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Interleukin-1α stimulation in monocytes by periodontal bacteria: antagonistic effects of *Porphyromonas gingivalis*

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Periodontal pathogenic bacteria are associated with elevated levels of interleukin-1α (IL-1α) but it is unclear if all species can induce cytokine production equally. *Porphyromonas gingivalis* may be able antagonize IL-1α induced by other species through the activity of its proteases or lipopolysaccharide (LPS). Monomac-6 cells and primary human monocytes were treated with culture supernatants from *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Campylobacter rectus*, *Actinobacillus actinomycetemcomitans*, *Prevotella intermedius*, *Veillonella atypical* and *Prevotella nigrescens*. IL-1α protein levels were measured after 6 h of incubation. In addition, monocytes were co-stimulated with supernatants from *P. gingivalis* and other bacteria. The role of *P. gingivalis* proteases was tested using Arg-X and Lys-X mutant strains. The role of LPS was investigated using purified *P. gingivalis* LPS and polymixin depletion. All species tested induced significant IL-1α production, but *P. gingivalis* was the weakest. Co-stimulation of monocytes with *P. gingivalis* antagonized the ability of other bacterial species to induce IL-1α production. This effect was at its greatest with *C. rectus* (resulting in a 70% reduction). Gingipain mutant strains and chemical inhibition of protease activity did not reduce antagonistic activity. However, 100 ng/ml of *P. gingivalis* LPS can reproduce the antagonistic activity of *P. gingivalis* culture supernatants. Periodontitis-associated bacterial species stimulate IL-1α production by monocytes. *P. gingivalis* can antagonize this effect, and its LPS appears to be the crucial component. This study highlights the importance of mixed infections in the pathogenesis of periodontal disease because reduction of pro-inflammatory cytokine levels may impair the ability of the host to tackle infection.

Key words: immune suppression; interleukin-1α; periodontal disease; *Porphyromonas gingivalis*

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The initiation and progression of periodontal disease depends on the presence of bacterial plaque. Tissue destruction is an indirect result of the presence of bacteria and their ability to trigger a host inflammatory response. The local host response to periodontal bacteria includes the recruitment of polymorphonuclear neutrophils and monocytes/macrophages (14, 28). Monocytes are a major part of the inflammatory infiltrate and can be found within the gingival connective tissues of patients with periodontitis (51). These cells play a critical role in the regulation of local inflammatory host responses, in part through their ability to secrete pro-inflammatory cytokines, including interleukin-1α (IL-1α) in response to gram-negative bacteria and their products (29, 42).

Specific cytokines appear to be key regulators of the inflammatory response and the progression of periodontal disease. One of the most important factors known to be induced by oral bacteria is IL-1 (17).

A number of lines of evidence support a pivotal role of IL-1α in mediating the host response to periodontal pathogenic bacteria and the associated tissue damage. First, levels of IL-1α are undetectable in the gingival crevicular fluid of healthy teeth but are highly elevated in severe periodontal disease (24, 37). Second, successful treatment results in the reduction of IL-1α concentration to pre-disease levels (23). Third, in a primate model of acute periodontal disease the use of IL-1 receptor antagonists to block IL-1 activity is able to reduce inflammation and tissue damage significantly (13). Finally, the ectopic expression of IL-1α in the oral epithelia of transgenic mice shows that expression of IL-1α is sufficient to induce all the cardinal features of periodontal disease (12). IL-1α is known to act in a number of ways to trigger tissue destruction. For example, it is able to simultaneously inhibit osteoblast activity, promote osteoclast activity and stimulate the production of matrix metalloproteinases (46–49).

More than 500 bacterial species colonize the subgingival area but studies suggest that the presence of relatively few key species may be causally related to periodontal disease (41). The most frequently isolated bacteria from such infections include *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Fusobacterium nucleatum*, *Treponema denticola*, *Campylobacter rectus* and *Veillonella atypica* (18, 22). The stimulation of IL-1α production by bacterial products has been studied by several groups. However, there has not been a systematic comparison of a full range of periodontal pathogenic bacterial species. Previous studies using primary monocytes or cell lines have investigated only either a single species or a number of strains of a single species or they have compared relatively small numbers of different species (21, 25, 33, 43).

The complexity and species diversity of the microflora found in periodontal pockets may have a number of implications. There is almost limitless scope for bacterial interactions, including synergism, commensalism and antagonism in the ecology of the subgingival microflora. In addition, the mixed nature of the infection will have a direct influence on the nature and extent of the host response to the bacterial challenge. The phenomenon of endotoxin tolerance has long been recognized whereby the prolonged exposure of cells to endotoxin attenuates the response to subsequent challenges. In the oral mucosa the down-regulation of Toll-like receptors in patients with chronic periodontitis suggests that induction of tolerance may be an important aspect of this disease (31). *P. gingivalis* lipopolysaccharide (LPS) exhibits unusual characteristics and can interact with the toll-like receptor 2 and is able to antagonize the effects of other bacteria (2, 6, 11, 34). *P. gingivalis* also produces proteases that may directly degrade CD14 and lead to further reduction in response to LPS from other bacteria (15, 44).

This study aims to compare the ability of supernatants from a number of the major periodontal pathogens including *P. gingivalis*, *F. nucleatum*, *C. rectus*, *A. actinomycetemcomitans*, *P. intermedia*, *V. atypica* and *P. nigrescens* for their ability to stimulate IL-1α production in the MonoMac-6 cell line and in primary monocytes. All the tested species induced IL-1α production but *P. gingivalis* induced notably lower levels. To investigate if *P. gingivalis* culture supernatant appears to be able to antagonize the IL-1α production stimulated by other pathogens, cells were exposed to mixtures of supernatants. *P. gingivalis* can antagonize cytokine production stimulated by a range of periodontal pathogens. Using bacterial mutants, chemical inhibition and fractionation to investigate the underlying mechanisms suggests that the LPS is the key constituent of the *P. gingivalis* supernatant. This study thus suggests that *P. gingivalis* has the potential to suppress the normal immune response to a wide range of periodontal pathogens.

