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Superoxide dismutase in Bacteroides gingivalis

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SUPEROXIDE DISMUTASE in BACTEROIDES GINGIVALIS.

by

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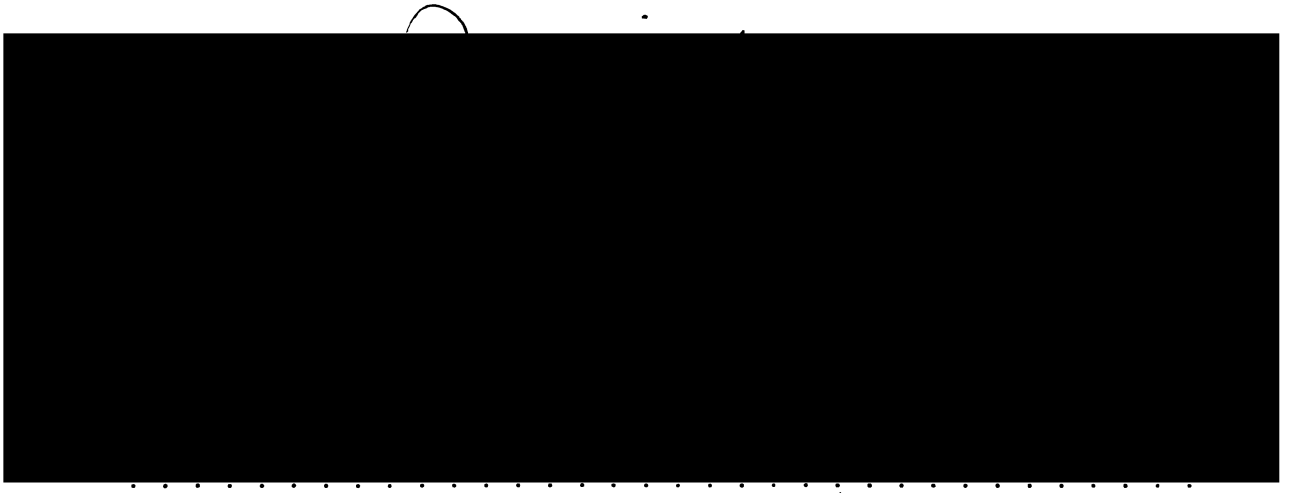

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INTRODUCTION

1. Cytotoxicity of Superoxide.

Activated oxygen radicals play a major role in inflammation. During phagocytosis of microrganisms by polymorphonuclear neutrophils, O_2^- is produced by a membrane bound superoxide synthetase. O_2^- is cytotoxic to living cells and organisms. In comparison to other free radicals particularly in comparison to the hydroxyl radical, superoxide seems unreactive, even benign (4,9). Yet its relative stability may contribute to its cytotoxicity. A very reactive radical, such as $OH^{\cdot}$, reacts rapidly with most classes of organic and inorganic compounds. It does not diffuse far from its point of origin before it reacts. Moreover, it would exhibit little selectivity with respect to its target. A less reactive radical, such as O_2^- , could ultimately do far significant damage because it can diffuse and strike specific targets.

Fluxes of O_2^-, have been found to inactivate viruses, kill bacteria, induce lipid peroxidation (30), depolymerize hyaluronate, inactivate enzymes, lyse erythrocytes, damage artificial vesicles and destroy mammalian cells in culture (4,9,29). Futhermore, there are indications that, in many of these actions, O_2^- could, in collaboration with H_2O_2 , generate a very potent oxidant such as OH· (hydroperoxy radical) in the presence of iron catalysts (4,9). Superoxide has also been found by White *et al* to be mutagenic (7). It could cause single strand breaks in DNA and produce chromosome breaks and rearrangements in the normal cell culture.

2. Superoxide Dismutase

Superoxide dismutases constitute an essential defense against the toxicity of superoxide radicals. There are three very distinct types of superoxide dismutases. The iron-containing superoxide dismutases (FeSOD) and the corresponding manganese-containing enzymes (MnSOD) are characteristic of prokaryotes and are closely related. The copper and zinc containing superoxide dismutase (CuZnSOD) is characteristic of eucaryotes (18).

Superoxide dismutases work by catalyzing the

dismutation of O_2^-. The mechanism of dismutation is mainly through the catalytic center, which undergoes a cycle of reduction and reoxydation during successive encounters with O_2^- (see equation below, mechanism I). Bielsky has recently reported another kind of dismutation (mechanism II) (4,9).

$$Mn^{++} + O_2^- \text{---> } Mn^+ + O_2 \qquad \text{Mechanism I}$$

$$Mn^+ + O_2^- + 2H^+ \text{---> } Mn^{++} + H_2O_2$$

$$Mn^{++} + O_2^- \text{---> } Mn\text{-}O_2^+ \qquad \text{Mechanism II}$$

$$Mn\text{-}O_2^+ + O_2^- + 2H^+ \text{---> } Mn^{++} + H_2O_2 + O_2$$

Generally speaking, there is a correlation between superoxide dismutase and oxygen tolerance (4,9,25). Initially it was observed that aerobes contained more superoxide dismutase and catalase than the aerotolerent organisms, while anaerobes had none. Later, however, the situation with anaerobes turned out to be more complex. The presence of superoxide dismutase in anaerobic bacteria was first reported by Hewitt and Morris (39) after it had been reported to be absent in obligate anaerobic bacteria. This should not have been suprising, since obligate anaerobes are frequently, if transiently, exposed to oxygen en route from one anaerobic niche to another. More interestingly, some

anaerobes induced higher levels of superoxide dismutase upon experimental exposure to superoxide. This adaptation of organisms to oxidative stress is significant , because any substantial shift in local oxidation-reduction balance arising from exogenous or endogenous oxidative stress threatens to impair the functioning and ultimately the viability of the affected cells (3). Therefore the ability of antioxidant defense systems to react adaptively in response to oxidative challenges is essential to organisms, including anaerobic organisms.

E. coli is one example. Aerobically grown *E. coli* contains three distinct isoenzymes of SOD, Mn-SOD, Fe-SOD and a hybrid SOD containing one subunit from each of the other enzymes. Anaerobically grown cells, in contrast, contain only Fe-SOD (4,9). Thus the synthesis of Mn-SOD in *E. coli* seems to be under rigorous control. The cells are also able to efficiently modulate the level of this enzyme to meet their defense requirements. The constitutive Fe-SOD of *E. coli* provides a backup defense against oxygen toxicity during sudden exposure to oxygen and represents a compromise between the expense of maintaining a large standing level of defensive enzyme and the damage sustained during the delay inherent in induction.

It has been proved that the inducer for superoxide

dismutase is not molecular oxygen per se but rather a product of its metabolism (4,9). Paraquat and its related compounds can enter *E. coli* and other cells and canbe readily reduced by a single electron to a stable but dioxygen-sensitive monocation radical ($PQ^{+\cdot}$), which then combines rapidly with molecular oxygen to produce intracellular superoxide free radical (29,30,31,32). Studies have shown that *E. coli* became much more tolerant to paraquat when grown in rich medium (trypticase soy plus yeast extract) than in a glucose-salts medium (minimum medium) (4,9). This was due to the rapid induction of superoxide dismutase in the rich medium, which was not possible in minimal medium. When rapid induction of superoxide dismutase was prevented by adding inhibitors of protein synthesis (i.e.,puromycin or chloramphenicol), the protective effect of the rich medium was eliminated. It has been proved that increased synthesis of superoxide dismutase in response to paraquat was due to true induction, rather than selection by paraquat of some super producers of superoxide dismutase. Studies have shown that intracellular O_2^- generated by paraquat and other redox active compounds induced the MnSOD but not the FeSOD enzyme (40,41). This increase in the biosynthesis of the MnSOD by paraquat was prevented by inhibitors of transcription or translation, but not by replication. Thus, the increase in MnSOD biosynthesis

in response to increases in intracellular O_2^-, as caused by paraquat, was due to *de novo* enzyme synthesis activated at the level of transcription and O_2^-, or a unique product derived therefrom, was the effector (4,9,40).

The role of superoxide dismutase is further confirmed by creating mutants in the SOD enzymatic activity and by the comparision of such mutants with the parent strain. The level of confidence in the correlation of observed changes with differences in the activity under consideration can be elevated by selecting revertants. McCord *et al* (4,9) had produced a mutant with a temperature-dependent intolerance for dioxygen. This mutant was found to have a parallel temperature-dependent defect in SOD. In a more recent study of dioxygen-sensitive mutants of *E. coli* (42), catalase, peroxidase and superoxide dismutase were examined. Whereas the parental strain responded to oxygenation or paraquat by induction of catalase, peroxidase and MnSOD, some of the mutants generated chemically were defective in the ability to induce catalase and peroxidase, while other mutants failed to induce all three enzyme activities. These mutants reverted to wild type oxygen-tolerance at rates in the range 1×10^{-5} to 1×10^{-4}, which exceeded the rate that was expected for point mutations and suggested endogenous mutagenesis, perhaps by the unscanvenged O_2^- and H_2O_2 (4).

