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Effects of Anaerobiosis and Aerobiosis on Interactions of Human Polymorphonuclear Leukocytes with the Dental Plaque Bacteria Streptococcus mutans, Capnocytophaga ochracea, and Bacteroides gingivalis

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Human polymorphonuclear leukocytes (PMN) were able to generate and release superoxide anions upon stimulation of Streptococcus mutans, Bacteroides gingivalis, and Capnocytophaga ochracea when incubated aerobically but not when incubated anaerobically. Lysozyme release and phagocytosis by PMN were independent of oxygen, and no difference between PMN incubated aerobically or anaerobically was observed (PMN stimulated by B. gingivalis released 7.6% total lysozyme when aerobic and 6.9% when anaerobic). There were variations in lysozyme release and phagocytosis for the three organisms, particularly for phagocytosis. B. gingivalis and C. ochracea yielded lower phagocytosis values than those for S. mutans, e.g., at 1 h 67% of the initial inoculum of S. mutans was phagocyted (versus only 40% for B. gingivalis). Transmission electron microscopy showed that both S. mutans and B. gingivalis were internalized into classical phagolysosomes. In contrast, C. ochracea showed two forms of internalization; C. ochracea either formed a classical phagolysosome or was tightly bound in the cytoplasm with no surrounding cell membrane. Intracellular killing of S. mutans and C. ochracea was unaffected by anaerobiosis, but killing of C. ochracea was much lower than that of S. mutans (1 × 10^7 to 2 × 10^7 bacteria killed compared with 5.1 × 10^7 bacteria killed at 6 h). In contrast, a greater number of B. gingivalis was killed in the presence of oxygen (5.3 × 10^7 bacteria were killed when aerobically incubated and 1.9 × 10^7 bacteria were killed when anaerobically incubated). These results suggest that the ability to survive anaerobically may enable some bacteria to evade PMN killing; however, abnormal phagocytosis may represent a more efficient way to evade both oxygen-dependent and -independent killing mechanisms, leading to enhanced virulence of the organism.

Polymorphonuclear leukocytes (PMN) are known to be the first line of defense for protection against infectious microorganisms (27). There are two major mechanisms involved in performing this task efficiently: oxygen-dependent and oxygen-independent killing mechanisms (5). The oxygen-dependent mechanism involves the utilization of oxygen in a respiratory burst to generate a variety of reactive products including superoxide anions and hydroxyl radicals (5). The oxygen-independent mechanisms involve a variety of antimicrobial components which are contained in the PMN granules, such as bacterial permeability-increasing factor, defensins, lysozyme, and lactoferrin (21, 27).

Much work has shown that oxygen-dependent killing mechanisms are very efficient and are involved in the killing of numerous organisms including Escherichia coli, Staphylococcus aureus, Actinobacillus actinomycetemcomitans, and Candida albicans (4, 14, 18). However, studies of chronic granulomatous disease or myeloperoxidase-deficient patients who lack oxygen-dependent killing products have shown that oxygen-independent mechanisms are also necessary for killing (27). The importance of the oxygen-independent killing mechanisms is further reinforced by the fact that many infectious organisms are anaerobic and may survive in conditions which may prevent the PMN from utilizing oxygen metabolic pathways to generate cytotoxic effects (8).

Human periodontal diseases are a group of inflammatory processes, associated with accumulation of bacterial plaque and loss of collagen attachment and bone resorption around the tooth (35). Disease progression is associated with an increasing pocket depth, a lowering of the oxygen tension within the pocket and, as would be expected, a shift from aerobic and facultative aerobic bacteria such as Streptococcus mutans and Actinomyces naeslundii to anaerobic organisms such as Veillonella and Bacteroides spp. (3, 15, 31). Therefore, if the polymorphonuclear leukocytes are to successfully protect the host, they must interact with a variety of organisms in a range of conditions varying from aerobic to anaerobic (12, 33). This study was undertaken to investigate the interactions of PMN incubated in aerobic and anaerobic conditions with three bacterial species which can be isolated from dental plaque, Bacteroides gingivalis (an obligate anaerobe) and Capnocytophaga ochracea (a facultative anaerobe), which have both been implicated in periodontal diseases (3, 26), and Streptococcus mutans (a facultative anaerobe), which has been associated with dental caries.