Materials and methods

Bacterial strains and growth conditions

Bacterial supernatants were taken from cultures of *P. gingivalis* W50 and W83, 381, *F. nucleatum* ATCC 25586, *C. rectus* ATCC 33238, *A. actinomycetemcomitans* Y4, *P. intermedia* NCTC 9336, *V. atypica* NCTC 11830 and *P. nigrescens* Mu14. Supernatants from a culture of *Streptococcus sanguinis* NCTC 7863 were used as a negative control and LPS from the non-oral bacterium *Escherichia coli* 026: B6 (Sigma-Aldrich, Poole, UK) was used as a positive control. In subsequent experiments, the *P. gingivalis* W50 protease mutant strain (*kgp*, Lys-gingipain) and the protease (Arg-gingipain) double-defective mutants (*rgpA*, *rgpB*) were utilized (1). To investigate the role of *P. gingivalis* gingipain cysteine proteinases in cytokine production, *P. gingivalis* supernatant was pre-treated with 1 mM of the proteinase inhibitor *N*α-p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) (Sigma-Aldrich) for 1 h at 4°C. Cultures were grown in the appropriate growth medium either aerobically in a 5% CO_2 incubator or in an anaerobic chamber containing an atmosphere of 80% N_2, 10% H_2 and 10% CO_2. Cultures were grown to the same density (approximately 5×10^7 colony-forming units/ml) and were harvested by centrifugation at 10,000 ***g*** for 15 min at 4°C. The culture supernatants were collected, filter-sterilized and stored at −80°C until used. For some studies, culture supernatants were heat-inactivated at 100°C for 20 min. The supernatants of bacteria were directly diluted in culture medium to final dilutions of 1 : 250 and 1 : 50.

Stimulation of human monocytes and the myelomonocytic cell line Monomac-6

Peripheral blood was obtained from healthy individuals by venous puncture and collected in ethylenediaminetetraacetic acid (EDTA) vacutainers (Becton Dickinson, BD Biosciences, Oxford, UK). All participants signed a written consent form. Protocols for the study were approved by the East London and City Health Authority London Research Ethics Committee. Human peripheral blood mononuclear cells were separated by density gradient centrifugation using Ficoll–Paque Plus (GE Healthcare, Chalfont St Giles, UK) according to the manufacturer's instructions. The cells were washed twice with phosphate-buffered saline containing EDTA (2 mM) and bovine serum albumin (2%), pH 7.3. Cell viability was determined by Trypan blue exclusion. Peripheral blood mononuclear cell count was performed with a haemocytometer and processed for magnetic labelling. The Monocyte Isolation Kit II (Milteny Biotech, Bisley, UK) was used. This technique relies upon an indirect magnetic labelling system allowing the isolation of unstimulated monocytes from human peripheral blood mononuclear cells (19). The purity of isolated monocytes was evaluated by flow cytometry on a fluorescence-activated cell sorter (FACScan) flow cytometer (Becton Dickinson) using a CD14 fluorescein isothiocyanate-conjugated antibody. Purified monocytes were re-suspended in RPMI–Glutamax (Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (Cambrex Biosciences, Wokingham, UK). Monocytes were then seeded in 96-well microtitre plates at 1×10^6

cells/ml and stimulated with bacterial culture supernatants at 37°C for 6 h in a 5% CO_2 atmosphere.

The myelomonocytic cell line Monomac-6 was obtained from the German Collection of Microorganisms and Cell Cultures (Mascheroder, Braunschweig, Germany). MonoMac-6 cells were cultured in RPMI-Glutamax (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (Invitrogen, Paisley, UK), 1% sodium pyruvate (Gibco BRL) and 9 μg/ml bovine insulin (Sigma-Aldrich). MonoMac-6 cells were cultured at a density of 1×10^6 cells/ml in 96-well flat-bottom plates and challenged with bacterial supernatants. The bacterial supernatants were added to the monocytes (200 μl/well) at dilutions of 1 : 250 (0.8 μl) and 1 : 50 (4 μl) and were incubated at 37°C in a 5% CO_2 humidified atmosphere. In preliminary experiments, a 6-h incubation period was found to be suitable for the detection of IL-1α production. To assay total IL-1α (cell-associated and secreted) cells were lysed by three freeze–thaw cycles. The cell-free culture supernatants were collected by centrifuging at 400 ***g*** for 5 min and stored at −80°C until the cytokine assays were performed. Control cultures were incubated in the absence of bacterial supernatant. All experiments were carried out in triplicate.

Cell viability assays

Cell viability was determined by measuring the release of lactate dehydrogenase using the CytoTox96® non-radioactive cytotoxicity assay (Promega, Southampton, UK). In brief, Monomac-6 cells or primary human monocytes were exposed to bacterial culture supernatants and then incubated for 6 h at 37°C; 50 μl supernatant per well was carefully removed and transferred into an optically clear 96-well plate. Reaction solution was added to each well and incubated for 30 min in darkness. The enzyme reaction was then stopped by the addition of 1 M HCl. The absorbance at 490 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Model 680; Bio-Rad Laboratories, Hemel Hempstead, UK). The activity of the enzyme released from damaged cells into the supernatant was measured, and the activity was expressed as a percentage of the total lactate dehydrogenase activity released from cells lysed by exposure to 0.1% Triton X-100 for 45 min. Values shown represent the mean ± standard deviation of three wells.

Protease assays

P. gingivalis wild-type (W50) and protease gene knockouts (E8 and K1A) were grown in brain–heart infusion broth with haemin and menadione under identical conditions to achieve maximal Arg-X and Lys-X active proteases. The enzyme assays for Arg-X and Lys-X protease activities using the synthetic substrates *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (DL-BR*p*NA) and *N*-α-acetyllysine-*p*-nitroanilide (Ac-Lys*p*-NA) respectively, were performed according to the published protocols (36).

