Genetic background of Porphyromonas gingivalis capsule biosynthesis

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Chapter 4

The capsule of *Porphyromonas gingivalis* reduces the immune response of human gingival fibroblasts

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Abstract

Periodontitis is a bacterial infection of the periodontal tissues. The Gram negative anaerobic bacterium *Porphyromonas gingivalis* is considered a major causative agent. One of the virulence factors of *P. gingivalis* is capsular polysaccharide (CPS). Non-encapsulated strains have been shown to be less virulent in mouse models than encapsulated strains.

To examine the role of the CPS in host-pathogen interactions we used the *epsC* mutant, the W83 wild type strain and the complemented mutant to challenge human gingival fibroblasts to examine the immune response by quantification of *IL-1β*, *IL-6* and *IL-8* transcription levels. For each of the cytokines significantly higher expression levels were found when fibroblasts were challenged with the *epsC* mutant compared to those challenged with the W83 wild type, ranging from two times higher for IL-1β to five times higher for IL-8.

These experiments provide the first evidence that *P. gingivalis* CPS acts as an interface between the pathogen and the host that may reduce the host's pro-inflammatory immune response. The higher virulence of encapsulated strains may be caused by this phenomenon which enables the bacteria to evade the immune system.

Background

Porphyromonas gingivalis is a major pathogen in destructive periodontal diseases including chronic and aggressive periodontitis that are characterized by breakdown of the tooth-supporting tissues [1-3]. *P. gingivalis* is a black pigmented, often encapsulated, strict anaerobic, Gram negative coccobacillus that occurs in the human oral cavity.

Among the variety of virulence factors that have been described for *P. gingivalis*, CPS has shown to be a major factor in experimental infections. Studies in a mouse infection model have revealed that encapsulated *P. gingivalis* strains are more virulent than non-encapsulated strains [4-7]. Non-encapsulated strains mostly cause non-invasive, localized abscesses whereas encapsulated strains cause invasive, spreading phlegmonous infections after subcutaneous inoculation of experimental animals.

Six distinct capsular serotypes have currently been described (K1-K6) [8,9] and a seventh serotype (K7) has been suggested by R. E. Schifferle (personal communication). Small differences in virulence have been found between capsular serotypes and strong variation in virulence has been described between strains of the same capsular serotype [10]. CPS of all serotypes has been tested for induction of immunological responses in macrophages and it has been revealed that the CPS of K1 serotype strains induces higher chemokine expression in murine peritoneal macrophages than the other serotypes [11]. These data suggest that the K1 CPS plays an important role in host-pathogen interaction. The chemical composition of the K1 CPS has been studied to a limited extent. It has been reported that the CPS of K1 (strain W50) comprises of mannuronic acid (ManA), glucuronic acid (GlcA), galacturonic acid (GalA), galactose and N-acetylglucosamine (GlcNAc), but the CPS structure has not been solved [12].

Although CPS is a major structure at the interface between the bacterial cell and the host, the exact role of *P. gingivalis* CPS is not yet clear. Adhesion to epithelial cells has been shown to be higher for non-encapsulated *P. gingivalis* and the level and mechanism of co-aggregation has been shown to be CPS dependent [5,13,14]. In many pathogens CPS has been found to be involved in evasion of the host immune system by circumvention of phagocytosis, opsonization and complement killing [15-17].

The aim of this study was to investigate *in vitro* differences in host response during infection with a wild type and an isogenic non-encapsulated mutant of a naturally encapsulated strain. The well-studied K1 serotype W83 strain was used as the wild type strain since its CPS biosynthesis locus has

been described [18,19]. Its non-encapsulated insertional *epsC* mutant described in chapter 2 of this thesis was used as well as its complemented, encapsulated, counterpart.

The *epsC* mutant is tested in a fibroblast infection model [20] since fibroblasts are the most abundant stromal cells in soft connective tissue of the gingiva [21] and among the first cells encountering periodontal infections by anaerobic bacteria like *P. gingivalis*. And above all, fibroblasts have been shown to be involved in the immune response in periodontitis [22,23]. Human gingival fibroblasts were infected with W83 and the *epsC* mutant and complemented mutant. Transcription of *IL-1β*, *IL-6* and *IL-8* was determined as host response parameters. This study provides the first direct evidence that *P. gingivalis* CPS reduces the host immune response, thereby potentially enabling evasion of the immune system to sustain successful long-term infection.

