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Synergistic effects of lipopolysaccharides from periodontopathic bacteria on pro-inflammatory cytokine production in an *ex vivo* whole blood model

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SUMMARY

Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* have been strongly associated with chronic periodontitis. This disease is characterized by an accumulation of inflammatory cells in periodontal tissue and subgingival sites. The secretion of high levels of inflammatory cytokines by those cells is believed to contribute to periodontal tissue destruction. The aim of this study was to investigate the inflammatory response of whole blood from periodontitis patients following challenges with whole cells of *P. gingivalis*, *T. denticola*, and *T. forsythia* or their lipopolysaccharides (LPS), individually and in combination. Whole blood collected from seven periodontitis patients was stimulated with whole cells or LPS and the production of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α) were quantified by enzyme-linked immunosorbent assays. The mono and mixed challenges with whole bacterial cells or LPS induced the secretion of high amounts of IL-1β, IL-6, IL-8, and TNF-α by the mixed leukocyte population from periodontitis patients. In addition, *P. gingivalis* LPS, *T. denticola* LPS, and *T. forsythia* LPS acted in synergy to induce high levels of IL-1β and TNF-α. This study suggests that *P. gingivalis*, *T. denticola*, and *T. forsythia* may contribute to the immunodestructive host response characteristic of periodontitis through synergistic effects of their LPS on the inflammatory response induced by a mixed population of leukocytes.

INTRODUCTION

Periodontal diseases are polymicrobial infections that lead to the destruction of tooth-supporting tissues, including periodontal ligament and alveolar bone. The gingival sulcus is home to a complex microbial ecosystem (Kroes *et al.*, 1999; Hutter *et al.*, 2003). While human subgingival plaque harbors over 700 bacterial species, only a few are involved in the initiation and progression of periodontal disease (Haffajee & Socransky, 1994; Paster *et al.*, 2001). Specific bacterial species and bacterial complexes occur more frequently in diseased sites while others are associated with healthy or stable periodontal tissues. *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, have been strongly implicated in advanced periodontal lesions by clinical measures of periodontitis, particularly pocket depth and bleeding on probing

(Socransky *et al.*, 1998; Kasuga *et al.*, 2000). These three anaerobic Gram-negative bacterial species, collectively known as the red complex, produce a broad array of virulence factors that allow them to colonize subgingival sites, disturb the host defense system, invade and destroy periodontal tissue, and promote an immunodestructive host response (O'Brien-Simpson *et al.*, 2004; Holt & Ebersole, 2005; Bodet *et al.*, 2007). The incidence and rate of progression of periodontitis involve complex interactions among periodontopathic bacterial species as well as between periodontopathic bacteria and host immune cells. These interactions lead to the secretion by host cells of various cytokines, including interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor alpha (TNF-α), which modulate periodontal tissue destruction (Okada & Murakami, 1998).

While all types of blood cells are present in normal gingival connective tissue (Hassell, 1993), an accumulation of inflammatory cells is observed during periodontitis (Yamalik *et al.*, 1991). Moreover, the number of leukocytes migrating to the gingival sulcus and periodontal pockets increases during the progression of inflammation (Attstrom, 1970; Kowashi *et al.*, 1980). All types of leukocytes, including neutrophils, monocytes/macrophages, T lymphocytes, and B cells, form the local cell infiltrate in periodontal diseased sites (Kornman *et al.*, 1997; Hillmann *et al.*, 2001). *P. gingivalis*, *T. denticola*, and *T. forsythia* have been observed within gingival tissues *in vivo* (Noiri *et al.*, 2001; Rautemaa *et al.*, 2004; Rudney *et al.*, 2005; Colombo *et al.*, 2007), indicating that they may reach deeper structures of connective tissues. These bacteria can thus interact with the heterogeneous leukocyte population of the gingival sulcus and periodontal tissue as the disease progresses. The present study used an *ex vivo* model based on whole blood collected from periodontitis patients to mimic the interactions between different immune cell types in the presence of plasma proteins in order to characterize the inflammatory response induced by periodontopathic bacteria. More specifically, we investigated the effects of mono- and poly-mixtures of whole cells and lipopolysaccharide (LPS) of *P. gingivalis*, *T. denticola*, and *T. forsythia* on the production of IL-1β, IL-6, IL-8, and TNF-α by the mixed leukocyte population contained in whole blood.