Determination of cytokine production

Cytokine production from the different experiments was determined in the cell-free culture supernatant using a commercially available specific ELISA (R&D Systems, Minneapolis, MN) according to standard procedures. The absorbance at 450 nm was read using a microplate reader (Model 680; Bio-Rad Laboratories) with a wavelength correction set at 570 nm. A standard curve was generated using a four-parameter logistic curve-fit (MICROSOFT OFFICE EXCEL) for each set of samples assayed. The values of the samples were assigned in relation to the standard curve.

Determination of cytokine degradation

To control for any effects of degradation of IL-1α by *P. gingivalis* supernatants, recombinant human (rh) IL-1α (50 ng/ml) was added to the cultures. The cytokine concentration in the supernatants was determined by ELISA and compared to the basal levels of a control incubated (for 6 h) in the absence of bacterial supernatant.

Preparation of LPS from *P. gingivalis*

P. gingivalis W50 was grown anaerobically in brain–heart infusion broth supplemented with haemin (5 μg/ml) for 24 h. The cells were harvested, washed with water and freeze-dried. The *P. gingivalis* LPS was prepared by the cold $MgCl_2$–ethanol procedure as described previously (10). LPS was lyophilized to determine the yield and stock solutions were prepared in pyrogen-free distilled water (1 mg/ml) and vortexed before use. No protein was detected in LPS preparations as determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and staining for protein using the enhanced colloidal gold procedure.

Determination of endotoxin levels in bacterial culture supernatants

Bacterial endotoxin was assessed using The Chromogenic Limulus Amoebocyte Lysate (LAL) Test (QCL1000, Cambrex Biosciences, Wokingham, UK). Purified *P. gingivalis* LPS and bacterial supernatants were dissolved in pyrogen-free water and serial dilutions were made. The absorbance of the sample was determined spectrophotometrically at 405 nm and the concentration of endotoxin was calculated from a standard curve. In the case of polymyxin B treatment, endotoxin was removed from bacterial supernatants using a polymyxin B column (Detoxigel; Pierce Biochemicals) according to the manufacturer's instructions. As assessed by LAL assay (using the purified LPS as a standard), the concentration of LPS in *P. gingivalis* and *C. rectus* supernatant was 35.7 and 38 μg/ml, respectively. In addition, pretreatment of bacterial supernatants with polymyxin B resulted in a dramatic reduction in LPS in both supernatants (0.55 and 0.39 μg/ml, respectively).

Statistical analysis

Student's *t*-test for paired values was used and data were considered significant at $P < 0.05$.

Results

Stimulation of IL-1α production by culture supernatants from periodontal pathogens

When the monocytic cell line Monomac-6 was exposed to bacterial supernatants of cultured periodontal pathogens a significant up-regulation of the total level of IL-1α was seen after 6 h (Fig. 1A). Unstimulated Monomac-6 cells or those exposed to uninoculated culture media showed very low levels of IL-1α production. The most pronounced inducers were *A. actinomycetemcomitans*, *F. nucleatum*, *C. rectus* and *V. atypica*, whereas *P. gingivalis* was consistently the least potent treatment. Some putative pathogenic species, including *P. intermedia* and *P. nigrescens*, produced only a modest induction of IL-1α. Stimulation of primary CD14-positive monocytes with bacterial supernatants in a similar manner caused IL-1α production to be significantly up-regulated after 6 h (Fig. 1B). The pattern of IL-1α production was somewhat different to that in Monomac-6 cells with responses to *A. actinomycetemcomitans* and *F. nucleatum* being relatively lower and responses to *P. intermedia* and

