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Depolarization of Polymorphonuclear Leukocytes by *Porphyromonas (Bacteroides) gingivalis* 381 in the Absence of Respiratory Burst Activation

M. JOHN NOVAK[1]†* AND HARVEY J. COHEN[2]

Departments of Dental Research and Microbiology[1] and Pediatrics and Cancer Center,[2] University of Rochester Medical Center, Rochester, New York 14642

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***Bacteroides* spp. may contribute to the chronicity of mixed infections by affecting the normal functions of polymorphonuclear leukocytes (PMN). This study evaluated the physiologic and biochemical responses of human peripheral blood PMN to a variety of strains of the oral periodontal pathogen *Porphyromonas (Bacteroides) gingivalis*. Strain 381 and ATCC type strain 33277 caused rapid and lasting depolarization of the electrochemical potential that exists across the PMN membrane by a mechanism that was independent of activation of the pertussis toxin-sensitive G protein or protein kinase C. Membrane depolarization did not initiate increases in intracellular calcium or respiratory burst activation, and activity was not inhibited by surface proteolysis or sugars. However, membrane depolarization was associated with inhibition of PMN responses to the chemotactic peptide *N*-formylmethionyl leucyl phenylalanine. Membrane-depolarizing activity was isolated with the outer membrane of strain 381 by surface extraction of the bacteria by using Zwittergent 3,14, followed by Sephacryl S-200 gel filtration chromatography. The partially purified outer membrane components were heat stable, were not inhibited by tosyl-lysine chloromethyl ketone, and inhibited *N*-formylmethionyl leucyl phenylalanine-stimulated superoxide production. The results suggest that outer membrane components of *P. gingivalis* 381 and 33277 have porinlike activity that can depolarize PMN membranes and immobilize PMN responses to chemotactic peptides. This may prove to be an important virulence characteristic of these strains.**

Bacteroides species are frequently isolated in large numbers from chronic purulent lesions as part of a mixed microbial flora (9, 40). Their ability to induce abscess formation as part of a mixed infection appears to be due to their local effects on the functions of polymorphonuclear leukocytes (PMN) (6, 17, 18, 30, 39, 41). The enteric pathogen *Bacteroides fragilis* appears to affect PMN microbicidal activity through production and release of short-chain fatty acids (39, 41), whereas the periodontal pathogen *Porphyromonas* (formerly *Bacteroides*) *gingivalis* (47) inhibits PMN functions by cell-associated molecules and by constituents of culture supernatants (25, 30, 46, 54, 58). The presence of increased numbers of *P. gingivalis* cells in periodontal pockets has been associated with chronic suppurative lesions and progressive periodontal destruction (48). It has been speculated that this destruction is mediated, in part, by alterations that occur in the function of the large numbers of PMN that are found in and around periodontal pockets (45, 58). The PMN migrate through the mucosal lining of the pocket and are essential for the defense of periodontal tissues against the mixed microflora that constitutes subgingival plaque (11). Few studies have been performed that have investigated the physiological and biochemical effects of different strains of *P. gingivalis* on human PMN. It has been reported that intact cells of *P. gingivalis* induce very little chemiluminescence when interacted with human PMN (36), suggesting that they do not activate the PMN microbicidal respiratory burst. The purpose of this study was to characterize the physiological and biochemical responses of human PMN to selected strains of the periodontal pathogen *P. gingivalis* to gain an insight into how the presence of this microorganism may affect PMN and, subsequently, periodontal disease progression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. gingivalis* 381, ATCC 33277, W83, and W50 and rifampin-resistant 3079.03-R1 were grown to the late-logarithmic–early-stationary phase in prereduced Schaedler broth (Oxoid, Basingstoke, England) containing hemin at 10 $\mu g \cdot ml^{-1}$. The purity of cultures was ascertained by phase-contrast microscopy and Gram staining and confirmed by aerobic and anaerobic subculturing on blood agar plates, followed by biochemical analysis using API ZYM chromogenic substrates (Analytab Products, Plainview, N.Y.). Cells were harvested by centrifugation, washed twice in sterile, isotonic saline, lyophilized, and stored at −20°C. Immediately prior to use, bacterial samples were suspended at 1 mg (dry weight) ml^{-1} in Kreb-Ringer phosphate, pH 7.4 (KRP) and dispersed by vortexing and intermittent 30-s bursts of sonication at 50 W at 4°C in a Braun-sonic 1510 homogenizer (B. Braun, AG, Melsungen, Germany) until a single-cell suspension was observed by phase-contrast microscopy. Suspensions of bacterial whole cells were used for all experiments, and except when otherwise indicated, studies were carried out with strain 381.

Preparation of PMN. Human peripheral blood PMN were isolated from healthy adults by dextran T-500 and Ficoll-Paque sedimentation (Pharmacia Fine Chemicals, Piscataway, N.J.), followed by hypotonic lysis of contaminating

* Corresponding author.
† Present Address: Department of Periodontics, University of Texas Health Sciences Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284-7894.

erythrocytes as previously described (3). The isolated PMN were suspended in phosphate-buffered saline (PBS; 0.135 M NaCl, 0.005 M KCl, 0.01 M sodium phosphate buffer, pH 7.4) at 5×10^7 ml^{-1} and kept on ice. Unless otherwise stated, all assays were performed in KRP (PBS, 1.0 mM $CaCl_2$, 1.3 mM $MgSO_4$, pH 7.4) in the presence of 5 mM glucose (KRPg).

