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Effect of Anaerobic Bacteria on Killing of *Proteus mirabilis* by Human Polymorphonuclear Leukocytes

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Killing of *Proteus mirabilis* by human polymorphonuclear leukocytes was tested in the presence of different *Bacteroides* species. In vitro experiments showed that anaerobic bacteria interfered with the killing of aerobic bacteria. However, this inhibitory effect was not a property of all *Bacteroides* species. *Bacteroides gingivalis* W83 showed the greatest inhibitory effect of the five *Bacteroides* strains tested. Killing of *P. mirabilis* was inhibited by the culture supernatant of *B. gingivalis* but not by washed cells. Two factors were found in the supernatant of *B. gingivalis* to account for the inhibitory effect. One was heat stable with a molecular weight of less than 3,500 and inhibited the killing activity of leukocytes, and the other was heat labile and partly inactivated the complement system. The killing experiments paralleled chemiluminescence measurements.

The pathogenic synergy of aerobic and anaerobic bacteria has long been recognized in a variety of infections, e.g., intra-abdominal sepsis, pleuropulmonary infections, and brain abscesses (2). A high mortality rate has been reported owing to mixed infections of aerobes and anaerobes after intestinal obstruction in patients with vascular disease or carcinoma (15).

Acute processes such as ruptured appendix, septic abortion, and early post-hysterectomy sepsis are predominantly associated with mixed infections by aerobes and anaerobes (2). Unsuccessful treatment of aerobic bacteria in the presence of anaerobic bacteria has been reported (11). Animal studies have confirmed the pathogenic synergy of aerobic and anaerobic bacteria. A mixed inoculum of anaerobes and aerobes in animals produced sepsis that could not be induced by either component individually (7, 8, 13, 14).

The precise mechanisms for this phenomenon have not yet been fully elucidated, but one group of investigators has given evidence for the hypothesis that anaerobic bacteria inhibit phagocytosis and killing of aerobic bacteria by human polymorphonuclear leukocytes (PMN) (3, 4). The purpose of this paper is to describe the killing of aerobes in the presence of anaerobic bacteria.

MATERIALS AND METHODS

Bacterial strains. The following anaerobes were used: *Bacteroides gingivalis* W83, *Bacteroides asaccharolyticus* VPI 4199, *Bacteroids melaninogenicus* subsp. *melaninogenicus* ATCC 15930, *Bacteroids fragilis* 5MB, and *B. fragilis* 1503. The aerobic strain in all experiments was *Proteus mirabilis* P154. All bacteria used in this study were clinical isolates except for *B. asaccharolyticus* VPI 4199, which was isolated from human feces. Anaerobes were maintained by weekly subculturing on 5% horse blood agar plates (no. 2 agar; Oxoid Ltd., London, England) supplemented with hemin (5 μg/ml; BDH, Poole, England) and menadione (2 μg/ml; E. Merck AG, Darmstadt, West Germany).

Preparation of bacteria. *P. mirabilis* was grown for 18 h in Hartley broth (Oxoid), washed three times in phosphate-buffered saline (PBS), and suspended in PBS. Viable counts of *P. mirabilis* were performed on CLED agar (Oxoid), and the bacterial suspension was kept at 4°C overnight. The next day, the bacterial suspension was adjusted to 10^8 CFU/ml.

Anaerobes were grown in cooked meat medium (CMM; Difco Laboratories, Detroit, Mich.) supplemented with hemin (5 μg/ml) and menadione (2 μg/ml) for 48 h at 37°C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) under an atmosphere of 80% N_2, 10% H_2, and 10% CO_2. The numbers of anaerobic bacteria were determined by optical density and adjusted to 5×10^8 CFU/ml. Culture supernatants were prepared by centrifugation (1300 × *g*, 20 min, 20°C) and filter sterilized through 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.). Cells were washed twice in PBS and suspended in CMM to give a concentration of 5×10^8 CFU/ml.

Dialysis. A volume of 5 ml of *B. gingivalis* supernatant was dialyzed against 20 ml of sterile CMM for 24 h at 4°C in dialysis tubing with a cutoff of 3,500 (Arthur and Thomas, Philadelphia, Pa.). The dialyzed fluid and dialysate were both tested.

