KILLING CAPACITY OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES IN AEROBIC AND ANAEROBIC CONDITIONS

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SUMMARY. The killing by human polymorphonuclear leukocytes of several species of bacteria, some of which were catalase positive, was examined *in vitro* in aerobic and anaerobic conditions. When all conditions other than the oxygen tension were identical, killing after 30 min was slightly greater in aerobic than in anaerobic conditions. However, after 60 and 120 min the difference between aerobic and anaerobic killing was smaller, and killing was nearly complete for all strains tested. These results conflict with the common opinion that oxygen is essential for efficient killing. Minor differences in experimental conditions can greatly influence results, and may be responsible for the discrepancy between this study and some previous studies on this subject.

INTRODUCTION

Polymorphonuclear leukocytes (PMNL) phagocytosing microorganisms produce a metabolic burst in which oxygen uptake is greatly increased (Baldrige and Gerard, 1933). The extra oxygen consumed is converted into oxygen radicals and hydrogen peroxide, which are potent bactericidal agents alone or in combination with myeloperoxidase (Klebanoff and Clark, 1978). There are two lines of evidence for the relation between oxidative metabolism and killing: first, in several studies killing was found to be diminished in an anaerobic atmosphere (McRipley and Sbarra, 1967; Holmes *et al.*, 1968; Mandell, 1974); second, PMNL of patients with chronic granulomatous disease (CGD), which are defective in oxidative metabolism, kill some bacteria less efficiently than do normal PMNL (Holmes, Page and Good, 1967; Quie *et al.*, 1967). Resistance of catalase-positive bacteria to killing by PMNL of CGD patients probably results from destruction of traces of H_2O_2 produced in these cells. Catalase-negative, H_2O_2 -producing bacteria may, in part, be killed in these PMNL by their own metabolic products (Mandell and Hook, 1969). Some catalase-positive bacteria are killed by PMNL from CGD patients and by normal PMNL in anaerobic conditions (Bjornson, Altemeier and Bjornson, 1976; Ingham *et al.*, 1981; Okamura and Sptiznagel, 1982; Segal *et al.*, 1982; Weiss *et al.*, 1982). PMNL clearly possess

bactericidal systems that do not depend upon oxidative metabolism but reports on the efficiency of these systems towards different bacteria vary between authors.

The purpose of this study was to investigate further the killing capacity of PMNL in anaerobic conditions with special attention to some variables which might explain discrepancies between published reports. Therefore, we examined the killing of several bacterial species, some of them catalase positive, by normal human PMNL in aerobic and anaerobic conditions.

MATERIALS AND METHODS

Bacterial strains. Clinical isolates of seven bacterial species were used in this study: five strains of *Escherichia coli*, and one strain each of *Bacteroides vulgatus*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Staphylococcus aureus*, *S. saprophyticus* and *Streptococcus faecalis*.

Aerobic bacteria were grown at 37°C in nutrient broth (Oxoid) in air. Anaerobic bacteria were grown in liquid BM medium (Shah *et al.*, 1976) supplemented with haemin 5 µg/ml (BDH, Poole, England) and menadione 2 µg/ml (E. Merck AG, Darmstadt, West Germany) at 37°C in an anaerobic chamber (Coy's Manufacturing Co., Ann Arbor, MI, USA) with an atmosphere of N₂ 80%, H₂ 10%, CO₂ 10% and O₂ < 20 ppm as measured with a trace oxygen monitor (Lockwood and McLory, Horsham, USA). Bacteria were maintained as a mixture of 1 ml of a pure culture and 1 ml of 60% glycerol, frozen at -70°C.

For each experiment, aerobic bacteria from the frozen stock were grown on nutrient agar, and a single colony inoculated into nutrient broth which was incubated for 18 h at 37°C. Bacteria were washed once and resuspended in phosphate-buffered saline (PBS). Viable counts were performed by plating serial dilutions on nutrient agar, and the bacterial suspension was kept at 4°C overnight. The next day the bacterial suspension was adjusted to a concentration of 1×10^8 cfu/ml.