Superoxide production. Continuous measurements of superoxide anion (O_2^-) production were performed in a Perkin-Elmer Lambda 4 dual-beam spectrophotometer equipped with a temperature-controlled circulating water bath set at 37°C as previously described (4, 32). The sample cuvette contained ferricytochrome *c* (100 nmol), PMN (10^6), and either whole bacteria (100 μg), *N*-formylmethionyl leucyl phenylalanine (FMLP; 1 nmol), or phorbol myristate acetate (PMA; 1 μg) in 1 ml of KRPg. The reference cuvette contained superoxide dismutase (20 μg) in addition to the above. All assays were run in the presence of cytochalasin B (5 μg). O_2^- production was monitored continuously by monitoring superoxide dismutase-unhibitable reduction of ferricytochrome *c* at 550 nm, and the rate of O_2^- produced was calculated by using the molar extinction coefficient for ferricytochrome c reduction of 2.11×10^4 M^{-1} cm^{-1} (28). All assays of O_2^- production were carried out in triplicate on at least three different occasions.

Membrane potential. Apparent membrane potential depolarizations were monitored by using the fluorescent lipophilic cationic dye 3-3′-dipentyloxacarbocyanine [diOC$_5$-(3)] as described by Seligmann and Gallin (44). Fluorescent measurements of 5×10^5 PMN ml^{-1} of KRPg in the presence of 50 nM diOC$_5$-(3) were performed in stirred cuvettes in a Perkin-Elmer 650-10S fluorescence spectrophotometer at 37°C. The excitation and emission monochromators were set at 470 and 500 nm, respectively, with band pass slits of 6 nm. Under these conditions, steady-state fluorescence was achieved within 10 min of equilibration of diOC$_5$-(3) with PMN. A decrease in fluorescence corresponds to an apparent membrane potential depolarization. The results are presented as tracings of the chart recorder following addition of stimuli under the indicated conditions and represent triplicate results obtained on at least two different occasions.

Intracellular calcium. Changes in intracellular calcium concentration following stimulation with FMLP or *P. gingivalis* were measured as follows. PMN were loaded with the acetoxymethyl ester of Quin 2 as previously described (37, 50). PMN at 5×10^7 ml^{-1} in PBS containing 1.0 mM $CaCl_2$, 5 mM glucose, and 0.5% bovine serum albumin were incubated with the acetoxymethyl ester of Quin 2 at 50 μM for 10 min at 37°C, after which they were diluted to 10^7 ml^{-1} with the same buffer and incubation was continued for an additional 20 min. The PMN were then diluted with 1 volume of PBS, centrifuged at 4°C, washed once with cold PBS, and suspended to 5×10^7 ml^{-1} in PBS. The fluorescence of Quin 2-loaded PMN was monitored as previously described (37, 50). PMN (5×10^7 ml^{-1} of KRPg) were stirred in a thermostatically controlled (37°C) sample compartment of a Perkin-Elmer 650-10S fluorescence spectrophotometer. The excitation wavelength was set at 339 nm, with the band pass slit at 4 nm, and the emmision wavelength was 492 nm with the band pass slit at 10 nm. Changes in fluorescence were monitored on a chart recorder following addition of 10^{-8} M FMLP or 100 μg *P. gingivalis* ml^{-1}. The intracellular calcium concentration was calculated from the maximum fluorescence obtained in PMN lysed with 1% Triton X-100 in the presence of 1 mM calcium and the minimum fluorescence of lysed PMN in the presence of 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] as previously described (50) and individual values were adjusted to account for volumetric changes.

Pretreatment of PMN with PT and staurosporine. In experiments intended to identify the role of G proteins in the interaction of *P. gingivalis* with human PMN, PMN were pretreated with *Bordetella pertussis* toxin (PT; List Biological Laboratories, Campbell, Calif.) by incubating 5×10^7 PMN ml^{-1} of KRPg with 1 μg of PT for 90 min at 37°C. Control cells were incubated with buffer alone. The role of protein kinase C was examined by adding the protein kinase C inhibitor staurosporine (Kamiya Biochemical Co., Thousand Oaks, Calif.) at a final concentration of 105 nM to PMN immediately prior to addition of *P. gingivalis*. Experiments were run in duplicate on three different occasions.

Inhibition of *P. gingivalis*-PMN interactions. *P. gingivalis* was added to PMN in the presence of a panel of sugars known to inhibit lectinlike interactions, i.e., *N*-acetylglucosamine, *N*-acetylgalactosamine, melibiose, mannose, and lactose, all at a final concentration of 50 mM (E. Y. Laboratories, Inc., San Mateo, Calif.). In addition, interactions were carried out in the presence of the amino acids L-lysine, L-glycine, and L-arginine at a final concentration of 5 mM. The effects of surface modification of *P. gingivalis* were examined by treatment with 0.1% trypsin (Sigma), hyaluronidase (Sigma), or neuraminidase (Sigma) at 1 U/ml for 60 min at 37°C. The effect of heat was investigated by heating *P. gingivalis* at 70°C in KRP for 60 min prior to addition to PMN. The potential role of hydrophobic interactions between *P. gingivalis* and PMN was investigated by substituting KRPg with a low-ionic-strength sucrose buffer containing 240 mM sucrose, 10 mM HEPES (*N*-2-hydroxyethylpiperazone-*N*′-2-ethanesulfonic acid), 0.5 mM $MgCl_2$, and 0.33 mM $CaCl_2$ (5). The effects of protease inhibitors on *P. gingivalis*-PMN interactions was evaluated by using tosyllysine chloromethyl ketone (TLCK) and phenylmethylsulfonyl fluoride at a final concentration of 4 mM (14).