McCord *et al* Examined several revertants and have found that most of these had partially or completely recovered the ability to induce the enzymatic activities missing in the mutants. In contrast, one revertant had recovered oxygen tolerance without a restoration of the lacking activities. The behavior of this revertant was clear when rates of respiration were measured and it alone was found to have lost the ability to respire. Lacking the ability to reduce dioxygen, it could not generate O_2^- and H_2O_2, and could dispense with the enzymatic scavangers of these intermediates (42). Therefore, superoxide dismutase is an enzyme that is induced when needed.

3. The Role of O_2^- and H_2O_2 In Inflammation

The killing of pathogens by phagocytes is a critical element of host defense. The ability of phagocytes to destroy invading bacteria, fungi, and parasites is indispensible for life, and when this function fails the organism rapidly succumbs to overwhelming infection. The mechanisms by which these cells kill their targets can be divided into two classes: those which require oxygen, and those which do not (4,9,15). With regard to the oxygen-dependent microbicidal mechanisms of phagocytes, it is now clear that superoxide is the key.

The neutrophil finds its target by chemotaxis. It is attracted by substances released by the interaction of tissue fluids with the microrganism at the site of infection (35). Interaction with tissue fluids also results in the opsonization of the microrganisms. Contact of phagocytes with opsonized particles triggers a dramatic and complex change in behavior, one of whose components is a marked increase in oxygen consumption (15,17,27,36). This is unlike ordinary respiration in that it is cyanide-insensitive and it does produce substances needed for optimal killing of the ingested microrganisms (21).

The respiratory burst is caused by an NADPH oxidase located in the plasma membrane. The burst is brought about by contact with opsonized particles. This NADPH oxidase reduces dioxygen to O_2^- and this O_2^- then gives rise to H_2O_2 by the dismutation reaction. As a consequence, both O_2^- and H_2O_2 are released by activated phagocytes and appear to be important for the microbicidal action of these cells. There is a human genetic defect which is expressed as chronic granulomatous disease, a situation marked by enhanced susceptibility to infection by the common enteric bacteria. Phagocytes from persons afflicated with this defect normally engulf, but fail to kill engulfed microrganisms. These infective phagocytic cells do not show the burst of

respiration ordinarily shown by activated phagocytes. The membrane NADPH oxidase has been shown to be defective also (4,17).

As described above, two microbicidal systems (4,9,17) have been proposed. One system does not depend on oxygen and is called myeloperoxidase system, the other does depend on oxygen and is called oxidizing radical system. While my study is mainly focused on the latter sysytem (oxidizing radicals), only a brief description of the myeloperoxidase bacterial killing mechanism is given below.

Myeloperoxidase: Myeloperoxidase is a heme enzyme that catalyzes a number of H_2O_2-mediated oxidations, the most important of which is probably the oxidation of chloride to hypochlorite.

$$Cl^- + H_2O_2 \text{ ---> } OCl^- + H_2O$$

A role for myeloperoxidase in bacterial killing by neutrophiles was first proposed by Klebanoff (43), who showed that the combination of myeloperoxidase, H_2O_2 and a halide ion constituted a remarkably effective microbicidal system. This system operates *in vivo* as well as *in vitro*, as indicated by the finding that neutrophils from patients with

inherited myeloperoxidase deficiency when compared with normal neutrophils show a pronounced delay in the killing of *S. aureus*. The peroxidase-halide-myeloperoxidase system is thus an important component of the microbicidal capacity of neutrophils.

Oxidizing radicals: This microbicidal system requires the products of the respiratory burst but does not use myeloperoxidase. This mechanism appears to employ reactive oxidizing radicals as the microbicidal agents.

It is known that both O_2^- and H_2O_2 are produced during the respiratory burst of phagocytes. These can react in the presence of Fe^{++} to form OH·(hydroperoxyl radical), suggesting the production of reactive oxidizing radicals. Neither O_2^- nor H_2O_2 is especially potent as a microbicidal agent, yet myeloperoxidase-deficient neutrophils express an efficient respiratory burst-dependent microbicidal capacity that must be explained in terms of a killing agent generated in a myeloperoxidase-independent reaction from O_2^-, H_2O_2, or both (9). The highly reactive OH· radical provides such an explanation, at least in principle.

There is good evidence that oxidizing radicals participate in killing by neutrophils. A

superoxide-generating system consisting of acetaldehyde and xanthine oxidase is able to kill bacteria by means of an agent. This killing is neutralized by oxidizing radical scavangers and the production of these radicals is blocked by either superoxide dismutase or catalase. It is of interest that bacterial killing by this system followed a time course similar to that seen with myeloperoxidase-deficient neutrophils. The addition of myeloperoxidase-deficient neutrophils and the addition of myeloperoxidase both increased the potency of the system and altered the time course of bacterial killing to one very similar to that of normal neutrophils. The killing of bacteria by neutrophils themselves could also be prevented by either superoxide dismutase or catalase, if the enzyme was introduced directly into the microrganism-containing phagocytic vesicles. This is accomplished in these experiments by means of enzyme-coated latex particles which were taken up into the same phagocytic vesicles as the microrganism. These results indicate that hydroperoxyl radicals, whose production is derived from superoxide radicals, are directly involved in the microbicidal activity of neutrophils (4,9).

4. The Anti-Inflammatory Effect Of SOD

It has been proposed that the superoxide dismutase is

an anti-inflammatory and anti-chemotactic agent (1,5,6,17,19,34). Several investigators have observed that the intravenous administration of superoxide dismutase to animals with induced inflammation suppresses the inflammatory response and inhibits leukocyte infiltration into the challenged site. Their data indicated that neutrophil-generated superoxide reacts with an inactivated plasma component, converting it to an active, stable, and potent chemotactic factor for neutrophils (19). Perez *et al* (31) suggested that the superoxide-generated chemotactic factor may be a metabolite of arachidonic acid. Recent studies (19) suggest that the chemotactic factor may be a leukotriene, LTB_4 produced via the lipooxygenase pathway from arachidonic acid. LTB_4 has been shown to be a potent chemotactic factor and mediator for neutrophil function. Such a factor would provide a self-amplifing mechanism whereby the first stimulated neutrophils at the site of potential inflammatory lesion could call additional leukocytes to the area. Thus the enzyme's anti-inflammatory action prevents the accumulation of inflammatory cells at the site of a potential lesion and all neutrophil-mediated cytotoxic events. Although many chemotactic factors exist and many play roles at various stages in the development of inflammation, it has been suggested that the superoxide-dependent factor is critically important in some

models (1,5,6). Its role may be that of the quantitatively dominant chemotactic factor, or it may play an early role whereby its generation results in the release of the quantitatively dominant chemotactic factor(s) (1,5,6).

5. Periodontal Disease and SOD

Peridontal disease is characterized by persistent infection and inflammation. Although inflammation with its attendant phagocytic cells is designed to eliminate the invading microrganisms, the ability of periodontal pathogens to survive under these conditions results in a complex situation. The host continues to challenge the organisms with hydrolytic enzymes and numerous toxic factors released by cells of the host's inflammatory response, and perhaps by the microrganisms as well. The activation of immune mechanisms results in exacerbation of the disease with all its manifestation (22,23,24). Common infections such as those in injured tissues are cleared readily, yet in periodontal disease, the organisms appear to be successful in evading the host mechanisms directed at eliminating them.

The importance of the gram-negative anaerobic bacteria in the etiology of periodontal disease is well known (13,16,22,23). Gram negative anaerobic rods (mostly asaccherolytic) constitute 75% of the subgingival flora in

adult periodontitis (24). The most common isolate is *Bacterioides gingivalis.* Numerous studies have demonstrated that *B.gingivalis* is a dominant organism in more severe stages of periodontal disease. When a silk ligature is placed it increases from a few percent to nearly 1/4 of the flora (Table 1) subgingivally in monkeys (24). This increase in *B.gingivalis* parallels loss of periodontal attachment and pocket formation. These longitudinal studies in monkeys support the role of *B. gingivalis* as an important pathogen in periodontitis.

Other findings substantiate the pathogenicity of *B. gingivalis*. This organism is required in the transmission of mixed anaerobic infections and will cause significant bone loss when inoculated into germ-free animals. *B. gingivalis* elaborates various cytotoxic and proteolytic substances including a potent collagenase. Endotoxin from this species stimulates bone resorption *in vitro* (24). *B. gingivalis* also stimulate a potent immune response in the host. Both high antibody titers and significant numbers of sensitized T cells reactive with antigens of this organism are found in patients with severe periodontitis. In addition, some strains of *B. gingivalis* resist phagocytosis and killing by neutrophils.

It has been suggested that neutrophils from the human

gingival crevice have a defective phagocytic capacity in comparison with peripheral blood neutrophils (24,26,28). When crevicular and peripheral blood neutrophils were challenged *in vitro* with inert particle, latex beads and *candida albicans* blastospores, little difference was found in their phagocytic capacity. It was postulated that the intrinsic defect of the crevicular neutrophil may be acquired *in vivo* and may be a general property of neutrophils from inflammatory periodontal sites (26).