MATERIALS AND METHODS

Isolation of PMN. Peripheral blood, obtained by venipuncture from healthy adult volunteers, was mixed with preservative-free heparin (1,000 U/20 ml). The PMN were isolated by Ficoll-Isopaque centrifugation (2), followed by dextran sedimentation (BDH Chemicals, Dagenham, United Kingdom) and lysis of residual erythrocytes (22). The PMN were counted, and viability was assessed by trypan blue exclusion. The concentration of cells was adjusted to 5 × 10^6

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PMN/900 μl in Hanks balanced salt solution (HBSS) with added HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) but without phenol red (Gibco, Uxbridge, United Kingdom). Samples (100 μl) of the suspension (5 × 10⁶ cells/μl) were added to 24-well plates (900 μl was added to each well containing a glass coverslip). The PMN were then allowed to adhere for 1 h at 37°C in 5% CO₂–95% air atmosphere.

Preparation of bacteria. The bacterial species used were S. mutans (B. gingivalis W83 (obtained from G. M. Bowden, University of Manitoba), and C. ochracea (ATCC 27872). The bacteria were grown on 10% horse blood agar plates and incubated in an anaerobic chamber (Mark III; Don Whitley, Otley, United Kingdom) before being scraped from the plate and suspended in phosphate-buffered saline (PBS) (Oxoid, Basingstoke, United Kingdom). The optical density at 640 nm was read with a LKB Ultraspec 4050, and the suspensions were adjusted to an optical density of 1.0, yielding a concentration of 1 × 10⁶ to 2 × 10⁶/ml. The bacteria were prepsorized for 30 min with 20% normal human serum (which contained antibodies to all the bacteria tested as measured by enzyme-linked immunosorbent assay in this laboratory) and has been shown to opsonize C. ochracea for phagocytosis by human PMN (10), while unopsonized organisms were incubated with an equivalent volume of HBSS. The viability of bacteria was checked after preopsonization by performing colony counts and was not found to be different from the bacteria incubated in the absence of serum.

Aerobic and anaerobic incubation. All reagents for the assays were incubated at 30°C in either an aerobic incubator (Astell-Hearson, Sidcup, United Kingdom) or an anaerobic chamber for 24 h prior to use. Cell monolayers were transferred to the anaerobic chamber or aerobic incubator for 1 h before use in the assays. PMN survival was not affected by 24-h incubations in anaerobic conditions, as viability rates at this time were 90% for aerobic incubation and 85% for aerobic conditions.

Superoxide assay. The superoxide assay was performed in 96-well plates containing 5 × 10⁵ PMN per well by the method of Pick and Mizel (20). A 200 mM solution of cytochrome c (Sigma, Poole, United Kingdom) was added to each well in the presence or absence of superoxide dismutase (SOD) (Sigma) at a concentration of 2 mg/ml. Samples (10 μl) of suspensions of unopsonized and opsonized organisms were then added, the plates were incubated for 1 h and centrifuged at 450 × g for 10 min, and the optical density at 540 nm was read with a Titrertek Multiscan MCC (Flow Laboratories, High Wycombe, United Kingdom). The supernatants were removed from the wells and replaced with 100 μl of HBSS containing 0.1% sodium dodecyl sulfate (BDH Chemical, Watford, United Kingdom). The protein content was estimated by using a Bio-Rad dye assay (Bio-Rad, Hemel Hempstead, United Kingdom) with bovine serum albumin as the standard (1 to 200 μg). The amount (nanomoles) of superoxide release was calculated by the following equation: (A₄₅₀ × 100)/(6.3) × milligrams of cell protein.