METHODS

Bacterial strains and growth conditions

The bacterial strains used were *P. gingivalis* ATCC 33277, *T. denticola* ATCC 35405, and *T. forsythia* ATCC 43037. *P. gingivalis* was grown in Todd-Hewitt broth (THB; BBL Microbiology Systems, Cockeysville, MD) supplemented with 0.001% hemin and 0.0001% vitamin K. *T. denticola* was grown in oral spirochete medium as previously described (Leschine & Canale-Parola, 1980). *T. forsythia* was grown in Brain Heart Infusion broth (BHI; BBL Microbiology Systems) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO) and 0.001% *N*-acetyl muramic acid (Sigma Chemical Co.). The bacterial cultures were incubated at 37°C in anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) for 24 h (*P. gingivalis*) or 4 days (*T. denticola* and *T. forsythia*).

LPS preparation

LPS were isolated from all three bacterial species using the protocol described by Darveau & Hancock, (1983), which is based on the protein digestion of a whole cell extract by proteinase K followed by successive solubilization and precipitation steps. The LPS preparations were freeze-dried and stored at −20°C until used. Contaminating protein, which was evaluated using a protein assay kit (Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin as a control, amounted to <0.001% (w/w) in all LPS preparations. Purity of the preparations was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver nitrate staining (data not shown). *T. denticola* possesses a lipooligosaccharide (although it will be called LPS in the present paper) whose properties are distinct from the classical LPS of the *Bacteroides* group (Schultz *et al.*, 1998).

Whole blood collection

Samples of venous blood were collected from the antecubital vein of seven generalized periodontitis patients using the Vacutainer™ system and sterile endotoxin-free blood collection tubes containing 150 IU of sodium heparin (Becton Dickinson, Franklin

Lakes, NJ). Informed consent was obtained from all the donors prior to the experiments. The protocol was approved by the ethics committee of Université Laval (#2006-078R-2). The patients were non-smokers and had clinically diseased gingiva with at least 30% of periodontal sites showing attachment loss ≥4 mm. Blood samples were taken before any treatment for periodontitis. Hematological analyses of the whole blood samples performed at the Centre Hospitalier de l'Université Laval (Quebec City, Canada) showed that all the donors had normal leukocyte counts (data not shown). Whole blood samples were diluted 1 : 3 in RPMI-1640 medium (HyClone, Logan, UT) and seeded in 4-ml aliquots in 6-well plates.

Whole blood stimulation

Bacterial cells were harvested by centrifugation at 11 000 *g* for 10 min and suspended in RPMI-1640 medium to a concentration of 1×10^9 bacteria/ml as determined using a Petroff-Hausser counting chamber. Polymicrobial suspensions were prepared by mixing equal volumes of individual bacterial suspensions. They were incubated for 15 min at 37°C prior to conducting the challenges. The mixtures tested were *P. gingivalis*/*T. denticola*, *P. gingivalis*/*T. forsythia*, *T. denticola*/*T. forsythia*, and *P. gingivalis*/*T. denticola*/*T. forsythia*. The effects of individual bacterial species were also tested. The bacterial suspensions were added to the whole blood to obtain a final concentration of 10^7 bacteria/ml. LPS mixtures were prepared by mixing equal volumes of 1 μg/ml LPS solutions. *P. gingivalis*, *T. denticola*, and *T. forsythia* LPS were added alone or in combination to the whole blood. The whole blood challenged with bacteria or LPS were incubated at 37°C in a 5% CO_2 humidified atmosphere with occasional gentle shaking. After 6 h, the mixtures were centrifuged at 11 000 *g* for 10 min and supernatants were stored at −20°C until used. Control blood was incubated in the absence of bacteria or LPS.