These observations have led to the hypothesis that superoxide dismutase may be the mechanism by which infecting periodontal organisms such as *B. gingivalis* protect themselves against phagocytosis. Decreased superoxide resulting from elevated SOD would also affect the generation of chemotactic factors resulting in what may appear to be defective neutrophil function in certain types of periodontal disease. The combination of low availability of oxygen in the periodontal pocket along with bacterial SOD would contribute to decreased chemotaxis as well as to reductions in phagocytic killings.

The objective of this research is, to determine if SOD is a measure of virulence of *B. gingivalis* by surveying its SOD content and its ability to synthesize the enzyme when challenged *in vitro* with the free radical, to determine the

subcellular location of the enzyme, and to isolate and purify the SOD elaborated by *B. gingivalis*. This information will be useful in devising counter-measures against bacterial SOD.

Table 1.1 Change in cultivable subgingival flora during ligature-induced periodontitis in monkeys[37].

	Stage I (Time = 0)	Stage II (1-3 weeks)	Stage III (4-7 weeks)
Number of sample sites	7	4	7
Total count ($x10^6$)[a]	7.78	11.05	5.48
	(2.6-73.6)	(0.5-27.6)	(0.8-11.4)
G + coci[b]	25.9+ 7.7	14.9+11.8	7.1+ 7.1
Anearobic	12.3+12.7	6.9+ 7.4	3.0+ 5.1
Facultative	13.6+10.9	8.0+ 7.1	4.1+ 3.2
G + Rods	17.8+12.7	18.9+ 5.8	5.3+ 6.3
Anearobic	4.3+10.5	7.2+12.5	3.9+ 6.4
Facultative	13.5+10.1	11.7+11.4	1.4+ 3.4
G - cocci	2.2+ 3.8	3.2+ 3.6	4.1+10.0
G - rods	49.0+14.6	56.8+30.7	77.1+14.2
Anaerobic	31.4+ 7.5	28.8+ 9.1	61.9+15.3
B.melaninogenicus ss.intermedius	17.2+ 9.5	6.8+10.1	0.9+ 1.3
B.asaccharolyticus (B.gingivalis)	1.3+ 3.5	5.3+ 9.2	34.2+15.7
Fusobacterium species	8.2+ 6.4	8.3+ 3.5	14.8+ 5.0
Facultative	17.6+ 8.2	28.1+ 5.2	15.2+14.6
Motile & surface translocating G - rods	6.6+ 2.9	20.0+ 3.5	14.3+20.9

a. Mean total colony-forming units with range of counts in parenthese.

b. Mean percentage of total cultivable flora + standard deviation.

MATERIALS AND METHODS

B. gingivalis is chosen as our study subject, since it is the predominant pathogen associated with rapidly destructive periodontitis. *B. gigngivalis* strain 33227 was obtained from ATCC. In the present study system, the induction of superoxide dismutase in *B. gingivalis* was examined in the presence of paraquat.

1. Materials

Paraquat, bovine erythrocyte cupper-zinc superoxide dismutase, dianisidine, ferricytochrome C, xanthine, xanthine oxidase, EDTA and molecular weight standard were products of Sigma Chemical Company. Microgranular diethylaminoethyl cellulose and sephadex G-100 were obtained from Phamacia. Riboflavin was obtained from Eastman Organic Chemicals. Potassium phosphate was from Baker Chemical Co.. Ammonium sulfate was bought from Mallinckradt Inc..

2. Culture Method

B. gingivalis 33227 was inoculated into tryptic soy broth supplemented with 0.25% yeast extract 10 ug/ml menadione and 0.01% dithiothreiotol as a reducing agent. Incubation was carried out anaerobically (85% N_2 10% H_2 and 5% CO_2) at 37°C for about 72-96 hours when *B. gingivalis* is in its late logarithmic growth phase. Estimation of culture viability was carried out immediately preceeding induction by paraquat. The cultures yielded approximately 10^8 colony forming units/ml.

3. General Analytical procedures

1) Assay for Protein Concentration:

Protein was measured by the Bio-Rad method using bovine serum as a standard.

2) Assay for Superoxide Dismutase Enzyme Activity:

Two methods were employed to assay SOD enzyme activity. Calibration of both methods using commercial SOD gave the same results.

i. Superoxide dismutase was measured in the supernatant of the disrupted cells by a modification of the method of McCord and Fridovich (47). The micro-assay was performed in 0.1M potassium phosphate, PH 7.8, containing 1mM of Tris-EDTA in a 1 ml cuvette. The reaction mixture contained 50mM of ferricytochrome C, 0.75mM xanthine, certain amount of bovine erythrocyte Cu-Zn SOD and sufficient xanthine oxidase to produce a rate of reduction of cytochrome C by 50% at OD 550 nm (i.e. to a rate of 0.0125 absorbance units, per minute). This amount of SOD was defined as 1 unit of enzymatic activity.

Less than 50% inhibition of cytochrome C reduction was defined as low SOD activity.

ii. The second method of assay is based on the method of Misra and Fridovich (8). Superoxide dismutase increases the rate of the aerobic photoxidation of dianisidine, sensitized by riboflavin. This rate enhancement appears to be due to the catalytic scavenging of oxygen, which would otherwise nullify the overall photoxidation by reducing an intermidiate oxidation state of dianisidine. The micro-assay was performed in 0.01M potassium phosphate buffer, PH 7.5 in a 3 ml quartz cuvette. The reaction mixture contain 1.3 x 10^{-5} M riboflavin, 2 x 10^{-4} M dianisidine, and were illuminated by cool light emmitted by fluorescent tubes for

4 minutes at room temperature. The absorbance at 460 nm was recorded in a spectrophotometer. Dianisidine was dissolved in ethanol to 1.2 x 10^{-3} and this ethanolic stock was added to the reaction mixture to give the desired final concentration. All other components were dissolved in 0.01M pottasium phosphate buffer at PH 7.5.

The data are presented in terms of units of SOD/mg protein.

4. Exposure to superoxide free radicals

Paraquat (PQ) was used in *E. coli* study to generate intracellular superoxide free radicals (O_2^-) to induce the production of SOD enzyme (4,9). Paraquat can enter bacterial cell walls and divert electron flow from the water-producing cytochrome pathway to an O_2^--producing pathway. PQ is rapidly reduced by diaphorases present in the cells, yielding O_2^- (29,30). The following equation may be used to present its mechanism of producing superoxide radicals (46):

$$PQ \longrightarrow PQ^{\cdot +} + O_2^{-\cdot}$$

$$O_2^{-\cdot} + PQ^{\cdot +} \longrightarrow PQ^{2+} + O_2^{2-}$$

$$2O_2^{-\cdot} \longrightarrow O_2 + O_2^{2-}$$

$$2H^+ + O_2^{2-} \text{---> } H_2O_2$$

5. Conditions for Exposure to Paraquat

In order to determine the optimal conditions for induction, responses to PQ was studied. In previous studies of *E. coli*, 30 minutes incubation time was found to be the optimal induction time (4).

1) The Effect of Various Paraquat Concentrations on the Induction of SOD in *B. gingivalis*

This was carried out by the addition of the following paraquat concentrations: 0.1 mM, 0.5 mM, 1.0 mM and 1.5 mM respectively to 10 ml of the bacterial culture. Cultures to which no paraquat was added were used as controls to verify the induction of SOD. After 30 munites incubation the cultures were centrifuged for 30 min at 6,500 r.p.m.. The supernatant was discarded and the bacterial pellet was washed twice with 5 ml of 0.01 M potassium phosphate buffer PH 7.5. The cells were resuspended in 2.5 ml of potassium phosphate buffer and were sonically treated with six 45-S bursts with a microtip on Artec Sonic Dismembrator Model 150 (Fisher Scientific Co.) operated at approximately 75w. Sonication was carried out under ice to prevent overheating. The sonic extract was centrifuged at 12,000 r.p.m. for 30

min. The supernatant was used for SOD enzyme and protein assays.

2) SOD Induction as a Function of Time of Exposure to Paraquat

The optimal paraquat concentration which yielded the highest SOD activity in *B. gingivalis* (i.e. 0.5 mM) was added to various tubes containing 10 ml of the bacterial culture. At intervals of 15 min, 30 min, 60 min, 120 min and 180 min, incubation periods, all culture tubes were immediately chilled in ice following the appropriate incubation periods. Cell free extracts were prepared as above and assayed for protein and SOD.

6. The Effect of Paraquat Elimination Following Induction of SOD (Deinduction)

Induction of SOD in *B. gingivalis* was carried out by adding optimum paraquat concentration (0.5 mM) and incubated for optimum incubation time (60 min), which yielded highest SOD activity. The cells were then chilled, collected by centrifugation, and twice washed in cold, sterile tryptic soy medium devoid of paraquat. The washed cells were resuspended in prewarmed (37^{o}C) tryptic soy medium (with suppletments) and allowed to resume growth. At intervals of

0, 15, 30, 60, 90 minutes respectively, samples were removed, the cells were washed and cell free extracts were prepared and assayed as above.