Lysosome (muramidase) assay. 1% agarose plates containing 2 mg of Micrococcus lysodeikticus (Sigma) were used to assay for lysozyme (19). Samples (8 μl) of the supernatants from the above incubations (superoxide assay samples) or of lysozyme standards were added to wells in the plates. The plates were incubated for 24 h, and the resulting zones of bacterial clearance were measured. The amount of lysozyme present in the samples was then calculated by correlation of the zone of clearance with the logarithmic concentration of lysozyme in the standards (5 to 500 mg/ml). The intracellular content of lysozyme of 5 × 10⁶ PMN was also measured following four cycles of freeze-thawing. The release of lysozyme was expressed as a percentage of the total content.

Phagocytosis assay. Phagocytosis assays of PMN were carried out in 24-well tissue culture plates. A 100-μl sample of opsonized organisms was added to each culture well, which contained a coverslip and 5 × 10⁶ PMN/900 μl. Preliminary studies showed that phagocytosis appeared complete by 1 h; therefore, the plates were incubated at 37°C for 1 h. The PMN were then washed from the coverslip and centrifuged to remove unbound organisms. The cell pellet was lysed with 1 ml of ice-cold water. The number of organisms in this fraction was counted microscopically, and phagocytosis was expressed as the number of the organisms ingested.

Bacterial killing assay. Cell monolayers were incubated, and the level of phagocytosis was assessed. After 1-, 3-, 6-, and 24-h incubations, the PMN were lysed and the resulting suspensions were serially diluted with sterile PBS (dilutions of 1:1,000 and 1:10,000). These samples were spread onto 10% horse blood agar plates and incubated anaerobically for 4 days. The number of colonies was then counted, and the number of organisms killed was calculated as follows: number of organisms killed = number of organisms phagocytosed − number of colonies counted. To ensure that bacterial death was a result of incubation with the PMN and not a result of adverse environmental conditions, colony counts of bacteria incubated in the absence of PMN were performed at each time point.

Transmission electron microscopy. Separate assays of phagocytosis were performed to provide samples for electron microscopy. After centrifugation of the PMN to remove unbound bacteria as described above, the cell pellet was resuspended in Karnovsky fixative modified so that the buffering solution was 0.2 M sodium phosphate and then processed as previously described (6). The samples were placed in an automatic tissue processor for dehydration (Lynx model "el" microscope tissue processor). On completion of the run, the samples were placed in gelatin capsules which contained medium white resin (London Resin, Basingstoke, United Kingdom) and incubated at 60°C in an oven for 20 h. The samples were then sectioned and stained for examination in the electron microscope.

Statistical analyses. Means and standard deviations were calculated for each set of experiments. The differences between two means were analyzed by Student's t test, and P values of less than 0.05 were considered statistically significant.

RESULTS

Superoxide release in response to live organisms. The reactions between PMN and live organisms were measured after aerobic and anaerobic incubation. As expected, no superoxide production could be detected for any of the stimulants under anaerobic conditions (Table 1). Superoxide release under aerobic conditions was detected for all bacteria. There was a marked variation in superoxide release stimulated by the different organisms; for instance, unopsonized S. mutans yielded 11.4 nmol of superoxide while superoxide release decreased slightly, although not significantly (P > 0.05) with B. gingivalis in the absence of cytochalasin. The inclusion of 200 nM cytochalasin B to
TABLE 1. Superoxide anion release by PMN after 1-h incubation with live organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>Cytochalasin</th>
<th>nmol of superoxide/mg of proteina</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>Aerobic</td>
</tr>
<tr>
<td>Unopsonized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. mutans</td>
<td>–</td>
<td>11.4 ± 2.1</td>
</tr>
<tr>
<td>B. gingivalis</td>
<td>–</td>
<td>7.0 ± 4.5</td>
</tr>
<tr>
<td>C. ochracea</td>
<td>–</td>
<td>16.6 ± 2.4</td>
</tr>
<tr>
<td>Opsonized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. mutans</td>
<td>–</td>
<td>9.3 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>14.1 ± 4.0</td>
</tr>
<tr>
<td>B. gingivalis</td>
<td>–</td>
<td>4.2 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>9.9 ± 2.7</td>
</tr>
<tr>
<td>C. ochracea</td>
<td>–</td>
<td>37.5 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>49.8 ± 8.6</td>
</tr>
</tbody>
</table>

a 5 × 10⁶ PMN were incubated with 10⁷ bacteria. 200 nM cytochalasin B was added 10 min before the addition of the organisms.