Results

Sedimentation

To ensure that the tested strains have the same chance to get in contact with the fibroblasts seeded on the bottom of a flask a sedimentation experiment was performed. Sedimentation of the *epsC* mutant in comparison to W83 was analyzed in the same buffer as used in the infection experiments. No significant sedimentation differences were found between W83 and the *epsC* mutant within the 6 hours needed for infection of the fibroblasts (data not shown).

Survival

Since infections were done with viable *P. gingivalis*, survival of the bacteria during the 6-hour aerobic period of infection in DMEM medium had to be ensured. Therefore a 6-hour survival experiment was performed in the 24-well plates used for the fibroblast challenge. On average 60-75% of W83, *epsC* mutant and complemented mutant cells survived for 6 hours in Dulbecco's modified Eagle's Medium (DMEM; Sigma Chemical Co.) supplemented with 10% fetal calf serum (FCS) (figure 1).

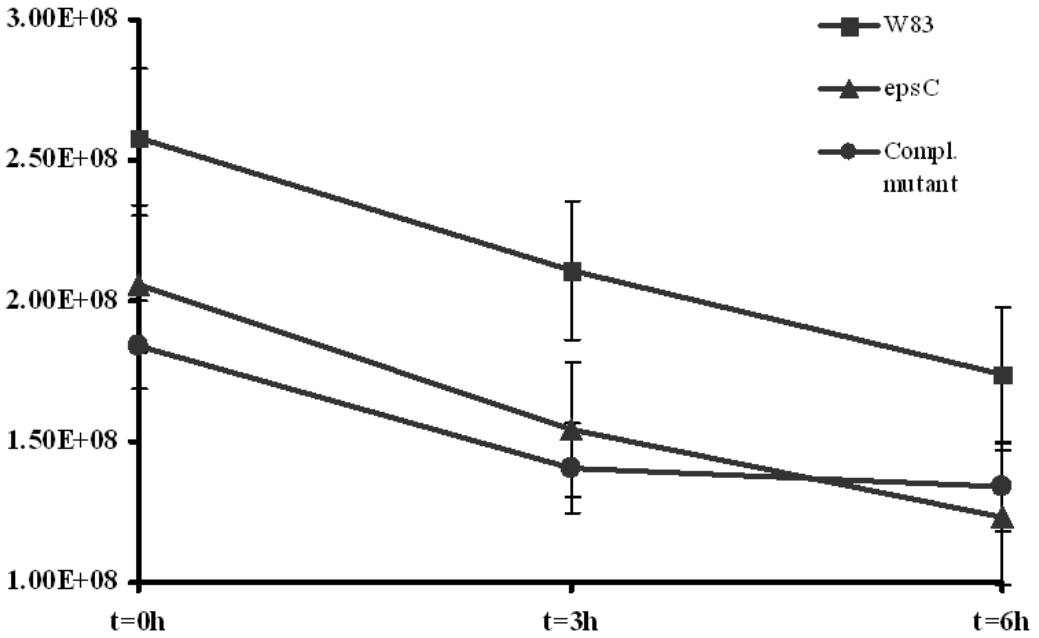

Figure 1. 6-hour survival of W83, the *epsC* mutant and the complemented mutant in aerobic experimental conditions. Survival of W83, the *epsC* mutant and the complemented mutant in 0.5 ml DMEM + 10% FCS under humidified 5% CO_2 in air conditions was determined by cfu-counts on BA + H/M plates. Survival was 67%, 60% and 73% for each strain respectively. Error bars represent the standard deviations of triplicate measurements.

Fibroblast response to *P. gingivalis* challenge

To study the effect of the lack of CPS on the host immune response six hour infection studies of human gingival fibroblasts with W83 and the *epsC* mutant were performed. Figure 2 shows *IL-1β*, *IL-6* and *IL-8* expression of infected gingival fibroblasts relative to the non-infected negative control which is set to 1 and normalized against expression of housekeeping gene *GAPDH*.

