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Suppression of Bactericidal Activity of Human Polymorphonuclear Leukocytes by *Bacteroides gingivalis*

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The direct effects of the culture supernatant of oral microorganisms on the bactericidal activity of human polymorphonuclear leukocytes (PMNs) were investigated. The bactericidal activity of PMNs, which were preincubated with the supernatant of *Bacteroides gingivalis*, *Bacteroides intermedius*, *Bacteroides melaninogenicus* or phosphate-buffered saline, was examined by counting the surviving bacteria. *B. gingivalis*-treated PMNs were found to have a diminished ability for killing bacteria in the presence or absence of serum. The chemiluminescence response of PMNs, which were preincubated with the culture supernatant of *B. gingivalis*, was strikingly reduced compared with that of PMNs preincubated with phosphate-buffered saline or other bacterial culture supernatants. The production of superoxide anion (O_2^-) by PMNs stimulated with either formyl-methionyl-leucyl-phenylalanine or phorbol myristate acetate was reduced in both cases after the PMNs were preincubated with the culture supernatant of *B. gingivalis*. However, it was observed that there was more reduction in superoxide anion (O_2^-) production stimulated with formyl-methionyl-leucyl-phenyalanine compared with that stimulated with phorbol myristate acetate. These results suggest that *B. gingivalis* releases a factor which interferes with the bactericidal activity of PMNs by modulating the generation of reactive oxygen species. These suppressive effects on bactericidal activity may be important in the pathogenesis of this microorganism.

Chronic inflammatory periodontal disease is initiated by bacterial infection. The role of specific gram-negative bacteria in the etiology and pathogenesis of human periodontal disease has been increasingly appreciated in recent years (20, 37). It is generally accepted that *Bacteroides gingivalis* is closely associated with adult periodontitis (7, 11, 21, 23). Furthermore, the interrelationship between a progression of inflammatory periodontal disease and polymorphonuclear leukocytes (PMNs), which act as one of the host defense systems against bacterial infection, has been widely investigated (2, 4, 9, 15, 27, 44). It has been reported that patients with a diminished function of PMNs often suffered from severe periodontal breakdown (5, 6, 39). Loesche et al. (16) recently reported that gingival crevicular PMNs collected from diseased sites had a diminished ability to form oxidized products after phorbol myristate acetate (PMA) stimulation. This result suggests that the functions of PMNs are affected by the bacteria found in subgingival plaque at the site of periodontal pockets. Van Dyke (43) reported that the soluble products of periodontopathic microorganisms modulated the receptors of the PMNs. It has been also reported that several factors from *B. gingivalis* affected the function of PMNs (12, 19, 22, 24). We recently reported (18) that the soluble factor from *B. gingivalis* suppressed the superoxide anion (O_2^-) production of PMNs via the modulation of cell surface receptors and internal cellular events. Because of this result, it was suggested that *B. gingivalis* impaired the bactericidal activity of PMNs.

It is already known that PMNs emit chemiluminescence (CL) during the process of phagocytosis, which correlates well with the bactericidal activity of PMNs (1, 14, 40). The purpose of this study was to investigate the effects of an important pathogen (*B. gingivalis*) on the bactericidal activity of PMNs by measuring the bacterial phagocytic killing, luminol-dependent CL, and superoxide anion (O_2^-) production.

MATERIALS AND METHODS

Bacterial strains and growth conditions. In this study, the following organisms were used: *B. gingivalis* 381, *B. gingivalis* ATCC 33277, *Bacteroides intermedius* ATCC 25611, *Bacteroides melaninogenicus* ATCC 25845, *Bacteroides asaccharolyticus* ATCC 27067, *Bacteroides fragilis* RIMD 0230001, *B. fragilis* ATCC 25285, *Capnocytophaga* strain 25, *Actinobacillus actinomycetemcomitans* ATCC 29522, *A. actinomycetemcomitans* ATCC 29523, *Streptococcus mutans* 6715, *Streptococcus sanguis* ATCC 10557, *Escherichia coli* W3350, *E. coli* IFO 3972, and *Salmonella typhimurium* B2245.