b Results are expressed as means ± standard deviations of four experiments. ND, No superoxide was detectable.

inhibit phagocytosis had no significant enhancing effect on superoxide release for either organism (P > 0.05 for each case).

Lysozyme release in response to live organisms. Table 2 shows that lysozyme release in response to live organisms was no different whether PMN were incubated aerobically and anaerobically. In contrast to superoxide anion release, under either condition, the maximum release was stimulated by opsonized organisms compared with unopsonized organisms. For B. gingivalis, lysozyme release increased from 7.3% ± 2.1% of the total lysozyme to 16.7% ± 5.9%. Opsonized bacteria showed levels of lysozyme release comparable with that stimulated by PMA.

Phagocytosis of opsonized organisms. The phagocytosis method used permitted the number of organisms ingested to be counted by lysing the PMN to free the internalized organisms. After 1 h, opsonized S. mutans was phagocytosed at high levels (67% ± 23% of initial inoculum) by PMN incubated aerobically and anaerobically (Table 3). There was no increase in the percent phagocytosed when assayed after 24-h incubation. The levels of phagocytosis of opsonized C. ochracea and B. gingivalis were only 22% ± 10% and 40% ± 6% of the initial inoculum, respectively, for both aerobically and anaerobically incubated PMN at 1 h (Table 3), which was significantly lower than that for S. mutans (P < 0.05).

However, after 24-h incubation, the levels of bacteria phagocytosed increased to 40% ± 5% of the inoculum with C. ochracea and 70% ± 10% with B. gingivalis for both aerobic and anaerobic conditions.

Intracellular killing of opsonized organisms. The killing of opsonized C. ochracea was essentially equal for PMN incubated aerobically and anaerobically at each time point measured (Fig. 1). All bacteria ingested were killed when the PMN were lysed with deoxycholate. Because this result appeared unlikely, the detergent alone was used to determine whether it was killing the bacteria that had resided within the PMN; it did not affect bacterial viability. This effect was not observed for the other bacteria. When PMN were lysed with water to release intracellular C. ochracea, maximum killing occurred at 6 h, with 1 × 10⁷ to 2 × 10⁷ bacteria killed (representing only 40% of the total ingested bacteria) for both aerobic and anaerobic conditions. PMN killing of opsonized S. mutans was also no different aerobically or anaerobically (Fig. 2). At 24 h, maximum killing occurred when 5.5 × 10⁷, representing 60% of the bacteria ingested, were killed. In contrast to the bacteria above, intracellular killing of B. gingivalis (Fig. 3) was more greater under aerobic conditions compared with anaerobic conditions. The number of B. gingivalis killed at 1 h under both conditions was approximately 1 × 10⁷ (10 to 12% of the bacteria ingested) and increased to 4 × 10⁷ to 5 × 10⁷ (70 to 80% of the ingested bacteria) at 6 h for PMN incubated aerobically. For PMN incubated anaerobically, the number killed increased only to 2.5 × 10⁷ (50% of ingested organisms) by 6 h (P < 0.005).