Anaerobic bacteria from the frozen stock were cultured on BM agar, supplemented with sheep blood 5%, haemin and menadione. A pure culture from a single colony was grown in liquid BM medium with haemin and menadione at 37°C in the anaerobic chamber until in log phase. The number of anaerobic bacteria was determined by measurement of optical density at 690 nm (OD₆₉₀) in a Gilford 250 spectrophotometer. The relation of OD₆₉₀ to cfu/ml had previously been determined by viable counts. The bacteria were washed once in PBS and the suspension was adjusted to a concentration of 1×10^8 cfu/ml.

Preparation of PMNL. PMNL were separated from heparinised venous blood of healthy volunteers by dextran sedimentation. The resulting buffy-coat leukocytes were removed, and washed three times with Hanks's balanced salt solution (GIBCO Laboratories, Grand Island, NY, USA) with gelatin, 0.1%, pH 7.3 (gel-HBSS). In chemiluminescence (CL) experiments, red cells were lysed in isotonic NH₄Cl before the washing (Weening, Roos and Loos, 1974). PMNL were resuspended in gel-HBSS to a concentration of 1×10^7 /ml. Pooled human serum from healthy donors was used in all experiments as the source of opsonins.

Killing studies. Reaction mixtures were prepared in sterile polypropylene tubes (12 × 75 mm, Rofa Mavi, Beverwijk, The Netherlands) each containing 0.5 ml of the PMNL suspension (1×10^7 cells/ml), 0.1 ml of serum, 0.1 ml of the bacterial suspension (1×10^8 cfu/ml) and 0.3 ml of gel-HBSS. Controls without PMNL were included in each experiment. In the experiments performed in aerobic conditions, the reaction mixture was prepared in air. The tubes were incubated at 37°C in a shaking waterbath. In the experiments performed in anaerobic conditions, gel-HBSS was introduced into the anaerobic chamber 18 h before the experiment and the pH was adjusted to 7.3 with NaHCO₃. PMNL, bacteria and serum were introduced into the anaerobic chamber 30 min before the experiment was set up and kept on ice. The reaction mixture was incubated in the anaerobic chamber at 37°C in an IKA Vibro-fix shaking apparatus (Rofa Mavi) at 400 rpm. In later experiments, as indicated in the results, double volumes of the reaction mixtures were prepared in anaerobic conditions; half of each mixture was removed from the anaerobic chamber and flushed with a mixture of air 90% and CO₂ 10%, and sealed with a rubber stopper. The flushing was repeated at each sampling. It was calculated from published data (Roos, 1980) that in this way the reaction tubes contained enough oxygen to support the

respiratory burst. The tubes were incubated in a Julabo Paramix shaking apparatus (Rofa Mavi) at 400 rpm at 37°C in air. The tubes in the anaerobic chamber were incubated in an identical Paramix apparatus at the same speed. After 0 and 30 min and then after 60 or 120 min or both, 0.1 ml of the reaction mixture was diluted in 9.9 ml of ice-cold distilled water to lyse PMNL. Viable counts were performed by plating serial dilutions on appropriate solid media.

Chemiluminescence studies. The same concentrations of PMNL and bacteria used in the killing experiments were used for measuring CL. PMNL were suspended in HBSS without phenol red (GIBCO Laboratories) but with gelatin. The reaction mixtures consisted of PMNL 0.1 ml, bacteria 0.1 ml serum 0.1 ml gel-HBSS 0.1 ml and 0.1 mM luminol (Lumac, Basel, Switzerland) in PBS 0.1 ml. The components were mixed in polystyrene vials (Lumac) and immediately incubated at 37°C in a shaking waterbath. For determinations of CL in anaerobic conditions, the mixture was prepared anaerobically, as in the killing experiments. The test vials were sealed with a rubber stopper before being removed from the anaerobic chamber and incubated in the shaking waterbath. 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 was recorded with a model 97S microcomputer (Hewlett Packard, Palo Alto, CA, USA).

