INFECTION AND IMMUNITY, Feb. 1992, p. 712–714
0019-9567/92/020712-03$02.00/0

Vol. 60, No. 2

Role of Superoxide Dismutase in Resistance of *Porphyromonas gingivalis* to Killing by Polymorphonuclear Leukocytes

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Porphyromonas gingivalis **in which the synthesis of superoxide dismutase (SOD) was induced by nitrate or by aeration was rendered resistant to killing by polymorphonuclear leukocytes. SOD purified from either anaerobically maintained or aerated cells also inhibited bacterial killing when added exogenously, and no difference between the effects of the two SODs was observed. These results suggest that SOD may form part of a defense mechanism that helps protect *P. gingivalis* against killing by polymorphonuclear leukocytes.**

Phagocytosis of bacteria by polymorphonuclear leukocytes (PMNLs) is accompanied by enhancement of PMNL oxidative metabolism. Oxygen metabolites such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxy radical ($^\cdot OH$) are major components of the bactericidal activity of PMNLs (14). Pathogenic organisms must therefore possess some mechanism(s) through which they circumvent the lethal effects of these products. Aerobic and many anaerobic bacteria synthesize enzymes, such as superoxide dismutase (SOD), catalase, and peroxidase, which neutralize these toxic reactive products (4). Thus, bacteria rich in these enzymes might resist killing by PMNLs. SOD is thought to protect the cells of *Nocardia asteroides* (9), *Listeria monocytogenes* (24), and *Shigella flexneri* (10) from phagocytic killing, whereas catalase activity has been suggested to protect *Staphylococcus aureus* (12) and *Neisseria gonorrhoeae* (3). However, neither catalase nor SOD in *Escherichia coli* was able to protect this bacterium from killing by PMNLs (21). This being the case, it is still unclear how pathogenic bacteria that are not killed by phagocytes avoid the lethal effects of PMNL oxidative metabolism.

Porphyromonas gingivalis, which seems to play a central role in the etiology of certain forms of periodontal disease, can colonize the periodontal pocket and invade gingival tissue (18, 22). It is reasonable to suggest that the virulence of bacteria may be connected with their ability to synthesize specific enzymes that neutralize the toxic products of the oxidative metabolism of the PMNLs. Our previous study demonstrated that *P. gingivalis* had SOD activity but no catalase or peroxidase activity (2). Furthermore, *P. gingivalis* SOD was found to be a unique enzyme whose apoprotein was capable of binding Mn or Fe, suggesting that this enzyme may be formed from a single apoprotein (1). In the present study, we assessed the influence of SOD on the killing of *P. gingivalis* by PMNLs by inducing its synthesis and by adding it exogenously.

P. gingivalis 381 was obtained from stock strain at the Research Laboratories of Oral Biology, Sunstar Inc., Osaka, Japan. The strain was maintained anaerobically by weekly transfer on plates containing Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% sheep blood, 1 mg of yeast extract (BBL) per ml, 5 μg of hemin per ml, and 1 μg of menadione per ml. The bacterial cells were cultured anaerobically in prereduced Trypticase soy broth (BBL) supplemented with 1 mg of yeast extract per ml, 5 μg of hemin per ml, and 1 μg of menadione per ml and were incubated to mid-logarithmic phase (A_{660} = 1.0) at 37°C in an anaerobic system 1024 (Forma, Marietta, Ohio) in an atmosphere of 80% N_2–10% CO_2–10% H_2.

Synthesis of SOD in *P. gingivalis* 381 was induced by aeration or by the addition of potassium nitrate to the growth medium. For aeration studies, cells grown anaerobically in 100 ml of broth were harvested aseptically by centrifugation at 3,500 × *g* for 30 min at 4°C, suspended in 100 ml of nonreduced fresh broth, transferred to sterile 500-ml flasks stoppered with cotton plugs, and incubated at 37°C in air while being vigorously shaken for 120 min. Controls were kept in an anaerobic chamber at 37°C for 120 min. For induction of SOD by potassium nitrate, *P. gingivalis* 381 was grown anaerobically in the presence and in the absence (controls) of 0.5 mM KNO_3 in broth.