Electron microscopy. Opsonized S. mutans and B. gingivalis were both observed inside the PMN, and phagocytosis appeared to follow the classical internalization pathway with bacteria present in the phagolysosomes (Fig. 4a and b). In contrast, opsonized C. ochracea showed two different forms of appearance within PMN. Some bacteria were present in phagolysosomes, while others were found tightly bound within the cytoplasm (Fig. 4c). This unusual form of internalization was observed for PMN incubated both aerobically and anaerobically (Fig. 4c and d, respectively). This form of uptake did not appear to be dependent on opsonins, because when unopsonized organisms were tested, the few internalized C. ochracea seen inside the PMN were in the cytoplasm and not in phagolysosomes (Fig. 4e).
DISCUSSION

The results show that PMN were capable of interacting with *S. mutans*, *C. ochracea*, and *B. gingivalis* under both aerobic and anaerobic conditions. PMN responded less well to *B. gingivalis* when incubated anaerobically, while the response to *S. mutans* and *C. ochracea* was not limited by an anaerobic environment, since *B. gingivalis* was phagocytosed and killed in equivalent numbers in anaerobic conditions to those of aerobic conditions. Thus, the virulence of anaerobic bacteria or facultative aerobes is unlikely to be due solely to the ability of the bacteria to survive under anaerobic conditions. It should be noted that the bacteria were killed by the PMN and not by the opsonization process. Although some strains of the bacterial species used in the present study such as *C. ochracea* (32) may be sensitive to complement-dependent lysis, there was no such effect with any of the bacteria used in our assays.

The lack of detectable superoxide anion under anaerobic conditions was not due to the failure of the organisms to stimulate a reaction because PMN incubated aerobically released superoxide anion in response to both bacteria (Table 1). One explanation for failure to detect the anion is...
FIG. 4. Electron micrographs of PMN with internalized bacteria. (a) Opsonized *S. mutans*; (b) opsonized *B. gingivalis*; (c and d) opsonized *C. ochracea* under aerobic and anaerobic conditions, respectively; (e) unopsonized *C. ochracea*. Bacteria in classical phagolysosome (A, a) and bacteria tightly bound in the cytoplasm (B, b) are indicated. Magnification, ×31,500.
that superoxide anions are internalized in the phagolysosome. However, the anion was still not detectable in anaerobic conditions when the membranes of the PMN were immobilized by cytochalasin B and phagocytosis did not occur. It is interesting to note that *B. gingivalis* showed a slight decrease in superoxide anion release when opsonized compared with that when unopsonized. This phenomenon could simply be due to the adherence of SOD from the opsonizing serum, but this is unlikely to be the sole reason, as it was not observed for all the bacteria tested, suggesting that there is an inherent difference in the bacteria which results in the superoxide anion decrease. Several studies have found that anaerobic bacteria, such as *Bacillus subtilis* and *Bacteroides distasonis* (9), contain SOD. The presence
of SOD may act as a mechanism by which the bacteria defend themselves against the oxygen-dependent killing mechanisms of the PMN. *B. gingivalis* has been shown to contain SOD and therefore may cause dismutation of the superoxide anion before it can interact with cytochrome c.

PMN release of lysozyme was limited for all bacteria tested even in the presence of opsonins. It should be noted that although in general, the release of lysozyme (indicating a release of lysosomal enzymes) and superoxide (indicating the occurrence of a respiratory burst and formation of oxygen metabolites) would be protective for the host by killing organisms (34), it has been shown that local release of lysosomal enzymes and oxygen radicals may cause tissue damage (24, 34). Thus, the observed lack of release of lysosomal enzymes may represent a mechanism by the PMN to prevent increased tissue damage. Similarly, the enhanced generation of radicals demonstrated by *C. ochracea* may increase localized tissue damage and, in this way, the organism might be said to have an indirect virulence factor(s).

