Immunobiological Properties of Lipopolysaccharides Isolated from *Fusobacterium nucleatum* and *F. necrophorum*

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Lipopolysaccharides (LPSs) were isolated from *Fusobacterium nucleatum* ATCC 10953 and *F. necrophorum* ATCC 25286 by the hot phenol/water procedure. *F. nucleatum* LPS was composed of 16% (w/w) carbohydrate, 10% (w/w) hexosamine and 40% (w/w) fatty acid, while *F. necrophorum* LPS was composed of 26% (w/w) carbohydrate, 12% (w/w) hexosamine and 28% (w/w) fatty acid. These LPS preparations induced mitogenic responses in spleen cells of BALB/c, BALB/c (nu/nu) and C3H/HeN mice, and these responses were suppressed by the addition of polymyxin B. The preparations also induced the polyclonal responses of C3H/HeN spleen cells. In addition, enhanced glucose utilization and interleukin-1 production by murine peritoneal macrophages were demonstrated. Neither spleen cells nor macrophages from the 'LPS-nonresponsive' C3H/HeJ mouse were activated by LPSs from the *Fusobacterium* species.

INTRODUCTION

Fusobacterium nucleatum and *F. necrophorum* are Gram-negative, obligatorily anaerobic filamentous bacteria. *F. nucleatum* is prominent in the bacterial flora of dental and subgingival plaques (Slots, 1979; Socransky *et al.*, 1982), with higher numbers than normal commonly found in sites with gingivitis or periodontitis (Moore *et al.*, 1982; Savitt & Socransky, 1984). It has induced periodontitis in monoinfected gnotobiotic rats (Irving *et al.*, 1978). *F. necrophorum* is rarely isolated from oral flora; however, it occasionally causes severe necrotic infections in man and animals (Duerden, 1983).

The pathogenicity of these *Fusobacterium* species may be related in part to their endotoxin (lipopolysaccharide; LPS) (Hofstad, 1982). It has been shown that the amount of endotoxin in gingival and periodontal tissues correlates with the severity of inflammation in these sites (Shapiro *et al.*, 1972). Simon *et al.* (1972) also reported that the number of anaerobic Gram-negative rods in gingival exudates correlated with the amount of endotoxin in crevicular fluid as well as with the degree of tissue inflammation. Endotoxic biological properties of various LPS preparations isolated from *Fusobacterium* species have been studied by several workers. LPS from *F. nucleatum* produces local and generalized Shwartzman reactions, and it is lethal to mice and chick embryos, and pyrogenic in rabbits (Mashimo *et al.*, 1985; Sveen, 1977; Sveen *et al.*, 1977). It can also activate human and guinea-pig complements (Hawley & Falkler, 1978; Nygren *et al.*, 1979), and stimulate bone resorption (Sveen & Skaug, 1980). LPS from *F. necrophorum* also possesses various endotoxic activities (Garcia *et al.*, 1975; Warner *et al.*, 1975). However, little is known about the immunobiological potencies of *Fusobacterium* LPS with

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Abbreviations: Con A, concanavalin A; FCS, foetal calf serum; IL-1, interleukin-1; KDO, 2-keto-3-deoxyoctonate (3-deoxy-D-manno-2-octulosonic acid); PFC, plaque-forming cell(s); SRBC, sheep red blood cell(s).

respect to lymphoid cells and macrophages. In this study, we isolated and characterized LPSs from *F. nucleatum* and *F. necrophorum* by the hot phenol/water procedure, and investigated their immunobiological properties.

METHODS

Bacterial strains and growth conditions. *F. nucleatum* ATCC 10953 was kindly supplied by Dr T. Umemoto, Department of Oral Bacteriology, Kanagawa Dental College, Yokosuka, Japan. *F. necrophorum* ATCC 25286 was obtained from the American Type Culture Collection, Rockville, Md, USA. The bacteria were grown in Brain Heart Infusion broth (Difco) supplemented with 0.5% (w/v) yeast extract (Difco) and cysteine. HCl (0.3 mg ml⁻¹) at 37 °C for 3 d in air/CO₂ (95:5, v/v). Organisms were harvested by centrifugation at 6000 g for 30 min, washed three times with pyrogen-free water, and lyophilized.