Extraction of cell surface components of *P. gingivalis*. Surface components were removed from *P. gingivalis* by using a detergent extraction procedure (8). Lyophilized *P. gingivalis* 381 was suspended at 10 g (dry weight) in 250 ml of extraction buffer (50 mM sodium citrate and 0.1% Zwittergent 3,14 [Calbiochem, La Jolla, Calif.] pH 4.5). The cells were incubated for 30 min at 42°C with intermittent stirring, centrifuged at $10,000 \times g$, washed in PBS, and suspended to their original concentration in KRP prior to addition to PMN. The integrity of the cells was confirmed by phase-contrast microscopy, and the presence of a capsule was determined by examination in an India ink preparation. The supernatant containing the extracted bacterial components was precipitated with 80% ethanol, and the precipitate was pelleted at $12,000 \times g$. The pellet was suspended in PBS containing 0.1% Zwittergent, and excess detergent was removed by passing the mixture over a Sephacryl S-200 gel filtration column (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) that had been preequilibrated with PBS containing 0.02% Zwittergent. Fractions were eluted with PBS–0.02% Zwittergent at 12 ml/h and tested for activity against PMN. Fractions were monitored at an optical density of 280 nm (OD_{280}) for protein content and by the anthrone reaction for neutral sugars (38).

RESULTS

Membrane potential. Changes in membrane potential occur subsequent to ligand-receptor binding for several stimuli,

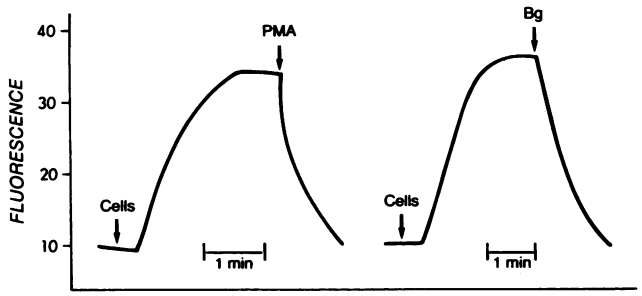

FIG. 1. Effects of PMA and *P. gingivalis* (Bg) on membrane depolarization. Depolarization was tested by monitoring the decrease in fluorescence of $diOC_5$-(3)-equilibrated PMN following addition of PMA or *P. gingivalis*.

including the tumorogenic agent PMA (21, 56). Apparent membrane potential depolarizations were monitored by using the lipophilic cationic dye $DiOC_5(3)$, and a typical tracing is shown in Fig. 1. Following addition of PMN (5×10^5 ml^{-1}) to the dye, a period of equilibration of dye and PMN results in an increase in fluorescence intensity due to binding of the dye to the PMN membrane. Addition of the control stimulus, PMA, was followed by an immediate and rapid decrease in fluorescence. Addition of intact, whole cells of *P. gingivalis* 381 to PMN resulted in a similar decrease in fluorescence intensity, suggesting that a similar change in membrane potential had occurred. Changes in membrane potential were also observed for *P. gingivalis* 33277 but not for any of the other strains tested. *P. gingivalis* 381, 33277, W83, W50, and 3079.03-R1 induced no superoxide production in human PMN, and of these five strains only 381 and 33277 induced membrane depolarization. Each strain was tested by using duplicate determinations on at least three different occasions with different donors. PMN responsive in this manner to *P. gingivalis* 381 and 3327 were not subsequently responsive to further depolarization by PMA (1 μg ml^{-1}) or gramicidin (10^{-4} M), and the changes observed were not due to any quenching effect of the dye by *P. gingivalis*, as assessed by studying the effects of *P. gingivalis* alone on dye fluorescence.

PMN superoxide generation. The activation and activity of the O_2^--generating system in human PMN has previously been demonstrated by using a continuous dual-beam spectrophotometric assay of superoxide dismutase-inhibitable reduction of cytochrome *c* by superoxide anion (4, 32). With this assay procedure, addition of *P. gingivalis* to PMN in the presence or absence of cytochalasin B did not result in production of any measurable level of O_2^- during a 60-min period for any of the strains tested. However, since depolarization of PMN has previously been shown to modulate subsequent responses to the chemotactic peptide FMLP (7, 27), the effects of membrane depolarization by *P. gingivalis* on PMN superoxide production to chemotactic and nonchemotactic stimuli were studied. Preexposure of PMN to *P. gingivalis* 381 whole cells for 2 min resulted in a marked reduction in superoxide production in response to FMLP but not in response to PMA (Table 1). In contrast to the reduced response to FMLP, membrane depolarization caused enhancement of PMA-induced superoxide production.