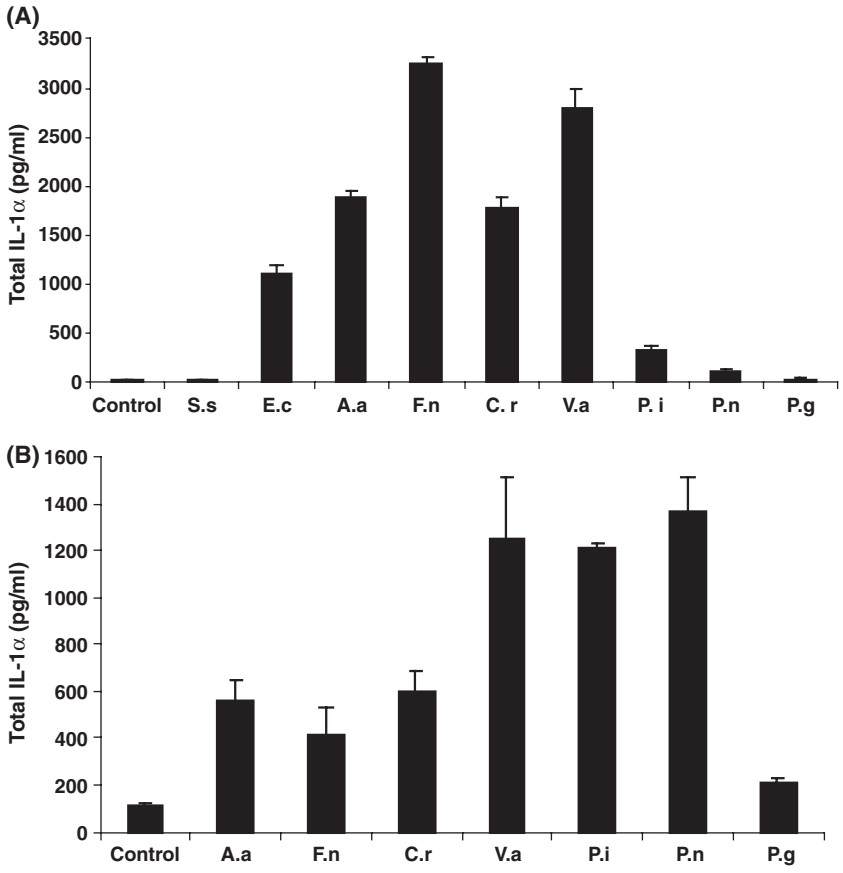

Fig. 1. Production of IL-1α by (A) Monomac-6 cells and (B) primary human monocytes in response to bacterial stimuli. Monomac-6 cells and freshly isolated monocytes were plated at a density of 1×10^6 cells/ml in 96-well dishes. Cells were exposed to culture supernatants from periodontopathogens. Bacterial supernatants were added to cell culture media at 1 : 250 dilution, After a 6-h incubation the cells were lysed and cell-free supernatants were harvested to assess total IL-1α (secreted and cell-associated) by ELISA. The data are means ± SD; $n = 3$. S.s, *Streptococcus sanguinis*; E.c, *Escherichia coli*; A.a, *Actinobacillus actinomycetemcomitans*; F.n, *Fusobacterium nucleatum*; C.r, *Campylobacter rectus*; V.a, *Veillonella atypica*; P.i, *Prevotella intermedia*; P.n, *Prevotella nigrescens*; P.g, *Porphyromonas gingivalis*.

P. nigrescens being significantly greater. However, the response to *P. gingivalis* was still lowest.

Monomac-6 cells and isolated human monocytes showed almost no toxic effects in the presence of bacterial supernatants except *A. actinomycetemcomitans* (Fig. 2-A,B). While *A. actinomycetemcomitans* did induce some toxicity, it was still able to significantly elevate levels of IL-1α. In the present study, most supernatants were diluted no less than 1 : 250 and for *P. gingivalis*, in combination with other supernatants, it was diluted 1 : 50 in medium. The toxicity testing provided no evidence that the inability of *P. gingivalis* to elicit a significant IL-1α response was related to its toxicity to the Monomac-6 cells or isolated human monocytes (Fig. 2A,B). Interestingly the levels of lactate dehydrogenase released in cells treated with both *C. rectus* and *P. gingivalis* were not increased compared to cells treated with either bacterium alone. It has been previously shown that *P. gingivalis* can partially neutralize the leukotoxicity of *A. actinomycetemcomitans* (26) and it is possible that a similar mechanism is involved in this situation. Although the amount of leukotoxin in the *A. actinomycetemcomitans* supernatants used was not defined, a 1 : 250 dilution induced cytotoxicity similar to the <5 ng/ml of purified leukotoxin shown in a previous study (27).

Antagonism of IL-1α production by *P. gingivalis*

To investigate if *P. gingivalis* could affect the IL-1α-stimulating activity of other periodontal species a fivefold excess of *P. gingivalis* was mixed with other bacterial supernatants before their addition to monocytes. Several species showed an altered capacity to induce IL-1α in the presence of *P. gingivalis* (Table 1). A small (21%) but significant ($P < 0.05$) synergistic effect was seen in combination with *A. actinomycetemcomitans* while all the other bacteria showed a reduction in IL-1α production. The *C. rectus* induction of IL-1α production appeared to be the most sensitive to the presence of *P. gingivalis*, showing a 72 ± 7% reduction in total IL-1α levels. One possible explanation for the antagonistic effects and the low IL-1α production by *P. gingivalis* is that the proteolytic activity of the supernatant acts to degrade any IL-1α produced. To evaluate the direct degradation of IL-1α by *P. gingivalis* supernatant, recombinant human IL-1α (50 ng/ml) was incubated with culture supernatant (at 1 : 250 and 1 : 50) at 37°C in phosphate-buffered saline for 6 h. Subsequent ELISA was able to detect 96% of the added IL-1α protein. This confirms that the proteolytic degradation of IL-1α was not a significant factor in this experimental system and did not account for the low cytokine production seen in the presence of *P. gingivalis* supernatant. The antagonistic activity of *P. gingivalis* is not restricted to the W50 strain; a comparison of the antagonistic ability of this strain with W83 and 381 showed no significant differences when used in combination with *C. rectus* (data not shown). Subsequent experiments used strain W50, which is the parental strain for the mutants that were tested in subsequent experiments.

The effects of co-stimulation with *P. gingivalis* and other pathogens were also seen in primary monocytes (Table 2). In all cases the addition of *P. gingivalis* significantly decreased total IL-1α production by between 69 and 84%. Interestingly the stimulating effect of *A. actinomycetemcomitans* in combination with *P. gingivalis* seen in Monomac-6 cells was not seen and instead, in primary monocytes, IL-1α production was reduced by 84%.

Mechanism of immune suppression

Role of P. gingivalis *virulence factors in inhibition of IL-1α stimulation by* C. rectus

Since the most dramatic antagonistic interaction in Monomac-6 cells was between *C. rectus* and *P. gingivalis* this combination of bacterial supernatants was used to further investigate the mechanism of antagonistic activity. Removal of LPS from *P. gingivalis* and *C. rectus* supernatants by polymyxin B almost completely eliminated the ability to stimulate IL-1α

