A lipid A-associated protein of *Porphyromonas gingivalis*, derived from the haemagglutinating domain of the RI protease gene family, is a potent stimulator of interleukin 6 synthesis

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There is evidence that the lipid A-associated proteins (LAPs) of enteric bacteria can induce the synthesis of interleukin 1 (IL-1) and therefore may be important virulence factors. *Porphyromonas gingivalis* is now recognized as a major pathogen in the chronic inflammatory periodontal diseases and it has previously been reported that a crude LAP fraction from this organism could induce IL-1 and interleukin 6 (IL-6) synthesis. In the present study the chemical and biological properties of the LAPs of this bacterium have been further characterized. Analysis by SDS-PAGE has shown that the LAPs comprise nine proteins of molecular masses 81, 68, 48, 47, 28, 25, 20, 17 and 16 kDa. These LAPs, at concentrations as low as 100 ng ml⁻¹, were shown to stimulate human gingival fibroblasts, human peripheral blood mononuclear cells and whole human blood to produce the pro-inflammatory cytokine IL-6. The cytokine-inducing activity of the LAPs was reduced after heat-inactivation and trypsinization, suggesting that the activity was not due to contaminating LPS. To establish which proteins in this mixture were the active cytokine inducers, the LAPs were separated by electrophoresis on polyacrylamide gels. The majority of the activity was associated with the 17 kDa LAP. N-terminal sequence analysis demonstrated that this protein was homologous to an internal region of a conserved adhesin domain contained within a family of *P. gingivalis* extracellular proteins including the RI protease, Lys-gingipain, porphypain and haemagglutinin A. In addition to a role in adherence, the adhesin domain(s) of these proteins may also have cytokine-inducing properties. Furthermore, it has also been shown that the previously observed degradation of cytokines by *P. gingivalis* may be attributable to the catalytic domain of the RI protease. Thus, different domains within the same molecule appear to have opposing actions on pro-inflammatory cytokine levels and the balance between these two activities may influence the cytokine status of the periodontium in patients with the common chronic inflammatory conditions known as the periodontal diseases.

Keywords: *Porphyromonas gingivalis* RI protease, lipopolysaccharide, lipid A-associated protein, interleukin 1 β , interleukin 6

Abbreviations: HGFs, human gingival fibroblasts; IL-1 β , interleukin 1 β ; IL-6, interleukin 6; LAL, *Limulus* amoebocyte lysate; LAP, lipid A-associated protein; PBMCs, peripheral blood mononuclear cells; P-LPS, *P. gingivalis* LPS.

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INTRODUCTION

The Gram-negative anaerobic bacterium *Porphyromonas gingivalis* is implicated as an important pathogen in the periodontal diseases (Kurimatsu *et al.*, 1995). Much attention has focused on the endotoxin and LPS of this organism and these components are believed to be major virulence factors of this bacterium. While acute exposure to LPS can produce extensive pathology, it is known that LPS is rapidly inactivated (Emancipator *et al.*, 1996) and repeated exposure produces selective tolerance (Ziegler-Heitbrock *et al.*, 1995). It has also been suggested that *P. gingivalis* LPS (P-LPS) has a weak ability to activate inflammation (Reife *et al.*, 1995). Therefore it is unclear what role LPS plays in chronic inflammation. LPS requires CD14 to produce its full agonist response and a recent report has established that the LPS from *P. gingivalis* has a low affinity for CD14 (Cunningham *et al.*, 1996), again suggesting that this bacterial component may be of limited pathological potential.