Intracellular calcium. Previous studies have demonstrated changes in the concentration of intracellular calcium in PMN in response to chemotactic peptides (12, 20, 37), lectins (22, 57), and gram-negative bacteria (26). These changes form an important part of the intracellular second-messenger system of human PMN (42) and appear to be an essential component of the signal transduction mechanism for PMN orientation, migration, and phagocytosis (10). The increase in the intracellular calcium concentration that occurred following addition of the chemotactic peptide FMLP (10^{-8} M) was as shown in Fig. 2. An increase in fluorescence was observed following addition of the chemotactic peptide to PMN, preequilibrated with Quin 2, in the presence of KRP. Fluorescence then returned to baseline levels with time. With addition of intact, whole cells of *P. gingivalis* 381, no increase in Quin 2 fluorescence above the baseline was observed (Fig. 2), suggesting that no detectable changes in the intracellular levels of ionized calcium had occurred.

TABLE 1. Effect of *P. gingivalis* 381 whole cells on superoxide generation by human PMN

Treatment	Mean ± SE maximum rate of O_2^- production (total)[a] with incubation with:	
	PMA (1 μg)	FMLP (1 μM)
Buffer	4.2 ± 1.0 (77.3 ± 19.7)[b]	8.0 ± 0.9 (16.5 ± 1.2)[c]
P. gingivalis	10.7 ± 1.7 (107.8 ± 10.8)[b]	4.4 ± 0.9 (8.1 ± 0.3)[c]

[a] These data are based on triplicate determinations repeated on at least three different occasions ($n = 3$). Maximum rates are in nanomoles of O_2^- min^{-1} 10^6 PMN^{-1}.
[b] Total O_2^- after 30 min.
[c] Total O_2^- after 15 min.

Effects of PT and staurosporine. Treatment of PMN with PT, a known inhibitor of G protein-mediated PMN activation by the synthetic bacterial chemotactic peptide FMLP, failed to inhibit membrane potential changes induced by *P. gingivalis* (Table 2). Since PT treatment completely inhibited FMLP-induced changes in membrane potential and FMLP-induced superoxide production (data not shown), it can be concluded that the mechanism by which *P. gingivalis* depolarizes PMN membranes is independent of the G protein-mediated FMLP activation pathway. Changes in PMN membrane potential are temporally related to activation of protein kinase C and the respiratory burst. Pretreatment of PMN with the protein kinase C inhibitor staurosporine had no effect on *P. gingivalis*-induced changes in membrane potential (Table 2), whereas it inhibited FMLP- and PMA-induced membrane potential changes and activation of the respiratory burst (data not shown). The data suggest that not only does *P. gingivalis* not depolarize PMN by activating the G protein-dependent, FMLP receptor-mediated pathway, but membrane depolarization occurs independently of protein kinase C activity.

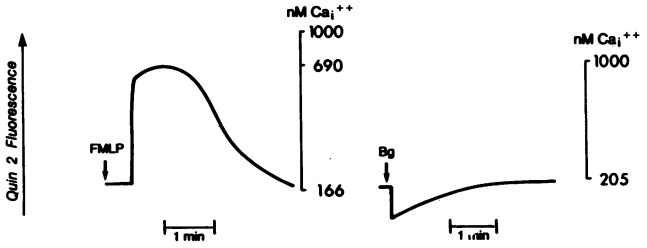

FIG. 2. Effects of FMLP and *P. gingivalis* (Bg) on PMN intracellular calcium concentration (Ca_i^{++}). FMLP or Bg was added to PMN preloaded with the acetoxymethyl ester of Quin 2, and changes in intracellular fluorescence were monitored.

TABLE 2. Inhibition of *P. gingivalis*-induced membrane depolarization of human PMN

Condition	Effect on membrane depolarization
N-Acetylglucosamine (50 mM)	None
N-Acetylgalactosamine (50 mM)	None
Melibiose (50 mM)	None
Mannose (50 mM)	None
Lactose (50 mM)	None
L-Lysine (5 mM)	None
L-Glycine (5 mM)	None
L-Arginine (5 mM)	None
Trypsin (0.1%)	None
Hyaluronidase (0.1%)	None
Neuraminidase	None
Heat (70°C, 1 h)	Inhibition
Low-ionic-strength buffer	Inhibition
Outer membrane extraction	Inhibition
PT	None
Staurosporine	None

Inhibition of *P. gingivalis*-PMN interactions. The interaction between *P. gingivalis* 381 and PMN that initiated depolarization of the PMN membrane could not be inhibited by any of the sugars or amino acids tested or by enzymatically modifying the bacterial surface (Table 2). Membrane-depolarizing activity of whole cells was inhibited, however, by heating the bacteria to 70°C and by removal of the outer layer of the bacteria by detergent extraction. Detergent extraction resulted in minimal changes in bacterial morphology, as viewed by phase-contrast microscopy, although most of the cells showed little evidence of an existing capsule when viewed in an India ink preparation. Inhibition of membrane depolarizaiton by low-ionic-strength sucrose buffer suggests that the interaction between *P. gingivalis* 381 and human PMN depends on hydrophobic forces.

