

Bactericidal Activity of Aerobic and Anaerobic Polymorphonuclear Neutrophils

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Human polymorphonuclear neutrophils (PMN) were made anaerobic by nitrogen washout (oxygen saturation <1%, Eh < -42 mV at pH 7.0), and the ability of the cells to kill bacteria was assayed and compared to the bactericidal activity of aerobic PMN. Anaerobic PMN were able to kill *Staphylococcus epidermidis*, *Enterococcus*, viridans streptococci, *Pseudomonas aeruginosa*, *Peptostreptococcus anaerobius*, *Bacteroides fragilis*, *Clostridium perfringens*, and *Peptococcus magnus* normally. Organisms that were not killed normally by anaerobic PMN included *Staphylococcus aureus* (strains Wood 46 and 502a), *Escherichia coli*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Salmonella typhimurium*. These findings suggest that mechanisms other than those dependent on hydrogen peroxide may be important in the killing of some bacteria.

Several systems have been found in polymorphonuclear neutrophils (PMN) that may be responsible for their bactericidal activity. Antibacterial cationic proteins (11), low intracellular pH (7), the production of hydrogen peroxide (3) and superoxide (1) have all been implicated to explain PMN bactericidal activity. The very potent hydrogen peroxide (or superoxide) system also involves iodination of organisms (4). This mechanism is operative only in situations where oxygen is available to leukocytes to be used for hydrogen peroxide production (6). To better understand the role of the various leukocyte antibacterial mechanisms, we examined the bactericidal activity of PMN under aerobic and anaerobic conditions towards 4 species of obligate anaerobes and 10 species of aerobic organisms.

MATERIALS AND METHODS

Human leukocytes. Heparinized venous blood from normal volunteers was sedimented for 60 min with an equal volume of 3% dextran. The supernatant fluid containing the leukocytes was collected, and erythrocytes remaining in the supernatant fluid were lysed with iced distilled water as previously described (9).

Bacteria. Aerobic organisms were cultured overnight in Trypticase soy broth, washed, and suspended in Hanks balanced salt solution without bicarbonate (HBSS). Anaerobic organisms were obtained from George Moore at the Virginia Polytechnic Institute Anaerobic Laboratories. The organisms were cultured overnight in pre-reduced brain heart infusion-supplemented liquid (Scott Laboratories, Inc., Fiskeville, R.I.). All manipulations

of anaerobic organisms were done under CO₂ gas by using the Virginia Polytechnic Institute Anaerobic Laboratory gas apparatus.

PMN bactericidal activity towards aerobes. A leukocyte button from 30 ml of blood (approximately 8×10^7 PMN) was resuspended in 3.4 ml of HBSS with 0.4 ml of autologous serum (with 10 U per ml of sodium heparin) plus 10^7 bacteria in 0.2 ml of HBSS (final volume 4 ml). Experiments were also performed with 5×10^8 and 10^9 *Staphylococcus epidermidis*. Samples (1 ml) were iced and placed in 25-ml siliconized Erlenmeyer flasks with air-tight rubber stoppers pierced with two no. 19 hypodermic needles. Flasks that were to be made anaerobic were connected to a tank of ultrapure nitrogen (Bluefield Industrial Gas Supply Co., Bluefield, Va.) and in series to a flask with the Eh indicator resazurin (anaerobic dilution blank, Scott Laboratories). A nitrogen flow of 200 to 500 ml/min was filtered through sterile cotton and humidified by bubbling through sterile, boiled, nitrogen-saturated, distilled water. Flasks were agitated on an oscillating platform for 2 h at 0 C, at which time the oxygen saturation was less than 1% (the lowest reading) when measured in sample flasks by using a polarographic oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio, model 53). No bacterial killing occurred at 0 C, either aerobically or anaerobically. Flasks containing resazurin indicator were colorless, indicating Eh values of less than -42 mV (pH was 6.9 to 7.1). Eh or oxidation reduction potential is a measure of the tendency of a system to give up electrons. This is expressed as the potential in millivolts that would exist between the system under study and a hydrogen half-cell at pH 0. After the 2 h of nitrogen washout, flasks were transferred to a 37 C shaker bath where incubation was initiated for 2 h to allow phagocytosis and killing to take place. Samples (0, 60, and 120 min) were aspirated through the rubber stopper to

determine total, sediment, and supernatant counts as previously described (9).