It is now established that endotoxin consists of LPS and proteins associated with the LPS in the outer membrane (Bjornson *et al.*, 1988). These proteins, variously known as lipid A-associated proteins (LAPs) (Morrison & Jacobs, 1976), endotoxin-associated proteins (Phillips *et al.*, 1989) and endotoxin proteins (Sultz, 1968), were first recognized when it was discovered that the B cell mitogenic activity of *Escherichia coli* endotoxin in the LPS non-responder C3H/HeJ mouse depended on the protein content of the endotoxin (Sultz, 1969; Sultz & Goodman, 1976). The LAPs are isolated from the endotoxin by hot-phenol extraction to give an operationally defined population of proteins. The LAPs extracted from a number of enteric bacteria have been shown to have potent immunomodulatory properties (Goodman & Sultz, 1979b; Sultz *et al.*, 1985). Furthermore, the enteric LAPs have been noted for their extreme stability to both heat and proteinases (Bjornson *et al.*, 1988; Goodman & Sultz, 1979a, b), which accounts for their ability to resist denaturation when extracted in phenol at 68 °C. This stability, linked to the rapid removal and inactivation of LPS by serum proteins (e.g. high-density lipoproteins) (Wurfel *et al.*, 1994, 1995), suggests that the LAPs may play an important role in the biological actions of endotoxin.

In earlier studies we had shown that a preparation of LAPs from *P. gingivalis* was able to stimulate human gingival fibroblasts (HGFs) and the myelomonocytic cell line (Mono-Mac-6) to produce interleukins 1 and 6 (IL-1 and IL-6) (Reddi *et al.*, 1995b). In this study we have characterized the LAPs and have identified the components in this population of proteins responsible for cytokine induction.

METHODS

Extraction of endotoxin. *P. gingivalis* W50 was grown on Wilkins Chalgren agar (Oxoid) supplemented with 5% (v/v) horse blood under anaerobic conditions for 72 h at 37 °C. The

bacterial cultures were Gram-stained to check for contamination with Gram-positive organisms, harvested from the plates by gentle scraping in sterile saline and sedimented by centrifugation at 30000 g for 30 min at 4 °C. We had previously demonstrated that *P. gingivalis* contained a large amount of bioactive material on its outer surface (Wilson *et al.*, 1993). To remove this bioactive extracellular material, the bacteria were resuspended in saline, stirred for 1 h at 4 °C and harvested by centrifugation. This process was then repeated. Endotoxin was then extracted from the surface-washed *P. gingivalis* using the method of Morrison & Leive (1975). Briefly, bacteria were suspended in sterile saline and mixed with an equal volume of water-saturated butanol for 10 min at 4 °C. The phases were separated by centrifugation at 35000 g for 20 min and the aqueous phase was removed. The butanol phase was washed a further two times with approximately half the volume of saline. The aqueous phases were combined and centrifuged to remove any insoluble residues before dialysis for 48 h with six changes of distilled water at 4 °C. The endotoxin was lyophilized and then washed twice by resuspending in water, followed by ultracentrifugation at 100000 g for 1 h. The resulting endotoxin pellet was recovered and lyophilized.

Preparation of LAPs. The LAPs were prepared by hot-phenol extraction as described previously (Westphal & Jann, 1965; Wober & Alaupovic, 1971). Briefly, the endotoxin was suspended in distilled water and mixed with an equal volume of phenol (Sigma; 90%, w/v) for 10 min at 68 °C. The phenol phases were separated by centrifugation at 35000 g and the aqueous phase was removed. The phenol phase was washed twice more with distilled water, before being dialysed against distilled water for 96 h. The LAPs were then lyophilized and stored at -70 °C until required.

Extraction of P-LPS. The aqueous phases from the phenol/water extraction were pooled and dialysed against distilled water for 96 h, then lyophilized. Protein and nucleic acids were removed by DNase/RNase and proteinase K treatment. The LPS was washed by resuspension in pyrogen-free water and collection by ultracentrifugation at 100000 g for 1 h at 4 °C. The wash was repeated before the LPS was lyophilized. P-LPS prepared by the cold MgCl₂ method (Darveau & Hancock, 1983) was also used.