Extraction and purification of LPS. LPS was isolated from lyophilized cells by the hot phenol/water extraction procedure of Westphal & Jann (1965). The crude extract was treated with nuclease, washed extensively with pyrogen-free water by ultracentrifugation, and lyophilized (Koga *et al.*, 1985). This preparation was referred to as 'partially purified' LPS. The partially purified preparation was dissolved in pyrogen-free water at a concentration of 2 mg ml⁻¹, and an equal volume of 90% (v/v) phenol was added. The mixture was stirred vigorously at 65 °C for 30 min, and the aqueous phase was then collected. This extraction procedure was repeated once more. The combined aqueous phases were dialysed extensively against distilled water, lyophilized, and the product termed 'purified' LPS. LPS from *Escherichia coli* K235 was isolated and purified as described previously (Koga *et al.*, 1985).

Chemical analyses. Hexose, hexosamine, heptose, 2-keto-3-deoxyoctonate (KDO), protein, fatty acid esters, and phosphorus were assayed by the colorimetric methods previously described (Koga *et al.*, 1985). For sugar and fatty acid analyses, LPS was hydrolysed for 6 h at 100 °C in 2 M-HCl. The hydrolysate was suspended in chloroform/methanol/water (4:10:5, by vol.) and mixed vigorously. The aqueous and chloroform phases were separated by centrifugation, and were processed for the sugar and fatty acid analyses, respectively. Neutral and amino sugars in the aqueous phase were identified as trimethylsilyl or alditol acetate derivatives by gas-liquid chromatography (GLC), and fatty acids in the chloroform phase were quantified as their methyl esters by GLC (Koga *et al.*, 1985).

Mitogenic and polyclonal B cell activation activities. Mitogenic activity of LPS for spleen cells from BALB/c, BALB/c (nu/nu), C3H/HeN and C3H/HeJ mice, and for thymocytes from BALB/c mice, was determined as previously described (Koga *et al.*, 1985). Spleen cells (5 × 10⁵) were suspended in 0.2 ml RPMI 1640 medium supplemented with penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹) and HEPES buffer (15 mM, pH 7.2) (hereafter, this medium is referred to simply as RPMI 1640 medium), and were cultured with various stimulants at 37 °C in a humidified atmosphere of air/CO₂ (95:5, v/v). Cultures were pulsed for the final 6 h of incubation with [³H]thymidine (0.25 µCi, 9.25 kBq; Amersham), and then the amount of [³H]thymidine uptake was determined. Concanavalin A (Con A; Sigma) was used as a T-cell mitogen. The effect of polymyxin B (Sigma) on mitogenic responses of BALB/c spleen cells to LPS was examined as follows. Spleen cells (5 × 10⁵) were cultured in 0.2 ml RPMI 1640 medium containing various amounts of polymyxin B (0–5 µg) and 5 µg of stimulant. The mitogenic responses were determined as described above.

In vitro polyclonal B cell activation activity of LPS for C3H/HeN and C3H/HeJ spleen cells was determined as previously described (Koga *et al.*, 1985). After incubation for 4 d at 37 °C in air/CO₂ (95:5, v/v), cells were removed from each culture well, washed, and assayed for direct anti-sheep erythrocyte plaque-forming cell (anti-SRBC PFC) responses by the haemolytic plaque technique of Cunningham & Szenberg (1968).

Glucose consumption by peritoneal macrophages. Macrophage activation was evaluated by the glucose consumption assay described by Ryan *et al.* (1979). Murine peritoneal macrophages were induced by intraperitoneal injection of 2 ml 10% (w/v) Trypticase peptone (BBL). Four days later, peritoneal exudate cells were collected by peritoneal lavage with 5 ml Hanks' balanced salt solution (GIBCO) containing 1% (v/v) foetal calf serum (FCS; Armour Pharmaceutical Co.), washed, and resuspended in RPMI 1640 medium containing 5% (v/v) FCS. Peritoneal macrophages (6 × 10⁵) were placed in 96-well microtitre plates and incubated for 3 h at 37 °C in air/CO₂ (95:5, v/v), then nonadherent cells were removed by washing with RPMI 1640 medium. The adherent cells were cultured in 0.2 ml RPMI 1640 medium with or without stimulant at 37 °C in air/CO₂ (95:5, v/v), and samples (5 µl) of the culture supernatant were assayed for glucose using a blood sugar GOD-Perid test kit (Boehringer Mannheim). The results were expressed as percentages of the initial glucose concentration in the culture supernatants. Each assay was done on triplicate cultures.