PMN bactericidal activity towards anaerobic bacteria. Leukocytes were prepared as described above. Organisms cultured overnight were resuspended in HBSS under CO_2 . PMN (2×10^7) were placed in 25-ml Erlenmeyer flasks with 0.1 ml of autologous serum, 0.85 ml of HBSS, and 0.05 ml of bacterial suspension (2.5×10^6 organisms) in HBSS. Flasks were iced and made anaerobic as described above. After 2 h one set of flasks was warmed and zero time cultures were obtained under CO_2 by using 0.001- and 0.01-ml calibrated platinum loops that were streaked on previously prepared brain heart infusion-supplemented agar roll tubes (obtained from Scott Laboratories and prepared by using a tube roller from VPI Research Support Service). Other flasks were opened after 60 min of incubation, and under CO_2 0.001- and 0.01-ml loop cultures were taken and streaked as above. The roll tubes were incubated for 24 to 48 h, and colonies were counted by using a dissecting microscope. Experiments were repeated two to four times, with each bacterial species utilizing leukocytes from different donors, and results are expressed as mean values.

RESULTS

Certain organisms were killed equally well by either aerobic or anaerobic PMN. Figure 1 shows results of studies done with *S. epidermidis* as a representative organism phagocytized and destroyed equally well by PMN with or without oxygen. PMN were able

to kill this organism equally well in nitrogen or in air at all three multiplications tested (0.125 bacteria:PMN; 6 bacteria:PMN, and 30 bacteria:PMN). Figure 2 shows that four species of aerobic bacteria, *S. epidermidis*, *Enterococcus*, *Streptococcus viridans*, and *Pseudomonas aeruginosa*, were also killed equally well in the presence or the absence of oxygen. Experiments performed with four species of obligate anaerobic bacteria showed that all of these organisms were killed by both aerobic and anaerobic PMN (Fig. 3).

In marked contrast to the above results, certain bacteria survived much better in anaerobic phagocytes. Figure 4 shows a representative study done with such an organism, *Staphylococcus aureus* (502a). More than 90% of the bacterial inoculum was killed by aerobic PMN, whereas less than 50% was killed by anaerobic PMN. Figure 5 shows seven organisms that were not normally killed by anaerobic PMN (normal killing by anaerobic PMN was defined as within 10% of that killed by aerobic PMN).

Aerobic and anaerobic studies performed without leukocytes showed that neither the aerobic nor the anaerobic bacteria were killed during the 2-h period of incubation. Phagocytosis appeared to be unaffected by the absence of oxygen as judged by supernatant bacterial counts and microscopy examination of stained smears made of the incubation mixture.

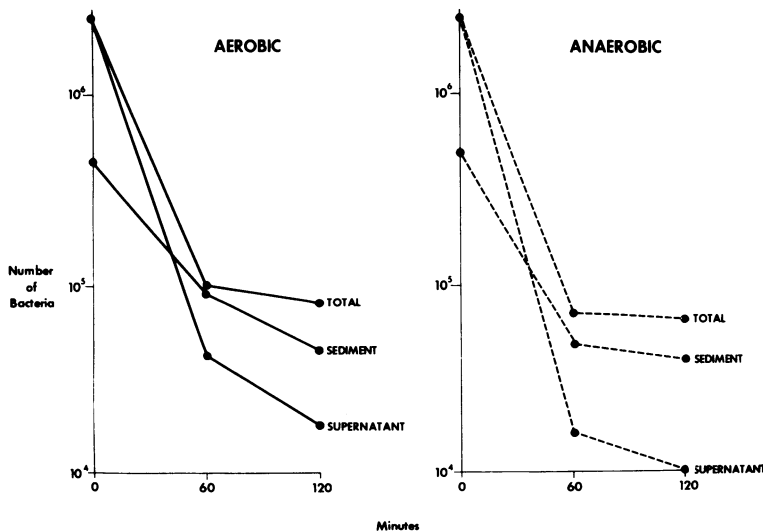


FIG. 1. Aerobic and anaerobic PMN and *S. epidermidis*. Leukocytes were incubated with *S. epidermidis*, and results of differential centrifugation tests are shown. Note that both aerobic and anaerobic leukocytes ingest (reflected as decrease in supernatant count) and kill (reflected as decrease in sediment and total count) *S. epidermidis* equally well.