B. gingivalis 381 was grown on CDC anaerobic blood agar (BBL Microbiology Systems, Cockeysville, Md.) in a N_2-H_2-CO_2 (80:10:10) atmosphere at 37°C. The organism was transferred to brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with yeast extract, hemin, and menadione. Culture supernatant was obtained by centrifugation (12,000 × *g* for 20 min) and was lyophilized. Lyophilized culture supernatant was suspended in phosphate-buffered saline (PBS) and extensively dialyzed against PBS before use. Other bacterial culture supernatants were also lyophilized and dialyzed against PBS before use in the same manner.

Dialysate medium was prepared in the following manner. Concentrated brain heart infusion broth was dialyzed against PBS at 4°C overnight, and the dialysate fluid was collected for growing *B. gingivalis*.

Preparation of human PMNs and guinea pig PMNs. Human PMNs were separated from the heparinized blood of a healthy adult by centrifugation (31) with Mono-Poly Resolving Medium (Flow Laboratories, Inc., Tokyo, Japan). More than 85% of the total cells were found to be PMNs, and the

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viable cells were more than 95%. Guinea pig PMNs were obtained from 0.2% oyster glycogen-induced peritoneal exudates. Purified PMNs were washed twice with Hanks balanced salt solution, and the cells were suspended in Hanks balanced salt solution containing bovine serum albumin (10 mg/ml).

Pretreatment of PMNs with bacterial culture supernatants. PMNs suspension (10^7 cells per ml) was preincubated at 37°C with the indicated doses of bacterial culture supernatants. After the PMNs were incubated, they were washed with PBS and suspended in PBS to the original cell concentration (10^7 cells per ml). The viability of the PMNs was examined by the trypan blue dye exclusion technique and by measuring the amount of lactate dehydrogenase release.

Measurement of the bactericidal activity of PMNs. Streptomycin-resistant *E. coli* K-12 JM83, which was kindly provided by K. Nakayama (Department of Microbiology, Faculty of Dentistry, Kyushu University, Fukuoka, Japan), was used for the phagocytic killing assay to avoid any contamination of other bacteria during the assay. The organism was maintained on an L-agar plate (agar, 1.5%; tryptone, 1%; yeast extract, 0.5%; NaCl, 0.5%) containing streptomycin sulfate (200 μg/ml; Meiji Seika Kaisha, Tokyo, Japan) and was transferred to L broth 20 h before the assay. The organism was washed and suspended in PBS, and the concentration was adjusted to 10^8 CFU/ml. The bactericidal activity of the PMNs was examined by a modified method of Iwahi and Imada (13). Briefly, 0.05 ml of *E. coli* suspension (10^8 CFU/ml), 0.25 ml of PMNs suspension (10^7 cells per ml), and 0.2 ml of Hanks balanced salt solution or 0.2 ml of normal human serum were added to Falcon 2054 polystyrene tubes and incubated at 37°C in a shaking water bath. The tubes containing serum were incubated for 10, 30, 60, or 120 min, and the tubes without serum were incubated for 120 min. After the samples were incubated, 0.1 ml of the samples was removed from the tubes and was diluted with 0.9 ml of distilled water to disrupt the PMNs. After appropriate dilution of the samples, 0.1 ml of each sample was poured on L-agar plates containing streptomycin and the number of viable bacteria was determined. One of the results was expressed as a killing index. A killing index was calculated as follows (30): killing index (%) = [(number of viable bacteria without PMNs − number of viable bacteria with PMNs)/number of viable bacteria without PMNs] × 100.

Preparation of opsonized zymosan. Serum-activated zymosan was prepared by a modified method of Seim (35). Zymosan A (Sigma Chemical Co., St. Louis, Mo.) suspended in PBS (20 mg/ml) was boiled for 10 min and washed prior to being opsonized to reduce clumping. The zymosan suspension was incubated with an equal volume of normal human serum at 37°C for 30 min. The particles were washed and suspended to the original volume in PBS.

Measurement of luminol-dependent CL response. A concentrated luminol solution (1 mM) was purchased from Analytical Luminescence Laboratory Inc. (San Diego, Calif.) and was diluted fivefold with PBS immediately before being used. The cuvette of reaction mixture consisted of 0.1 ml of diluted luminol solution, 0.1 ml of PMNs suspension (10^7 cells per ml), and 0.1 ml of opsonized zymosan (20 mg/ml) and was placed into a Lumiphotometer TD-4000 (LABO Science Co., Tokyo, Japan). The assay cuvette was maintained at 37°C, and the intensity of light emitted in the cuvette was recorded automatically. The intensity of CL was indicated as a relative light unit (rlu). Percent inhibition of CL response was calculated as follows: percent inhibition = [(rlu of PBS-treated PMNs − rlu of sample-treated PMNs)/rlu of PBS-treated PMNs] × 100.

