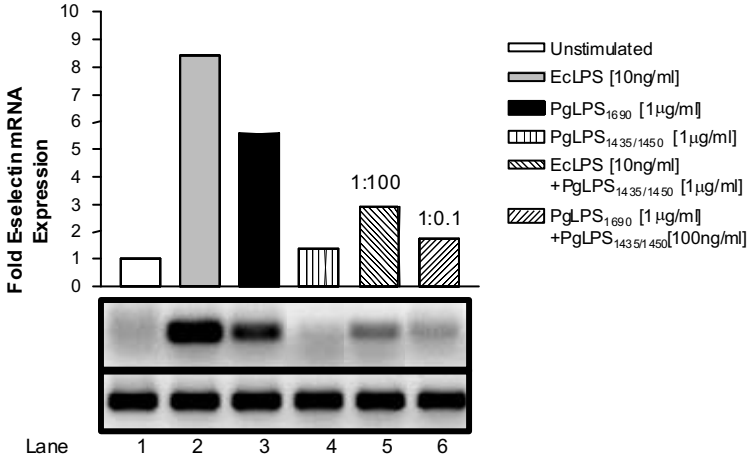


**Fig 5** *P. gingivalis* $LPS_{1435/1450}$ antagonizes both *E. coli* LPS-induction and *P. gingivalis* $LPS_{1690}$ induction of E-selectin mRNA expression in HUVEC. HUVEC cells (passaged four times) were plated in six-well culture dishes coated with collagen and treated with the indicated LPSs and dosages for four hours. Total RNA was harvested and RT-PCR analysis was performed to detect E-selectin and β-actin mRNA expression. Resulting RT-PCR products (lower panels) were imaged and subjected to densitometric analysis. E-selectin mRNA expression was normalized to β-actin mRNA expression and the resulting values were expressed as fold E-selectin mRNA induction relative to the unstimulated control (upper panel). Results shown are representative of three independent experiments.

In contrast to our previous data with purified *P. gingivalis* LPS that contains predominantly lipid A mass ions at m/z 1435 and 1450 ($Pg_{1435/1450}$, Fig. 2B), a significant E selectin response was observed for ($Pg_{1690}$, Fig. 5, lane 3). In addition, $Pg_{1435/1450}$, which has been demonstrated to be an antagonist for *E. coli* LPS-stimulated E selectin expression (Cunningham et al, 1996), was a potent antagonist for E selectin expression in response to Pg1690 (Fig 5, lane 6). Several studies have shown (Darveau et al, 1995; Cunningham et al, 1996; Darveau et al, 2002) that 50 to a hundred fold excess of $Pg_{1435/1450}$ over *E. coli* LPS yields slightly greater then 50% inhibition of the E selectin mRNA response (Fig 5, lane 5). In contrast, E selectin mRNA (Fig 5, lane 6) expression was inhibited at significantly lower ratios of $Pg_{1435/1450}$ to $Pg_{1690}$. For example, E selectin mRNA expression was inhibited by greater than 50% when a ten-fold excess of $Pg_{1690}$ was mixed with $Pg_{1435/1450}$ (Fig 5, lane 6). This represents approximately a thousand fold difference and complete reversal of the ratios of antagonistic LPS required to elicit inhibition of E selectin expression and demonstrates that $Pg_{1435/1450}$ LPS is potent antagonist against another LPS species made by the same bacterial population.

## DISCUSSION

This work demonstrates that the innate host inflammatory potential of *P. gingivalis* LPS preparations (at least as measured by E selectin expression on human endothelial cells) is highly dependent upon LPS extraction procedures and *in vitro* growth conditions. *P. gingivalis* LPS contains multiple lipid A species (Bainbridge et al, 2002; Darveau et al, 2004; Kumada et al, 1995; Yi and Hackett, 2000) that can be preferentially extracted to enrich the resultant LPS preparation in a subset of the lipid A types (Darveau et al, 2004). The culture media components and conditions of growth responsible for modulation of the lipid A composition of *P. gingivalis* are currently being examined.

The ability to obtain two *P. gingivalis* LPS preparations ($PgLPS_{1435/1450}$ and $PgLPS_{1690}$) which differ in their lipid A content provided the opportunity to examine innate host responses to individual lipid A species found in *P. gingivalis*. Surprisingly, these two different preparations yielded opposite effects on E selectin expression on human endothelial cells. One preparation, $PgLPS_{1435/1450}$, was similar to our other LPS preparations containing similar lipid A mass ions (Bainbridge et al, 2002; Darveau et al, 2004) and was an E selectin antagonist. The other preparation, $PgLPS_{1690}$, was an E selectin agonist.

One significant consequence of *P. gingivalis* LPS containing two different lipid A species which have opposing effects on endothelial cells is to modulate the innate host response through alteration of the relative amounts of these lipid A species. Examination of the structures of these two different lipid A mass ions reveals that the removal of one of the ester linked fatty acids from $PgLPS_{1690}$ will yield mass ions of either m/z 1435 or m/z 1450 (depending upon whether the C15i3OH or the C163OH fatty acid is removed, see Fig 2). Removal of ester linked fatty acids has been reported in other bacteria (Basu et al, 1999) and may alter innate host responses (Kawasaki et al, 2004) although antagonistic effects of one lipid A species on the other has not been previously observed. The ability to significantly modify the innate host response by minor alterations in lipid A structure may represent one adaptation made by this bacterium in response to changing local host microenvironments.

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