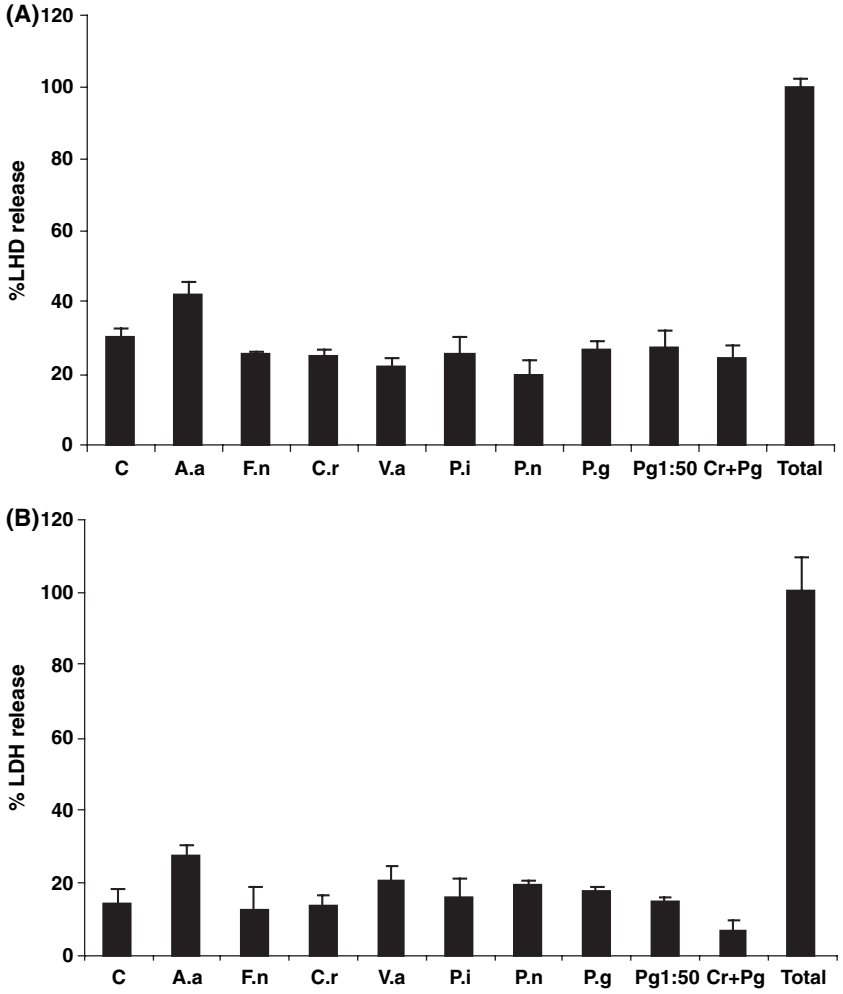

Fig. 2. Cytotoxicity of bacterial culture supernatants to (A) Monomac-6 cells and (B) freshly isolated human monocytes. Monomac-6 cells and freshly isolated monocytes were plated at a density of 1×10^6 cells/ml in 96-well dishes. Cells were exposed to culture supernatants from periodontopathogens for 6 h. Cytotoxicity was determined by the release of the cytosol enzyme lactate dehydrogenase (LDH). The activity was expressed as a percentage of the total LDH activity released from cells lysed by exposure to 0.1% Triton X-100 for 45 min. The data are means ± SD; $n = 3$. See legend of Fig. 1 for abbreviations of bacteria.

Table 1. Overall changes in total IL-1α levels in Monomac-6 cells treated with different bacterial supernatants in the presence of *Porphyromonas gingivalis*

Bacteria	Singles	Co-stimulation	% change
Control	30.11±7.56	–	–
Porphyromonas gingivalis	35.89 ± 4.23	45.15 ± 18.05	26.84 ± 12.4↑
Actinobacillus actinomycetemcomitans	1874.89 ± 89.45	2272.88 ± 47.90	21.22 ± 16.5*↑
Fusobacterium nucleatum	3423.25 ± 344.99	2468.14 ± 405	27.90 ± 16.0*↓
Campylobacter rectus	1754.78 ± 138.16	500.27 ± 74.48	71.49 ± 7.43*↓
Veillonella atypica	3103.12 ± 641.74	2423.10 ± 364	21.91 ± 9.89↓
Prevotella intermedia	284.45 ± 84.21	159.57 ± 38.42	43.90 ± 9.26↓
Prevotella nigrescens	121.24 ± 15.74	64.40 ± 32.80	46.33 ± 1.44*↓
Escherichia coli LPS	1109.86 ± 89.87	464.90 ± 39.83	64.80 ± 2.59*↓

↑ % increase, ↓ % decrease, *$P < 0.05$.
Monomac-6 were plated at a density of 1×10^6 cells/ml in 96-well dishes (100 μl/well). Cells were treated with a range of periodontopathogens or LPS from *E. coli* (10 ng/ml) with (co-stimulation) or without (single) supernatant from *P. gingivalis* for 6 h. After 6 h of incubation the cells were lysed and cell-free supernatants were harvested to assess total IL-1α (secreted and cell-associated) by ELISA. The table shows the percentage difference in levels of IL-1α with and without the addition of *P. gingivalis* supernatant. Changes induced by co-stimulation represent mean changes in three separate experiments with mean ± standard error. Figures for co-stimulation were adjusted for the stimulating effect of *P. gingivalis* alone.

production by 94 and 99%, respectively. Heating of supernatants resulted in an increase in the stimulating ability of the supernatants by 44 and 48 %, respectively, and suggests that the active components are not heat-sensitive (Fig. 3).

The LPS and protease contents of *P. gingivalis* cultures are known to vary significantly with time in culture. Thus cultures grown between 1 and 6 days were compared for their ability to induce IL-1α from Monomac-6 cells and antagonize *C. rectus* activity. Cultures did not show any significant change in the ability to induce IL-1α over the culture period but did show a progressive increase ($R^2 = 0.95$) in their ability to antagonize *C. rectus* activity (Fig. 4).

Using specific protease mutants lacking the Lys-gingipain *kgp* (K1A) or Arg-gingipains *rgpA rgpB* expression (E8) it was possible to test their role in the antagonism of *C. rectus* activity. Enzyme-specific activity assays were used to confirm the phenotypes of these mutants (Table 3). A complementary approach used the proteinase inhibitor TLCK or heating to block protease activity in the wild-type *P. gingivalis* supernatants. Both K1A and E8 *P. gingivalis* protease-mutant strains remained capable of antagonizing *C. rectus*-mediated IL-1α production by respectively 68.4 ± 4.9% and 49.6 ± 2.8% (wild-type inhibition was 68.9 ± 4.7%). In addition, heat-treatment or chemical blockage of the cysteine protease activity of *P. gingivalis* did not remove the inhibitory activity. Together these data suggest that protease activity does not play a significant role in the antagonistic activity of *P. gingivalis*.