At multiplicity of infection (MOI) 1000:1 of both strains a small induction of the tested genes could be detected compared to the non-infected control, but significant induction for all three genes was found when MOI 10.000:1 was used for infection. At MOI 1000:1 *IL-6* and *IL-8* expression showed a significantly higher induction (150-fold and 37-fold induction respectively) in the cells challenged with the *epsC* mutant when compared to the wild-type (6-fold and 2-fold induction respectively), *IL-1β* did not show a difference compared to the wild-type. However, when gingival fibroblasts were

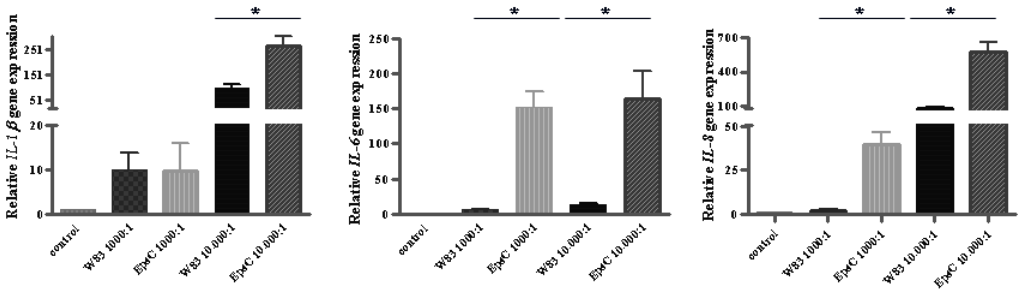

Figure 2. Relative expression of *IL-1β*, *IL-6* and *IL-8* genes in human gingival fibroblasts (HGF1) infected with *P. gingivalis* W83 and the *epsC* mutant. After a 6-hour challenge with *P. gingivalis* cells at MOI 1000:1 or 10.000:1 as indicated on the Y-axis, the expression levels of *IL-1β*, *IL-6* and *IL-8* in human gingival fibroblasts were measured using RT-PCR and represented as a relative value compared to a non-infected control sample which is set to a value of 1. Significant differences $p<0.01$ are indicated by an asterisk.

challenged with MOI 10.000 bacteria all three tested genes showed a significantly higher induction in the cells challenged with the *epsC* mutant than with W83 (figure 2).

A separate experiment which also included the complemented mutant showed that when fibroblasts were challenged with the complemented mutant the response was almost completely restored to wild-type levels (figure 3).

Discussion

The aim of this paper was to understand the role of *P. gingivalis* CPS in the response of human gingival fibroblasts *P. gingivalis* CPS has been regarded as an important virulence factor. It has been shown to induce inflammatory mediators in *in vitro* studies [11]. The capsule also plays an important role in shielding of immune response inducers in several bacterial species [25-27]. Since a distinct CPS biosynthesis locus in *P. gingivalis* has been described and shown to be functional [18,19], studying the role of *P. gingivalis* CPS in the immune response by use of a mutant became feasible. For this purpose the non-encapsulated *epsC* mutant was constructed (chapter 2) to block capsule synthesis.

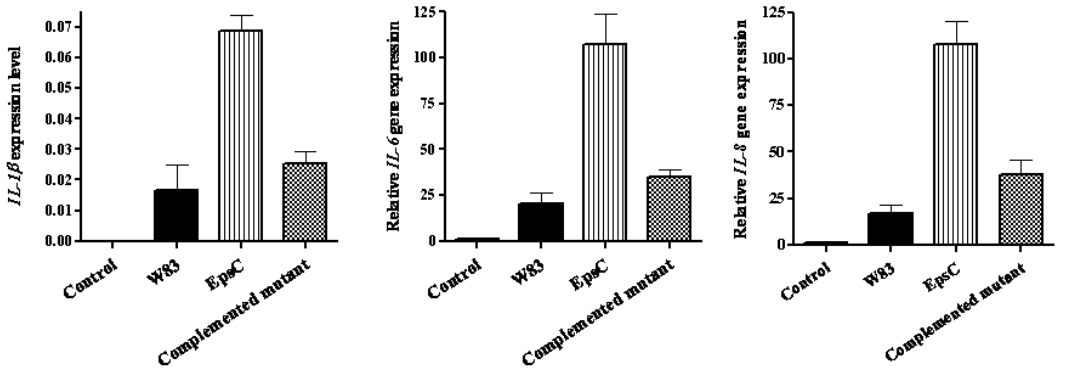

Figure 3. Effect of complementation of the *epsC* mutant on the immune response mutant of human gingival fibroblasts (HGF2). After a 6-hour challenge with *P. gingivalis* cells at MOI 10.000:1, the expression levels of *IL-1β*, *IL-6* and *IL-8* in human gingival fibroblasts were measured using RT-PCR and if possible represented as a relative value compared to a non-infected control sample which is set to a value of 1. Relative *IL-1β* expression could not be calculated as *IL-1β* was not detected in the non-infected control. Complementation almost restored the wild-type situation for *IL-1β* (83%), *IL-6* (83%) and *IL-8* (77%)