Analysis of LAPs. The protein content of the LAPs was estimated using the detergent-compatible Protein Assay kit (Bio-Rad). The LPS contamination in the LAP preparations was determined by the *Limulus* amoebocyte lysate (LAL) assay used as described in the European Pharmacopoeia (1997). P-LPS was also assessed in the LAL assay. To remove any contaminating LPS, the LAP preparations were applied to a polymyxin B affinity column (Detoxigel column; Pierce). The LAL assay was used to assess the removal of LPS by this treatment.

The heterogeneity of the LAPs was analysed by SDS-PAGE using the method of Laemmli (1970); 12% gels were used and the proteins were visualized with colloidal Coomassie blue stain (Sigma).

Heat and trypsin treatment of LAPs. LAPs (10 µg ml⁻¹) were digested with trypsin-coated beads (2.5 U ml⁻¹) (Sigma), in 50 mM Tris/HCl, pH 7.6, at 37 °C for 2 h. LAPs, at the same concentration, were heat-treated for 45 min at 85 °C. Materials treated in these ways were tested for activation of IL-6 synthesis by cultured cells.

Stimulation of IL-6 synthesis by HGFs, human peripheral blood mononuclear cells (PBMCs) and human whole blood.

HGFs, prepared from explants of gingivae obtained during routine oral surgery, were used between passages 6 and 12, as described previously (Reddi *et al.*, 1995b). The cells were dispensed into 24-well culture plates at 30000 cells per well and incubated overnight in DMEM containing 2% (v/v) foetal calf serum, at 37 °C in 5% CO₂/air to allow adherence. The cells were then exposed to various concentrations of the LAPs, P-LPS or *E. coli* LPS overnight. As a control, cells were incubated with media alone.

Heparinized whole blood, collected from healthy individuals, was diluted five times with RPMI-1640 media; 0.5 ml of this blood mixture was dispensed into the individual wells of 24-well culture plates. LAPs or LPS were then added at a range of concentrations to each of the wells and the plates were incubated at 37 °C in 5% CO₂ for 18 h. The culture supernatants were collected by transferring the contents of each well to sterile microfuge tubes and centrifuging at room temperature for 10 min at 9000 g. The resultant supernatants were removed and diluted five times in ELISA wash buffer prior to assay of IL-6.

Human PBMCs enriched for monocytes were prepared from buffy coats of mixed human blood (obtained from the South London Blood Transfusion Service, St George's Hospital, London) by Ficol-Hypaque centrifugation and differential adherence. These PBMCs were dispensed into 24-well plates at a density of 2×10^6 cells per well in RPMI-1640 containing 2% (v/v) foetal calf serum and were incubated for 24 h with either various concentrations of the LAPs or LPS. The medium was then collected into microfuge tubes and centrifuged at 10000 g to remove any cells and then assayed for IL-6.

IL-6 assay. IL-6 immunoassay was performed as described by Taktak *et al.* (1991). Briefly, microtitre plates were coated with immunoaffinity-purified, polyclonal goat antibodies to human IL-6 at 1 µg ml⁻¹ in PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.2) overnight at 4 °C. The unbound antibody was removed from the plates before washing three times with 0.01 M phosphate/0.05% (w/v) NaCl containing 0.1% (v/v) Tween 20 (wash buffer). One hundred microlitres of culture supernatant sample was added to the wells. The standard for IL-6 was the International Standard for IL-6 preparation 89/548, used over a concentration range from 4 to 9000 pg ml⁻¹ in threefold dilutions. The plates were incubated for 2 h at room temperature before washing three times in wash buffer. Biotinylated, affinity-purified, polyclonal goat anti-IL-6 antibodies were diluted to a concentration of 1 µg ml⁻¹ and 100 µl was added to each well before incubation at room temperature for 1 h. The plates were washed three times before a 1/5000 dilution of avidin-horseradish peroxidase (Dako) was added to each well and incubated for 15 min at room temperature. Following three more washes, 100 µl 0.2 mg o-phenylenediamine (Sigma) ml⁻¹ in 0.1 M citric acid/phosphate buffer, pH 5.0, with 0.4 µl 30% H₂O₂ (Sigma) ml⁻¹ was added to each of the wells, and incubated in the dark until the colour started to develop. The reaction was stopped using 150 µl 1 M H₂SO₄ (Sigma) and the A₄₉₂ values were measured on a Titertek Multiskan spectrophotometer (Flow Laboratories).