RESULTS

Killing studies. The percentage of the inoculum of the six facultative bacterial species killed after 60 min in aerobic and anaerobic conditions is shown in table I. None of the strains tested was affected by serum alone. A significantly greater proportion of the inoculum was killed in anaerobic than in aerobic conditions. (Wilcoxon signed rank test applied to the logarithms of the ratio of the percentage killed aerobically to the percentage killed anaerobically, $n = 24$ $p < 0.01$). However, as a result of the difference in CO₂ tension — 0.04% in air versus 10% in the anaerobic chamber — the pH of the reaction mixture fell to 6.5 in anaerobic conditions and rose to 8.0 in aerobic conditions. This difference might have influenced our results. Moreover, CO₂ might influence phagocytosis in other ways than by its effect on pH; therefore we equalised the CO₂ tensions in aerobic and anaerobic conditions in subsequent experiments, and buffered the reaction mixture with NaHCO₃ so that the pH remained 7.3 throughout the experiment.

The difference between killing in aerobic and in anaerobic conditions could also be the result of differences in type and speed of agitation; therefore, the influence of shaking on the killing of *E. coli* strain EB2 was tested at two different shaking

TABLE I
Killing of six species of facultative bacteria by PMNL in air and in anaerobic conditions after 60 min

Bacteria	Percentage of inoculum killed*	
	in air	in N ₂ + H ₂ + CO ₂ (80:10:10)
<i>E. coli</i>	56.2 ± 19.6	96.1 ± 3.3
<i>K. pneumoniae</i>	55.8 ± 19.9	72.5 ± 15.3
<i>Sal. typhimurium</i>	93.8 ± 7.9	96.0 ± 3.5
<i>S. aureus</i>	54.5 ± 9.4	82.3 ± 14.2
<i>S. saprophyticus</i>	44.8 ± 10.2	97.3 ± 1.7
<i>Str. faecalis</i>	42.5 ± 10.2	97.0 ± 5.6

* Values are expressed as means ± standard error of at least four experiments

FIG.—Killing of a strain of *E. coli* by PMNL shaken at 110 rpm (●—●) or at 400 rpm (○—○). Values are expressed as mean $\pm$ standard errors of eight experiments.

rates—110 rpm and 400 rpm in two Paramix shaking apparatuses. The killing at 400 rpm was significantly greater (fig., Wilcoxin signed rank test, $P < 0.01$).

When both aerobic and anaerobic test tubes contained CO₂ 10% and were shaken at 400 rpm in identical Paramix shaking devices, killing after 30 min seemed greater in aerobic than in anaerobic circumstances although no statistical significance could be established. After 60 and 120 min the difference between aerobic and anaerobic killing was smaller, and in both conditions >90% of the inoculum of all species except *S. aureus* and *B. vulgatus* was killed (table II).