Extraction of *P. gingivalis* surface components and their effects on PMN superoxide production. The detergent extraction procedure resulted in removal of a number of outer membrane proteins, as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the ethanol precipitates (Fig. 3). Removal of excess detergent by chromatographing the ethanol-precipitated extract on a Sephacryl S-200 column decreased the detergent concentration to the point where it failed to affect the PMN and resulted in an elution profile containing a major protein peak, as determined by the OD_{280}s of individual fractions (Fig. 4). Analysis of the individual fractions for the ability to depolarize PMN membranes resulted in the identification of a peak fraction with membrane-depolarizing activity (Fig. 4). Addition of the elution buffer alone failed to have any effect on membrane potential changes. The fractions also failed to induce superoxide production in PMN over and above the low background level induced by the elution buffer alone. However, when added to PMN prior to activation by FMLP, inhibition of FMLP-stimulated superoxide production similar to that observed for intact *P. gingivalis* 381 was observed (Fig. 5). Inhibition was observed in both protein peaks, but the total inhibition of superoxide production in the first peak was associated with cell lysis and not with membrane depolarization. This was probably due to the higher concentration of detergent used in the loading buffer, which eluted close to the void volume. Membrane depolarization and inhibition of FMLP-stimulated superoxide production were observed for the peak fraction of the second peak. Further

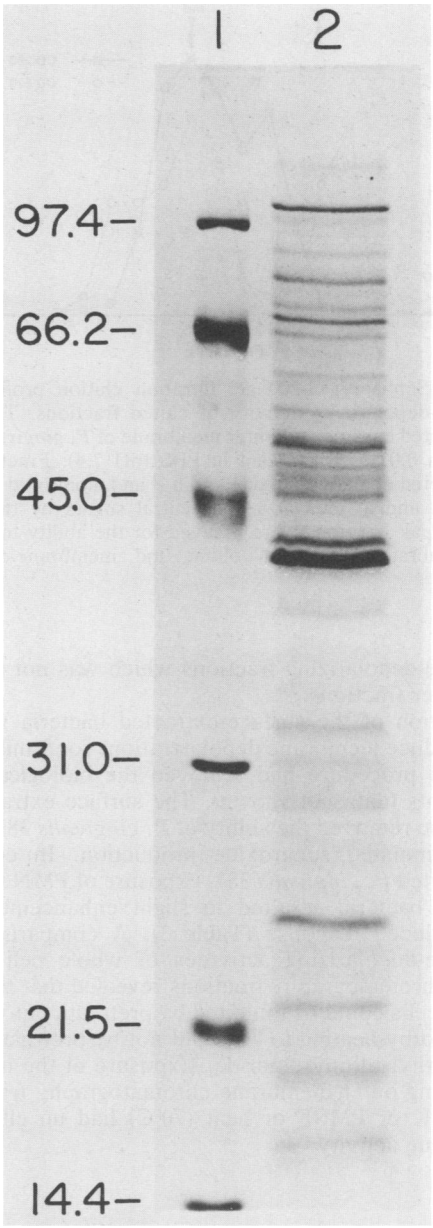

FIG. 3. SDS-PAGE protein profiles of surface extract of *P. gingivalis* 381 stained with Coomassie brilliant blue R-250. Lane 1 contained low-molecular-weight standards, whose sizes are indicated in kilodaltons. Lane 2 contained the ethanol-precipitated detergent surface extract of *P. gingivalis* 381 (200 μg of protein) heated at 100°C for 5 min in SDS prior to application to the SDS-containing 12% polyacrylamide gel.

purification was performed by pooling the protein-containing fractions, concentration them by ethanol precipitation as previously outlined, suspending them in PBS containing 0.1% Zwittergent, and passing them over the Sephacryl S-200 column again. This resulted in removal of the carbohydrate seen in Fig. 4 and a single protein peak with two fractions demonstrating membrane-depolarizing activity (Fig. 6). Analysis of fractions 15 through 20 by silver-stained SDS–12% polyacrylamide gel electrophoresis (PAGE) (Fig. 7) revealed a unique band of approximately 31 kDa in the

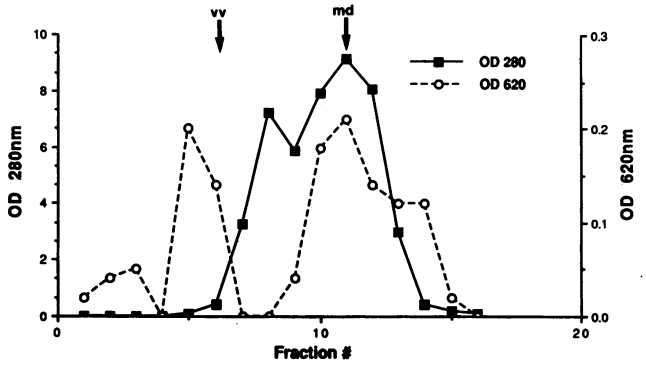

FIG. 4. Sephacryl S-200 gel filtration elution profile showing membrane-depolarizing activity of eluted fractions. The Zwittergent-extracted capsule and outer membrane of *P. gingivalis* 381 was eluted with 0.02% Zwittergent in PBS (pH 7.4). Fractions (6 ml) were collected at a flow rate of 12 ml h^{-1} and monitored at an OD_{280} for protein and at an OD_{620} for neutral sugars by the anthrone reaction. Peak fractions were assayed for the ability to depolarize PMN membranes. vv, void volume; md, membrane-depolarizing fraction.

membrane-depolarizing fractions which was not so evident in the other fractions.