Electrophoretic separation of LAPs. The LAPs were separated by a modification of the conventional SDS-PAGE technique. Approximately 200 µg *P. gingivalis* LAPs was dissolved in non-reducing buffer (0.0625 M Tris/HCl, 10% glycerol, 0.025% bromophenol blue) containing 1% SDS and, rather than boiling, was incubated at room temperature for 1 h.

Proteins were then separated using conventional SDS-PAGE. This modification separated proteins in a similar manner to the conventional SDS-PAGE with denatured proteins. To identify the regions in the gels containing proteins, unstained gels were compared with Coomassie-blue-stained gels and the regions containing distinct protein bands were then cut from the gel and placed into sterile PBS and homogenized. The samples were mixed overnight at 4 °C to allow the protein to diffuse from the gel. The gel was then removed from the samples by centrifugation down a 0.1 mm spin column (Whatman) and the samples were dialysed to remove the SDS and then tested for their capacity to stimulate PBMCs to secrete IL-6.

Sequence analysis. LAPs were separated by conventional SDS-PAGE as previously described. The separated proteins were then blotted onto ProBlott membranes (Applied Biosystems) according to the method of Matsudaira (1987). The N-terminal sequences of the LAPs were determined by Edman degradation, using a model 477A pulsed liquid protein sequencer (Applied Biosystems). The sequence data obtained were compared with protein and nucleotide databases using the National Centre for Biotechnology Information BLAST network service.

Assessment of IL-1β degradation by purified RI protease.

Purified *P. gingivalis* RI protease (Curtis *et al.*, 1996) was diluted to a concentration of 4 U ml⁻¹ in assay buffer (0.1 M Tris/HCl, pH 8.0, 10 mM Ca²⁺, 10 mM cysteine.HCl). One unit of RI protease catalyses the formation of 1 µmol *p*-nitroaniline min⁻¹, using the substrate benzoyl-DL-arginine *p*-nitroanilide at 30 °C. Human recombinant IL-1β (a gift from Dr A. Shaw, Glaxo, Geneva) was added to 100 µl of the enzyme suspension to give a final concentration of 2.5 µg ml⁻¹ and incubated anaerobically at 37 °C. Samples of 15 µl were removed from the tube after incubation times of 15 min, 30 min, 1 h, 2 h and 4 h. Human recombinant IL-1β was also incubated for 4 h with RI protease which had been heat-inactivated by boiling for 30 min in assay buffer. The protease activity was immediately stopped at each sample time by adding an equal volume of 20% (v/v) formic acid protein denaturant. The samples were dried on a Speedivac (Savant) and stored at -70 °C until they were analysed. To ensure complete inactivation of the RI protease prior to electrophoresis, each of the samples was resuspended in 15 µl of the protease inhibitor leupeptin (1 mg ml⁻¹) and incubated at room temperature for 1 h before heating at 100 °C for 3 min. Samples were analysed by Western blotting. The reaction mixtures were separated on 18% SDS-PAGE gels before transferring to Immobilon-P PVDF membranes (Millipore) by the method of Towbin *et al.* (1979). After incubating for 1 h in blocking buffer, the membrane was incubated with a 1:1000 dilution of sheep anti-human-IL-1β polyclonal antibodies. The bands were detected using a 1:1000 dilution of peroxidase-conjugated rabbit anti-sheep antibodies (Dako) detected by incubation with Sigma Fast 3,3'-diaminobenzidine.