Assay of superoxide anion (O_2^-) production by PMNs. Production of superoxide anion (O_2^-) was measured spectrophotometrically by a superoxide dismutase-inhibitable reduction of ferricytochrome *c* (8). A reaction mixture consisting of 890 μl of ferricytochrome *c* suspension plus 100 μl of PMNs suspension (10^7 cells per ml) was prewarmed at 37°C. Ten microliters of stimulant, either formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma) or phorbol myristate acetate (PMA; Sigma) was added to the reaction mixture at final concentrations of 10^{-8} and 10^{-7} M, respectively, and the superoxide dismutase-inhibitable reduction of ferricytochrome *c* was measured with a dual-wavelength (550 versus 540 nm) spectrophotometer (Hitachi 557; Hitachi Ltd., Tokyo, Japan). The amount of O_2^- produced was calculated with a molar extinction coefficient of 19.1×10^3 per cm.

Effect of protease inhibitors. Stock solutions of inhibitors were dissolved either in PBS or in dimethyl sulfoxide. The culture supernatant of *B. gingivalis* was treated with inhibitors at 30°C for 60 min before the addition of PMNs suspension. The inhibitors tested were phenylmethylsulfonyl fluoride, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, leupeptin, and $ZnCl_2$ from Sigma.

Protein concentrations of the culture supernatants were measured by the method of Lowry et al. (17).

Statistical analysis. The data are expressed as means ± standard deviations of triplicate experiments. Statistical comparison was performed by Student's *t* test. Differences were considered significant from the control if the *P* value was <0.05, <0.01, or <0.001.

RESULTS

Phagocytic killing of *E. coli* by PMNs. PMNs pretreated with the culture supernatant of *B. gingivalis* (1 mg/ml) were found to have a diminished ability to kill bacteria compared with PMNs pretreated with PBS (Fig. 1). The killing index of PBS-treated PMNs was 98.7%, and the killing index of *B. gingivalis*-treated PMNs was 55.0% after being incubated for 60 min. From this result, it was calculated that the inhibition of the killing index by the culture supernatant of *B. gingivalis* was 43.7%. However, the bacteria used in this assay were sensitive to complements in the serum. The bactericidal activity of the PMNs was examined in the presence or absence of serum (Table 1). In the presence of serum, PMNs could effectively kill *E. coli* in 30 min. The culture supernatants of both *B. intermedius* and *B. melaninogenicus* had little or no suppressive effect. When serum was omitted from the assay system, PMNs could not kill bacteria as effectively, even after 120 min of incubation, but with this system, we could measure the direct bactericidal activity of the PMNs. PMNs pretreated with the culture supernatant of *B. gingivalis* killed only a limited number of bacteria, and this number was significantly different from bacteria killed by PMNs pretreated with PBS ($P < 0.01$). The two other bacterial culture supernatants had no suppressive effect on the bactericidal activity of the PMNs.

Effect of *B. gingivalis* culture supernatant on CL response. Figure 2 shows the effect of *B. gingivalis* 381 culture supernatant on the CL response of PMNs induced by serum-activated zymosan. PMNs pretreated with PBS evoked a strong CL response after the addition of opsonized zymosan. PMNs pretreated with 1 mg of the culture supernatant of *B. gingivalis* per ml elicited a diminished CL