Induction of interleukin-1 (IL-1) production by peritoneal macrophages. Peritoneal macrophages (3 × 10⁶) prepared as described above were cultured in 1 ml RPMI 1640 with or without stimulant at 37 °C in air/CO₂ (95:5, v/v) for 24 h. The culture supernatant was harvested by centrifugation, and passed through a filter membrane (0.45 µm pore size, Millipore). IL-1 activity in the culture supernatant was quantified by measuring the incorporation of [³H]thymidine into C3H/HeJ mouse thymocytes (Vacheron *et al.*, 1983). The cells (1.5 × 10⁶) were cultured for

72 h at 37 °C in RPMI 1640 containing 10% (v/v) FCS, Con A (1 µg ml⁻¹) and test sample. They were pulsed for the final 24 h with 0.5 µCi (18.5 kBq) [³H]thymidine.

RESULTS

Chemical composition of LPSs from Fusobacterium spp.

Purified LPS from *F. nucleatum* ATCC 10953 (*F. nucleatum* LPS) contained (w/w) 15.9% carbohydrate, 10.4% amino sugar, 40.0% fatty acid and 4.9% phosphorus. Purified LPS from *F. necrophorum* ATCC 25286 (*F. necrophorum* LPS) contained 26.1% carbohydrate, 11.5% amino sugar, 27.9% fatty acid and 3.1% phosphorus. These LPS preparations contained less than 2% protein as determined by the Lowry procedure. The major sugars in *F. nucleatum* LPS were galactose and heptose, whereas those in *F. necrophorum* LPS were glucose and heptose. The major fatty acids in *F. nucleatum* LPS were myristic (C14:0), β -hydroxymyristic (3-OH C14:0) and β -hydroxypalmitic (3-OH C16:0) acids. *F. necrophorum* LPS did not contain β -hydroxypalmitic acid (data not shown).

Mitogenic and polyclonal B-cell activation activities of LPS

Purified LPSs from *F. nucleatum* and *F. necrophorum* were mitogenic for spleen cells of BALB/c and BALB/c (nu/nu) mice, but not for BALB/c thymocytes (Table 1). Similar activities were also found with the reference *E. coli* LPS. These results indicate that LPSs from *Fusobacterium* species, like *E. coli* LPS, are B-cell mitogens. Purified LPS preparations from *F. nucleatum* and *F. necrophorum* were clearly mitogenic for spleen cells of C3H/HeN mice but not for those of C3H/HeJ mice (Fig. 1*a, b*). However, 'partially purified' LPSs from both species were significantly mitogenic for C3H/HeJ spleen cells (Fig. 1*c, d*). This may have been due to contaminating endotoxic proteins, since these 'partially purified' LPS preparations contained significant amounts of protein (5.9% and 4.3% for *F. nucleatum* and *F. necrophorum*, respectively).

We examined the effect of polymyxin B, which inhibits mitogenic activity of *Enterobacteriaceae* LPS, on the mitogenicity of LPSs from the *Fusobacterium* species (Fig. 2). Polymyxin B markedly suppressed the mitogenic activity of purified LPSs from *Fusobacterium* species as well as that of LPS from *E. coli*, but it only weakly inhibited the mitogenicity of Con A, used as a reference T-cell mitogen.

Purified LPSs from both *F. nucleatum* and *F. necrophorum* enhanced *in vitro* direct PFC responses of C3H/HeN spleen cells to SRBC, but not those of C3H/HeJ spleen cells (Table 2). These results suggest that the LPS preparations induced polyclonal IgM synthesis in C3H/HeN but not in C3H/HeJ mice. Both LPS preparations showed *in vitro* adjuvant activity for C3H/HeN spleen cells, but not for C3H/HeJ spleen cells (data not shown).

Activation of macrophages by LPS

The ability of the purified LPSs to enhance the metabolic rate of macrophages was evaluated by measuring glucose consumption. Both *F. nucleatum* and *F. necrophorum* LPSs enhanced glucose utilization by BALB/c peritoneal macrophages, to the same extent as *E. coli* LPS (Fig. 3). C3H/HeJ macrophages were not stimulated by these LPS preparations (Fig. 4).