Bioassay to show the loss of activity of IL-1β following digestion with RI protease.

Induction of IL-6 release from human monocytes was used to assay the biological activity of IL-1β following digestion with the RI protease. Human monocytes were prepared and plated out as described earlier. The digested IL-1β time-course samples, diluted 1:1250 in DMEM, were added to the wells and incubated overnight at 37 °C in 5% CO₂/air. The cell media were then collected and assayed for IL-6 by ELISA. IL-1β which had not been incubated with RI protease was added to the cells to act as a control.

RESULTS

Analysis of *P. gingivalis* LAPs

The yield of LAPs from *P. gingivalis* was of the order of 0.1% (weight of LAPs/dry weight of bacteria). Using the detergent-compatible Protein Assay the mean protein content of the isolated LAP preparations was 11% (w/w). Previous studies have shown that the remainder of the material is lipid (Reddi *et al.*, 1995b). The preparation of LAPs had an endotoxin activity of 30 IU μg^{-1} in the LAL assay, which was reduced to <0.01 IU μg^{-1} after passage down a Detoxigel column. Colloidal Coomassie blue staining of the SDS-PAGE gel (Fig. 1) revealed nine protein bands corresponding to approximate molecular masses of 81, 68, 48, 47, 28, 25, 20, 17 and 16 kDa. The P-LPS had an activity in the LAL assay of 3×10^6 IU mg^{-1} . Silver staining of the SDS-PAGE gel of P-LPS showed a typical ladder pattern.

IL-6-stimulating activity of LAPs

The addition of the LAPs to cultured HGFs, monocytes or whole blood caused the dose-dependent production of IL-6 with a maximum effect at 5 $\mu\text{g ml}^{-1}$ with HGFs and PBMCs. In some experiments the LAPs demonstrated significant IL-6-stimulating activity at concentrations as low as 100 ng ml^{-1} (Fig. 2). Exposure of the LAPs to heat or trypsin caused a significant decrease in the IL-6-inducing activity when added to human monocytes at 1 $\mu\text{g ml}^{-1}$ (1945 ± 120 and 1922 ± 533 pg ml^{-1} , respectively, compared to 3982 ± 598 pg ml^{-1} in un-

Fig. 1. SDS-PAGE analysis of *P. gingivalis* LAPs stained with Coomassie blue. Lane 1 shows the molecular mass markers and lane 2 shows *P. gingivalis* LAP preparation (10 $\mu\text{g ml}^{-1}$) boiled for 5 min in reducing buffer. The molecular masses of the proteins (kDa) are as follows: 1, 81; 2, 68; 3, 48; 4, 47; 5, 28; 6, 25; 7, 20; 8, 17; 9, 16.

Fig. 2. IL-6 release by (a) HGFs, (b) whole blood and (c) human monocytes, stimulated with various concentrations of *P. gingivalis* LAPs. This is a typical result expressed as the means $\pm$ SD ($n = 3$).

Table 1. IL-6 production by human monocytes induced with LPS from *E. coli* (E-LPS) and *P. gingivalis* (P-LPS) and LAP from *P. gingivalis* (P-LAP)

Cell stimulant	LPS (IU ml^{-1})	IL-6 production* (pg ml^{-1})
E-LPS	7	6000 ± 98
P-LAP	30	4010 ± 62
P-LPS	30	624 ± 20
P-LPS	300	642 ± 5
P-LPS	3000	2824 ± 82

* Mean of triplicate experiments $\pm$ SD.

treated LAPs; mean of triplicate experiments $\pm$ SD). However, neither treatment completely abolished activity.

Role of LPS contamination in the IL-6-inducing activity of the LAPs

To assess the importance of the LPS contamination to the biological activity of the LAPs, the cytokine-inducing activity of both the P-LPS and the LAPs was compared with their LPS content as defined by their activity in the LAL assay. It was shown that 3000 IU P-LPS was required to stimulate a similar response to a LAP sample containing only 30 IU P-LPS (Table 1).