SOD activity was assayed by a modification of the method of McCord and Fridovich (13). Protein content was determined by Bradford's method (5) with bovine serum albumin as the standard.

Both SODs (anaero-SOD and aero-SOD) were purified from extracts of either anaerobically maintained or aerated *P. gingivalis* 381 cells by the method of Amano et al. (1).

The assay for phagocytosis and bacterial killing by PMNLs was performed by a method modified from that of Quie et al. (17). Blood from healthy adult volunteers was drawn into heparin (9 U/ml). PMNLs were prepared from the heparinized blood by centrifugation in discontinuous Ficoll-Hypaque gradients (Flow Laboratories Inc., North Ryde, New South Wales, Australia) (8). Residual erythrocytes were lysed in 0.87% ammonium chloride at 4°C, and the samples were washed three times in Hanks balanced salt solution. The purity and viability of PMNLs recovered was more than 95% PMNLs as monitored by exclusion of trypan blue dye. Pooled serum from three healthy individuals was used for the opsonin in the assay. The serum contained antibodies to *P. gingivalis* as measured by enzyme-linked immunosorbent assay in this laboratory. After growth of *P. gingivalis* 381 to mid-logarithmic phase, cells were centrifuged at 3,500 × *g* for 30 min and washed three times with Hanks balanced salt solution. Bacterial suspension (10^7 CFU/ml; adjusted on the basis of an A_{660} of 1.0 = 8×10^8 CFU/ml) opsonized in 10% human serum was added to PMNLs (5×10^6 PMNLs per ml) in the same medium. In each assay, controls from which serum or PMNLs or both

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were omitted were included. Some experiments were carried out with the addition of 30 U of anaero-SOD or aero-SOD or with no addition (control). The mixtures were incubated at 37°C in a CO_2 incubator on a rotator (RT-50; Taiyo Scientific Industrial Co., Tokyo, Japan). At specified times (0, 30, 60, and 120 min), the tube containing the phagocytic mixture was centrifuged at 150 × *g* for 10 min. Duplicate samples were removed from the supernatant fraction for determination of the number of extracellular bacteria, and 0.1 ml of the washed PMNL suspension was placed in 0.9 ml of sterile distilled water and incubated for 10 min to disrupt the PMNLs. Triplicate preparations of the supernatant fluid and the disrupted PMNLs were appropriately diluted and plated onto Trypticase soy agar plates supplemented with 5% sheep blood, 1 mg of yeast extract per ml, 5 μg of hemin per ml, and 1 μg of menadione per ml. The samples were incubated at 37°C for 5 days in an anaerobic chamber, and the colonies were then counted. Phagocytosis is expressed according to the following formula: phagocytic index = $[(N_{cont} - N_{extra})/N_{cont}] \times 100$, where N_{cont} and N_{extra} are the number of viable bacteria without PMNLs and the number of viable extracellular bacteria, respectively. The results of the intracellular killing test are expressed as a killing index, i.e., the percentage of bacteria killed during the incubation period, as follows: killing index = $[(N_{cont} - N_{extra} - N_{intra})/(N_{cont} - N_{extra})] \times 100$, where N_{intra} is the number of viable intracellular bacteria. The mean of three experiments was calculated, and differences were compared by using Student's *t* test. Serum, in the absence of PMNLs, did not affect the viability of *P. gingivalis* at any time. In order to distinguish bacterial adherence from phagocytosis, bacteria which had been opsonized with either chilled PMNLs at 4°C or PMNLs pretreated with cytochalasin B (6) were incubated. However, the number of bacterial cells which adhered to both types of treated PMNLs was negligible.