Previous studies of phagocytosis by PMN in a variety of organisms, including *E. coli*, *Proteus mirabilis* (16) and *Bacteroides fragilis* (1), have shown that oxygen was not necessary and that PMN phagocytosis levels were equally significant in both aerobic and anaerobic conditions. The present study confirmed these results and suggests that any difference between aerobic and anaerobic killing by PMN was not due simply to differences in phagocytosis. *S. mutans* was ingested efficiently by PMN, in contrast to *C. ochracea* and *B. gingivalis*. The limited uptake of these bacteria was of interest since both organisms have been linked to the pathogenesis of periodontal diseases (3, 26). The mechanisms by which the bacteria evade phagocytosis are not clear, although several have been suggested. Studies have shown that black-pigmented *Bacteroides* spp. may survive in the periodontal pocket because they can resist phagocytosis because of the presence of a capsule (28) and also have the tact immunoglobulins G and A at the hinge region (13). It has also been suggested that the bacteria not only release the proteases into the extracellular medium but also contain them in their cell wall. This would allow the bacteria to degrade bound antigen-specific immunoglobulin G and therefore protect themselves from phagocytosis (26). Studies with *Capnocytophaga* species have shown that they contain and secrete a toxinlike substance which inhibits PMN phagocytosis and locomotion (25, 30). It has been postulated that the effect of the toxin was produced by immobilization of the PMN cell membrane; therefore, the limited phagocytosis observed in this study could result from the release of such a toxin. The limited phagocytosis in this present study represents a mechanism by which *C. ochracea* and *B. gingivalis* may avoid PMN intracellular antimicrobial killing.

PMN were capable of killing *S. mutans* and *C. ochracea* equally well when incubated aerobically or anaerobically, although the susceptibility of each organism to killing was different. The present findings show that *S. mutans* was efficiently killed by PMN, which was similar to the results of other studies with *Streptococcus viridans* and *Streptococcus pneumoniae* (16, 29). The intracellular killing rate of *C. ochracea* was found to be low. This was not due to the failure of PMN contents to kill bacteria, since the PMN granules were able to kill the organism very effectively (data...
not shown). Previous work has shown that PMN isolated from the gingival crevices of juvenile periodontitis patients contained bacterial cells that resemble Capnocytophaga cells within their cytoplasm, not in phagolysosomes (7). The present study has shown that a similar appearance (Capnocytophaga cells within cytoplasm) can be induced in vitro. Thus, the failure of intracellular killing in PMN may be related to the fact that the bacteria found in the cytoplasm do not come into contact with PMN antimicrobial phagosomes or to the low killing rate of the organisms (17). However, unlike C. ochracea, C. trachomatis was found in classical loose phagosomes or in the cytoplasm, but the organisms were tightly bound by the vacuole membrane. In contrast, a smaller number of B. gingivalis was killed under anaerobic conditions. This difference in killing is unlikely to be due to the intolerance of B. gingivalis for oxygen, since incubation of bacteria alone without PMN did not result in a decrease of bacterial survival in aerobic conditions. Anaerobiosis was not found to affect the PMN killing of B. fragilis and B. vulgatus incubated either aerobically or anaerobically (1, 11). The difference between those studies and the present findings may represent either strain differences or assay conditions. The experiments in the present study were performed in an anaerobic chamber, while both the studies cited above used anaerobic jars and may therefore have failed to achieve complete anaerobiosis.

The present study shows that S. mutans and C. ochracea are handled equally well by PMN in aerobic and anaerobic conditions, although it would appear that in general PMN interact poorly with B. gingivalis and C. ochracea. The ability of the PMN to efficiently handle S. mutans suggests that this bacteria has limited infectivity, even under anaerobic conditions as is found in periodontal diseases. In contrast to S. mutans, the limited ability of PMN to interact with B. gingivalis and the low-level intracellular killing in anaerobic conditions may increase the virulence of this bacterium. Thus, it may be that B. gingivalis is only partially controlled by the PMN in normal oxygen-rich shallow pockets and the conversion to oxygen-deficient deep pockets would allow the bacteria to proliferate, resulting in increased tissue breakdown and disease progression. PMN also interacted poorly with C. ochracea, particularly for intracellular killing which may be due to a low level of phagocytosis and abnormal internalization. The virulence of this bacteria was not enhanced by aerobicosis. It may be that under anaerobic conditions C. ochracea is able to release toxins, which further decrease the efficiency of PMN interactions with harmful anaerobic bacteria, such as B. gingivalis, thus enhancing its virulence under anaerobic conditions.

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REFERENCES