Fig. 3. IL-6 production by human monocytes stimulated with LAP fractions. LAPs were separated on SDS-PAGE and extracted from the polyacrylamide gel as described in Methods. Monocytes were treated with 10 µl (▨) or 100 µl (□) of each fraction to identify the cytokine-stimulating components. A limited amount of stimulation was caused by fractions 7 and 9 but most of the activity is in fraction 8, which corresponds to the 17 kDa protein. Results are expressed as means of triplicate wells $\pm$ SD.

IL-6-stimulating activity of fractionated LAPs

Electrophoresis of the undenatured LAPs in the presence of SDS resulted in a separation similar to that seen when the proteins were boiled in SDS sample buffer (Fig. 1). Unstained lanes in the polyacrylamide gel were compared with the Coomassie-blue-stained lanes in a parallel gel and areas corresponding to stained bands were cut out, eluted and dialysed before testing for activity on human PBMCs. As the protein content of each sample was too low to measure, 10 or 100 µl of each fraction (from a total volume of 500 µl) was added to cells. The majority of the IL-6-stimulating activity coincided with the band corresponding to the 17 kDa protein. The 16 and 20 kDa proteins showed some limited activity and all other protein bands were inactive (Fig. 3).

N-terminal sequencing

The active 17 kDa protein was sequenced and had the following N-terminal sequence, ADFTETFESSTHG-EAP(A)EETTID. This sequence is highly homologous to a region of a putative haemagglutinating domain which is common to several gene products of *P. gingivalis*. These include the PrpRI (at A1139–D1161) and homologous arginine-specific proteases (Aduse-Opoku *et al.*, 1995; Pavloff *et al.*, 1995), Kgp (A1156–D1178) (Pike *et al.*, 1994), PrtP (A1157–D1179) (Barkocy-Gallagher *et al.*, 1996), HagA (A687–D707, A1141–D1163, A1597–D1619 and A2053–A2075) (Naiming *et al.*, 1996; Progulsk-Fox *et al.*, 1993) and the TonB-linked adhesin (A522–D544) (Aduse-Opoku *et al.*, 1995). The 15 kDa protein had the following, novel, sequence, AQGDNPDKDTDG(F)N.

Fig. 4. Induction of IL-6 by IL-1 β following RI protease treatment. The figure shows IL-6 production from PBMCs treated with: U, untreated IL-1 β ; HI, IL-1 β treated with heat-inactivated RI protease; 0–4, IL-1 β treated with RI protease for 0–4 h as described in the text. Inset shows a Western blot of IL-1 β treated as described above, detected with anti-human IL-1 β polyclonal antibody.

IL-1 β degradation by purified RI protease

Western blotting revealed that after 1 h incubation of IL-1 β with RI protease there was a significant decrease of the intact cytokine in the sample. After 4 h incubation there was no detectable intact IL-1 β . In contrast, incubation of IL-1 β with heat-inactivated RI protease for 4 h resulted in no degradation (Fig. 4). These data demonstrate that RI protease is able to degrade IL-1 β . The results from the bioassay (Fig. 4) showed a time-dependent decrease in the IL-6-stimulating activity of the IL-1 β , suggesting that IL-6 has completely lost all biological activity within 1 h.

DISCUSSION

P. gingivalis is now recognized as an important pathogen in certain forms of the chronic inflammatory periodontal diseases (Kurimatsu *et al.*, 1995). Two of the major recognized virulence factors of this bacterium are its endotoxin/LPS (Watanabe *et al.*, 1996) and its cell-associated and exported proteinases (Lantz *et al.*, 1991; Kurimatsu *et al.*, 1995). These proteinases are also major antigens in patients with periodontal disease (Cridland *et al.*, 1994). In this study we have revealed an unexpected association between these apparently distinct putative virulence factors.