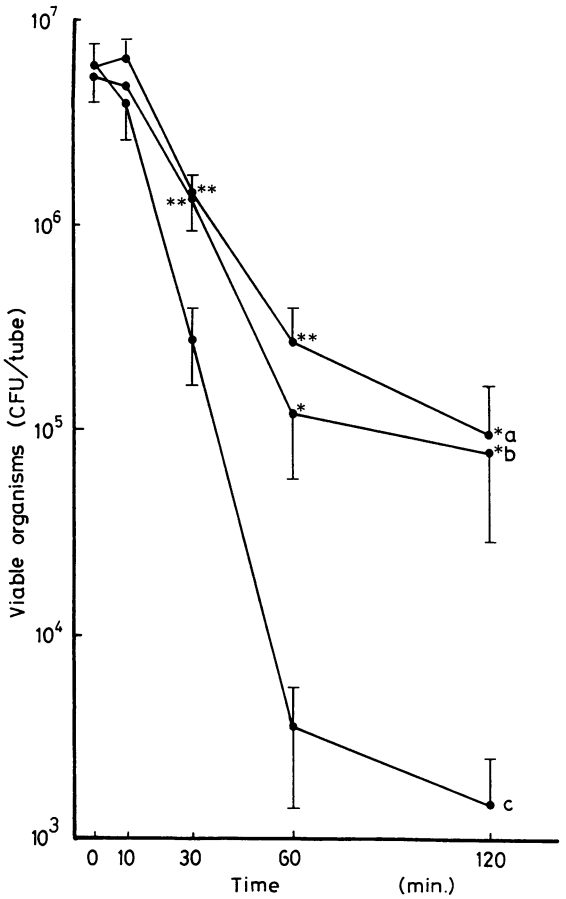

FIG. 1. Effect of the culture supernatant of *B. gingivalis* 381 on the killing of *E. coli* by PMNs. Human PMNs were preincubated with PBS or 1 mg of *B. gingivalis* 381 culture supernatant per ml at 37°C for 60 min. After the PMNs were incubated, they were washed twice with PBS and suspended in PBS. *E. coli* suspension was incubated with these PMNs suspensions at 37°C in the presence of 5% human serum. At the indicated intervals, samples were removed and the viable organisms were counted. Datum points: a, no PMNs; b, *B. gingivalis*-treated PMNs; c, PBS-treated PMNs. Data are expressed as the mean bacteria numbers of triplicate experiments performed in duplicate. Symbols: ** and *, *P* values that are significantly different from those of PBS-treated PMNs. **, $P < 0.01$; *, $P < 0.05$.

response, and this suppression was found to be dose dependent. To minimize the effect of the medium itself, *B. gingivalis* was cultured in the dialysate medium. The culture supernatant of *B. gingivalis* prepared with the dialysate medium also produced a suppressive effect (Fig. 2). When PMNs were pretreated with brain heart infusion broth dialyzed against PBS at 37°C for 60 min, the CL response of these cells was almost the same as that of PMNs pretreated with PBS (data not shown). Figure 3 shows the effect of the preincubation time on the suppressive effect of *B. gingivalis*. The results showed that the suppression of CL response was dependent on the preincubation time. No dead cells were observed when PMNs were incubated with the culture supernatant of *B. gingivalis* for 120 min. The culture supernatant of *B. gingivalis* suppressed the peak CL response as well as the total CL response (Table 2), and the suppressions were found to be well correlated with each other. This suppressive effect was also observed in other strains of *B. gingivalis*, (e.g., *B. gingivalis* ATCC 33277). A total of 14

TABLE 1. Effect of bacterial culture supernatant on the killing of *E. coli* by PMNs

Treatment of PMNs with[a]:	Killing index (%)[b]	
	In the presence of serum[c]	In the absence of serum
PBS	88.8 ± 1.6	43.4 ± 5.6
B. gingivalis 381	60.5 ± 4.9[d]	6.0 ± 4.7[d]
B. intermedius ATCC 25611	84.6 ± 6.7	42.5 ± 4.6
B. melaninogenicus ATCC 25845	91.1 ± 2.2	38.4 ± 5.8

[a] Human PMNs were preincubated with 1 mg of each culture supernatant per ml at 37°C for 60 min.
[b] See Materials and Methods for the equation for calculating the killing index. Data are expressed as the mean killing index of triplicate experiments performed in duplicate.
[c] Five percent normal human serum was added to the test tube.
[d] *P* values that are significantly different from those of PBS-treated PMNs ($P < 0.01$).

kinds of bacterial culture supernatants, including oral and nonoral and gram-positive and gram-negative bacteria, were used to examine the suppressive effects on the CL response of PMNs. The culture supernatant of *B. gingivalis* 381 exhibited a strong suppressive activity on human PMNs, but other bacterial culture supernatants had either weak suppressive activity or no suppressive activity at all (Table 3). A similar result with smaller standard deviations was obtained when guinea pig PMNs were used instead of human PMNs.