IL-1 activity in the culture supernatant of peritoneal macrophages was assessed by measuring the incorporation of [³H]thymidine by C3H/HeJ thymocytes. Both *F. nucleatum* and *F. necrophorum* LPSs stimulated C3H/HeN macrophages but not C3H/HeJ macrophages, to produce IL-1 activity (Table 3).

DISCUSSION

Chemical analyses revealed that *F. nucleatum* and *F. necrophorum* possess a 'classical' LPS which contains KDO, heptose and β -hydroxymyristic acid. The sugar and fatty acid compositions that we found for *F. nucleatum* LPS are essentially similar to those reported previously (Hase *et al.*, 1977; Hofstad, 1982; Hofstad & Skaug, 1980; Kristoffersen & Hofstad,

Fig. 1. Mitogenic responses of C3H/HeN (a, c) and C3H/HeJ (b, d) mouse spleen cells to purified LPSs (a, b) and 'partially purified' LPSs (c, d). Spleen cells (5×10^5) were cultured for 48 h with $1 \mu\text{g}$ (▨), $5 \mu\text{g}$ (▧) or $10 \mu\text{g}$ (■) stimulant per well, or without stimulant (□), in 0.2 ml RPMI 1640 medium. Data are expressed as the mean $\pm$ SD of triplicate cultures.

Table 1. Mitogenic responses of BALB/c spleen cells, thymus cells and BALB/c (nu/nu) spleen cells to purified LPSs from *Fusobacterium* spp.

Spleen cells or thymocytes (5×10^5 per well in 0.2 ml RPMI 1640 medium) were cultured for 48 h with or without stimulant. Values are expressed as the mean $\pm$ SD of triplicate cultures.

Stimulant	Dose (μg per well)	$[^3\text{H}]$ Thymidine uptake (c.p.m.)		
		BALB/c spleen cells	BALB/c thymus cells	BALB/c (nu/nu) spleen cells
None	—	937 $\pm$ 229	495 $\pm$ 91	971 $\pm$ 195
Con A	5	3945 $\pm$ 1574	26539 $\pm$ 3751	643 $\pm$ 111
	2.5	30560 $\pm$ 6509	16651 $\pm$ 2560	594 $\pm$ 36
	1	19377 $\pm$ 2894	1040 $\pm$ 598	996 $\pm$ 77
<i>E. coli</i> K235 LPS	10	9595 $\pm$ 2414	777 $\pm$ 425	10501 $\pm$ 1416
	5	19561 $\pm$ 3463	570 $\pm$ 274	9840 $\pm$ 721
	1	15561 $\pm$ 2146	369 $\pm$ 54	7678 $\pm$ 672
<i>F. nucleatum</i> ATCC 10953 LPS	10	10393 $\pm$ 1159	640 $\pm$ 447	11739 $\pm$ 2278
	5	11843 $\pm$ 1353	612 $\pm$ 360	7016 $\pm$ 689
	1	3357 $\pm$ 131	660 $\pm$ 220	2994 $\pm$ 496
<i>F. necrophorum</i> ATCC 25286 LPS	10	5782 $\pm$ 855	396 $\pm$ 56	6923 $\pm$ 850
	5	3742 $\pm$ 395	430 $\pm$ 35	4779 $\pm$ 209
	1	1859 $\pm$ 225	289 $\pm$ 47	2219 $\pm$ 189

Fig. 2

Fig. 3

Fig. 2. Effects of polymyxin B on mitogenic responses of BALB/c mouse spleen cells. Cells (5×10^5) were cultured for 48 h with 5 μ g of Con A (○), *E. coli* LPS (●), *F. nucleatum* LPS (▲) or *F. necrophorum* LPS (■) in 0.2 ml RPMI 1640 medium containing various amounts of polymyxin B. Control spleen cell cultures to which no polymyxin B had been added responded as follows: Con A, 17361 c.p.m.; *E. coli* LPS, 12352 c.p.m.; *F. nucleatum* LPS, 11486 c.p.m.; *F. necrophorum* LPS, 7466 c.p.m.