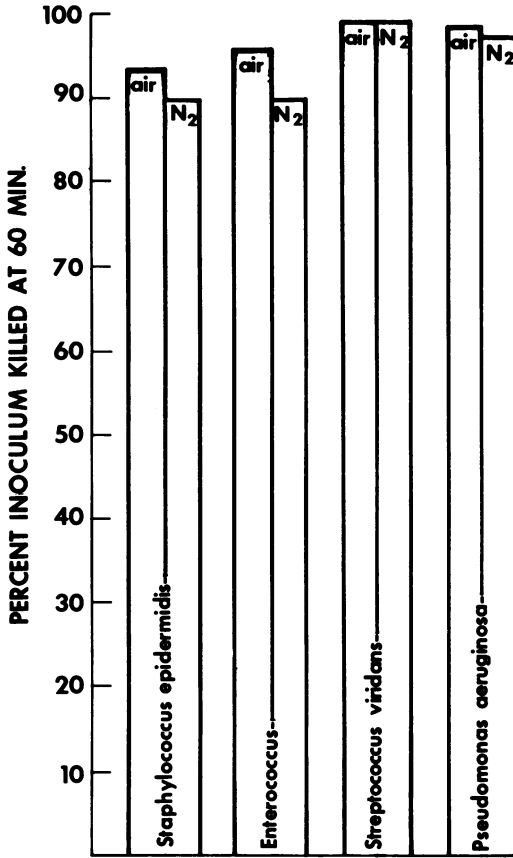


FIG. 2. Killing of bacteria by aerobic and anaerobic PMN. Leukocytes were incubated with bacteria aerobically (air) or anaerobically (N₂), and differential centrifugation tests were done. These four species were killed equally well by aerobic or anaerobic PMN.

DISCUSSION

In 1966, Selvaraj and Sbarra (10) showed that *Escherichia coli* were ingested but not killed normally by anaerobic leukocytes, and, in 1967, McRipley and Sbarra (6) reported that anaerobiosis interfered with intracellular killing of *P. aeruginosa* and *Staphylococcus albus*. Our results differed from theirs in that, in our hands, anaerobic PMN were able to effectively kill both *S. epidermidis* and *P. aeruginosa*. Differing ratios of bacteria to phagocyte were not the explanation for the discrepancy as we found with experiments performed with three different bacterial inocula.

A variation in the sensitivity of various bacteria to leukocytes that were partially anaerobic was noted. For example, leukocytes that are only slightly anaerobic (shorter

nitrogen washout times) showed defects in the killing of virulent *S. aureus*, but these partially anaerobic PMN were able to kill *E. coli* normally. After essentially complete anaerobiosis was achieved by the methods described, PMN were then unable to kill these *E. coli*.

It is interesting to relate the microorganisms found by Johnston and Baehner (2) in patients with chronic granulomatous disease of childhood (CGD) to results of these studies. The six organisms that we found to be killed abnormally by anaerobic PMN were responsible for 71.5% of isolates from purulent foci of patients with CGD. In contrast, the eight bacterial species that were killed normally by anaerobic PMN made up only 10% of those organisms cultured from patients with CGD.

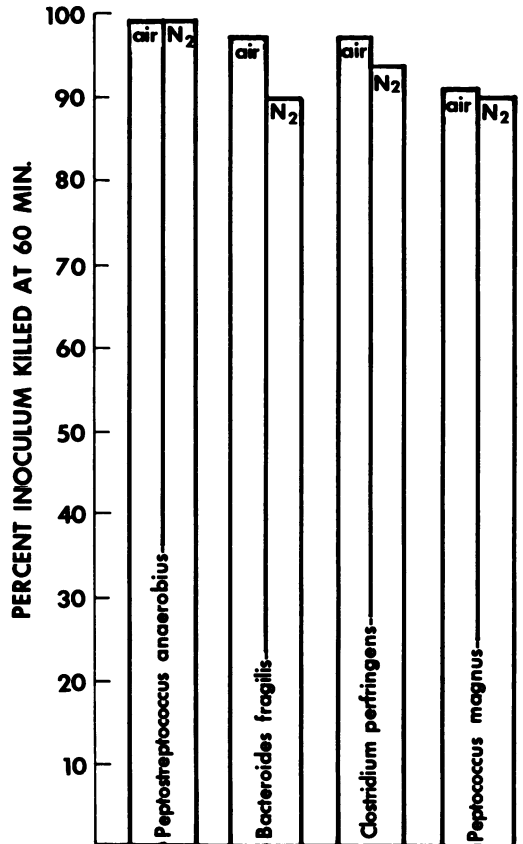


FIG. 3. Killing of anaerobic bacteria by aerobic and anaerobic PMN. Leukocytes were incubated with one of four species of obligate anaerobes, aerobically (air), or anaerobically (N₂), and counts of viable bacteria were done. These four species were killed equally well by aerobic or anaerobic PMN.