7. Induction of SOD by Paraquat in the Presence of SOD in the Culture Medium

The following SOD units were added to 10 ml of *B. gingivalis* cultures viz 1 unit, 5 units and 10 units. Induction of SOD was then attempted by adding optimum paraquat concentration (i.e. 0.5 mM) and induction was carried out for 60 minutes. Culture tubes to which no SOD was added were used as controls. Cell free extracts were prepared as above and were used for protein and SOD assays.

8. Subcellular Location of SOD in *B. gingivalis*

First, sonicates were centrifuged at speeds of 15,000 g, SOD activity was assayed in the supernatant and pellet respectively. The sonicates were then reconstituted.

An additional procedure in this study determined the location of the enzyme in membrane layers. And this is based on the method previously used for the separation of the inner and outer membrane layers of *V. cholera*. The cell suspension were treated with DNAse, sonicated, and the cell lysate were centrifuged to remove debris. The supernatant

was then layered on a sucrose step gradient consisting of 1 ml 65% sucrose and 5 ml 50% sucrose in HEPES buffer. The gradients were centrifuged at 38,000 r.p.m. for 1 hour. The total membrane fraction appear just below the interface between the 65% and 15% sucrose. The total membrane fraction was layered on a 10 ml linear sucrose gradient 45-60% in HEPES, and centrifuged for 24 hours at 38,000 r.p.m.. The inner and outer membrane separated and equilibrated near the middle of the tube. They were carefully isolated and assayed for SOD and protein.

9. Metal Requirement for SOD Activity

Various concentration of azide (0.1 mM, 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM and 1.0 mM), o-phenanthroline (1 mM, 10 mM and 50 mM), and diethyl diethiocarbarmate (1 mM), hydralazine, isonicotinic acid, 8-hydroxyquinoline, picolinic acid, were added respectively to the reaction mixture containing *B. gingivalis* SOD and then measured SOD activity in the presence of these chelating agents.

The metal requirement of the enzyme was further analysed by dialyzing the SOD against 1 mM EDTA solution exhaustively to produce its apoprotein. Then 1 mM $FeSO_4$, and 1 mM $MnSO_4$ in 0.01M potassium buffer solution were used

respectively to add back the metals for the enzyme SOD activity, by dialyzing the bacterial SOD against these metal-buffer solution.

10. Purification of *B. gingivalis* SOD

Large volumes of induced *B. gingivalis* cultures (about 70 liters) were collected and washed twice with 0.01 M potassium phosphate PH 7.5. The bacterial pellets were then sonicated twice, 4 minutes each time with a macrotip on. The sonicates were spinned down at 12,000 r.p.m. for 60 minutes. The pellets were dissolved in minimum volume of 0.01 M potassium phosphate buffer at PH 7.5, then 2% tritonX-100 were used to extract the enzyme for 72 hours at $4^{o}C$ temperature. Cell debris was removed by centrifugation at 12,000 r.p.m. for 30 minutes. The supernatant was brought to 60% saturation, at $23^{o}C$, with solid ammonium sulfate. The precipitate which formed within 1 hour was removed by centrifugation and the supernatant solution was brought to 80% saturation with $(NH_4)_2SO_4$. The precipitate, collected by centrifugation,was suspended in a minimum volume of 0.01 M potassium phosphate buffer, PH 7.5 and was dialyzed against several changes of the same buffer at $4^{o}C$. The dialysate was clarified by centrifugation and 17.6 ml of the supernatant were passed onto a column of DEAE-cellulose (25 ml of DEAE-cellulose packed in a 60-ml disposable syringe), which

had been equilibrated at 4°C with the above buffer. Batches of DEAE-cellulose were successively washed with 50 ml of the potassium phophate buffer and with 50 ml of 0.05 M $(NH_4)_2SO_4$ in the potassium phosphate buffer and were finally eluted with 100 ml of 0.2 M $(NH_4)_2SO_4$. The eluted fractions containing superoxide dismutase activity were dialyzed exhaustively against several changes of 0.01 M potassium phosphate PH 7.5. These pooled fractions were purified further by gradient DEAE-cellulose chromatography. The samples (15 ml) were applied to a column (2.5 X 25 cm) and washed with 25 ml of 0.01 M potassium phosphate buffer PH 7.5, and then with 100 ml of 0.1 M $(NH_4)_2SO_4$ in potassium phosphate buffer. Next, a linear gradient of $(NH_4)_2SO_4$ (0.1->0.25 M in the potassium phosphate buffer, 200 ml each) was then applied and 6 ml fractions were collected. The fractions containing the SOD activity were pooled together. The samples were applied onto a Sephadex G-100 Gel filtration column in order to get higher purification fold. Sephadex G-100 was prepared as 100 mg/100 ml, set in room temperature for 3 days for complete swelling of Sephadex gels. Then the prepared Sephadex G-100 was packed into a 0.75 X 25 cm column and the partially purified samples were applied onto the column. The samples were successively washed with sufficient 0.01 M potassium phosphate buffer PH

7.5. The fractions containing the SOD enzyme were pooled together.

11. Partial Characterization of *B. gingivalis* SOD

1) Molecular Weight Determination

Molecular weight was determined by gel filtration on a Sephadex G-100 column (0.75 X 25 cm). The column was equilibrated with 0.01 M potassium phosphate buffer PH 7.5, and calibrated with the following molecular weight standards: bovine albumin (66,000), carbonic anhydrase (29,000) and a-lactalbumin (14,200).

2) Subunit Molecular Weight Determination

The purified enzyme of *B. gingivalis* was analyzed by polyacrylamide gel electrophoresis with the presence of 7% polyacylamide sodium dodecyl sulfate and 2% mercaptoethanol. Zones of protein were localized by staining with coomassie blue R-250 while zones of SOD enzyme were visualized by the silver stain method. The standards used to make a plot of log molecular weight versus mobility of protein band were bovine albimin (66,000), egg albumin (45,000), pepsin (34,700), trypsinogen (24,000), b-lactoglobulin (18,400), and lysozyme (14,300).

RESULTS

1. Effect of Varying Paraquat Concentration on SOD Induction (Fig.3.1)

Paraquat has been shown to generate O_2^- in various organisms (4). In previous studies, paraquat was shown to induce SOD synthesis in *E. coli* (4,9). Optimal induction of SOD was seen at 0.5 mM paraquat. In preliminary experiments in our laboratory, we had noted increased SOD production in *B. gingivalis* after exposure to paraquat. In the present experiments, we determined an optimal concentration of paraquat to be used in subsequent studies.

SOD induction by paraquat occurred following the addition of 0.1 mM paraquat (2.64 units/mg protein). The addition of 0.5 mM paraquat to the bacterial culture gave an additional 5-fold increase of SOD activity (10.00 units/mg protein). There was only a slight increase to 11.00 units/mg protein following the addition of 1.0 mM paraquat (Fig.3.1).

This observation is consistent with that of others (4,9). In all subsequent experiments, the organism was exposed to 0.5 mM paraquat.

2. Effect of Time of Exposure of *B. gingivalis* to 0.5 mM Paraquat

In order to determine the kinetics of induction, *B. gingivalis* was incubated with 0.5 mM paraquat for various time periods. As shown in Fig.3.2, low levels of SOD were noted immediately
following the addition of paraquat. This was followed by a sharp increase in SOD activity with increasing exposure time to paraquat. This increase reched its maximum following 60 minutes exposure to paraquat (18.5 SOD units/mg protein). This was followed by a decline after 120 minutes of exposure to paraquat. The lower level of activity was maintained even after 180 minutes exposure. This result of with *B. gingivalis* is different from that of previous studies of Fridovich on *E. coli*. They have found 30 mins to be the optimal induction time (4). This difference may be due to the intrinsic differences between these two organisms. In the subsequent studies, 60 minutes induction time was used as maximal induction time.

3. Deinduction

It has been known that lower level organisms synthesize proteins, including enzymes, only when needed. When inducers were removed, the synthesis would stop. This has been demonstrated in *E.coli* (48). In the present study, we have shown that removal of paraquat from the medium caused deinduction of SOD in *B. gingivalis*.

As shown in Fig.3.3, following induction of SOD in *B.gingivalis*, a marked decline in the level of SOD was noted 15 minutes following removal of paraquat. The level of enzyme continued to decrease as a function of incubation time in the absence of inducer. There was no further decrease after 90 minutes of deinduction.

4. Addition of SOD to the Bacterial Medium Prior to Induction by 0.5 mM Paraquat and 60 minutes Incubation

Since it is possible that superoxide dismutase production increases at inflammed sites of periodontal tissue and more than one pathogen may be present, it is important to determine ifthe presence of SOD affects the induction of SOD in *B. gingivalis*.

Results of this experiment show that SOD induction in *B.*

gingivalis was not significantly affected by SOD added to the medium (Table 3.1.). These data are consistent with the idea that paraquat generates O_2^- intracellularly (4).