Preparation of PMN. PMN were separated from heparinized blood of healthy adults by dextran sedimentation. Erythrocytes were lysed in isotonic NH_4Cl

TABLE 1. Composition of the medium used for growth of *Bacteroides* species for the CL measurements

Component	%
Glucose	0.5 (wt/vol)
Mineral solution[a]	5.0 (vol/vol)
Hemin	0.0001 (wt/vol)
Resazurin solution	0.0001 (wt/vol)
$FeSO_4 \cdot 7H_2O$	0.0004 (wt/vol)
$(NH_4)_2SO_4$ (6 mM solution)	5.0 (vol/vol)
Methionin	0.75 (wt/vol)
Cystein · HCl · H_2O (2.5% solution)	2.0 (vol/vol)
$NaHCO_3$ (8% solution)	5.0 (vol/vol)
Trypton (Difco)	1.0 (wt/vol)

[a] Mineral solution contained per 100 ml: KH_2PO_4, 1.8 g; NaCl, 1.8 g; $CaCl_2 \cdot 2H_2O$, 0.053 g; $MgCl_2 \cdot 6H_2O$, 0.04 g; $MnCl_2 \cdot 4H_2O$, 0.02 g; and $CoCl_2 \cdot 6H_2O$, 0.002 g (pH 7.0).

(21). PMN were washed and suspended in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) with 0.1% gelatin (gel-HBSS) at a concentration of 10^7 leukocytes per ml.

Opsonization. Pooled human serum from 10 healthy donors was used in all experiments. To test the need for heat-labile opsonic factors, serum was heated at 56°C for 30 min before being added to the phagocytic mixture. Opsonization in the absence of the classical complement pathway was studied in serum chelated with 10 mM Mg-EGTA (magnesium–ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid) (1). The effect of the supernatant of *B. gingivalis* W83 on the inactivation of the complement system was tested as follows. The supernatant of *B. gingivalis* W83 was incubated in several dilutions with pooled human serum for 30 min at 37°C. Fifty percent hemolytic units were determined as described by Mayer (10). The effect of CMM was tested in a control experiment.

Killing. Incubation mixtures were prepared in sterile polypropylene tubes (15 by 95 mm; Thovadec, Nieuwkoop, The Netherlands), each containing 0.5 ml of the leukocyte suspension (10^7 cells per ml), 0.1 ml of serum, 0.1 ml of *P. mirabilis* suspension (10^8 cells per ml), 0.2 ml of gel-HBSS, and 0.1 ml of either anaerobic cell suspension (5×10^8 cells per ml), supernatant, or whole culture. Controls of serum, *P. mirabilis* alone, and CMM were included. The tubes were incubated at 37°C in a shaking water bath. After 0, 30, 60, and 120 min, 0.1 ml of the phagocytic mixture was suspended in 9.9 ml of cold distilled water, and viable bacterial counts were performed by plating serial dilutions on CLED agar plates.

Chemiluminescence. The CMM used for growth of anaerobes itself reduced the chemiluminescence (CL) counts. Therefore, to test the CL response of the PMN, we developed a medium in which all bacterial strains used in our experiments could grow sufficiently but which did not affect CL counts (Table 1). This medium was a modification of a minimal medium for *B. fragilis* (20). The same concentrations of PMN and of aerobic and anaerobic bacteria used in the killing experiments were used for measuring the CL. Leukocytes were suspended in HBSS without phenol red (GIBCO Laboratories) but with gelatin. The reaction mixture consisted of 0.1 ml of leukocytes, 0.1 ml of *P. mirabilis* suspension, 0.1 ml of *Bacteroides* cell suspension or supernatant, 0.1 ml of serum, and 0.1 ml of 0.1 mM luminol (Lumac, Basel, Switzerland) in PBS. Suitable controls were included. These components were mixed in polystyrene vials (Lumac) and immediately incubated at 37°C in a shaking water bath. CL was measured at 37°C in a Lumacounter model 2080 (Lumac) in the integral mode with a preset of 10 s, and the mean of counts during five periods of 2 s each was recorded on a model 97S microcomputer (Hewlett Packard, Palo Alto, Calif.).