Figure 1 shows the results for phagocytosis and killing of *P. gingivalis* 381 by PMNLs during the incubation period after SOD was induced by growth in the presence of 0.5 mM potassium nitrate or by aeration. The specific activity of SOD in *P. gingivalis* 381 was increased 2.3- and 2.0-fold by growth in the presence of nitrate and by aeration, respectively. *P. gingivalis* 381 was phagocytosed at high levels (ca. 90%) after 120 min by PMNLs, but the levels of phagocytosis were not affected by the induction of SOD. Human PMNLs killed 86% of *P. gingivalis* in 120 min, whereas inductions of SOD by aeration and the addition of KNO_3 reduced the ability of PMNLs to kill the bacteria to 75% ($P < 0.05$) and 54% ($P < 0.01$), respectively, after 120 min. Privalle and Fridovich (16) showed that *E. coli* synthesized Fe-SOD induced by nitrate under anaerobic conditions. We also confirmed that the SOD induced by nitrate had the properties of Fe-SOD, on the basis of its inactivation by H_2O_2 and its inhibition by NaN_3 (data not shown). In contrast, we have reported that aeration of *P. gingivalis* induces mainly Mn-SOD (1). It appears possible that *P. gingivalis* synthesizes SOD containing different metals, depending on the treatment. However, the effects of both these inductions of SOD on resistance to killing by PMNLs were not found to differ when compared roughly on the basis of SOD-specific activity. The effects of aero-SOD or anaero-SOD, added exogenously, on the phagocytosis and killing of *P. gingivalis* by PMNLs are shown in Fig. 2. The levels of phagocytosis were not changed when both SODs were added to the phagocytic mixture. Both SODs inhibited bacterial killing ($P < 0.01$), but there was no difference between the inhibitory effects of these two enzymes. Thus, it is suggested

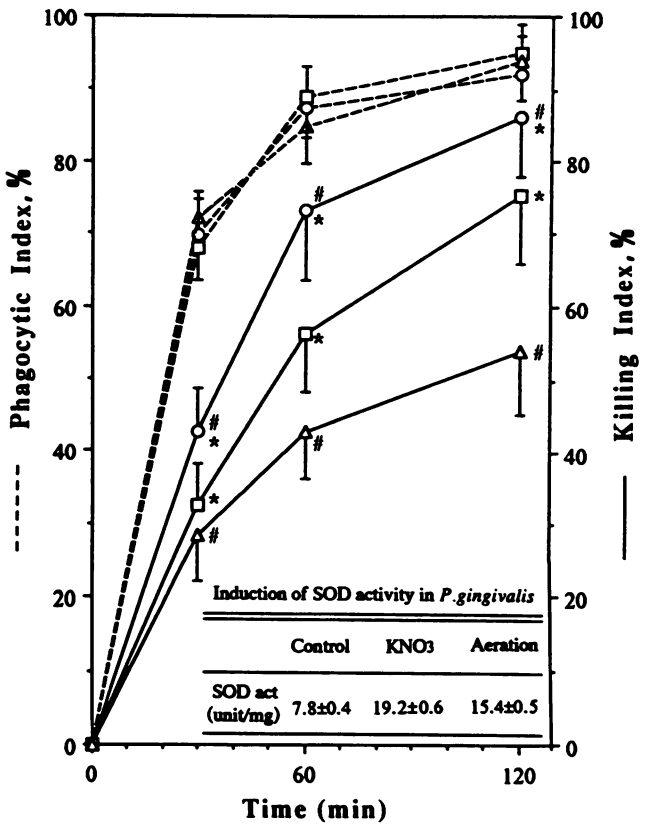

FIG. 1. Effects of SOD induction on phagocytosis (- - -) and killing (——) of *P. gingivalis* by PMNLs. SOD induction experiments were performed by aeration of *P. gingivalis* 381 (□) and by the addition of KNO_3 to the growth medium (△). ○, control (no treatment). Opsonized *P. gingivalis* organisms (10^7 CFU/ml) were incubated with PMNLs (5×10^6/ml). Variance is the mean ± standard deviation of three experiments. Means were significantly different from each other; $P < 0.05$ (☆) and $P < 0.01$ (#).

that the biological functions of Fe- and Mn-SODs in *P. gingivalis* as well as their biochemical properties, such as amino acid sequences, electrophoretic mobilities, and acceptability of either Fe or Mn to form holoenzymes (1), may be the same.