Heat sensitivity and the effect of protease inhibitor. The suppressive activity of *B. gingivalis* culture supernatant was not affected by incubation at 56°C for 30 min. Eighty percent of the suppressive activity was lost after the culture supernatant was heated at over 80°C for 30 min (data not shown). When protease inhibitors (phenylmethylsulfonyl fluoride [4 mM], *N*-α-*p*-tosyl-L-lysine chloromethyl ketone [0.04 mM], leupeptin [0.5 μg/ml], and $ZnCl_2$ [1 mM]) were added to the culture supernatant of *B. gingivalis*, no reduction of the suppressive activity was observed.

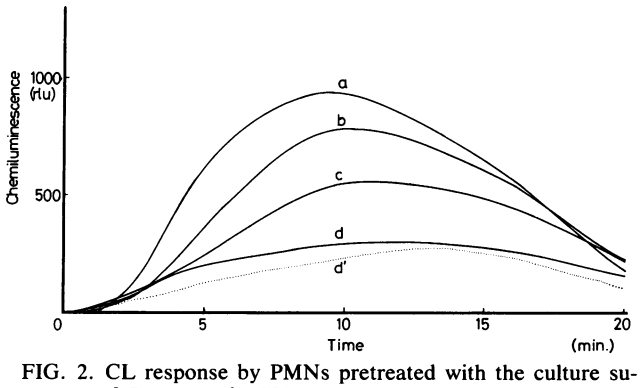

FIG. 2. CL response by PMNs pretreated with the culture supernatant of *B. gingivalis* 381. Human PMNs were preincubated with PBS or the culture supernatant of *B. gingivalis* 381 at 37°C for 60 min. The final protein concentrations are as follows. Datum points: a, 0 μg/ml (PBS); b, 100 μg/ml; c, 500 μg/ml; d, 1,000 μg/ml; d', 1,000 μg/ml (d' was prepared with dialysate medium). After the PMNs were incubated, they were washed twice with PBS and suspended in PBS. The PMNs were stimulated by serum-activated zymosan in the presence of luminol solution, and the CL responses of each 30 s were recorded for 20 min. Data are expressed as the mean CL response of triplicate experiments performed in duplicate.

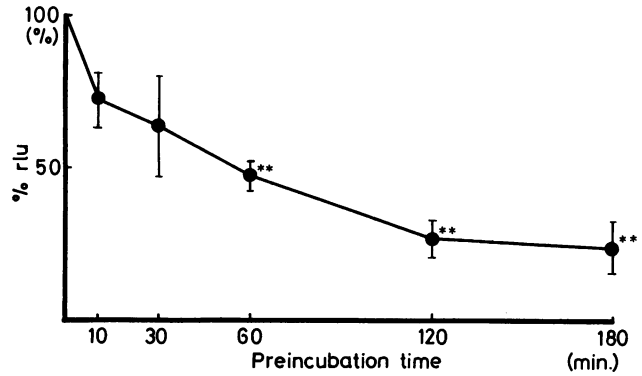

FIG. 3. Effects of time of preincubation with *B. gingivalis* 381 culture supernatant on CL response. Human PMNs were preincubated with PBS or 1 mg of *B. gingivalis* 381 culture supernatant per ml at 37°C for the indicated periods. After the PMNs were incubated, they were washed twice with PBS and suspended in PBS. The PMNs were stimulated by serum-activated zymosan in the presence of luminol solution. Data are expressed as the mean CL response of triplicate experiments performed in duplicate. % rlu = (rlu of *B. gingivalis*-treated PMNs/rlu of PBS-treated PMNs) × 100. Symbol: **, *P* values that are significantly different from those of PBS-treated PMNs ($P < 0.01$).

Effect of *B. gingivalis* culture supernatant on superoxide anion (O_2^-) production by PMNs. After the PMNs were pretreated with the culture supernatant of *B. gingivalis*, there was approximately a 63.0% reduction in the amount of superoxide anion (O_2^-) production by PMNs stimulated with FMLP (Table 4). Similar results were obtained when these cells were stimulated with PMA, but the reduction of superoxide anion (O_2^-) production was less effective (42.7%). It was observed that there was no reduction in the amount of superoxide anion (O_2^-) production by PMNs pretreated with the culture supernatant of *B. intermedius* or *B. melaninogenicus*.

DISCUSSION

The results of this study show that *B. gingivalis* produces a factor which inhibits bacterial phagocytic killing, CL response, and superoxide anion (O_2^-) production by PMNs.