Lactate dehydrogenase. Lactate dehydrogenase (LDH) activity was determined with a diagnostic kit (LDH-L; Fisher Scientific Co., Pittsburgh, Pa.) and a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The change in absorbance per minute was measured at 340 nm.

RESULTS

Effect of anaerobes on the killing of *P. mirabilis*. Figure 1 shows the viable counts of *P. mirabilis* after incubation with PMN. A reduction of 99% in the viable counts of *P. mirabilis* was observed after 120 min. When CMM was added to the phagocytosis mixture, killing of *P. mirabilis* was reduced by 20%. These results indicate that CMM slightly inhibited the killing of aerobic

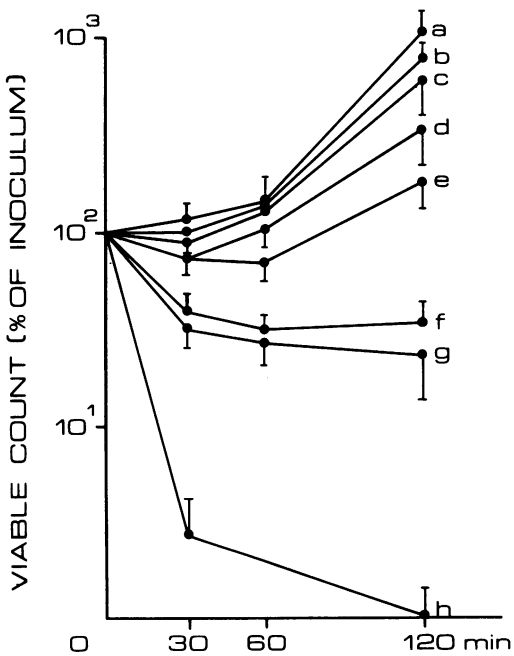

FIG. 1. Killing of *P. mirabilis* by PMN in the presence of different *Bacteroides* species: (a) *B. gingivalis* W83; (c) *B. fragilis* 1503; (d) *B. asaccharolyticus* VPI 4199; (e) *B. melaninogenicus* ATCC 15930; and (f) *B. fragilis* 5MB. Controls: (b) No leukocytes, (g) CMM, (h) no anaerobic bacteria. Values are expressed as means ± standard errors of four experiments.

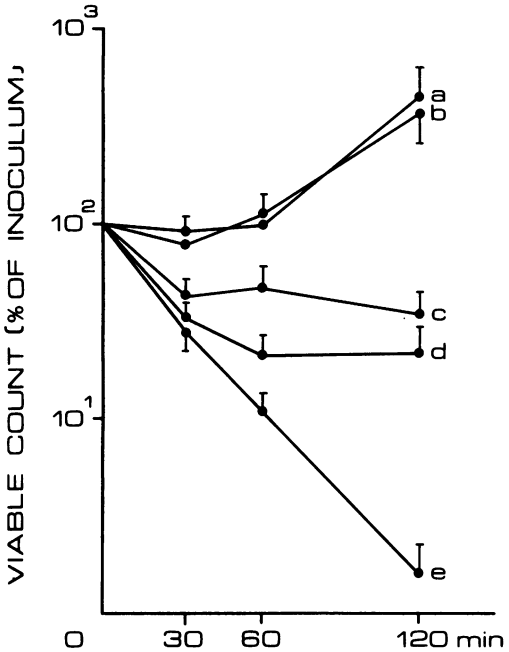

FIG. 2. Killing of *P. mirabilis* by PMN in the presence of: (a) *B. gingivalis* W83 supernatant; (b) strain W83 supernatant heated at 100°C for 30 min; and (c) washed cells of strain W83. Controls: (d) CMM, (e) *P. mirabilis* alone. Values are expressed as means ± standard errors of four experiments.

bacteria. All cultures of anaerobes inhibited the killing of *P. mirabilis* except for *B. fragilis* 5MB, which had no more effect than CMM alone. The viable counts of *P. mirabilis* in the presence of *B. gingivalis* and *B. fragilis* 1503 was almost the same as the counts of *P. mirabilis* without PMN. Among all anaerobes, tested *B. gingivalis* W83 and *B. fragilis* 1503 showed the highest inhibitory activity on the killing of *P. mirabilis*. However, *B. fragilis* 1503 lost its inhibitory activity after passage on blood agar. We decided, therefore, to investigate *B. gingivalis* W83 in more detail.