Cytokine production

Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) were used to quantify the levels of IL-1β, IL-6, IL-8, and TNF-α in the cell-free supernatants according to the manufacturer's protocols. The absorbance at 450 nm was read using a microplate reader (model 680; Bio-Rad) with the wavelength correction set at 550 nm. Cytokine concentrations were determined in triplicate. The sensitivities of the commercial ELISA kits were 31.2 pg/ml for IL-8, 15.6 pg/ml for TNF-α, 9.3 pg/ml for IL-6, and 3.9 pg/ml for IL-1β.

Statistical analyses

Differences between whole blood stimulations were determined using a randomized block design analysis of variance (ANOVA). Data were considered significant at $P < 0.05$.

RESULTS

Secretion of cytokines by the *ex vivo* whole blood model following stimulation by bacteria

The capacity of periodontal pathogens to induce pro-inflammatory cytokine secretion by whole blood from periodontitis patients was examined. Whole blood samples from seven generalized periodontitis patients were challenged with cells of *P. gingivalis*, *T. denticola*, and *T. forsythia*, alone or in combination, at an infectious dose of 10^7 bacteria/ml for 6 h (Table 1). The mono and polymicrobial challenges all resulted in significant increases in IL-1β, IL-6, IL-8 and TNF-α production compared to unstimulated whole blood ($P < 0.05$). In general, IL-6 and IL-8 were the cytokines produced in higher amounts. High inter-individual variabilities in the amounts of inflammatory mediators secreted following bacterial challenge were observed for IL-1β, IL-8, and TNF-α, while the variability was less important for IL-6. This could be due, at least in part, to cytokine gene polymorphisms or the consequence of a more or less recent bacterial challenge or sub-infectious state in certain subjects, resulting in leukocyte pre-activation. The polymicrobial challenges did not cause a significant synergistic effect on cytokine production compared to the challenges by individual bacterial species.

Secretion of cytokines by the *ex vivo* whole blood model following stimulation by LPS

LPS from *P. gingivalis*, *T. denticola*, and *T. forsythia* whether alone or in combination, all induced significant IL-1β, IL-6, IL-8 and TNF-α secretion compared to

Table 1 Secretion of TNF-α, IL-1β, IL-6, and IL-8 in human whole blood from seven periodontitis patients following mono- and polymicrobial challenges by *P. gingivalis* ATCC 33277 (*P. g*), *T. denticola* ATCC 35405 (*T. d*), and *T. forsythia* ATCC 43037 (*T. f*) for 6 h at a final concentration of 10^7 cells/ml. The bacterial combinations tested were *P. gingivalis*/*T. denticola*, *P. gingivalis*/*T. forsythia*, *T. denticola*/*T. forsythia*, and *P. gingivalis*/*T. denticola*/*T. forsythia*. Cytokine concentrations were assessed by ELISA