The proteins of the outer membranes of Gram-negative bacteria, known as LAPs or endotoxin-associated proteins, are an operationally defined group of proteins which co-isolate with LPS in endotoxin preparations and which can be separated from the LPS by extraction with hot phenol (Reddi *et al.*, 1995b). The properties of the LAPs have been extensively reviewed by Hitchcock & Morrison (1984). One important property is their stability to heat and proteases (Goodman & Sultz,

1979a; Bjornson *et al.*, 1988; Johns *et al.*, 1988). Enteric bacterial LAPs appear to be important virulence factors. For example, immunization of *Salmonella*-hyper-susceptible mice with LAP-LPS complexes, but not with LPS alone, protects against the lethality of *Salmonella typhimurium* infections (Killion & Morrison, 1986). The LAPs from several bacterial species have potent adjuvant activity (Mangan *et al.*, 1993). Indeed, Hogan & Vogel (1987) have suggested that the LAPs represent a 'second signal' for the activation of macrophages. Enteric LAPs are potent inducers of cytokine synthesis (Hogan & Vogel, 1988) and can prevent monocytes from undergoing apoptosis (Mangan *et al.*, 1993). Surprisingly, the identity of the proteins which constitute the LAPs of these various bacteria has not been defined and, to date, no protein sequence data on the LAPs have been reported.

Given these properties of the LAPs and their stability, it is possible that such proteins from oral Gram-negative bacteria could play a role in the pathology of the periodontal diseases (Reddi *et al.*, 1995b). We have previously shown that the LAPs of *Actinobacillus actinomycetemcomitans*, *P. gingivalis* and *Prevotella intermedia* were able to induce HGFs or the human myeloid cell line, Mono-Mac-6, to produce pro-inflammatory cytokines (Reddi *et al.*, 1995a). In addition, the LAPs of *A. actinomycetemcomitans*, *P. gingivalis* and *Eikenella corrodens* were able to stimulate the breakdown of cultured bone (Reddi *et al.*, 1995a). Comparison of the LPS and LAPs of *A. actinomycetemcomitans* demonstrated that the latter were significantly more active inducers of cytokine synthesis than the former and when the LPS and LAPs of *P. gingivalis* and *Prev. intermedia* were tested they appeared to be equally potent and efficacious. While care was taken to ensure that the LAPs were not contaminated with residual LPS, these studies did not investigate the role of LPS in the biological activity of the LAPs.

In the present study we have attempted to define the mechanism of action of the LAPs isolated from the major periodontopathogen *P. gingivalis* and identify the active proteins. We have shown that preparations of LAPs from this bacterium stimulate HGFs, human PBMCs and whole human blood to synthesize the pro-inflammatory cytokine IL-6. Care was taken to remove any contaminating LPS in the LAPs preparation by use of a polymyxin B affinity column, and measurement of LPS contamination by the LAL assay showed that this treatment was effective in removing P-LPS. To determine if the capacity of the LAPs to induce cytokine synthesis was due to residual LPS, use was made of the knowledge that LPS-dependent effects can be blocked by the peptide antibiotic polymyxin B (Morrison & Jacobs, 1976) or by a neutralizing antibody (MY4) to the LPS receptor CD14 (Viriyakosol & Kirkland, 1996). We were unable to demonstrate inhibition of P-LPS with these agents (data not shown). This suggests that while polymyxin B can bind to P-LPS and remove it from the LAPs, this binding does not inhibit the biological activity of P-LPS. This confirms the findings of Hamada *et al.* (1988), who

showed that polymyxin B did not block the mitogenic activity of P-LPS. Exposure of the LAPs to heat or trypsin resulted in significant, but not total loss of activity. This clearly shows that LPS (which is stable to heat and proteases) is not responsible for the cytokine-inducing activity of the LAPs. The failure to completely block the biological activity of the LAPs may be explained by the known stability of the proteins. In other experiments we have directly compared the relative activities of the LPS contamination in the LAP preparation with that of known concentrations of P-LPS. This showed that 3000 IU P-LPS was required to produce the same cytokine-inducing activity as a LAP preparation contaminated with 30 IU LPS.