Inhibitory effect of supernatant and cells of *B. gingivalis*. *B. gingivalis* cells as well as supernatant, both unheated and heated at 100°C for 30 min, were tested for their inhibitory activity on the killing of *P. mirabilis*. Washed cells of *B. gingivalis* in CMM did not show a greater inhibitory effect than CMM alone (Fig. 2). The inhibitory activity was found to reside in the supernatant of *B. gingivalis*. When the supernatant was heated, no reduction of the inhibitory effect was observed under the experimental conditions described. Dialysis experiments showed that dialysate and dialyzing fluid had virtually the same effect as the supernatant, indicating that the inhibitory factor has a molecular weight of less than 3,500 (Table 2).

Touw et al. (18) showed that *B. gingivalis* grown under conditions similar to those of our experiments releases butyrate in the culture fluid. These authors demonstrated that butyrate is cytotoxic for animal cells. We therefore tested whether a concentration of butyrate similar to that produced by the bacteria inhibited the killing capacity of PMN. No inhibition of killing owing to butyrate was observed.

Effect of *B. gingivalis* supernatant on PMN. PMN were preincubated with *B. gingivalis* supernatant at 37°C for 30 min. After centrifugation, the supernatant was discarded, and the PMN were suspended to the original volume in gel-HBSS. In a control experiment, the PMN were washed once in HBSS after preincubation in CMM and suspended in gel-HBSS. Preincubation with *B. gingivalis* supernatant resulted in a killing inhibition equivalent to that found

TABLE 2. Killing of *P. mirabilis* by PMN under different experimental conditions

Killing of *P. mirabilis* in the presence of[a]:	Viable count (%) of inoculum of *P. mirabilis* after (min)[b]:			LDH activity (U/liter)[b]
	30	60	120	
P. mirabilis alone	20 ± 3	15 ± 3	3 ± 1	41.0 ± 0.5
CMM	50 ± 6	40 ± 5	15 ± 3	44.0 ± 1.5
B. gingivalis W83 supernatant	150 ± 4	200 ± 24	600 ± 98	42.6 ± 0.4
Preincubated PMN with W83 supernatant	150 ± 3	200 ± 21	700 ± 120	42.0 ± 0.6
PMN washed after preincubation with W83 supernatant	30 ± 3	15 ± 3	3 ± 1	NT[c]
Dialyzing fluid of W83 supernatant	130 ± 5	150 ± 16	500 ± 3	NT
Dialysate of W83 supernatant	130 ± 8	200 ± 25	600 ± 50	NT
Heat-inactivated serum	120 ± 3	200 ± 3	400 ± 28	NT
Mg-EGTA–treated serum	120 ± 5	150 ± 3	400 ± 60	NT

[a] Normal serum was used unless otherwise stated.
[b] Values are expressed as means ± standard errors of four experiments.
[c] NT, Not tested.