5. Subcellular Location of SOD in *B. gingivalis*

In all bacteria except *N. asteroides*, superoxide dismutase activity has been shown to be entirely localized in the cytosol. In *N. asteroides*, the enzyme is bound to the bacterial outer membrane (2,11,13,14). *E. coli* has two forms of SOD, the manganese form and iron form, both of which are located in the cytosol (9). The result of this experiment is interesting. In *B. gingivalis*, SOD is not secreted into the medium and is released only on disruption followed by detergent extraction. It appears that the superoxide dismutase of *B. gingivalis* is largely associated with the bacterial outer membrane (Table 3.2.a and Table 3.2.b).

6. Metal Requirement for SOD Activity

It is clear that eucaryotes contain copper-zinc superoxide dismutase, while procaryotes contain iron or/and manganese SOD. Anaerobes such as E. coli contain only the iron enzyme when cultured under anaerobic conditions (9). *E. coli* synthesizes the manganese enzyme and a hybrid iron-manganese form of superoxide dismutase in the presence

of paraquat (9). In the present studies, it has been shown that the enzyme of *B. gingivalis* was inhibited by o-phenanthroline and azide, but not by diethyl diethiocarbamate, suggesting that Fe^{2+} and Mn^{2+} are required. The $k_i$app of these inhibitors were also determined (Table 3.3). The k_i of O-phenanthroline is smaller than that for azide. Stripping the enzyme of metals resulted in decreased enzymatic activity in SOD. When Fe^{2+} or Mn^{2+} was added back to the apoprotein, a three to four fold increase in SOD activity resulted (Table 3.4).

7. Purification of *B. gingivalis* SOD

The manganese and iron enzymes were first purified from *E.coli* by Fridovich and his colleagues using conventional protein purification procedures (9). In these cases, ammonium sulphate precipitation was used as the major initial purification step. Later a membrane-bound Fe, Mn and Zn SOD had been purified from *N. asteroides* by Beaman *et al* (49). This was done by ammonium sulphate precipitation followed by anion-exchange cellulose. With this method about 50-fold purification was obtained (49). In the present investigation, we used procedures similar to those of Beaman and his colleagues. The scheme for the purification of the enzyme is shown in Table3.5. About 292 fold purification of

B. gingivalis SOD was obtained in this experiment. This is one of the highest level of purity achieved for any bacterial SOD.

8. Partial Characterization of *B. gingivalis* SOD

The copper-zinc superoxide dismutase from eucaryotes is a homodimer of about 32,000 daltons (9). The molecular weights determined for the enzyme from a variety of sources are all substantially in agreement (9). By contrast, the manganese and iron forms of the enzyme from procaryotes have slightly higher molecular weight of about 40,000 daltons (4). While all the copper/zinc and the iron superoxide dismutases have been shown to be dimeric, the manganese enzyme has been found to form tetramers (4,9). Interestingly, the only other known membrane-bound SOD is in *N. asteroides*. This SOD has a molecular weight of 100,000 daltons. It is a tetramer, with each monomer having a molecular weight of 25,000 (49). In this experiment we determined the molecular weight and subunit structure of *B. gingivalis* Fe-Mn SOD.

a. Molecular Weight Determination

Molecular weight of *B. gingivalis* SOD was determined by molecular sieve chromotography (49). The elution profile of

the molecular weight markers and the enzyme on gel filtration from Sephadex G-100 column are shown in Fig.3.5. The molecular weight of the enzyme is approximately 56,000 dalton.

b. Subunit Molecular Weight Determination

In order to determine the subunit structure, the enzyme was subjected to PAGE in SDS (49). The PAGE pattern is shown in Fig.3.4. The result of this experiment shows that dissociation of the enzyme resulted in a single homogeneous band consistent with a molecular weight of 14,000 dalton, suggesting that this bacterial SOD is a tetramer with each monomer having a molecular weight of 14,000 dalton.

Therefore, our results with *B. gingivalis* are somewhat similar to the result of studies on *N. asteroides*.

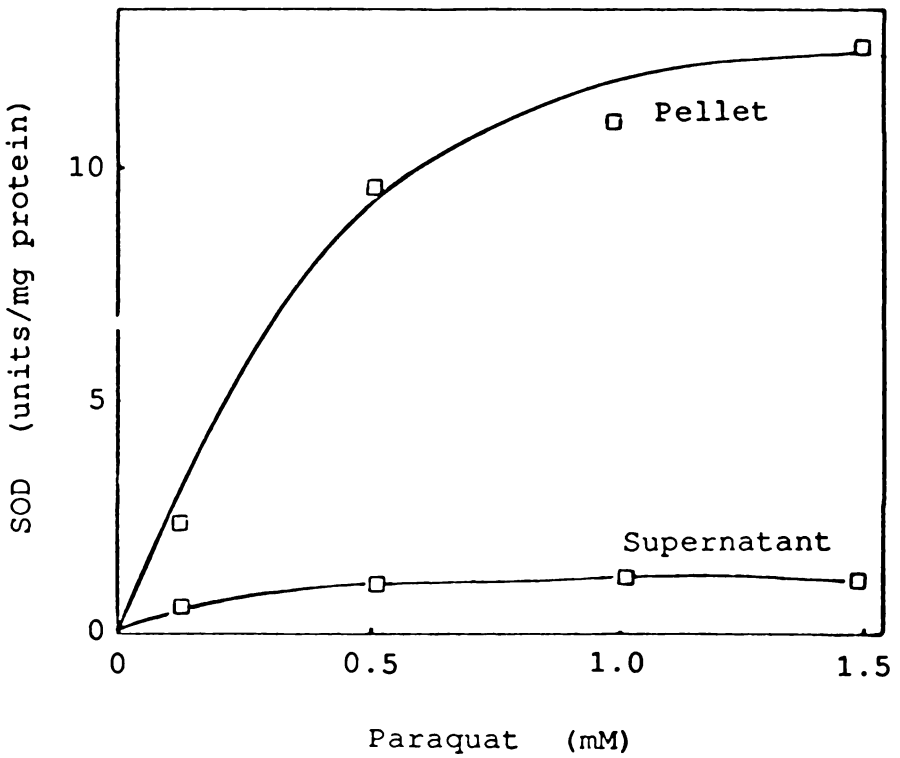

Fig. 3.1 SOD Induction as a Function of Inducer's Concentration

Legend to Fig.3.1.:

Effect of varying the concentration of paraquat: various concentrations of paraquat were added to *B. gingivalis* cultures in TSY medium in the late logarithmic growth phase. After 30 mins of incubation, cell free extracts and pellet were prepared and assayed for total superoxide dismutase (SOD) activity. This figure presents specific activity as a function of paraquat concentration in the growth medium.

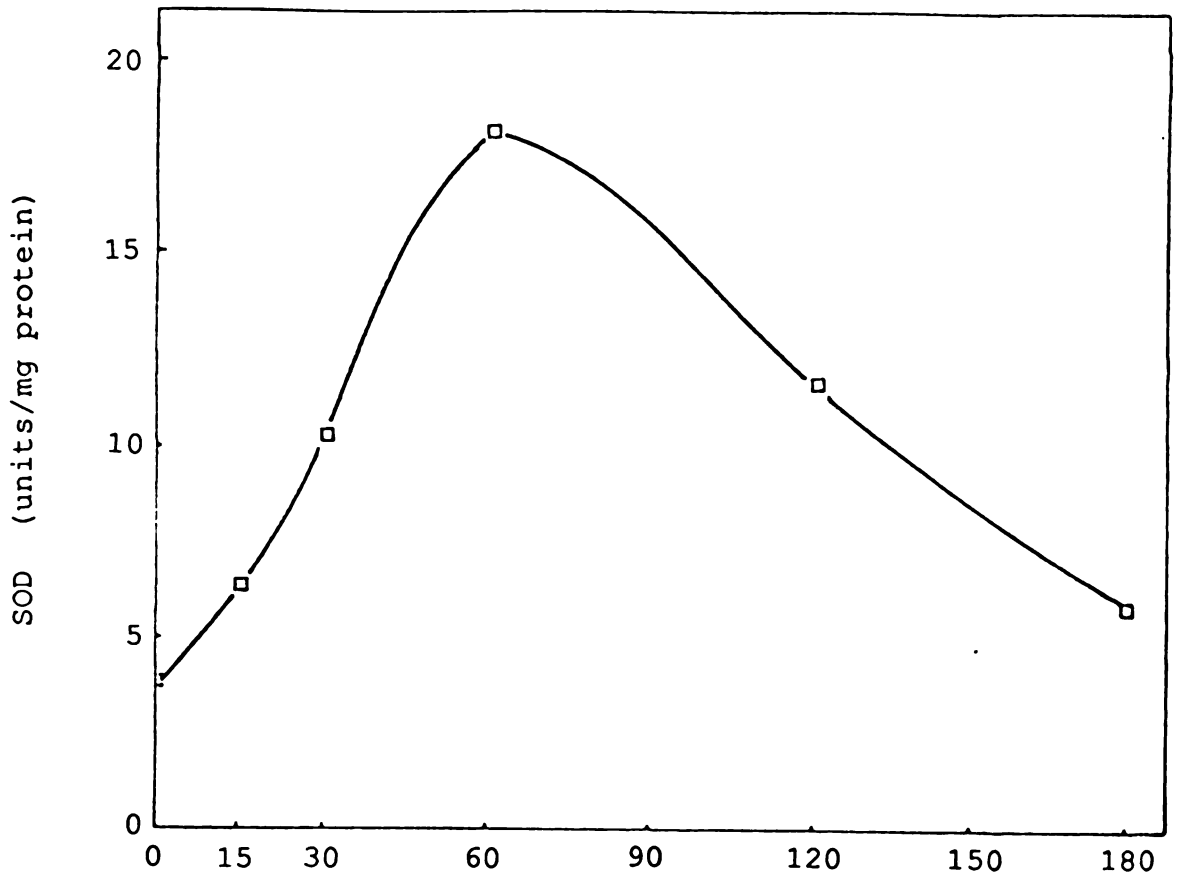

Fig. 3.2 SOD Induction in *B. gingivalis* as a Function of Time Exposure to Paraquat