The process of bacterial killing by phagocytes can be divided into several stages, i.e., opsonization of bacteria by serum factors, attachment of the opsonized bacteria, and digestion of these particles (3). It was observed that there was a reduction in bacterial phagocytic killing by PMNs in the presence of serum when the PMNs were pretreated with the culture supernatant of *B. gingivalis* (Table 1). The bacterial killing was less effective in the absence of serum, but similar suppressive effects were obtained. These results show that the bactericidal activity of PMNs is affected by the culture supernatant of *B. gingivalis*.

Bacteria attach to phagocytes and then activate a mem-

TABLE 2. Dose-dependent suppression of CL response by the culture supernatant of *B. gingivalis* 381

Concentration of *B. gingivalis* culture supernatant (μg/ml)[a]	Peak CL		Total CL[b]	
	rlu[c]	% Inhibition[d]	rlu × 10^5[c]	% Inhibition[d]
1,000	307.7 ± 59.4[e]	67.9	2.59 ± 0.41[e]	62.6
500	580.3 ± 146.4	39.4	4.42 ± 1.05	36.2
100	788.7 ± 126.4	17.6	5.88 ± 0.85	15.2
PBS	957.3 ± 187.4	0	6.93 ± 1.08	0

[a] Doses represent final protein concentrations.
[b] Total CL during 20 min of measurement.
[c] Mean ± standard deviation of triplicate experiments performed in duplicate.
[d] See Materials and Methods for the equation for calculating percent inhibition.
[e] *P* values that are significantly different from those of PBS-treated PMNs ($P < 0.01$).

TABLE 3. Effect of bacterial culture supernatants on CL response

Bacterial culture supernatant[a]	Peak rlu[b]	% Inhibition[c]
B. gingivalis 381	156.8 ± 45.6[d]	71.9
B. intermedius ATCC 25611	587.7 ± 183.6	0
B. melaninogenicus ATCC 25845	401.8 ± 164.9	27.9
B. asaccharolyticus ATCC 27067	535.6 ± 141.0	3.9
B. fragilis RIMD0230001	491.8 ± 164.3	11.8
B. fragilis ATCC 25285	614.2 ± 211.1	0
Capnocytophaga strain 25	443.0 ± 277.6	20.5
A. actinomycetemcomitans ATCC 29522	522.2 ± 173.8	6.3
A. actinomycetemcomitans ATCC 29523	436.0 ± 83.2	21.8
S. mutans 6715	608.7 ± 118.8	0
S. sanguis ATCC 10557	538.0 ± 68.2	3.5
E. coli W3350	570.0 ± 138.9	0
E. coli IFO 3972	407.7 ± 62.5	26.8
S. typhimurium B2245	535.7 ± 144.2	3.9
PBS	557.3 ± 212.3	0

[a] Human PMNs were preincubated with 1 mg of each culture supernatant per ml at 37°C for 60 min.
[b] Mean ± standard deviation of triplicate experiments performed in duplicate.
[c] See Materials and Methods for equation for calculating percent inhibition.
[d] *P* values that are significantly different from those of PBS-treated PMNs ($P < 0.01$).

TABLE 4. Dose-dependent suppression of superoxide anion (O_2^-) production by the culture supernatant of *B. gingivalis* 381[a]

Stimulants	Concentration of *B. gingivalis* culture supernatant (μg/ml)[b]	Superoxide (nM/min per 10^6 cells)[c]	% Inhibition[d]
FMLP (10^{-8} M)	1,000	0.68 ± 0.11[e]	63.0
	500	1.01 ± 0.43[f]	45.1
	100	1.35 ± 0.19	26.6
	PBS	1.84 ± 0.30	0
PMA (10^{-7} M)	1,000	4.45 ± 0.52[e]	42.7
	500	4.68 ± 0.47[f]	39.7
	100	6.79 ± 1.07	12.5
	PBS	7.76 ± 0.59	0

[a] Doses represent final concentrations of the stimulants.
[b] Human PMNs were preincubated with PBS or indicated doses of *B. gingivalis* culture supernatant at 37°C for 60 min.
[c] Mean ± standard deviation of triplicate experiments performed in duplicate.
[d] Percent inhibition was calculated by the same method as that for percent inhibition of CL response.
[e] *P* values that are significantly different from those of PBS-treated PMNs ($P < 0.01$).
[f] *P* values that are significantly different from those of PBS-treated PMNs ($P < 0.05$).