Interaction of the surface-extracted bacteria with PMN did not induce membrane depolarization, confirming that the extraction procedure had removed the biologically active components (data not shown). The surface extraction procedure also removed the ability of *P. gingivalis* 381 to inhibit FMLP-stimulated superoxide production. In contrast to nonextracted *P. gingivalis* 381, exposure of PMN to surface-extracted bacteria resulted in slight enhancement of the FMLP-induced response (Table 3). A comparison of the membrane-depolarizing activities of whole cells and the isolated chromatography fractions revealed that the activity of whole cells could be inhibited by pretreatment with 4 mM TLCK and by heating to 70°C but not by pretreatment with phenylmethylsulfonyl fluoride. Exposure of the membrane-depolarizing outer membrane chromatography fraction to 4 mM TLCK or PMSF or heat (70°C) had no effect on its depolarizing activity.

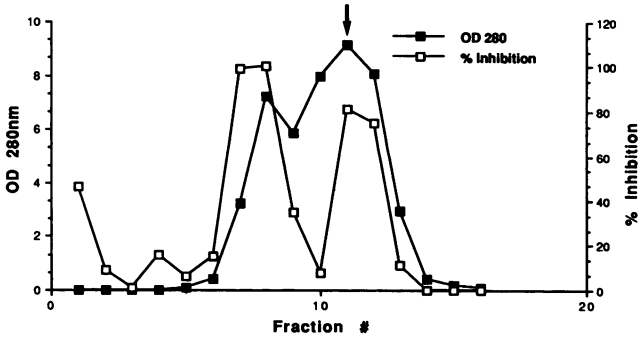

FIG. 5. Sephacryl S-200 gel filtration elution profile showing inhibitory activities of fractions for FMLP-stimulated superoxide production. A 0.02-ml volume of each fraction was added to PMN immediately prior to stimulation by FMLP (final concentration, 10^{-6} M). Inhibition is expressed as a percentage of the maximal response following addition of 0.02 ml of elution buffer and FMLP. OD, optical density.

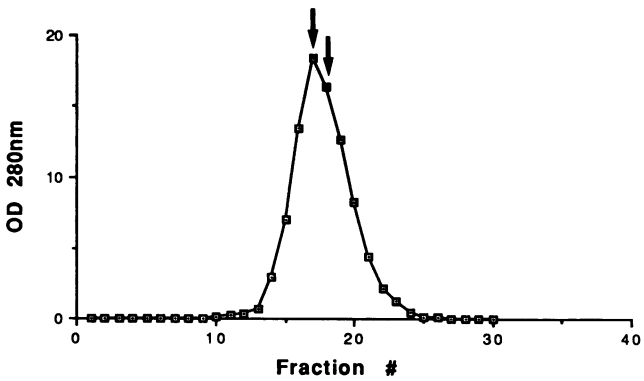

FIG. 6. Sephacryl S-200 gel filtration elution profile of the pooled protein-containing fractions of Fig. 4. Pooled fractions were concentrated by ethanol precipitation, suspended in PBS containing 0.1% Zwittergent, and eluted with PBS containing 0.02% Zwittergent as 3-ml fractions at a flow rate of 12 ml/h. Arrows indicate fractions 17 and 18 with membrane-depolarizing and FMLP-inhibitory activities.

DISCUSSION

Binding of soluble or particulate stimuli to the plasma membrane of human PMN leads to a series of physiologic and biochemical changes that have been termed stimulus-response coupling (19, 20, 49). These changes, that are subsequent to ligand-receptor binding, include changes in the electrochemical potential that exists across the plasma membrane (the membrane potential), mobilization of intracellular second messengers, such as calcium, and activation of physiologic responses, such as the respiratory burst, phagocytosis, and degranulation (19, 20, 41, 49). It has been shown that bacteria can activate PMN responses through either binding of surface opsonins to specific PMN receptors or direct binding of bacterial surface lectins to PMN membrane sugars (35). We have previously demonstrated that the gram-negative periodontal pathogen *Fusobacterium nucleatum* can activate normal stimulus-response coupling mechanisms in human PMN with depolarization of the membrane potential, changes in intracellular calcium concentrations, phagocytosis, and activation of the microbicidal respiratory burst (26). In this study of five different strains of the oral pathogen *P. gingivalis*, two of the strains tested, 381 and ATCC type strain 33277, caused profound and lasting depolarization of the PMN membrane potential. However, none of the strains tested caused a change in the concentration of intracellular calcium or activated the respiratory burst. The PMN membrane-depolarizing activity of these strains was extractable from the cell surface with a zwitterionic detergent that removed the capsule and some outer membrane proteins but did not result in cell lysis. Following removal of excess detergent by gel filtration chromatography, PMN membrane-depolarizing activity could be reconstituted from the column fractions, thus demonstrating that cell surface components of the two *P. gingivalis* strains was responsible for the membrane-depolarizing activity. It was further demonstrated that the membrane-depolarizing activity of the whole cells and the partially purified surface components was associated with inhibition of the PMN responses to the chemotactic peptide FMLP but not with inhibition of the PMN response to the nonchemotactic phorbol ester PMA.