LPS is another important component of the bacterial supernatant. The ability of purified *P. gingivalis* LPS to stimulate IL-1α production by Monomac-6 cells was tested. The *P. gingivalis* LPS caused a significant concentration-dependent stimulation of IL-1α production (Fig. 5). Purified LPS was a relatively poor inducer of IL-1α from Monomac-6 cells with *P. gingivalis* supernatant able to induce slightly more than 100 ng/ml purified LPS. In addition, measurement of *P. gingivalis* culture supernatants by the LAL assay showed that the 1 : 250 dilution used to stimulate cells contained 142 ng/ml.

Since purified LPS has only a limited ability to induce IL-1α it was possible to test its antagonistic activity when mixed with *C. rectus* supernatant. While 10 ng/ml purified LPS had no significant effect, the addition of 100 or 1000 ng/ml was able to significantly antagonize stimulation of

Table 2. Overall changes in total IL-1α levels in human monocytes treated with a range of bacterial supernatants in the presence of *Porphyromonas gingivalis*

Bacteria	Singles	Co-stimulation	% change
Control	117.72 ± 0.87	–	–
Porphyromonas gingivalis	208.54 ± 27.79	255.28 ± 34.57	22.41 ± 6.2↑
Actinobacillus actinomycetemcomitans	559.22 ± 88.06	88.78 ± 37.51	83.86 ± 2.49↓
Fusobacterium nucleatum	417.77 ± 113.8	123.17 ± 11.81	69.19 ± 7.30↓
Campylobacter rectus	594.66 ± 90.11	144.49 ± 64.17	75.20 ± 7.43↓
Veillonella atypica	1242.16 ± 268.84	206.81 ± 25.52	82.82 ± 4.14↓
Prevotella intermedia	1209.46 ± 16.34	239.52 ± 10.86	80.19 ± 2.60↓
Prevotella nigrescens	1369.58 ± 140.96	346.22 ± 37.83	74.56 ± 2.40↓

↑ % increase, ↓ % decrease, $*P < 0.05$.
Freshly isolated monocytes were treated as described in the footnote to Table 1.

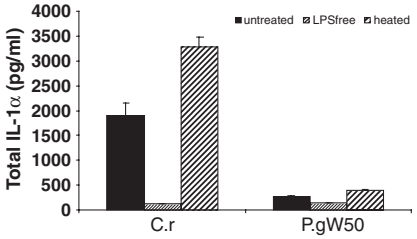

Fig. 3. The effect of LPS depletion and heat treatment on periodontopathogen-induced IL-1α production by Monomac-6 cells. The bacterial supernatants were passed through polymixin B columns or heated to boiling point for 30 min. The cells were exposed to untreated or LPS-depleted or heated bacterial supernatants for 6 h. After a 6-h incubation the cells were lysed and cell-free supernatants were harvested to assess total IL-1α (secreted and cell-associated) by ELISA. The data are means ± SD; $n = 3$. C.r, *Campylobacter rectus*; P.gW50, *Porphyromonas gingivalis* wild-type.

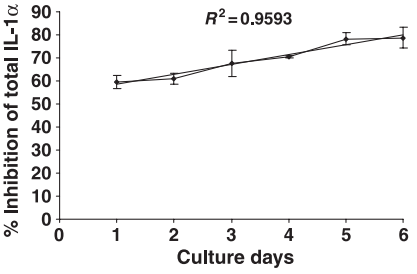

Fig. 4. Culture length and inhibition of IL-1α production stimulated by *C. rectus* in Monomac-6 cells. Cultures were grown as described in the Materials and methods. Supernatants were withdrawn at the indicated times (1- to 6-day-old). Monomac-6 cells were plated at a density of 1×10^6 cells/ml in 96-well dishes. Cells were exposed to *P. gingivalis* with or without *C. rectus* for 6 h. After incubation the cells were lysed and cell-free supernatants were harvested to assess total IL-1α (secreted and cell-associated) by ELISA. The data are means ± SD; $n = 3$.

IL-1α by *C. rectus* (Fig. 6). The level of IL-1α inhibition seen with 100–1000 ng/ml LPS was not significantly different from *P. gingivalis* culture supernatant (1 : 50) containing 714 ng/ml LPS ($P = 0.16$ and $P = 0.07$, respectively). In addition, polymixin B treatment of *P. gingivalis* supernatant reduced LPS concentrations to 11.2 ng/ml at the (1 : 50) dilution used, and subsequently inhibited the *C. rectus*-stimulated IL-1α production by only 10% (Fig. 6). Collectively these findings provide evidence that the LPS in *P. gingivalis* culture supernatants appears to be essential and present in sufficient quantities to account for the observed inhibition of *C. rectus*-induced IL-1α production by Monomac-6 cells.

To understand more about the possible mechanisms by which *P. gingivalis* LPS acts to antagonize *C. rectus* activity, cells were pre-treated with 1 μg/ml purified *P. gingivalis* LPS. Priming of Monomac-6 cells with *P. gingivalis* LPS largely abolished responsiveness to *C. rectus* (Fig. 7). There was no obvious effect when pre-treated cells were exposed to a combination of *C. rectus* and *P. gingivalis* compared to *C. rectus* alone. The result of LPS pre-treatment suggests that Monomac-6 cells were rendered either directly or indirectly unresponsive to *C. rectus*.

Table 3. Arg-X and Lys-X protease activities in isogenic mutants of *Porphyromonas gingivalis*

		Enzyme activities U/ml per mg protein	
Strain	Genotype	Arg-X	Lys-X
W50	wild-type	5.36 ± 0.14	1.70 ± 0.18
K1A	kgp::erm	1.90 ± 0.27*	0.20 ± 0.11*
E8	rgpA::tetQ	0.30 ± 0.07*	1.74 ± 0.19
TLCK-treated	wild-type	0.48 ± 0.11*	0.18 ± 0.02*

$*P < 0.05$ compared to wild-type W50.
Culture supernatants of *P. gingivalis* W50 and K1A and E8 were assayed for Arg-X and Lys-X activity using the substrates DL-BRpNA and *N*α-acetyl-L-lysine-*p*-nitroanilide (Ac-Lys*p*NA) respectively. Arg-X and Lys-X activities were normalized with respect to total protein in the culture supernatants and activities are expressed as U/ml per mg protein. The figures shown are the mean ± standard deviations of triplicate assays and statistical differences were calculated by Student's *t*-test.