brane oxidase, which in turn triggers the respiratory burst and produces chemically reactive molecules such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxy radical (OH ·). The generation of these reactive oxygen species can be quantified as CL in the presence of chemiluminogenic probes such as luminol (1, 14, 40, 41). The luminol-dependent CL response, therefore, is likely to prove a useful method for examining the intracellular killing by PMNs. Pretreatment of PMNs with the culture supernatant of *B. gingivalis* strikingly reduced the CL response stimulated by serum-activated zymosan. There was more reduction of CL response according to the duration of preincubation with the culture supernatant of *B. gingivalis* (Fig. 3). This result suggests that the culture supernatant of *B. gingivalis* activates the cellular process which leads to the suppression of the bactericidal activity of PMNs. This hypothesis is supported by the fact that pretreatment of PMNs with the culture supernatant at 4°C had a weak suppressive effect on CL response (data not shown).

We also examined the effects of *B. gingivalis* culture supernatant on the generation of a superoxide anion, which is one of the potent bactericidal agents of PMNs. PMA is thought to directly activate protein kinase C, which leads to an activation of NADPH oxidase to induce superoxide anion (O_2^-) production (3, 26, 28). From the results obtained by the use of PMA, some of the suppressive effect of *B. gingivalis* seems to be associated with intracellular events. However, it was observed that superoxide anion (O_2^-) production stimulated by FMLP was more suppressed than that stimulated by PMA. We had reported that *B. gingivalis* modulated the FMLP receptors of PMNs (18). These results suggested that *B. gingivalis* impaired the surface receptors and the intracellular mechanisms of PMNs. We also obtained the preliminary result that the culture supernatant of *B. gingivalis* inhibited phagocytosis of the fluorescein-coated beads by PMNs. It was thought that the inhibition of bacterial killing by *B. gingivalis* was dependent on the impairment of surface receptors, phagocytosis, and the generation of superoxide anion of PMNs.

It has been suggested that succinic acid, which is a major by-product of *Bacteroides* species metabolism and a small-molecular-weight fatty acid, is responsible for inhibiting superoxide anion (O_2^-) production in PMNs (29, 30). Our preliminary characterization of this factor of *B. gingivalis* suggests that the thermostable protein-like substance seems to be responsible for the inhibition of bactericidal activity. Moreover, succinic acid can inhibit the respiratory burst of PMNs only at a pH of 5.5, while the factor of *B. gingivalis* presented in this paper is effective at a neutral pH. Therefore, the suppressive factor of *B. gingivalis* seems to be quite different from that of succinic acid.

It has also been reported (36) that oral treponemal factors inhibit superoxide anion (O_2^-) production by human peripheral blood PMNs. A suppressive factor was obtained from the phenol extract and the culture supernatant of oral treponema. These suppressive factors from oral treponema and *B. gingivalis* have not been fully characterized, but there is the possibility that these factors have the same active components for the inhibition of superoxide anion (O_2^-) production.

Recently, researchers have focused on *B. gingivalis* proteases which hydrolyze a number of proteins, including collagen, immunoglobulins, iron-binding proteins, and complement factors (10, 25, 33, 34, 42, 45).

From our results on heat sensitivity and the protease inhibitor experiment, it would seem unlikely that the suppressive factor of *B. gingivalis* is of a protease nature. However, in the protease inhibitor experiment, we were unable to use a large amount of inhibitors because of the toxic effect on PMNs. Therefore, we are not able to rule out the possibility that the suppressive effect was dependent on protease activity. Further work on the isolation and purification of the suppressive factor of *B. gingivalis* is necessary to characterize the nature of this suppressive factor.

The association of members of the black-pigmented *Bacteroides* group, particularly *B. gingivalis* and *B. intermedius*, with active lesions of destructive periodontal disease has been recognized (7, 38). However, it was also reported that the mean percentage of *B. gingivalis* in the active destructive sites was only 2.5% (7). Therefore, one should consider that perhaps other noxious microorganisms are also associated with the destruction of periodontium. Immunosuppressed rats, which were treated with cyclophosphamide, developed a marked alveolar bone loss (32). In these immunosuppressed rats, it was observed that the numbers of mononuclear leukocytes and PMNs were reduced and several gram-negative bacteria had invaded the periodontium. The significant inhibition of bactericidal activity of PMNs by the factor of *B. gingivalis* may alter the ability of PMNs to defend against other noxious microorganisms in the infected sites, which will lead to an enhancement of the periodontal disease process.

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