	Amount of cytokine secreted (pg/ml)			
Conditions of stimulation	TNF-α	IL-1β	IL-6	IL-8
Patient 1				
Control	ND	10 ± 1	121 ± 6	448 ± 23
P. gingivalis	4451 ± 226	3391 ± 275	6412 ± 260	15 255 ± 352
T. denticola	3192 ± 165	1778 ± 108	6046 ± 266	13 184 ± 223
T. forsythia	4290 ± 198	3858 ± 209	6613 ± 249	14 592 ± 275
P. g/*T. d*	2747 ± 210	1994 ± 141	6446 ± 317	15 087 ± 185
P. g/*T. f*	3616 ± 87	3202 ± 253	6541 ± 267	12 436 ± 211
T. d/*T. f*	3197 ± 209	2743 ± 180	6561 ± 308	17 726 ± 307
P. g/*T. d*/*T. f*	3355 ± 117	3024 ± 227	6792 ± 243	18 609 ± 169
Patient 2				
Control	ND	8 ± 1	ND	410 ± 26
P. gingivalis	6490 ± 175	3764 ± 216	6368 ± 175	4970 ± 181
T. denticola	1140 ± 92	329 ± 20	5826 ± 261	11 682 ± 316
T. forsythia	4825 ± 245	3573 ± 197	6508 ± 233	6481 ± 211
P. g/*T. d*	2108 ± 111	816 ± 64	6104 ± 326	10 124 ± 109
P. g/*T. f*	4325 ± 204	3522 ± 218	6376 ± 178	5493 ± 245
T. d/*T. f*	2020 ± 108	1092 ± 77	5880 ± 341	11 020 ± 283
P. g/*T. d*/*T. f*	2053 ± 89	1152 ± 132	6068 ± 294	10 733 ± 190
Patient 3				
Control	ND	6 ± 1	ND	552 ± 28
P. gingivalis	1479 ± 142	139 ± 9	5617 ± 256	6364 ± 167
T. denticola	4413 ± 238	471 ± 18	6205 ± 297	23 954 ± 256
T. forsythia	6609 ± 257	2925 ± 115	6831 ± 350	18 446 ± 192
P. g/*T. d*	1835 ± 86	116 ± 13	5517 ± 216	13 854 ± 282
P. g/*T. f*	3664 ± 142	709 ± 45	6476 ± 311	20 444 ± 178
T. d/*T. f*	3482 ± 196	607 ± 34	6263 ± 247	19 393 ± 224
P. g/*T. d*/*T. f*	1362 ± 87	143 ± 11	5161 ± 197	6187 ± 114
Patient 4				
Control	37 ± 4	5 ± 1	196 ± 14	459 ± 28
P. gingivalis	4666 ± 104	2077 ± 198	6001 ± 306	11 726 ± 124
T. denticola	1508 ± 80	782 ± 64	4767 ± 209	9042 ± 98
T. forsythia	4057 ± 253	512 ± 39	5652 ± 347	11 677 ± 187
P. g/*T. d*	3624 ± 87	1852 ± 105	5076 ± 260	11 076 ± 145
P. g/*T. f*	3459 ± 123	705 ± 55	5668 ± 341	11 092 ± 117
T. d/*T. f*	2259 ± 147	615 ± 47	4898 ± 183	9572 ± 211
P. g/*T. d*/*T. f*	3346 ± 208	1035 ± 85	5074 ± 216	11 153 ± 280
Patient 5				
Control	86 ± 5	7 ± 1	135 ± 8	586 ± 34
P. gingivalis	7434 ± 268	4828 ± 305	4328 ± 203	3830 ± 75
T. denticola	2420 ± 94	1465 ± 114	3079 ± 143	7060 ± 263
T. forsythia	2292 ± 107	1221 ± 93	4288 ± 155	6505 ± 147
P. g/*T. d*	5942 ± 281	5180 ± 322	4208 ± 241	7021 ± 169
P. g/*T. f*	6980 ± 302	4704 ± 241	4880 ± 210	5740 ± 126
T. d/*T. f*	3414 ± 186	1989 ± 165	4043 ± 272	7129 ± 248
P. g/*T. d*/*T. f*	5773 ± 249	4300 ± 237	3830 ± 124	5629 ± 160
Patient 6				
Control	709 ± 53	6 ± 1	188 ± 7	522 ± 27
P. gingivalis	5831 ± 334	3349 ± 182	6233 ± 222	5259 ± 93
T. denticola	2281 ± 88	245 ± 13	4791 ± 171	5788 ± 135

Table 1 (Continued)

Conditions of stimulation	Amount of cytokine secreted (pg/ml)			
	TNF-α	IL-1β	IL-6	IL-8
T. forsythia	1906 ± 101	286 ± 21	4629 ± 67	5114 ± 82
P. g/*T. d*	1622 ± 137	437 ± 34	4874 ± 215	4420 ± 162
P. g/*T. f*	2531 ± 159	806 ± 59	5969 ± 182	6838 ± 237
T. d/*T. f*	2626 ± 210	562 ± 33	5819 ± 270	8518 ± 165
P. g/*T. d*/*T. f*	2749 ± 173	957 ± 70	6001 ± 165	8637 ± 217
Patient 7				
Control	301 ± 22	7 ± 1	193 ± 14	489 ± 30
P. gingivalis	1857 ± 95	359 ± 31	5072 ± 265	2983 ± 85
T. denticola	5430 ± 240	3528 ± 216	5568 ± 142	4838 ± 127
T. forsythia	4862 ± 199	3865 ± 187	5608 ± 277	2810 ± 90
P. g/*T. d*	3505 ± 187	1767 ± 124	5945 ± 260	6110 ± 129
P. g/*T. f*	3852 ± 205	2940 ± 217	5883 ± 305	4947 ± 168
T. d/*T. f*	6152 ± 224	4554 ± 231	5835 ± 281	5023 ± 208
P. g/*T. d*/*T. f*	7009 ± 316	3323 ± 177	6073 ± 183	4432 ± 170