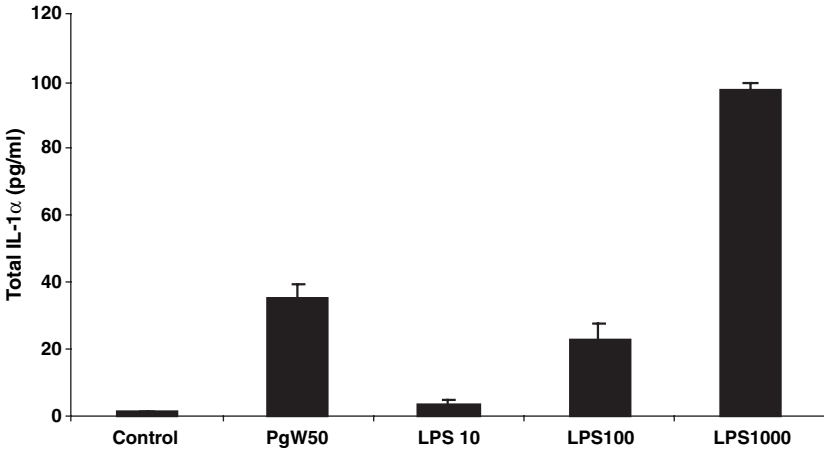

Fig. 5. The levels of total IL-1α in Monomac-6 cells treated with *P. gingivalis* supernatant or its LPS. Monomac-6 cells were plated at a density of 1×10^6 cells/ml in 96-well dishes. Cells were exposed to *P. gingivalis* supernatant or LPS from *P. gingivalis* (10, 100, 1000 ng/ml). After a 6-h incubation the cells were lysed and cell-free supernatants were harvested to assess total IL-1α (secreted and cell-associated) by ELISA. The data are means ± SD of triplicate assays; statistical differences were calculated using Student's *t*-test.

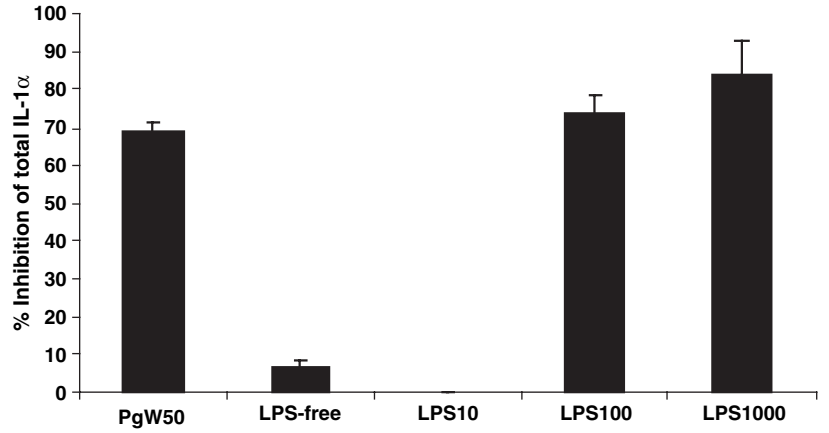

Fig. 6. The role of *P. gingivalis* LPS in *C. rectus* mediated IL-1α production by Monomac-6 cells. Monomac-6 cells were plated at a density of 1×10^6 cells/ml in 96-well dishes. Cells were exposed to *C. rectus* with purified *P. gingivalis* LPS (10, 100, 1000 ng/ml) or *P. gingivalis* supernatant. After a 6-h incubation the cells were lysed and cell-free supernatants were harvested and assayed for total IL-1α (secreted and cell-associated) by ELISA. The data are means ± SEM of three independent experiments; statistical differences were calculated using Student's *t*-test ($P < 0.05$).

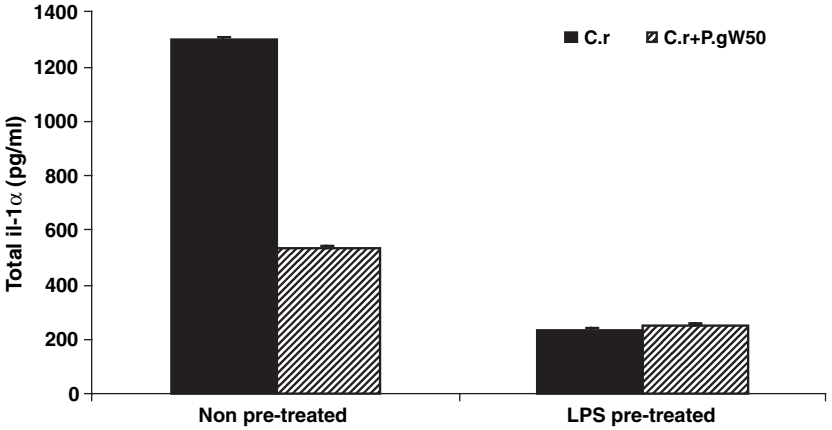

Fig. 7. The role of *P. gingivalis* LPS in *C. rectus*-mediated IL-1α production by Monomac-6 cells. Monomac-6 cells were pretreated for 1 h with *P. gingivalis* LPS (1 μg/ml) or medium alone. Monomac-6 cells were plated at a density of 1×10^6 cells/ml in 96-well dishes and subsequently challenged with supernatants of *C. rectus* or *P. gingivalis* or both for 6 h. After a 6-h incubation the cells were lysed and cell-free supernatants were harvested to assess total IL-1α (secreted and cell-associated) by ELISA. The data are means ± SD of triplicate assays and statistical differences were calculated using Student's *t*-test. C.r, *Campylobacter rectus*; P.gW50, *Porphyromonas gingivalis* wild-type.