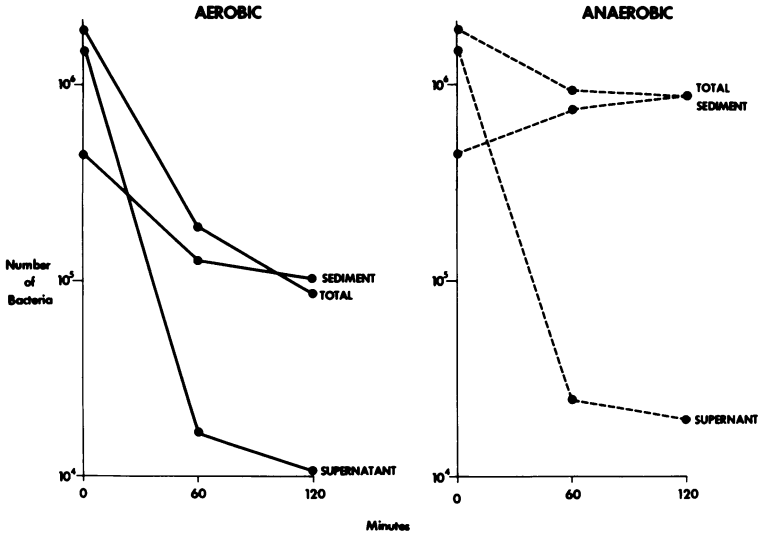


FIG. 4. Aerobic and anaerobic PMN and *S. aureus*. Leukocytes were incubated with *S. aureus*, and results of differential centrifugation tests are shown. Note that both aerobic and anaerobic PMN ingest (reflected as decrease in supernatant count) the organism equally well, but anaerobic PMN fail to kill normally (shown by higher total and sediment counts for anaerobic PMN).

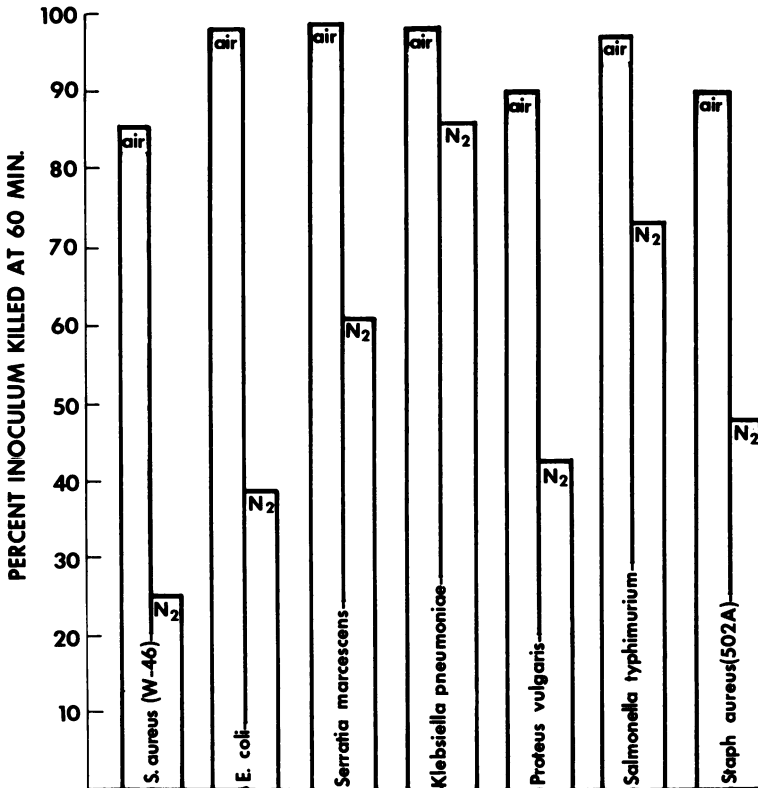


FIG. 5. Killing of bacteria by aerobic and anaerobic PMN. Leukocytes were incubated with bacteria aerobically (air) or anaerobically (N₂), and differential centrifugation tests were done. These seven species were not killed normally by anaerobic PMN.

Thus, it is possible that there may be yet another explanation for the spectrum of organisms that cause disease in these patients. The explanation often given (5, 8) is that organisms killed normally by PMN from patients with CGD are those that produce hydrogen peroxide which is not destroyed by endogenous catalase. This hydrogen peroxide plus myeloperoxidase and a halide results in self-destruction of the microbe. However, there may be certain organisms that do not require any hydrogen peroxide at all in order to be killed by PMN. These organisms may be killed by other bactericidal mechanisms of PMN.

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