Cutler et al. (6, 7) have shown that phagocytosis of virulent *P. gingivalis* by PMNLs requires a specific antibody in human serum and that this antibody-dependent phagocytosis is also highly dependent on serum complement. *P. gingivalis* 381 is moderately virulent in the mouse abscess model (23). Since we used nonheated serum which was pooled from healthy individuals but contained the specific antibody to *P. gingivalis*, this strain appeared to be phagocytosed at high levels even in the absence of serum from patients with adult periodontitis. However, *P. gingivalis* 381 was not highly sensitive to the killing effect of PMNLs. Okuda and Takazoe (15) showed that the encapsulated strains were more resistant to killing than the nonencapsulated strains. Several investigators suggested that the degradation of serum opsonins, including complement and immunoglobulin, by *P. gingivalis* plays a role in resistance to phagocytosis (11, 19, 20). The resistance of *P. gingivalis* to killing by PMNLs is probably partly due to nonoxidative mechanisms. However, another possible explanation for this resistance is higher activity of SOD in *P. gingivalis* than in

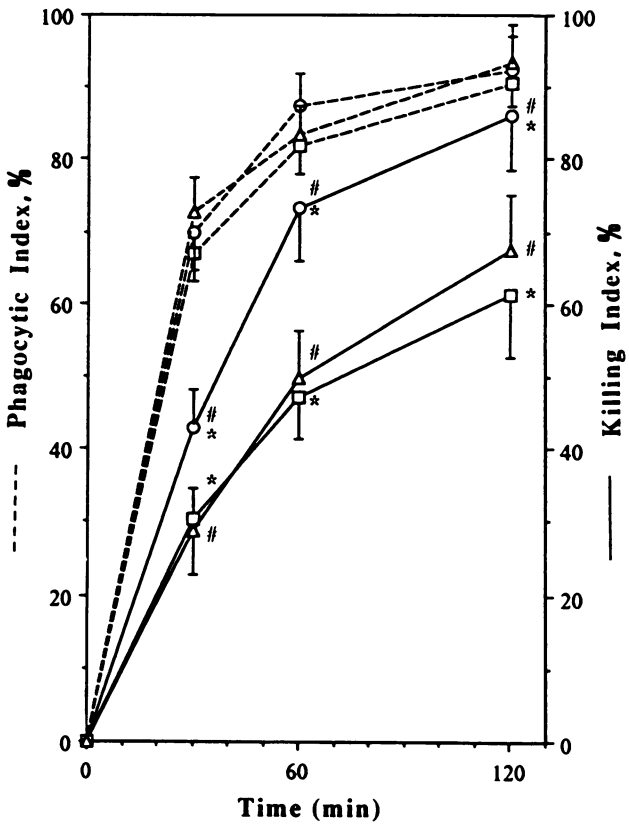

FIG. 2. Effects of exogenously added SOD on phagocytosis (---) and killing (——) of *P. gingivalis* by PMNLs. Assay of bacterial killing was performed as described for Fig. 1 in the presence of 30 U of anaero-SOD (△), 30 U of aero-SOD (□), or no addition (○). Variance is the mean ± standard deviation of three experiments. Means were significantly different from each other; $P < 0.01$ (☆ and #).

black-pigmented oral anaerobic rods (2). Thus, our results suggest that SOD may form part of a defense mechanism that helps protect *P. gingivalis* from killing by PMNLs.

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