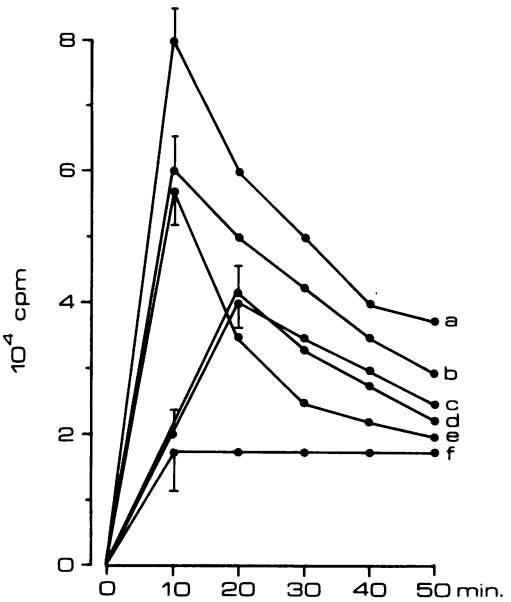

FIG. 3. Effect of whole cultures of different *Bacteroides* species on the CL of PMN with *P. mirabilis*: (b) *B. melaninogenicus* ATCC 15930; (c) *B. fragilis* 1503; (d) *B. asaccharolyticus* VPI 4199; (e) *B. fragilis* 5MB; and (f) *B. gingivalis* W83. Control: (a) *P. mirabilis* alone. Values are expressed as means ± standard errors of peak CL counts of three experiments.

when PMN were tested in the presence of *B. gingivalis* supernatant, but washing the leukocytes removed this inhibition (Table 2). To estimate the effect of *B. gingivalis* supernatant on the release of lysosomal enzymes from PMN, we measured the LDH release of PMN in the supernatant of the killing mixtures and leukocytes treated with and without *B. gingivalis* supernatant. No differences were found in the LDH release of PMN (Table 2).

Effect of *B. gingivalis* supernatant on serum complement. *P. mirabilis* was effectively killed by PMN in the presence of normal human serum. Inactivation of the serum for 30 min at 56°C or chelation of calcium in the serum with Mg-EGTA resulted in a complete inhibition of the killing of *P. mirabilis*, indicating that this strain activated complement via the classical pathway. The hypothesis that the effect of the supernatant of *B. gingivalis* on the killing of *P. mirabilis* is an effect on complement was tested. One ml of *B. gingivalis* supernatant inactivated 15.3 50% hemolytic units; CMM alone inactivated only 2.2 50% hemolytic units per ml. After heating the supernatant for 30 min at 100°C, the inactivation of complement was reduced to 3.3 50% hemolytic units per ml. These results indicate that *B. gingivalis* produced a heat-labile extracellular inactivator of complement.

CL. Figure 3 shows the CL response of PMN towards *P. mirabilis* in the presence of whole cultures of different anaerobes. The lowest CL counts were obtained with *B. gingivalis*. Higher counts were found for *B. fragilis* 5MB, *B. melaninogenicus*, *B. asaccharolyticus*, and *B. fragilis* 1503. The effects of broth cultures, washed cells, supernatant, and heated supernatant of *B. gingivalis* are shown in Fig. 4. Broth culture, supernatant, and heated supernatant of *B. gingivalis* resulted in a very low CL response. Washed cells of *B. gingivalis* alone or in combination with *P. mirabilis* produced a high CL response.

DISCUSSION

A pathogenic synergy between aerobic and anaerobic bacteria in rats (13, 14, 22), in jaundiced rabbits (12), and in guinea pigs (7, 8) has been described. Two hypotheses have been pro-

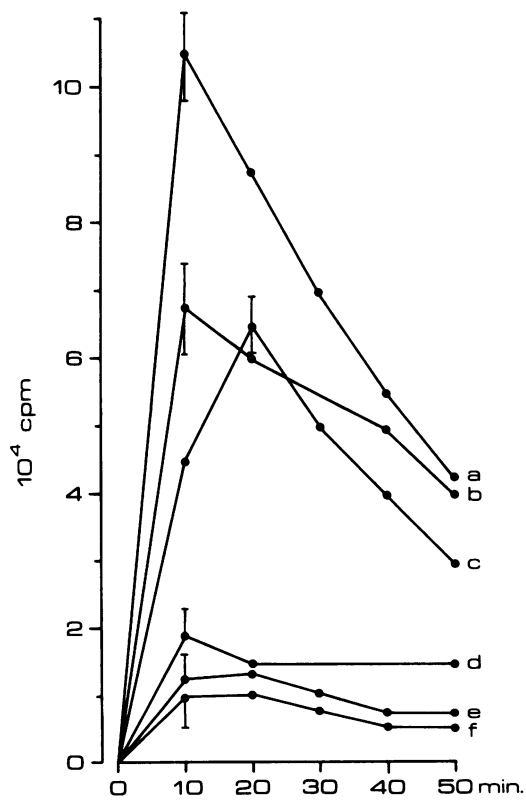

FIG. 4. CL of PMN with *P. mirabilis* in the presence of: (a) washed cells of *B. gingivalis* W83; (c) washed cells of strain W83 without *P. mirabilis*; (d) culture of strain W83; (e) supernatant of strain W83; and (f) supernatant of strain W83 heated at 100°C for 30 min. Control: (b) *P. mirabilis* alone. Values are expressed as means ± standard errors of peak CL counts of three experiments.