The *epsC* mutant was expected to have altered immunological properties. To examine the role of CPS, both the wild-type and the *epsC* mutant were used in an *in vitro* challenge of primary human gingival fibroblasts. Since the *epsC* mutant has altered physical properties, it was important to compare the sedimentation rate and viability of both the wild type and the mutant strain since these could have influenced the amount of living bacterial cells that are in contact with the fibroblasts. No differences were observed between the strains during the 6 hours of infection.

From the infection experiments of the gingival fibroblasts it became apparent that pro-inflammatory mediators *IL-1β*, *IL-6* and *IL-8* expression levels were up-regulated after a 6-hour challenge with both wild-type W83 and the *epsC* mutant in comparison to the non-infected control, especially when MOIs of 10.000:1 were used.

A challenge with the *epsC* mutant induced a significantly higher pro-inflammatory immune response than a challenge with the wild type W83, as shown by *IL-1β*, *IL-6* and *IL-8* gene expression. So, even though purified *P. gingivalis* CPS has been shown to stimulate pro-inflammatory cytokine expression in murine peritoneal macrophages [11] the absence of capsule induces extra cytokine induction when viable *P. gingivalis* cells were used to challenge fibroblasts.

Capsular polysaccharides of several bacteria have been implicated in down-regulation of pro-inflammatory cytokine production, including *Klebsiella pneumonia* [29]. *Bacteroides fragilis* capsular polysaccharide complex has been shown to induce IL-10 expression, a regulating cytokine which may cause suppression of the immune system [30].

An explanation of our results may be that the CPS prevents more potent immune inducers to be recognized by Toll-like receptors on the fibroblasts. It has been shown that the capsular antigen in *Salmonella typhi*, referred to as Vi-antigen, is able to prevent Toll-like receptor 4 recognition of LPS, thereby reducing expression of pro-inflammatory TNF-α and IL-6 [31-33]. In *E. coli* the capsule may cover short (10 nm) bacterial adhesins, which do not penetrate the 0.2-1.0 µm capsular layer, preventing them from being recognized by the immune system [26]. Likewise, *P. gingivalis* strain W83 was described as to have a small amount of short fimbriae that might be mostly covered by the CPS [34].

Another or additional explanation of our findings could be immune suppression by *P. gingivalis* CPS, meaning that CPS would actively modulate the immune response of the fibroblasts, leading to lower inflammatory cytokine expression levels, potentially enabling *P. gingivalis* to evade the immune system.

For several bacteria it has been described that capsular biosynthesis can be modulated depending on environmental conditions [35,36]. Although presently no regulation of *P. gingivalis* capsule expression has been described, we can not exclude the possibility that in the *in vivo* situation capsule expression is regulated. However, the reduced pro-inflammatory host's immune response by the encapsulated strain may explain the documented differences between natural *P. gingivalis* strains in spreading. Whereas non-encapsulated strains are tackled directly by the immune system in localized abscesses, the more virulent encapsulated strains can evade this defense and cause phlegmonous infections [4-7].

Conclusions

The absence of CPS results in increased induction of *IL-1β*, *IL-6* and *IL-8* in human gingival fibroblasts upon *in vitro* infection with viable *P. gingivalis* cells. *P. gingivalis* CPS acts as a functional interface between the pathogen and the host. The CPS-related reduced pro-inflammatory response may explain why naturally non-encapsulated strains cause localized abscesses and encapsulated strains spreading phlegmonous infections.

Methods

Bacterial maintenance

P. gingivalis strains were grown either on 5% horse blood agar plates (Oxoid no. 2, Basingstoke, UK) supplemented with hemin (5 µg/ml) and menadione (1 µg/ml) (BA+H/M plates) or BHI+H/M, both, at 37°C in an anaerobic atmosphere of 80% N2, 10% H2, and 10% CO2. Mutants were grown in the presence of 5 µg/ml erythromycin. Complemented mutants were grown in the presence of and 1 µg/ml tetracycline. Purity of *P. gingivalis* liquid and plate-grown cultures was routinely checked by Gram staining and microscopic examination.