Legend to Fig.3.2.:

Time course of induction of superoxide dismutase (SOD) by paraquat: 0.5 mM paraquat was added to *B.gingivalis* cultures in TSY medium in late logarithmic growth phase. At intervals thereafter, aliquots of cells were collected and cell-free extracts were prepared and assayed for protein and superoxide dismutase. This figure presents the specific activity of superoxide dismutase as a function of time of exposure of *B. gingivalis* to the paraquat.

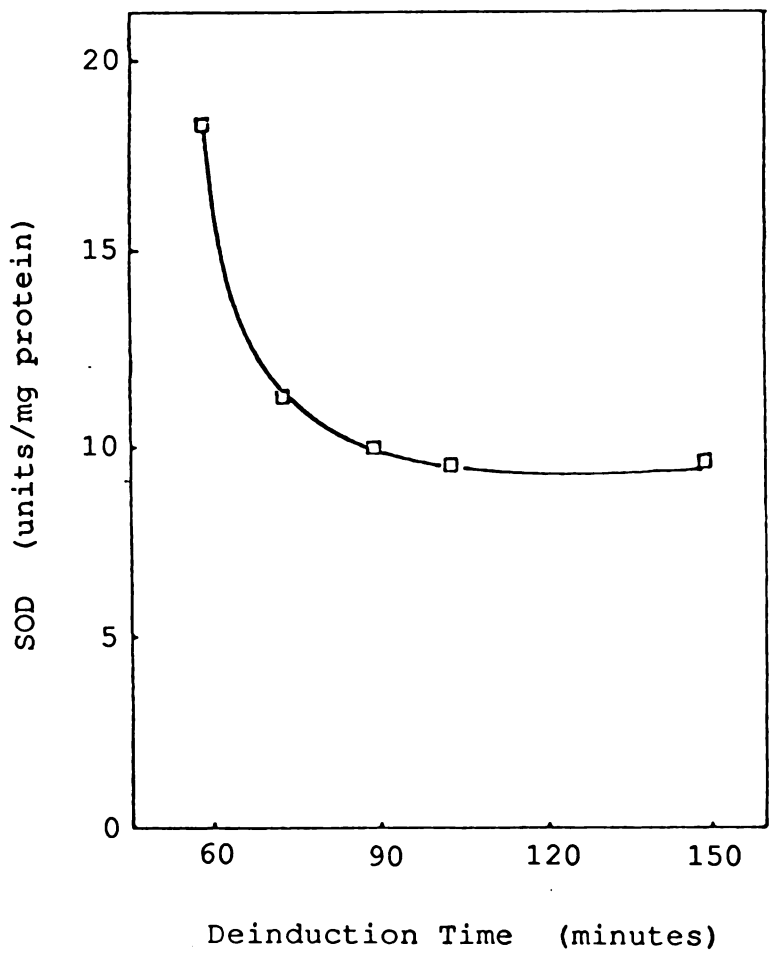

Fig. 3.3 Deinduction of SOD in *B. gingivalis* Following Induction by Exposure to Paraquat for 60 Minutes

Legend to Fig.3.3.:

Deinduction after removal of paraquat: *B. gingivalis* was induced with respect to superoxide dismutase (SOD) by aerobic growth for 60 min in TSY medium containing 0.5 mM paraquat. The cells were chilled, collected by centrifugation, and washed twice in cold, sterile TSY medium devoid of paraquat. The washed cells were resuspended in prewarmed (37^{o}) TSY medium and allowed to resume growth. At intervals, samples were removed and the cells were washed and cell-free extracts were prepared and assayed. This figure presents total activity as a function of time after resumption of growth in the TSY medium devoid of paraquat.

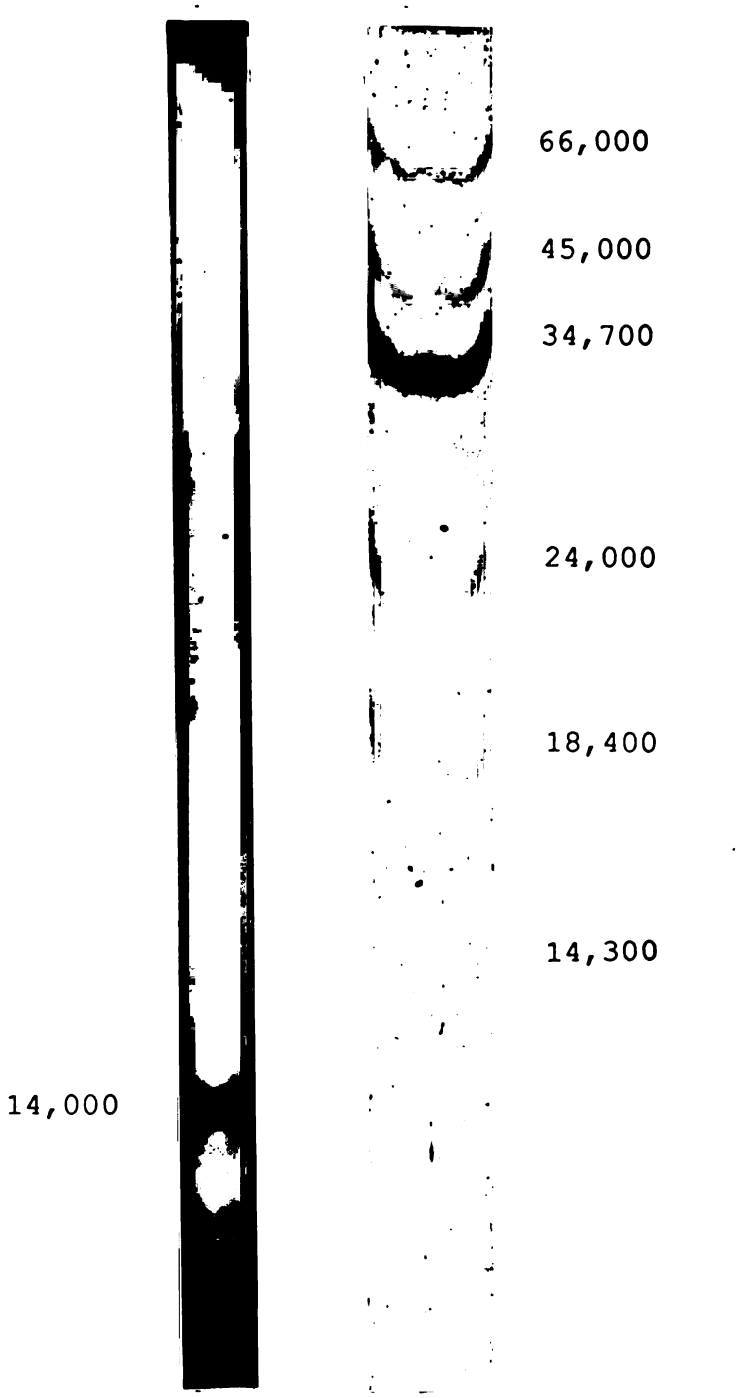

Fig. 3.4 Subunit Molecular Weight Determination of SOD by SDS-Page

Legend to Fig.3.4.:

purified superoxide dismutase was subject to 7% polyacylamide gel eletrophoresis. Electrophresis was carried out in 1.5 M Tris-cl buffer PH 8.8, containing 10% (w/v) SDS. The protein molecular weight markers were bovine albumin, mw = 66,000, egg albumin, mw = 45,000, pepsin, mw = 34,700, trypsinogen, mw = 24,000, *b*-lactoglobulin, mw = 18,400, lysozyme, mw = 14,300. Zones of protein were stained with Coomassie blue R-250, while superoxide dismutase (SOD) were visualized by the silver stain method eafter Coomassie blue stainning first.