posed for this phenomenon: (i) obligate anaerobes interfere with the phagocytosis and killing of aerobic bacteria (4); or (ii) obligate anaerobes depend on the second or helper organism to produce a required growth factor which is necessary for the growth of anaerobes (9, 10). In this paper the first hypothesis was tested by studying the killing of aerobic bacteria in the presence of anaerobic bacteria and by the CL response of the PMN to these bacteria.

Obligate anaerobes inhibited the killing of a facultative aerobe, confirming the finding of Ingham et al. (4). However, the inhibitory effect was not a property of all *Bacteroides* species tested. *B. gingivalis* W83, *B. asaccharolyticus* VPI 4199, and *B. fragilis* 1503 showed a higher inhibitory effect than other *Bacteroides* species tested. The heat-stable factor found in the supernatant of *B. gingivalis* W83 impaired the killing capacity of PMN, but this effect was reversed by washing the leukocytes with PBS.

Chelation of calcium in the serum with Mg-EGTA inhibited the killing activity of the leukocytes toward *P. mirabilis*, indicating that this strain activated complement via the classical pathway. Tofte et al. (16) have shown a significant reduction in the opsonization of *Escherichia coli* in the presence of *Bacteroides* species. This might be explained by a very efficient opsonization of one species, which leads to a depletion of opsonins for the second species. Jones and Gemmell (5) have recently reported that competition for serum opsonins is the basis for the impairment of phagocytosis. Although the inactivation of complement by *B. gingivalis* is clear, the effect of this phenomenon on phagocytosis is doubtful. The amount of *B. gingivalis* supernatant in the phagocytosis mixture inactivated only 20% of the complement present in this mixture. The remaining complement may be enough to opsonize *P. mirabilis*. However, the complement inactivation by *B. gingivalis* W83 was not the only factor in the killing inhibition. The supernatant of *B. gingivalis* W83 heated at 100°C for 30 min no longer inactivated the complement system but still inhibited the killing of aerobic bacteria. The heat-stable factor, therefore, seems to be the one which effectively inhibits the killing.

Cytotoxicity of *B. gingivalis* W83 on the PMN could not be shown by release of LDH. Ingham et al. (4) have suggested that the inhibitory activity of anaerobes depends on a low redox potential. In some preliminary experiments, we found no significant changes of redox potential in the various anaerobic or killing mixtures during 120 min. Furthermore, the inhibitory effect of *B. fragilis* 1503 decreased after in vitro passage on blood agar. Kasper et al. (6) have found a relative loss of capsular antigen after in vitro passage by *B. fragilis*. The decrease in the inhibitory activity of *B. fragilis* 1503 may have been due to the loss of capsular antigen. This, however, has not been studied.

We do not know the nature of the heat-stable and heat-labile factors measured in the killing and CL response. Touw et al. (18) have reported that supernatants of *B. gingivalis* and *B. asaccharolyticus* are cytotoxic for Vero cells. It has been shown that the cytotoxic effect is due to the butyrate concentration present in the supernatants of these strains. However, in our study we found that butyrate was not the factor responsible for the inhibition of the killing. Furthermore, Touw et al. (17) described a heat-stable cytotoxin other than butyrate that was present in the supernatant of *B. gingivalis* and *B. asaccharolyticus* and able to inhibit the maxtrix formation of chick embryo cartilage cells. This cytotoxin also has a molecular weight smaller than 3,500 (van Steenbergen and de Graaff, unpublished results). We are currently investigating whether this cytotoxin is identical with the heat-stable product that inhibits phagocytosis and killing of *P. mirabilis*.

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