Although membrane depolarization is generally associated with ligand-receptor coupling and cell activation in PMN, the change in the electrochemical potential that occurs

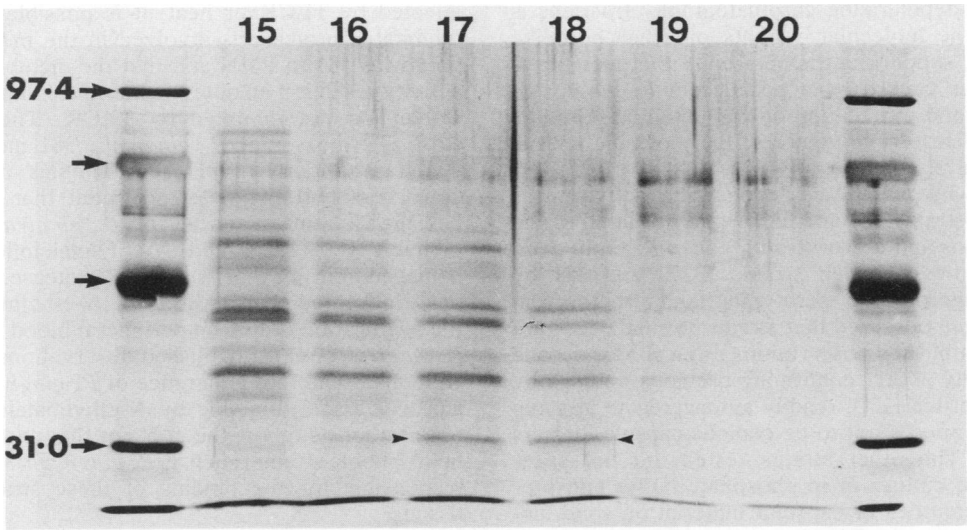

FIG. 7. Silver-stained SDS–12% polyacrylamide gel. Fractions 15 through 20 of the Sephacryl S-200 column elution profile shown in Fig. 6 were equilibrated to an OD_{280} of 0.1. Samples were boiled for 5 min in sample buffer containing 2% SDS. Molecular size standards were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin 42.7 kDa), and carbonic anhydrase (31.0 kDa). The 31.5-kDa bands present in the membrane-depolarizing fractions, 17 and 18, are marked with arrowhead.

during membrane depolarization or hyperpolarization is related to ion fluxes across the cell membrane. Investigators have demonstrated a close relationship between membrane potential changes and activation of the respiratory burst (21). Membrane depolarization is absent in PMN from patients with chronic granulomatous disease who lack NADPH oxidase activity and an associated respiratory burst (43, 44, 56), and depolarization occurs at concentrations identical stimulus to those that activate the respiratory burst (7). Depolarization of the membrane potential by a stimulus that does not itself activate the respiratory burst appears to blunt the oxidative burst to secondary stimulation (27). It has also been demonstrated that depolarization of the PMN membrane with gramicidin D, a pore-forming ionophore that passively exchanges intracellular potassium for extracellular sodium, does not activate the respiratory burst or initiate changes in intracellular calcium concentration but inhibits the increases in intracellular calcium in response to stimulation with FMLP (7). Depolarization of the PMN membrane by *P. gingivalis* in our study was not accompanied by measurable activation of the respiratory burst, suggesting that the changes in electrochemical potential were not linked to NADPH oxidase activity as previously described (43, 44, 56). This conclusion is supported by the inability of the protein kinase C inhibitor staurosporine to inhibit *P. gingivalis*-induced membrane potential changes. It is also evident from these studies that *P. gingivalis*-induced changes in membrane potential are not effected through the PT-sensitive FMLP activation pathway, since G protein inactivation had no effect on PMN membrane depolarization. However, the observation that *P. gingivalis* whole cells and partially purified outer membrane components were able to depolarize PMN membranes and inhibit FMLP-stimulated superoxide production suggests that the responsible outer membrane components act in a pore-forming manner similar to that of gramicidin D (7).

TABLE 3. Effects of surface extraction of *P. gingivalis* on FMLP-stimulated superoxide production

Condition	Mean ± SE O_2^- production (nmol of O_2^- 10^6 PMN^{-1})[a]	
	Maximum rate	Total (30 min)
Extracted *P. gingivalis*		5.3 ± 0.6
FMLP (1 μM)	13.2 ± 0.5	33.7 ± 1.6
Extracted *P. gingivalis* + FMLP	14.9 ± 0.2	45.5 ± 0.1

[a] These data are based on duplicate determinations.

The outer membranes of many gram-negative bacteria contain proteins that act as passive diffusion pathways for ions and macromolecules, and these proteins are termed porins (1, 33). In some cases, these porins can translocate from the bacterial outer membrane and enter eukaryotic cells or artificial membranes to produce an ion channel (2, 24). For this to occur, the donor bacterium must be in contact with the acceptor membrane or the purified membrane protein, part of which is hydrophobic, must be transported in a suitable vehicle. Protein 1, an anion-selective porin of *Neisseria gonorrhoeae*, has been shown to translocate into eukaryotic cells and induce changes in the membrane potential of human PMN (16). The method of isolation of the porin from *N. gonorrhoeae* was similar to that used in our study, with the same nonionic detergent used in the extraction procedure. Porins isolated from other gram-negative organisms, including the periodontal pathogen *Eikenella corrodens*, have been shown to alter phagocyte function and specifically decrease the oxidative burst of PMN (51–53). It is possible that the depolarization of the PMN membrane that occurred in our study, with whole bacterial cells or separated chromatography fractions, was due to the presence of a porinlike molecule. Porins are generally resistant to heat and are known to transfer to acceptor membranes, demonstrating greater fluidity (2), following bacterium-cell contact. It is possible that the membrane depolarization that occurred when *P. gingivalis* whole cells contacted the PMN was facilitated by a porinlike protein that translocated from *P. gingivalis* to the PMN membrane. The presence of a unique 31.5-kDa protein band