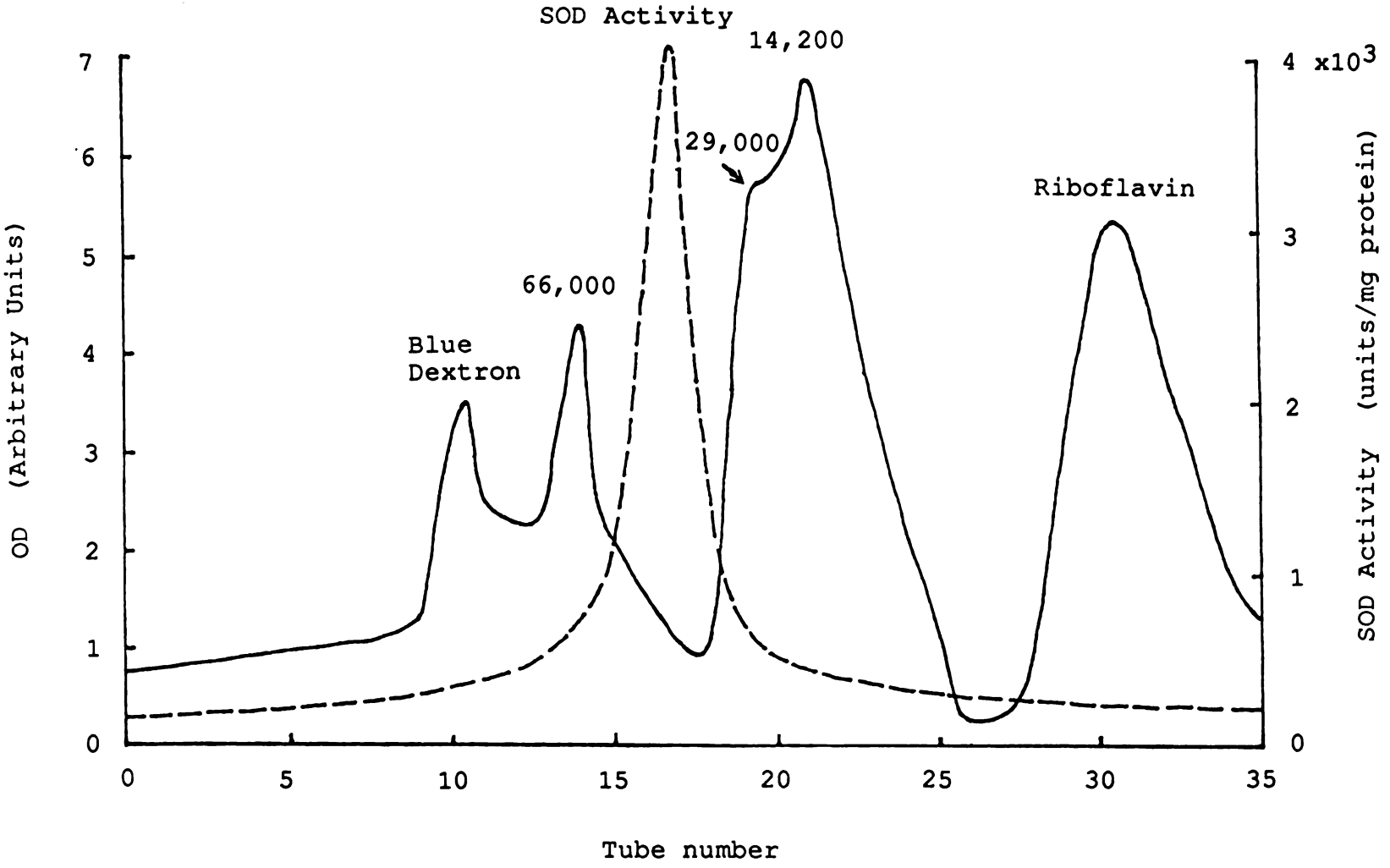

Fig. 3.5 The Elution Profile of SOD of Gel Filtration on Sephadex G-100 Column

Legend to Fig.3.5.:

Elution profile on Sephadex G-100 column: the column was calibrated by running purified protein standards: bovine albumin (66,000), carbonic anhydrase (29,000) and a-lactalbumin (14,200). The column was then loaded with partially purified *B. gingivalis* superoxide dismutase (SOD). Fractions containing the superoxide dismutase (SOD) activity were pooled together.

Table 3.1 Induction of SOD by paraquat in the presence of SOD in the culture medium

SOD Added to Culture Medium (units)	SOD (units/mg protein)
0	18
1	22
5	22
10	16

Different amounts of bovine erythrocytes Cu-Zn superoxide dismutase were added to bacterial cultures in the presence of 0.5 mM paraquat to decrease medium superoxide level. The effect of added SOD on bacterial SOD induction was examined by measuring *B. gingivalis* SOD after 60 mins.

Table 3.2.a Induction of SOD in Pellet Form in *B.gingivalis*

Preparation	Enzyme Activity (units/mg protein)
Total sonicate	38.0
Supernatant	Not detected
Pellet	56.5

Induction was carried out under optimal conditions. Bacterial cells were sonicated as described in the text. The sonicates were then centrifuged down at 15,000 g for 1 hr, and SOD was assayed in the sonicate, the pellet and supernatant fraction.

Table 3.2.b Association of SOD with the Outer Membrane

Membrane Fraction	Supernatant Activity SOD (units/mg protein)
Outer	10.66
Inner	2.70

The separation procedures used are described in the Material and Methods section. The cell suspension were prepared and layered on a sucrose step gradient. Membrane fractions were separated by centrifugation.

Table 3.3 Prosthetic metal requirement for *B. gingivalis* SOD

Inhibitors	k_i (mM)
o-phenanthroline	0.15
azide	0.80
diethyl dithiocarbamate	no inhibition
hydralazine	no inhibition
isonicotinic acid	no inhibition
8-hydroxyquinoline	no inhibition
picolinic acid	no inhibition

The enzyme activity was determined in the presence of various concentrations of o-phenathroline, azide, diethyl dithiocarbamate, hydralazine, isonicotinic acid, 8-hydroxyquinoline and picolinic acid. The inhibitors constants were determined by constructing Lineweaver-Burk plot.

Table 3.4. Restoration of enzyme activity to SOD apoprotein by the addition of Fe^{2+} and Mn^{2+}.

Preparations	Enzyme activity (units/mg protein)
before stripping	130
after stripping	98
plus Fe	300
plus Mn	400

This is a metal-removal and reconstitution study of the SOD of *B. gingivalis*. The procedure used for making the apoprotein was to dialyse against EDTA. And the enzyme was reconstituted by dialysing against either Fe^{2+} or Mn^{2+}.

DISCUSSION

Periodontal disease is caused by bacterial infection and persists and progresses because of the ability of infecting organisms to survive in host tissue (24). *B. gingivalis* appears to play an important role in the etiology and pathogenesis of periodontal disease. It is able to survive in periodontal pockets and in experimental abscess despite a massive migration of polymorphonuclear leukocytes to the site of infection, and it is also able to resist phagocytosis and neutrophils (16,22,23,24).

The superoxide free radical is produced as a potent microbicide and it stimulates chemotaxis (5,6). Most organisms counter the toxicity of superoxide by producing the enzyme superoxide dismutase. Many bacteria synthesize superoxide dismutase in defense against superoxide generated during phagocytosis. The ability of some bacteria to produce superoxide dismutase and hydrogen peroxide metabolizing enzymes constitutes an important aspect of these microbes pathogenicity and virulence. Tally and co-workers (20) showed that tolerance to small amounts of oxygen may be a

prerequisite for the virulence demonstrated by certain anaerobic organisms. Gregory *et al* (10) surveyed a large group of anaerobes for the presence of SOD. They found the enzyme in 23 0f 28 strains of the genus Bacteroides. The enzyme was also found, though with a much smaller frequency, in several clostridia, anaerobic cocci and anaerobic gram positive and in non-sporing rods. Among the oxygen tolerant species, the gram-negative organisms had higher levels of SOD than the gram-positive species, with Bacteroides SP. showing a high level of inducibility of the enzyme in the presence of oxygen.

In the present study, exposure of *B. gingivalis* to superoxide free radical generated by paraquat resulted in the copious induction of superoxide dismutase. The induction was dependent on the concentration of the inducer and the length of time of the exposure. Removal of the bacterium from the inducing medium resulted in de-induction, indicating that *B. gingivalis* synthesized the protective enzyme only when needed. By producing this enzyme, *B. gingivalis* may be able to survive in the hostile, superoxide enriched environment of inflammed periodontal tissue. If this observation is applied to pathogenic bacteria in general, many organisms that have not been shown to possess significant amounts of this enzyme may in fact be protected in the hostile environment of inflammed tissues by this

enzyme, although they may not show enzyme activity when cultured *in vitro* under favorable conditions in the absence of superoxide.

Hassan and Fridovich (40,41) noted a sharp decline in SOD activity 30 minutes following removal of paraquat (de-induction) in *E. coli*. These investigators suggest that the very rapid increase in SOD following addition of paraquat and the sharp decline of activity when paraquat was removed, strongly indicate that induction occurs in all of the cells. This same situation was observed in the case of *B. gingivalis*.