Fig. 3. Ability of purified LPSs from *F. nucleatum* and *F. necrophorum* to increase glucose utilization by BALB/c peritoneal macrophages. Macrophages (obtained from 6×10^5 peritoneal exudate cells) were cultured with 10 μ g of *E. coli* LPS (●), *F. nucleatum* LPS (▲) or *F. necrophorum* LPS (■) per well, or without stimulant (○), in 0.2 ml RPMI 1640 medium. Glucose consumption was assayed as described in Methods.

Table 2. Polyclonal B cell activation by purified LPSs from *Fusobacterium* spp.

Spleen cells (1×10^6) from C3H/HeN and C3H/HeJ mice were incubated with stimulant in 0.2 ml RPMI 1640 medium supplemented with 10% (v/v) FCS. After 4 d incubation, PFC responses were determined. Values are expressed as the mean $\pm$ SD of four different cultures.

Source of stimulant LPS	Dose (μ g per well)	No. of PFC per well	
		Spleen cells:	
		C3H/HeN	C3H/HeJ
None	—	9 $\pm$ 3	6 $\pm$ 3
<i>E. coli</i> K235	10	46 $\pm$ 5	6 $\pm$ 4
	2	69 $\pm$ 6	4 $\pm$ 2
	0.2	53 $\pm$ 13	5 $\pm$ 2
<i>F. nucleatum</i> ATCC 10953	10	61 $\pm$ 7	6 $\pm$ 2
	2	51 $\pm$ 5	4 $\pm$ 2
	0.2	38 $\pm$ 5	5 $\pm$ 2
<i>F. necrophorum</i> ATCC 25286	10	53 $\pm$ 4	8 $\pm$ 3
	2	46 $\pm$ 8	4 $\pm$ 3
	0.2	24 $\pm$ 6	4 $\pm$ 1

1970; Mashimo *et al.*, 1985; Wollenweber *et al.*, 1984). LPS from *F. necrophorum* ATCC 25286 did not contain β -hydroxypalmitic acid, in agreement with the findings of Hofstad & Skaug (1980) for LPSs from other *F. necrophorum* strains.

LPSs from *Fusobacterium* species have been reported to possess various endotoxic activities (Garcia *et al.*, 1975; Hawley & Falkler, 1978; Mashimo *et al.*, 1985; Nygren *et al.*, 1979; Sveen,

Fig. 4. Glucose consumption of BALB/c (a), C3H/HeN (b) and C3H/HeJ (c) peritoneal macrophages treated with purified LPSs from *F. nucleatum*, *F. necrophorum* and *E. coli*. Macrophages (obtained from 6×10^5 peritoneal exudate cells) were cultured for 72 h in the presence of 10 µg (■) or 2 µg (▨) of stimulant per well in 0.2 ml RPMI 1640 medium. Glucose consumption was assayed as described in Methods.

Table 3. Induction by LPSs from *Fusobacterium* spp. of IL-1 secretion by macrophages from C3H/HeN and C3H/HeJ mice

Macrophages (3×10^6) were cultured for 24 h in the presence of stimulant in 1 ml RPMI 1640 medium. The culture supernatant (1:2 dilution; 0.1 ml) and an equal volume of C3H/HeJ thymocytes suspension (1.5×10^7 ml⁻¹) were seeded into a 96-well microculture plate. Cultivation was carried out in the presence of Con A (0.2 µg per well). The basal values of [³H]thymidine incorporation were 409 ± 163 c.p.m. for C3H/HeN macrophage supernatants and 538 ± 66 c.p.m. for C3H/HeJ macrophage supernatants.

Stimulant added to macrophage culture	Concn (µg ml ⁻¹)	Relative [³ H]thymidine uptake	
		Macrophage supernatant: C3H/HeN	C3H/HeJ
None	—	1.0	1.0
Silica	100	10.6	3.2
<i>E. coli</i> K235 LPS	50	16.9	1.1
	25	19.3	1.2
	10	13.7	0.9
<i>F. nucleatum</i> ATCC 10953 LPS	50	6.0	1.3
	25	8.7	1.1
	10	5.7	0.9
<i>F. necrophorum</i> ATCC 25286 LPS	50	7.1	1.0
	25	7.3	0.9
	10	4.0	1.0