Thus based upon the measured LPS content of the LAPs, the inhibition of activity by heat and trypsin and the direct comparison of the activity of the LAPs with purified P-LPS, we conclude that the cytokine-inducing activity of the LAPs of *P. gingivalis* is due to these proteins and not to LPS contamination.

Analysis of the LAPs by SDS-PAGE revealed the presence of nine Coomassie-blue-stained protein bands of molecular masses ranging from 81 to 16 kDa. This raises the question of which of these proteins is responsible for the stimulation of IL-6 synthesis. Unfortunately, because of the small amounts of proteins that can be obtained, and the problems with the solubility and 'stickiness' of the LAPs, it was impossible to isolate the individual proteins by conventional methods of protein purification. Other methodologies were investigated, and it was found that a modification of the conventional SDS-PAGE technique in which the LAPs were incubated in the SDS-PAGE sample buffer at room temperature, and not boiled, as is the normal practice, enabled these proteins to be separated (with the same pattern of bands as seen in conventional SDS-PAGE) while retaining their biological activity. Using this technique, it was found that the cytokine-inducing activity of the LAPs was due predominantly to the 17 kDa protein with the 16 and 20 kDa proteins also showing limited activity. This finding is similar to that of Porat *et al.* (1992), who reported that the active component in the LAPs of *E. coli* was a 17 kDa protein.

N-terminal sequence analysis of the first 24 residues of the biologically active 17 kDa protein demonstrated near identity with an internal region (A1139-D1161) of the RI protease β chain and homologous haem-agglutinating arginine-specific proteases (Aduse-Opoku *et al.*, 1995), internal sequences of Lys-gingipain (Kgp; Pike *et al.*, 1994), porphypain (Prtp; Barkocy-Gallagher *et al.*, 1996), haemagglutinin A (Progulske-Fox *et al.*, 1993; Naiming *et al.*, 1996) and the TonB-linked adhesin of *P. gingivalis* (Aduse-Opoku *et al.*, 1997). Furthermore, peptides in the size range 14–16 kDa with this same N-terminal sequence, thought to be derived via proteolytic processing of the respective precursors, have been identified in preparations of RI (Aduse-Opoku *et al.*, 1995) and Prtp (Barkocy-Gallagher *et al.*, 1996) purified from *P. gingivalis*.

Recent studies have shown that supernatants and biofilms of *P. gingivalis* can hydrolyse and inactivate a number of pro- and anti-inflammatory cytokines including IL-6 (Fletcher *et al.*, 1997, 1998). Another study has shown that *P. gingivalis* arginine-specific proteases and Lys-gingipain are able to inactivate the pro-inflammatory cytokine TNF- α (Calkins *et al.*, 1998). In this study we have examined the direct action of the purified RI protease on IL-1 β and have discovered that it degrades this cytokine and, in doing so, negates IL-1 bioactivity.

In conclusion, we have isolated the operationally defined fraction of proteins associated with the cell wall of *P. gingivalis* and termed LAPs, and have found that the active cytokine-inducing component of this mixed population of outer-membrane proteins is related, at the sequence level, to the adhesin domain common to products of the RI family of *P. gingivalis*. The critical determinants of the adhesin domain have yet to be fully characterized. However, it is reasonable to suggest the possibility that the IL-6-stimulating component found in these *P. gingivalis* LAPs contains determinants which can mediate binding to host cells and, in doing so, can trigger the production of IL-6. This production may be balanced by the capacity of the RI protease to proteolyse and inhibit the activity of cytokines. The exact balance of these two opposing effects is not clear but reveals the potential complexity of cytokine network interactions in the periodontal diseases.

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