The data are the means ± standard deviations of triplicate assays.
ND, not detected.

unstimulated whole blood ($P < 0.05$) (Table 2). In six out of seven patients, *T. denticola* LOS was much more stimulatory in regard to secretion of IL-1β, IL-8 and TNF-α, compared to LPS from *P. gingivalis* and *T. forsythia*. Stimulation of whole blood by combination of LPS did not result in significantly higher IL-6 or IL-8 levels compared to the challenge using LPS from the individual bacterial species. However, the *P. gingivalis*/*T. denticola*/*T. forsythia* LPS mixture had a significant ($P < 0.05$) synergistic effect on IL-1β and TNF-α secretion compared to the LPS individually. In addition, the *P. gingivalis*/*T. denticola* LPS mixture induced higher IL-1β levels than the individual LPS. As for bacterial cells, we also observed high inter-individual variability in the amounts of IL-1β, IL-8, and TNF-α secreted following challenges of the *ex vivo* whole blood model with LPS (Table 2).

DISCUSSION

P. gingivalis, *T. denticola*, and *T. forsythia* are frequently detected together in periodontal sites, and their relative proportion increases significantly in the subgingival plaque of periodontitis patients (Socransky *et al.*, 1998; Kasuga *et al.*, 2000; Kuboniwa *et al.*, 2004; Lopez *et al.*, 2004). Binding interactions between these three bacterial species have been reported (Grenier, 1992a; Haffajee & Socransky, 1994; Yao *et al.*, 1996; Hashimoto *et al.*, 2003), and this may contribute to the establishment of periodontopathogenic plaque. Indeed, *P. gingivalis* and *T. denticola* form synergistic mixed biofilms when incubated together (Kuramitsu *et al.*, 2005; Yamada *et al.*, 2005). Cell extracts from *T. forsythia* stimulate the growth of *P. gingivalis* (Yoneda *et al.*, 2005), while *P. gingivalis* and *T. denticola* have a mutually beneficial nutritional relationship (Grenier, 1992b; Grenier & Mayrand, 2000). Synergistic effects on virulence by combinations of *P. gingivalis*, *T. denticola*, and *T. forsythia* has been characterized using various animal models. Mixed infections with *P. gingivalis* and *T. denticola* cause a much more pronounced inflammatory response than monoinfections (mouse pneumonia model) (Kimizuka *et al.*, 2003) while *T. denticola* significantly enhances the virulence of *P. gingivalis* (murine lesion model) (Kesavalu *et al.*, 1998). In a rabbit abscess model, *T. forsythia* strains are highly virulent and invasive when combined with *P. gingivalis* (Takemoto *et al.*, 1997), whereas in mice, *P. gingivalis* and *T. forsythia* have a synergistic effect on abscess formation (Yoneda *et al.*, 2001). A mixture of *P. gingivalis*, *T. denticola* and *T. forsythia* increases alveolar bone resorption in rats much more than each species on it own (Kesavalu *et al.*, 2007). However, little is known regarding the impact of these bacterial interactions on the inflammatory response in humans. While we previously reported that these bacterial species act in concert to increase the

Table 2 Secretion of TNF-α, IL-1β, IL-6, and IL-8 in human whole blood from seven periodontitis patients following challenges by *P. gingivalis* ATCC 33277 LPS (*P. g* LPS), *T. denticola* ATCC 35405 LPS (*T. d* LPS), and *T. forsythia* ATCC 43037 LPS (*T. f* LPS) for 6 h at a final concentration of 1 μg/ml. The LPS combinations tested were *P. gingivalis*/*T. denticola*, *P. gingivalis*/*T. forsythia*, *T. denticola*/*T. forsythia*, and *P. gingivalis*/*T. denticola*/*T. forsythia*. Cytokine concentrations were assessed by ELISA