TABLE II
Killing of bacteria by PMNL in different atmospheric conditions

Bacteria	Conditions*	Percentage of inoculum killed† after		
		30 min	60 min	120 min
<i>E. coli</i> EB 5R	O ₂	92.5 $\pm$ 2.8	97.8 $\pm$ 0.8	97.0 $\pm$ 2.7
	AnO ₂	82.0 $\pm$ 9.5	86.0 $\pm$ 9.3	94.1 $\pm$ 2.7
<i>E. coli</i> EB 12	O ₂	45.5 $\pm$ 11.8	86.7 $\pm$ 7.2	98.5 $\pm$ 1.0
	AnO ₂	24.0 $\pm$ 10.1	94.2 $\pm$ 2.1	96.6 $\pm$ 0.7
<i>E. coli</i> EB 14	O ₂	80.7 $\pm$ 5.3	96.8 $\pm$ 1.3	99.5 $\pm$ 0.3
	AnO ₂	52.5 $\pm$ 19.7	95.5 $\pm$ 2.2	97.3 $\pm$ 1.3
<i>E. coli</i> EB 15A	O ₂	72.3 $\pm$ 16.7	99.1 $\pm$ 0.4	99.4 $\pm$ 0.4
	AnO ₂	68.0 $\pm$ 19.9	92.4 $\pm$ 3.9	92.7 $\pm$ 4.3
<i>Sal. typhimurium</i>	O ₂	93.5 $\pm$ 1.6	99.4 $\pm$ 0.2	99.5 $\pm$ 0.2
	AnO ₂	92.3 $\pm$ 1.7	94.2 $\pm$ 2.0	90.5 $\pm$ 3.5
<i>S. aureus</i>	O ₂	92.4 $\pm$ 3.8	96.9 $\pm$ 1.3	98.3 $\pm$ 0.5
	AnO ₂	80.5 $\pm$ 9.2	88.2 $\pm$ 3.8	82.0 $\pm$ 1.3
<i>S. saprophyticus</i>	O ₂	93.4 $\pm$ 2.3	98.7 $\pm$ 0.3	99.5 $\pm$ 0.2
	AnO ₂	89.1 $\pm$ 3.6	97.5 $\pm$ 0.8	98.5 $\pm$ 0.5
<i>Str. faecalis</i>	O ₂	95.2 $\pm$ 1.4	99.3 $\pm$ 0.3	99.5 $\pm$ 0.2
	AnO ₂	97.4 $\pm$ 0.7	99.5 $\pm$ 0.1	99.7 $\pm$ 0.1
<i>B. vulgatus</i>	O ₂	19.5 $\pm$ 1.5	85.7 $\pm$ 7.7	93.0 $\pm$ 3.9
	AnO ₂	57.5 $\pm$ 2.5	76.6 $\pm$ 7.9	89.0 $\pm$ 7.3

* O₂ = air plus 10% CO₂; AnO₂ = N₂, 80%; H₂ 10%; CO₂ 10%.

† Values are expressed as mean $\pm$ standard error of at least three experiments.

TABLE III

Chemiluminescence of PMNL with four strains of bacteria in aerobic and anaerobic conditions

Bacteria	CL values* in	
	Aerobic conditions	Anaerobic conditions
<i>E. coli</i> EB1	50868	224
<i>E. coli</i> EB4	82484	196
<i>K. pneumoniae</i>	163276	1080
<i>Sal. typhimurium</i>	170884	2032
PMNL alone	8882	216

* Values represent total of counts after 10, 20, 30 and 40 min.

Chemiluminescence studies. To ascertain that the anaerobic conditions of the killing experiments were sufficient to prevent the metabolic burst, CL was compared in aerobic and anaerobic conditions. CL did not occur in anaerobic conditions when PMNL were challenged by *K. pneumoniae*, *Sal. typhimurium* and two strains of *E. coli*, whereas these strains provoked a large CL response in aerobic conditions (table III); therefore, killing in anaerobic conditions was shown to be oxygen independent.

DISCUSSION

In this study the killing capacity of PMNL from healthy human donors was tested simultaneously in aerobic and anaerobic conditions. This comparison has been made several times before. Table IV surveys the published literature on this subject, together with our own experimental data and indicates that some species of bacteria, which were killed in this study, have been reported to be resistant to anaerobic killing. These disparate results may be due to differences in strains and experimental conditions such as serum concentration, composition of the anaerobic atmosphere, and the ratio of PMNL to bacteria. Furthermore, agitation, which is often considered to be of minor importance, can greatly influence results, as is clearly shown by the effect of different speeds of shaking of the reaction mixture on killing in our experiments.

Shaking influences the mixing of air or gas in the phagocytosis mixtures, the physical impact of the collisions between bacteria and PMNL which could influence phagocytosis (Tomita and Kanegasaki, 1982), the clumping of the bacteria which could protect them against bactericidal components of the PMNL (Segal *et al.*, 1982), and the clumping of the PMNL which could reduce their functional phagocytosing surface. In most publications the description of the agitation used is very summary.