1977; Sveen *et al.*, 1977). In addition to these findings, the present study shows that the purified LPS preparations from *F. nucleatum* and *F. necrophorum* are B-cell mitogens, because they stimulated spleen cells of BALB/c and BALB/c (nu/nu) mice, but not BALB/c mouse thymocytes (Table 1). Moreover, the purified LPS preparations induced *in vitro* direct PFC responses to SRBC (Table 2). These LPS preparations stimulated spleen cells of the C3H/HeN mouse, but not those of the C3H/HeJ mouse, an LPS-nonresponder (Fig. 1a, b). In addition, polymyxin B, which inhibits the mitogenic activity of *Enterobacteriaceae* LPS (Jacobs & Morrison, 1977), inhibited the mitogenic activity of *Fusobacterium* LPS (Fig. 2). These results indicate that LPS from *F. nucleatum* and *F. necrophorum* possesses biological activities similar to those of LPS from *Enterobacteriaceae*.

The genus *Fusobacterium* is a member of the family *Bacteroidaceae* (Holdeman *et al.*, 1984). However, LPS from *Bacteroides* species has been reported to be quite different from that of *Enterobacteriaceae*, in not containing KDO, heptose or β -hydroxymyristic acid (Hofstad, 1982; Joiner *et al.*, 1982; Koga *et al.*, 1985; Nair *et al.*, 1983; Wollenweber *et al.*, 1980, 1984). Moreover, the biological activities of *Bacteroides* LPSs are atypical; they give weak responses in most tests of endotoxic activity (Sveen, 1977; Sveen *et al.*, 1977), and can act as potent mitogens for spleen cells from endotoxin-nonresponder C3H/HeJ mice (Joiner *et al.*, 1982; Koga *et al.*, 1985; Williamson *et al.*, 1984). Williamson *et al.* (1984) showed that the polysaccharide moiety of LPS from *Bacteroides* is biologically active and mediates the stimulation of B-cells from C3H/HeJ mice via macrophages. Thus, the present study reveals that *Fusobacterium* LPS differs from *Bacteroides* LPS in biological activities as well as chemical properties, although *Fusobacterium* species belong to the family *Bacteroidaceae*.

The 'partially purified' LPS preparations from both *Fusobacterium* strains were mitogenic for C3H/HeJ spleen cells (Fig. 1c, d). The LPS-protein complex is known to be strongly mitogenic for B cells of the C3H/HeJ mouse (Morrison *et al.*, 1976; Sultz & Goodman, 1976). The protein content of these 'partially purified' preparations was 4–6%, suggesting that they may have been contaminated with some mitogenic proteins. In this regard, we found that an endotoxin preparation extracted from *F. nucleatum* by the butanol/water procedure reported by Morrison & Leive (1975) was rich in protein, and strongly mitogenic for C3H/HeJ spleen cells (data not shown).

The present study also revealed that LPSs from *Fusobacterium* species enhance glucose utilization by murine macrophages (Figs 3 and 4). Macrophages activated by LPS secrete various biologically active substances (Vogel & Mergenhagen, 1982). Among these substances, IL-1 possesses many activities, such as stimulation of thymocyte proliferation and activation of B cells (Dinarello, 1984). Gowen *et al.* (1983) showed that an IL-1-like factor stimulated bone resorption in organ cultures of mouse calvaria. In the present study, we found that the LPS preparations from *Fusobacterium* species stimulated IL-1 production by mouse peritoneal macrophages. Furthermore, we have recently found that LPS from *Fusobacterium* species stimulates bone resorption in the mouse calvaria system (unpublished data). These findings suggest that *Fusobacterium* LPS may contribute to alveolar bone loss in the development of chronic periodontitis.

Obligatorily anaerobic Gram-negative filamentous rods such as *Fusobacterium* and *Bacteroides* species are opportunistic pathogens in man and animals. These types of infection are frequently of mixed aetiology. Price & McCallum (1986) demonstrated that the growth factor produced by *F. necrophorum*, which maintains the growth of *Bacteroides intermedius*, is LPS. Enhancement of the growth of oral *Bacteroides* species by *F. necrophorum* or other *Fusobacterium* species may occur in the subgingival flora in the gingival crevice of patients with periodontal diseases.

Taking these findings together, the endotoxic substances from *Fusobacterium* species may contribute to the destruction of infected tissues like the periodontium, possibly as pathogenic mediators.

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