	Amount of cytokine secreted (pg/ml)			
Conditions of stimulation	TNF-α	IL-1β	IL-6	IL-8
Patient 1				
Control	ND	10 ± 1	121 ± 6	448 ± 23
P. gingivalis LPS	3011 ± 146	3939 ± 187	6593 ± 202	18 429 ± 347
T. denticola LPS	3051 ± 123	3084 ± 206	6581 ± 123	14 693 ± 430
T. forsythia LPS	1658 ± 49	1130 ± 83	6332 ± 151	9984 ± 297
P. g LPS/*T. d* LPS	4342 ± 193	4986 ± 247	6679 ± 180	19 483 ± 284
P. g LPS/*T. f* LPS	2174 ± 66	2768 ± 111	6502 ± 247	13 803 ± 531
T. d LPS/*T. f* LPS	4095 ± 289	4027 ± 184	6611 ± 177	16 262 ± 316
P. g LPS/*T. d* LPS/*T. f* LPS	4439 ± 194*	4915 ± 169*	6729 ± 118	18 200 ± 293
Patient 2				
Control	ND	8 ± 1	ND	410 ± 26
P. gingivalis LPS	354 ± 21	162 ± 3	5772 ± 176	3870 ± 142
T. denticola LPS	1549 ± 114	648 ± 12	6231 ± 123	14 899 ± 267
T. forsythia LPS	334 ± 15	104 ± 4	5643 ± 212	2298 ± 94
P. g LPS/*T. d* LPS	2147 ± 127	1172 ± 64	6669 ± 187	14 113 ± 273
P. g LPS/*T. f* LPS	739 ± 83	124 ± 8	5503 ± 169	3467 ± 105
T. d LPS/*T. f* LPS	1806 ± 106	697 ± 20	6248 ± 214	7468 ± 149
P. g LPS/*T. d* LPS/*T. f* LPS	2130 ± 151*	933 ± 41*	6504 ± 148	8737 ± 234
Patient 3				
Control	ND	6 ± 1	ND	552 ± 28
P. gingivalis LPS	1126 ± 81	97 ± 5	6803 ± 259	15 227 ± 346
T. denticola LPS	1813 ± 151	133 ± 11	6747 ± 210	21 703 ± 255
T. forsythia LPS	1475 ± 87	91 ± 4	6243 ± 156	8637 ± 137
P. g LPS/*T. d* LPS	3391 ± 126	233 ± 14	6896 ± 183	17 841 ± 268
P. g LPS/*T. f* LPS	1254 ± 132	93 ± 6	6303 ± 251	8942 ± 203
T. d LPS/*T. f* LPS	3380 ± 163	210 ± 15	6751 ± 223	20 246 ± 349
P. g LPS/*T. d* LPS/*T. f* LPS	4258 ± 208*	341 ± 17*	7121 ± 238	23 008 ± 280
Patient 4				
Control	37 ± 4	5 ± 1	196 ± 14	459 ± 28
P. gingivalis LPS	688 ± 54	95 ± 3	5507 ± 214	4010 ± 173
T. denticola LPS	1605 ± 102	250 ± 19	5869 ± 180	9130 ± 241
T. forsythia LPS	751 ± 42	99 ± 5	5977 ± 165	2428 ± 88
P. g LPS/*T. d* LPS	2738 ± 168	500 ± 23	6091 ± 182	11 278 ± 361
P. g LPS/*T. f* LPS	1238 ± 87	132 ± 9	6721 ± 355	4193 ± 176
T. d LPS/*T. f* LPS	2727 ± 162	276 ± 14	6227 ± 231	7506 ± 214
P. g LPS/*T. d* LPS/*T. f* LPS	3081 ± 119*	466 ± 26*	6817 ± 186	7749 ± 187
Patient 5				
Control	86 ± 5	7 ± 1	135 ± 8	586 ± 34
P. gingivalis LPS	1051 ± 67	201 ± 13	4793 ± 221	6496 ± 264
T. denticola LPS	3036 ± 143	709 ± 50	5369 ± 176	7814 ± 166
T. forsythia LPS	709 ± 38	107 ± 3	4907 ± 147	4066 ± 190
P. g LPS/*T. d* LPS	2906 ± 173	1653 ± 37	5794 ± 191	9453 ± 258
P. g LPS/*T. f* LPS	467 ± 49	118 ± 10	5256 ± 230	6688 ± 246
T. d LPS/*T. f* LPS	2738 ± 189	589 ± 31	6175 ± 172	8470 ± 250
P. g LPS/*T. d* LPS/*T. f* LPS	4430 ± 271*	1147 ± 72*	6181 ± 141	8733 ± 183
Patient 6				
Control	709 ± 53	6 ± 1	188 ± 7	522 ± 27
P. gingivalis LPS	2822 ± 84	1472 ± 109	6813 ± 226	5310 ± 157
T. denticola LPS	5093 ± 265	2534 ± 86	6924 ± 159	9004 ± 261