Sedimentation of *P. gingivalis*

W83 and the *epsC* mutant were grown anaerobically for 18 hours in BHI+H/M at 37°C. After 3 wash steps in phosphate buffered saline (PBS) the OD690 was standardized to 5 in DMEM with 10% FCS. 10 ml of this culture was added to 40 ml DMEM with 10% FCS in a 100 ml flask to set the OD690 to 1. The cultures were incubated standing still at 37°C for six hours. At regular time intervals, a 200 µl sample was taken 0.5 cm from the liquid surface and the decrease of the OD690 values was determined as a measure for sedimentation.

Survival of P. gingivalis

W83, the *epsC* mutant and the complemented mutant were grown anaerobically for 18 hours in BHI+H/M at 37°C. After 2 wash steps in phosphate buffered saline (PBS) the pellets were resuspended in DMEM with 10% FCS to an OD690 of 0.05 as used in fibroblast infections at MOI 10.000:1. 500 µl of these suspensions was incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Samples for cfu-counts were taken at t = 0 hours, t = 3 hours and t = 6 hours and dilutions were plated on BA+H/M plates.

Human gingival fibroblasts

The gingival fibroblasts (HGF1 and HGF2) used in this study were collected from extracted third molars of two periodontally healthy subjects with a high pro-inflammatory immunological response when challenged with *P. gingivalis* [20]. Donors had given written informed consent, and the study was approved by the VUmc Medical Ethical committee.

Growth curve

Pre-cultures of W83 and the *epsC* mutant were grown anaerobically for 18 hours in BHI+H/M at 37°C. The pre-cultures were diluted to an OD690 of 0.05 *in duplo* in fresh BHI+H/M and incubated anaerobically at 37°C. Every few hours the OD690 was measured and a sample was taken for cfu-counts.

Infection of gingival fibroblasts with *P. gingivalis*

Bacteria were grown overnight for 18 hours in BHI+H/M. The bacterial cells were washed three times in PBS and then used to infect gingival fibroblasts at MOIs of 1000:1 and 10.000:1 (bacteria cells: fibroblasts) in a total volume of 500 µl DMEM with 10% FCS in 24-well plates. The plates were incubated for 6 hours at 37°C in a humidified atmosphere of 5% CO2 in air. The cells were washed twice with cold PBS. Then 350 µl lysis buffer (1% β-mercapthanol in RLT buffer) was added to the cells according to the protocol of Qiagen RNeasy® mini kit (Qiagen Benelux B.V.) after which the plate was stored at -80°C for later use.

RNA isolation and reverse transcription

mRNA was isolated from the gingival fibroblast lysates according to the manufacturer's protocol of Qiagen RNeasy® mini kit (Qiagen Benelux B.V.). The mRNA concentrations of the samples were determined using the Nanodrop ND_1000 (Isogen Life Science). mRNA was reverse transcribed using the Fermentas first-strand cDNA synthesis kit (Fermentas GmbH, St. Leon-Rot, Germany) according to the manufacturer's protocol.

Real-Time PCR

cDNA synthesized from mRNA isolated from gingival fibroblasts after infection with *P. gingivalis* was analyzed in quadruple using Real-Time PCR with gene-specific primers on a ABI Prism 7000 Sequence Detecting System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Reactions were performed with 2 ng cDNA in a total volume of 8 µl containing SYBR Green PCR Master Mix (Applied Biosystems) and 0.99 pM of each primer. After activation of the AmpliTaq Gold DNA polymerase for 10 minutes at 94°C, 40 cycles were run of a two step PCR consisting of a denaturation step at 95°C for 30 seconds and annealing and extension step at 60°C for 1 minute. Predicted product sizes were in the 100-200 bp range. Subsequently the PCR products were subjected to melting curve analysis to test if any unspecific PCR products were generated. The PCR reactions of the different

amplicons had equal efficiencies. Samples were normalized for the expression of housekeeping gene *GAPDH*, which is not affected by the experimental conditions, by calculating the Δ Ct (Ct housekeeping gene - Ct gene of interest) and expression of the different genes is expressed as 2-($^{\Delta}$Ct). Fold increase in gene expression (induction) was expressed by 2 -($^{\Delta\Delta}$Ct), wherein ΔΔCt = ΔCtchallenged- average Ct-value non-challenged.

Statistical analysis

Differences in gene induction between multiple groups were tested by one-way analysis of variance (ANOVA) and Bonferroni's Multiple Comparison Test. Tests were performed with GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. Differences were considered significant at $p < 0.01$.

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