in the membrane-depolarizing chromatography fractions is consistent with the molecular weights of other porinlike molecules (1, 33), supporting the possibility that membrane depolarization was caused by a porin from *P. gingivalis*. Further isolation and purification of this potential porinlike molecule is necessary to characterize the molecule and its mode of action fully.

It is unclear why membrane-depolarizing activity was observed only in strains 381 and 33277 and not in all of the strains tested. Loos and coworkers (23) have recently demonstrated similarities between strains 33277 and 381 by genomic DNA fingerprinting, serotyping, and streptomycin resistance. We have observed that strains 381 and 33277 are hydrophobic (unpublished observations using *N*-hexadecane in a hydrophobicity assay, confirming previous findings by Gibbons and Etherden [13]), readily autoaggregate at room temperature, and appear not to be copious capsule formers on solid media. The other strains tested did not show autoaggregation in culture or in phosphate buffer and produced copious quantities of capsular material on solid medium. It is distinctly possible that depolarizing activity is present in the highly encapsulated strains but is masked by the capsule. Although all strains of *P. gingivalis* appear to produce variable quantities of a capsular material, it is unclear why strains 381 and 33277 are hydrophobic. A difference in the virulence of these two strains compared with that of fresh isolates has been observed in animal models. Strain 381 causes formation of localized abscesses with pus formation and location of the bacteria in clumps, whereas fresh clinical isolates and the W50 and W83 strains used in this study produce disseminating infection by individual bacteria with little pus formation (31, 55). In these in vivo studies, the more hydrophobic bacteria appeared to be associated with more pus formation and therefore may have had a more direct effect on the PMN than did the hydrophilic strains (55).

The effects of *P. gingivalis* 381 on human PMN were not inhibited by the panel of sugars or amino acids tested or by surface trypsinization of the bacteria, indicating no lectinlike interaction. However, membrane depolarization of the PMN was inhibited by detergent extraction of the outer membrane components from the bacteria, by heating of the bacteria to 70°C, by pretreatment of the bacteria with 4 mM TLCK, and by performance of the assay in the presence of a low-ionic-strength buffer. This suggests that a heat-labile surface component of *P. gingivalis* 381 removed during detergent extraction is involved in its initial interaction with PMN and that this interaction is driven by hydrophobic forces that are reduced in the presence of a low-ionic-strength buffer. These findings also suggest that a TLCK-sensitive surface protease(s), is involved in this initial interaction.

In contrast to the results obtained with intact microorganisms, evaluation of fractions for the chromatographed detergent extract of *P. gingivalis* 381 showed that membrane-depolarizing activity was not inhibitable by TLCK or heat, thus eliminating the possibility that surface proteases are responsible for effecting PMN membrane depolarization. Grenier and coworkers (14) have demonstrated that most of the proteases of *P. gingivalis* are associated with the outer membrane, are inhibited by 4 mM TLCK, and are heat labile. They have also been able to localize at least one of the proteases, a glycylprolyl protease, to the cell surface of *P. gingivalis* 33277 (15), a strain that showed membrane-depolarizing activity similar to that of *P. gingivalis* 381 in our study. Since the membrane-depolarizing activity of the chromatography fractions of the detergent extract were not affected by TLCK or heat, it is possible that the TLCK-inhibitable protease is involved in the initial binding of *P. gingivalis* 381 to PMN and that the membrane-depolarizing activity resides in an outer membrane component that is heat stable and not inhibited by TLCK. The possibility that surface proteases of *P. gingivalis* 381 mediate binding to PMN is supported by recent findings of Nishikata and coworkers (34), who demonstrated that the binding and hemagglutinating activities of *P. gingivalis* 381 were inhibitable by TLCK and *N*-ethylmaleimide and that the hemagglutinin may be a surface protease. We have previously observed that addition of *N*-ethylmaleimide inhibits the aggregation of human peripheral blood PMN induced by *P. gingivalis* 381 (unpublished observations), and Naito and Gibbons found that adherence of *P. gingivalis* 381 to collagen was also inhibitable by *N*-ethylmaleimide (29). These observations support the concept that surface proteases of hydrophobic strains, such as *P. gingivalis* 381 and 33277, are responsible for the binding of these strains to cells and tissues.

The differences in the abilities of *P. gingivalis* strains to induce membrane depolarization of human PMN in this study reflect differences observed between the strains in animal models of virulence. The membrane-depolarizing strains are associated with purulent, localized abscesses, suggesting that they exert an effect on PMN in vivo. The results of this study show that it is distinctly possible that membrane depolarization immobilizes PMN responses to chemotactic stimuli. Combined with the inability of *P. gingivalis* strains to activate the microbicidal respiratory burst, immobilization of PMN by *P. gingivalis* may constitute a significant virulence factor.

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