Table 2 (Continued)

Conditions of stimulation	Amount of cytokine secreted (pg/ml)			
	TNF-α	IL-1β	IL-6	IL-8
T. forsythia LPS	2677 ± 142	547 ± 22	6686 ± 243	1659 ± 84
P. g LPS/*T. d* LPS	4006 ± 201	2831 ± 132	6837 ± 167	4865 ± 111
P. g LPS/*T. f* LPS	3289 ± 119	1769 ± 95	6996 ± 188	4204 ± 143
T. d LPS/*T. f* LPS	6310 ± 242	3344 ± 143	7198 ± 258	5552 ± 130
P. g LPS/*T. d* LPS/*T. f* LPS	6686 ± 308*	3692 ± 170*	7186 ± 140	7569 ± 169
Patient 7				
Control	301 ± 22	7 ± 1	193 ± 14	489 ± 30
P. gingivalis LPS	549 ± 39	111 ± 4	1586 ± 107	770 ± 35
T. denticola LPS	1470 ± 117	493 ± 16	4732 ± 269	3649 ± 142
T. forsythia LPS	462 ± 30	97 ± 2	4241 ± 224	1177 ± 76
P. g LPS/*T. d* LPS	652 ± 45	242 ± 8	4822 ± 169	1668 ± 62
P. g LPS/*T. f* LPS	564 ± 38	99 ± 6	4809 ± 193	1648 ± 108
T. d LPS/*T. f* LPS	2121 ± 106	573 ± 54	6157 ± 251	4089 ± 213
P. g LPS/*T. d* LPS/*T. f* LPS	2210 ± 91*	929 ± 62*	6574 ± 178	566 ± 34

ND, not detected.

The data are the means ± standard deviations of triplicate assays.

*$P < 0.05$ compared to monoinfected control.

inflammatory response of a macrophage/epithelial cell co-culture model (Bodet *et al.*, 2006), there is no evidence of a synergistic effect on the production of inflammatory mediators by host cells. We used a different model in the present study to characterize the effect of polymicrobial *P. gingivalis*, *T. denticola*, and *T. forsythia* infections on cytokine production by a mixed leukocyte population from periodontitis patients. The *ex vivo* whole blood model has several advantages. It contains all the relevant cell populations that are likely to come in contact with periodontopathogens during periodontitis and takes into consideration the complex cell/cell interactions that occur *in vivo*. Moreover, whole blood is a more physiological environment for investigating the production of inflammatory mediators in response to challenges by bacterial cells or their components since cellular interactions are preserved in the presence of various plasma proteins (soluble CD14, LPS-binding proteins, hormones, soluble cytokine receptors, etc.).