In this study when all conditions other than the oxygen tension were standardised, differences in killing in anaerobic and aerobic conditions were very small. With all strains tested, except *S. aureus* and *B. vulgatus*, after 120 min >90% of the inoculum was killed in either anaerobic or aerobic conditions. Evidence is accumulating that PMNL are equipped with very effective, though highly selective, oxygen-independent cytotoxic agents (Elsbach and Weiss, 1983). Moreover, in CGD, which is characterised by the absence of a respiratory burst, the susceptibility to infection of individuals varies considerably (Johnston, 1980).

TABLE IV
Survey of publications in which killing by aerobic and by anaerobic PMNL is compared

Authors	Buffer	Serum concentration	Experimental conditions			Bacterial species examined	Phagocytic killing* in	
			Aerobic atmosphere	Anaerobic atmosphere	Shaking	Ratio of PMNL to bacteria	Aerobic conditions	Anaerobic conditions
McRipley and Sbarra, 1967	KRPM	20%	air	N ₂	SWB	1:2 to 1:4	8	44
Mandell, 1974	Gel-HBSS	10%	air	N ₂	SWB	1:8 8:1	<i>Pseudomonas aeruginosa</i>	0.8
							<i>Shigella sonnei</i>	1.7
							<i>S. albus</i>	10
							<i>S. epidermidis</i>	65
							Enterococci	7
							<i>Str. viridans</i>	4
							<i>Ps. aeruginosa</i>	1
							<i>Peptostreptococcus anaerobius</i>	1
							<i>B. fragilis</i>	3
							<i>Clostridium perfringens</i>	10
Bjornson et al., 1976 Ingham et al., 1981	HBSS	10%	air	N ₂ :H ₂ :CO ₂ 85:10:5	R	5:1 1:5	<i>Peptococcus magnus</i>	3
							<i>Peptococcus magnus</i>	7
							<i>S. aureus</i> W46	9
							<i>E. coli</i>	15
							<i>Serratia marcescens</i>	2
							<i>K. pneumoniae</i>	1
							<i>Proteus vulgaris</i>	39
							<i>Sal. typhimurium</i>	15
							<i>S. aureus</i> 502A	10
							<i>B. fragilis</i>	54
Okamura and Spitznagel, 1982 Present study	HBSS Gel-HBSS	10%	air air + 10% CO ₂	N ₂ :H ₂ :CO ₂ 85:10:5 N ₂ :H ₂ :CO ₂ 80:10:10	R P	1:100 1:2	<i>B. thetaiotaomicron</i>	2
							<i>P. mirabilis</i>	14
							<i>E. coli</i>	0.1
							<i>K. aerogenes</i>	0.2
							<i>Str. faecalis</i>	0.2
							<i>B. fragilis</i>	0.07
							<i>Sal. typhimurium</i>	0.07
							<i>E. coli</i> EBR	0.1
							EB12	2.2
							EB14	13.5
Present study	Gel-HBSS	10%	air + 10% CO ₂	N ₂ :H ₂ :CO ₂ 80:10:10	P	1:2	EB15A	3.2
							<i>B. vulgatus</i>	7.5
							<i>Sal. typhimurium</i>	23.4
							<i>S. aureus</i>	10.6
							<i>S. saprophyticus</i>	5.8
							<i>Str. faecalis</i>	3.1
								11.3
								2.5
								0.7
								0.5

SWB = Shaking water bath; R = rotation; P = paramix shaking apparatus, 400 rpm; KRPM = Krebs Ringer Phosphate Medium; HBSS = Hanks's Balanced Salt solution; Gel-HBSS = Hanks's Balanced Salt Solution with gelatin (0.1%);
 * Percentage of inoculum surviving after 60 min, except for the data of Ingham et al., 1981, which are the percentages surviving after 5 h. If values were not given in the text by the authors, they have been calculated from the figures.

† Serum concentration not given.

Experimental results suggest that the mechanisms PMNL employ against microorganisms *in vitro* vary, depending upon prevailing circumstances and the bacteria encountered. If this applies *in vivo*, PMNL may be more efficient in host defence than in-vitro assessments indicate.

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