Discussion

This study demonstrated that a broad range of oral bacteria stimulate the expression of IL-1α in Monomac-6 cells and primary monocytes as previously reported (21, 38, 43, 44). The weakest stimulators of IL-1α were the non-pathogenic *S. sanguinis* and surprisingly *P. gingivalis*. Most previous studies considered the stimulation of cytokine production for each bacterial species in isolation. Therefore, we compared IL-1α production by monocytic cells when treated with single bacterial supernatants to those treated with mixtures. A number of previous studies suggested that *P. gingivalis* might evade the normal responses through its ability to suppress the normal innate immune mechanisms (b9). In the present study *P. gingivalis* supernatants inhibited the capacity of most studied pathogens to stimulate IL-1α production by monocytes. The most pronounced inhibition was seen in combination with *C. rectus*, where the overall level of IL-1α production was reduced by approximately 72% in the presence of *P. gingivalis*. In the case of *A. actinomycetemcomitans*, co-stimulation of the cells with *P. gingivalis* caused a synergistic effect in IL-1α production by Monomac-6 cells but a reduction in primary human monocytes. The basis for this difference is not clear but could reflect increased sensitivity to *P. gingivalis* stimulation by primary monocytes compared to Monomac-6 cells. There is no reason to believe that all oral bacterial species that are weak stimulators of cytokine production act to suppress immune responses. In preliminary experiments with *T. denticola* we have shown that culture supernatants elicit only a very small level of IL-1α production on their own but are highly synergistic when combined with other periodontal pathogenic species (data not shown).

P. gingivalis expresses a number of potential virulence factors. In this study the culture supernatant contains a mixture of bacterial endotoxin, bacterial metabolic products and enzymes. The most frequently studied components are the LPS and the Arg- and Lys-gingipain cysteine proteinases, which are found in large quantities in culture supernatants (7, 40). In previous studies it has been shown that proteolytic cleavage by *P. gingivalis* can degrade and inactivate cytokines including IL-6, IL-8 and tumour necrosis factor-α (3, 4, 30, 35) and the CD14 receptors (15, 44). However, the possibility that the antagonistic actions of *P. gingivalis* reported here were the result of proteolytic breakdown of IL-1α is not supported by our data. When cultures containing *P. gingivalis* supernatant were supplemented with IL-1α protein at known concentrations almost all could be detected by ELISA after 6 h. The reasons that we did not observe proteolytic degradation in our system could reflect the presence of serum proteins, including protease inhibitors and the relatively short (6 h) period of incubation. Moreover, the *P. gingivalis* protease-mutant strains still retained their capacity to antagonize *C. rectus*-mediated IL-1α production. In addition, heat-treatment or chemical inhibition of protease activity in the wild-type *P. gingivalis* strain supernatant did not abolish its inhibitory capacity, but was in fact slightly enhanced. This may reflect the normal complexing of the gingipains with LPS in the supernatant, which acts to mask LPS activity. The act of heating unmasks the LPS and results in increased inhibitory activity (45). Overall the data suggest that gingipains do not play a significant role in the antagonism of activity *C. rectus* by *P. gingivalis* as measured by changes in IL-1α production.

To investigate the involvement of LPS in the observed inhibition of IL-1α production the activity of purified

P. gingivalis LPS was tested. This purified LPS could not stimulate high levels of IL-1α production by monocytes, which is in agreement with previous findings (39). In contrast, depletion of LPS from *C. rectus* culture supernatants using polymyxin B columns largely abolished its ability to stimulate IL-1α production. Adding purified *P. gingivalis* LPS was sufficient to suppress IL-1α stimulation in response to the *C. rectus*. Interestingly LPS concentrations in *P. gingivalis* supernatants are similar to the level of purified LPS needed to inhibit IL-1α production. In addition, *P. gingivalis* supernatant that was depleted of LPS using polymyxin B was unable to block *C. rectus*-induced IL-1α production.

One possible explanation for the observed antagonistic effect is that although *P. gingivalis* LPS is a weak stimulus for pro-inflammatory cytokines it is a potent inducer of IL-1 receptor antagonist (IL-1RA) (34). Indeed, it has been shown that high levels of IL-1RA may down-regulate IL-1α production in response to LPS in monocytes (16). Alternatively, the antagonistic effect could be attributed to competition of the two species for common signalling receptors, such as CD14 and Toll-like receptors (11, 20, 50, 52). Although it has not been conclusively shown that *P. gingivalis* and *C. rectus* possess any components that initiate common signalling pathways, the present work supports a putative LPS cross-talk between these two species. We have shown that priming of Monomac-6 cells with *P. gingivalis* LPS results in a significant decrease in the capacity for *C. rectus* to stimulate IL-1α production either alone, or in combination with *P. gingivalis*. Previous studies have demonstrated that *P. gingivalis* LPS can antagonize the LPS of *E. coli* as well as other species (50). There is evidence that binding of *P. gingivalis* LPS to toll-like receptors 2 and 4 is involved in this inhibition (5, 8, 32). It is also important to draw a distinction between the phenomenon of endotoxin tolerance, which is normally thought to require a prolonged stimulation of immune cells, and this study, which involves a more acute short-term assay (31). It is possible that the same mechanisms are involved but equally, studies suggest that the observed antagonism could reflect the direct signalling by *P. gingivalis* LPS rather than a generalized suppression of immune responsiveness (5, 11).

In summary this work demonstrates an antagonism between *P. gingivalis* and *C. rectus* in terms of IL-1α production that appears to rely on the integration of positive and negative signals through cell signalling rather than any direct interaction between components in the supernatants. This study suggests that *P. gingivalis* plays a role in the short-term inhibition of innate immune responses. Although *P. gingivalis* LPS exhibits a weak cytokine-stimulating capacity, it may cause an early inactivation of immune cell responses to other bacteria. In this situation the other periodontal bacterial species may more likely to persist, therefore favouring the establishment of chronic inflammatory lesions typical of periodontitis.

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