Whole cells of *P. gingivalis*, *T. denticola*, and *T. forsythia*, and their LPS, alone and in combination, significantly upregulated the secretion of IL-6 and IL-8 in the *ex vivo* whole blood model. This suggests that these bacteria likely play a major role in IL-6 and IL-8 induction during periodontitis. Indeed, IL-6 and IL-8 levels are higher in the diseased gingiva and gingival crevicular fluid of patients with periodontitis than in the gingiva and crevicular fluid of periodontally healthy subjects (Takahashi *et al.*, 1994; Okada & Murakami, 1998; Garlet *et al.*, 2003; Lee *et al.*, 2003; Kurtis *et al.*, 2005). IL-6, a multifunctional cytokine, plays an important role in regulating the immune response to periodontal pathogens and is notably involved in osteoclast differentiation and bone resorption. By stimulating IL-6 production by leukocytes, *P. gingivalis*, *T. denticola*, and *T. forsythia* contribute to periodontitis-associated bone destruction. In addition, IL-6 participates in the recruitment of leucocytes to sites of inflammation by increasing the local production of chemokines, such as MCP-1 and IL-8 (Romano *et al.*, 1997). These chemokines help direct the migration of polymorphonuclear leukocytes, monocytes, and macrophages to sites of inflammation. The induction of IL-8 by periodontal pathogens may contribute to amplifying the influx of inflammatory cells to diseased periodontal sites, thus favoring the development of an intense inflammatory reaction.

Periodontal pathogens of the red complex, individually or in combination, induced the secretion of IL-1β and TNF-α by a mixed leukocyte population from periodontitis patients. Interestingly, we showed that a mixture of LPS isolated from those bacteria had a synergistic effect on IL-1β and TNF-α production by this model. IL-1β and TNF-α are critical determinants of the progression of periodontitis (Graves & Cochran, 2003). These two primary cytokines can induce the expression of adhesion molecules and secondary

mediators that facilitate and amplify the inflammatory response, matrix metalloproteinase production, and bone resorption (Graves & Cochran, 2003). Local inhibition of these two cytokines in periodontal tissues significantly reduces the inflammatory response and bone loss in ligature-induced periodontitis in monkeys (Assuma *et al.*, 1998). Stashenko *et al.* reported a correlation between the IL-1β and TNF-α levels in the periodontal tissues of diseased sites, suggesting the expression of these two mediators is coordinated (Stashenko *et al.*, 1991). The induction of IL-1β and TNF-α, which are pleiotropic inflammatory mediators, can play a major role in the initiation of the upregulation of the inflammatory response and can also stimulate the production of secondary mediators such as IL-6, IL-8, MCP-1, and prostaglandins which, in turn, can amplify the degree of inflammation. Periodontal tissue destruction may be an overreaction by the host immune system to periodontal pathogens resulting in excessive production of IL-1β and TNF-α (Graves & Cochran, 2003). Our data suggest that LPS of the periodontopathic bacteria that comprise the red complex may be involved in this phenomenon by acting in synergy to induce high levels of IL-1β and TNF-α. Kimizuka *et al.* reported that mixed *P. gingivalis* and *T. denticola* infections result in a higher IL-1β and TNF-α response in a mouse pneumonia model than monoinfections (Kimizuka *et al.*, 2003). However, we present the first evidence for a synergistic effect of periodontopathic bacteria on the human inflammatory response and provides support for previous studies showing the synergistic effects of these bacteria in animal models. Our hypothesis is that combinations of structurally different LPSs could activate various intracellular pathways by interacting with different receptors, such as Toll-like receptors, and lead to the synergistic production of inflammatory mediators. Considering the high diversity of bacterial species found in periodontal pockets, the synergistic phenomenon that we observed may not be unique and therefore additional combinations of LPS from Gram-negative bacteria may also exert synergistic effects in the whole blood model.

In summary, polymicrobial challenges involving *P. gingivalis*, *T. denticola*, and *T. forsythia* resulted in an increase in pro-inflammatory cytokine production in the *ex vivo* whole blood model. While non-myeloid cells are not present in this model, the synergistic stimulation of IL-1β and TNF-α secretion by LPS may be an important mechanism that contributes to the activation of various periodontal tissue destruction pathways that in turn results in bone loss and connective tissue breakdown.

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