Since recent studies have shown that SOD increased significantly in inflammed human gingiva and in gingival fluid of periodontally diseased sites, it would be interesting to add SOD to the bacterial culture prior to induction with paraquat. Results of this experiment showed that SOD induction in *B. gingivalis* did not seem to be affected by the added SOD. This may be due to the fact that paraquat requires reduction before it can participate in the ganeration of O_2^-. It has been shown that the induction of SOD by paraquat in *E. coli* is supported by the presence of diaphorase in that organism (9). In contrast, paraquat did not induce SOD in *S. mutans*, an organism lacking diaphorase.

Our studies suggest that the induction of SOD by paraquat in *B. gingivalis* results from intracellular generation of $O_2^{-}\cdot$ by paraquat.

Periodontal microganisms inhabit a milieu which can be expected to be enriched in superoxide secreted by neutrophils which arrive as part of the inflammatory process. The organism may synthesize large quantities of the enzyme and secrete it into its surroundings to decrease the surrounding levels of the free radical, or the enzyme may be present within the cell in the cytosol or concentrated in the cell wall. The cell wall site is strategically the most advantageous to the organism, if it is to prevent the free radical from entering and damaging its genetic material. Secreted SOD would affect chemotactic mechanisms, as discussed above, while cell-wall associated enzyme could allow the organism to survive ingestion by a phagocyte, at least for a short while. *Nocardia asteroides*, a strict aerobic and facultative intracellular pathogen, has its SOD associated with its outer membrane and it also secretes the enzyme. This may explain the ability of this organism to elicit an oxidative burst in neutrophils, without being killed (2,11,12,13,14). The data in our study demonstrate that *B. gingivalis* synthesizes large amounts of SOD when challenged with superoxide *in vitro*, and the enzyme is tightly complexed with the cell wall (outer membrane). These

observations are consistent with the finding that capsular material from *Bacteroides gingivalis* may inhibit phagocytosis. This unique subcellular location of the enzyme of *B. gingivalis* is very advantageous and protective.

Superoxide dismutase is a metalloenzyme. Its biochemical properties may contribute to the survival of the organism. The metal prosthetic group of the enzyme has been shown to play a role in the resistance to phagocytosis. Fe-SOD appears to be associated with the greatest tolerance to oxygen or to phagocytosis. Superoxide dismutase from *E. coli* have been shown to contain Mn or Fe. Yost and Fridovich (40,41) showed that *E. coli* containing Fe-SOD is more resistant to phagocytosis than the bacterium with Mn-enzyme. Fe-SOD confers greater resistance to phagocytosis, a fact that is consistent with the observation that the virulence of many organisms is enhanced by Fe. This idea is supported by the recent observation that *Nocardia asteroides,* contains in addition to the Mn-containing enzyme, an Fe-SOD (2,11,12,13,14,15). The data in the present study have demonstrated that the SOD of *B. gingivalis* is inhibited by azide and by o-phenanthroline, but not by diethyl dithiocarbamate, indicating this SOD is not a copper-zinc enzyme, namely not a tissue enzyme, and Mn and Fe are the prosthetic metals required for *B. gingivalis* SOD activity. This is consistent with other bacterial SODs. It is of

interest to note that this enzyme requires both Mn and Fe and the metals are interchangeable, while most other enzymes require only one metal as a prosthetic group. Moreover, *B. gingivalis* possesses Fe-SOD, and the k_i of o-phenanthroline is smaller then that of azide, suggesting that Fe may be a more important cofactor. The Fe-SOD may contribute to the virulence of the organism and its ability to survive in environments challenged with superoxide because of inflammation.

In conlusion, several factors seem to aid the resistance and virulence of *B. gingivalis* to the killing effect of neutrophil superoxide. These factors are: 1. The immediate adaptive response to exposure to superoxide by producing SOD, 2. Maintainance of high levels of SOD activity by hyperproducers even when superoxide was removed, 3. Advantageous cell wall location of the enzyme. This could prevent the microrganism from being subject to subsequent neutrophil phagocytic attack.

SUMMARY

Superoxide generated by neutrophils is an important agent of bacterial killing. Superoxide dismutase is the primary defense against the deleterious effects of this radical. Increasing the rate of intracellular O_2^- production, by different means (either by paraquat or some other redox compounds), leads to an adaptive increase in the synthesis of superoxide dismutase. Blocking this adaptive response with inhibitors of protein biosynthesis or with a nutritionally restricted medium, causes the cells to die.

The anaerobic bacteroide *B. gingivalis* is a major pathogen associated with rapidlly destructive periodontal disease. It survives intense inflammation associated with the disease and resists phagocytosis. It is therefore of interest to examine the activity of superoxide dismutase (SOD) in *B. gingivalis* to determine the mechanism of its survival in inflammed tissues. We have found that exposure of the organism to paraquat, a intracellular superoxide producer, resulted in copious induction of superoxide

dismutase in this bacteria. The activity of the enzyme was dependent both on the inducer's concentration and the length of exposure. The enzyme activity was found to be associated with the outer membrane of the bacterial cell-wall. We have purified the detergent extracted enzyme about 292 fold. The enzyme requires Mn and/or Fe for activity and has four 14 KD subunits. The ability of the *B. gingivalis* to adapt to oxidation stress to produce an antioxidant defense enzyme, and the unique association of the enzyme with the cell wall may be crucial to the survival of the organism in the hostile, superoxide-rich milieu of the inflammed host tissue.

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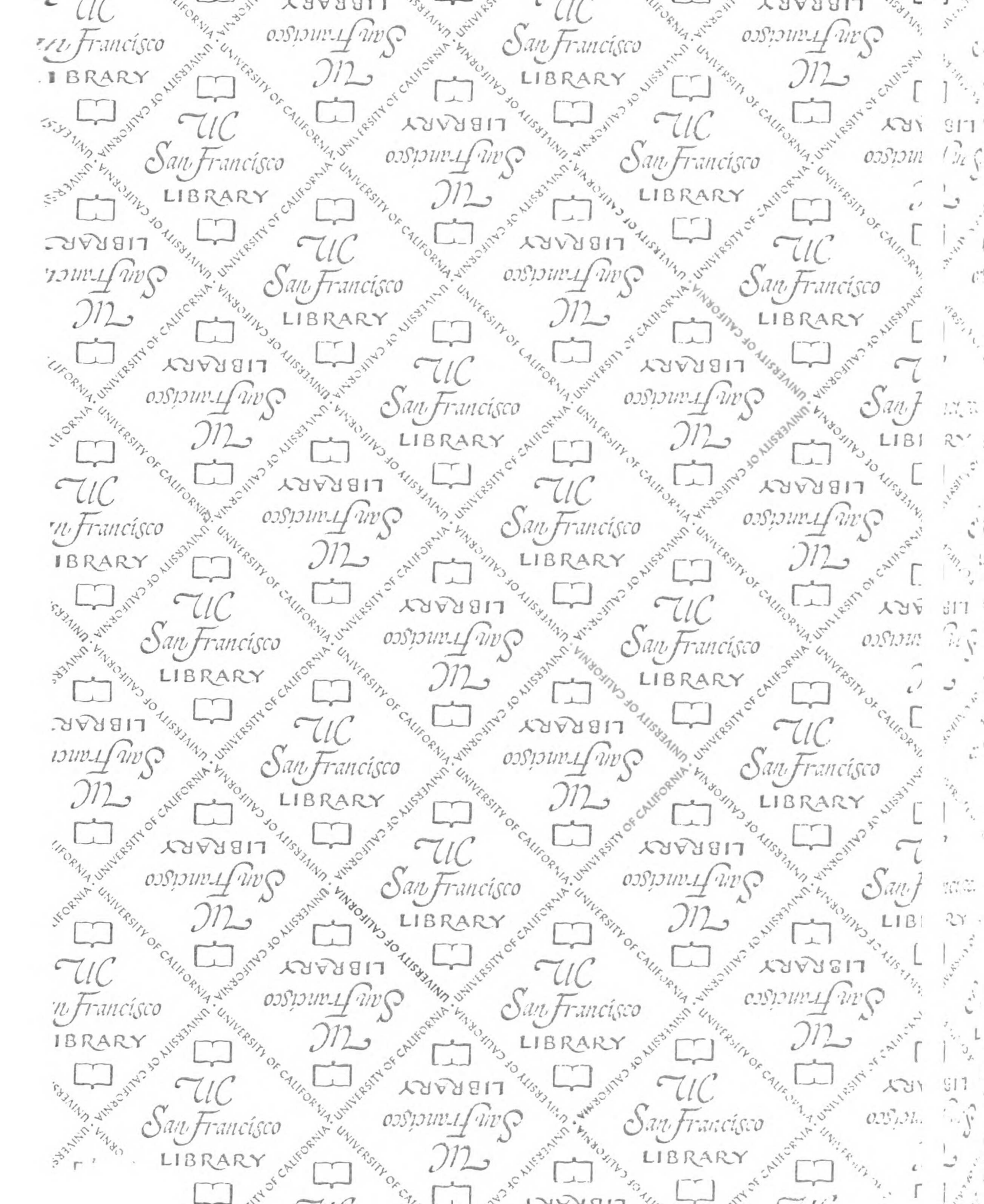
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