ELUCIDATION OF THE PHAGOCYTOSIS MECHANISM WITH THE AID OF LUMINOUS BACTERIA

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SUMMARY. Phagocytosis of the luminous bacterium *Vibrio cholerae* var. *albensis* caused a similar decrease both in viable count and in the in-vivo luminescence. These effects of polymorphonuclear neutrophil leukocytes (PMN) were oxygen-dependent processes. Exposure of PMN to oxygen caused a prompt decrease in the luminescence of bacteria that had been ingested in anaerobic conditions. Cell-free supernates from active PMN suspensions caused a decrease in luminescence and as much as 10% of the killing could be attributed to extracellular killing. Similarly, bacteria entrapped on a membrane filter showed a decrease in luminescence upon addition of active PMN, even though they could not be ingested.

INTRODUCTION

Phagocytosis of bacteria by leukocytes is accompanied by a burst of non-mitochondrial respiration. Although this oxygen consumption is not required for phagocytosis (Koch, 1978), it is required for the killing of certain bacteria (Iyer, Islam and Quastel, 1961; McRipley and Sbarra, 1967; Mandell, 1974; Curnutte and Babior, 1975; Ingham *et al.*, 1981). In addition, cells from patients with the rare congenital syndrome of chronic granulomatous disease do not show any evidence of the respiratory burst (Holmes, Page and Good, 1967) and kill bacteria less efficiently than do normal cells (Quie *et al.*, 1967; Segal *et al.*, 1982).

The efficient microbicidal activity of the polymorphonuclear leukocyte (PMN) depends upon the formation of highly reactive products of oxygen metabolism such as superoxide (O_2^-), hydrogen peroxide, singlet oxygen (O_2') and the hydroxyl radical (Babior, 1978; Klebanoff, 1980). The mechanism of activation of the bactericidal system has not been elucidated, and it is not clear whether the bacteria are killed extracellularly and then ingested or the killing is an intracellular process.

The bacterial luminescence system offers a unique model to study the interaction between the phagocyte and bacteria. The luminescence level reflects both the cellular metabolic activity and the viability of the phagocytosed bacteria (Barak, Ulitzur and Merzbach, 1983*a* and *b*). The bioluminescence test for phagocytosis is simple, rapid and non-destructive to the experimental system and, therefore, allows a continuous

determination of the kinetics of the process (Barak *et al.*, 1983b). The luminous bacterium *Vibrio cholerae* var. *albensis* resembles the pathogenic *V. cholerae* strains (Bauman *et al.*, 1980; Blake, Weave and Hollis, 1980) and thus represents a realistic model for phagocytosis. The present communication shows the important role of oxygen in the PMN bactericidal mechanism and presents evidence for and an estimation of the extent of extracellular killing.

MATERIALS AND METHODS

Bacteria. *V. cholerae* var. *albensis* was grown in nutrient broth (Difco Laboratories, Detroit, MI) containing NaCl 1% (w/v), KCl 0.1% (w/v) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4% (w/v) (NBC; pH 7.2). The bacteria were cultivated, with shaking, at 37°C to a late log phase of growth showing a turbidity of 180 Klett-Summerson units (filter 66) and a luminescence of 2×10^{11} quanta/s/ml. Cells were washed by centrifugation at 10 000 *g* for 10 min at 4°C and suspended in phosphate-buffered saline (PBS; NaCl 0.8% w/v, K_2HPO_4 0.12% w/v, KH_2PO_4 0.034% w/v; pH 7.3), containing CaCl_2 0.17% w/v, MgSO_4 0.05% w/v, bovine serum albumin 0.1% w/v (BSA; Sigma Chemical Co., St Louis, MO) and glucose 0.18% w/v. This buffer was designated PBSC. Viable counts were made in duplicate, after an appropriate dilution in saline by plating on NBC agar (NBC solidified with 1% w/v agar; Difco). The colonies were counted after incubation for 24 h at 37°C.

Human serum was a pool from several healthy donors. Sera were separated from blood, clotted for 1 h at room temperature, by centrifugation at 2500 *g* for 15 min and stored at -20°C in 1.5-ml volumes until required.

Polymorphonuclear leukocytes were obtained from 60 ml of heparinised blood taken from three healthy donors. Red cells were removed by sedimentation with dextran 6% w/v and leukocytes separated on a Ficoll-Hypaque gradient as described by Ferrante and Thong (1978). Cell viability checked by trypan blue exclusion was > 95%.

Opsonisation procedure. Bacteria (10^8 cells/ml) were opsonised in serum 5% v/v in PBSC for 30 min at 30°C and washed twice by centrifugation in PBSC.

Phagocytosis assay. The opsonised bacteria and the PMN were added to the PBSC buffer at different concentrations (the usual ratio of PMN to bacteria was 2:1) in a final test volume of 1 ml in a clean scintillation vial. The vials were shaken gently at 37°C, and the bioluminescence was determined periodically. For viable counts, 0.1-ml amounts were diluted in PBSC containing Triton X-100 0.1% v/v, vortex mixed to disrupt the PMN and plated as described above. Tests with entrapped bacteria were performed after 2×10^6 bacteria contained in 1-ml volumes were entrapped on 3- μm membrane filters (Schleicher and Schull, 25 mm diameter) by filtration under vacuum followed by three washings with 5 ml of PBSC.

Anaerobic phagocytosis assay. Opsonised bacteria were added to pre-boiled PBSC containing dithionite (Sigma) 100 $\mu\text{g}/\text{ml}$ and vials were sealed hermetically, as described by Levi and Ulitzur (1983). Each vial was tested for anaerobiosis before and after injection of PMN by determining its in-vivo luminescence by a scintillation counter. Any vial that showed detectable light was rejected. At different intervals, vials with or without PMN were exposed to air; samples of 1 ml were removed, and their luminescence was determined. At the same sampling times 2-ml samples were centrifuged at 150 *g* for 10 min at 4°C. The supernates were removed and diluted for viable counts. The sediments were suspended in 1 ml of PBSC containing Triton X-100 0.1% v/v; the PMN were disrupted by vortex agitation, and the sample diluted for viable counts.

Bioluminescence was determined in a scintillation counter (Packard, Model 2001) operating without coincidence at the ^3H setting at 30°C. When the luminescence level was above 10^5 quanta/s, a sensitive photometer photomultiplier calibrated by the Hastings and Weber (1963) standard was used.

RESULTS

The effect of PMN on the potential luminescence and viability of *V. cholerae* cells

FIG. 1.—The effect of exposure to oxygen on luminescence, after different periods of anaerobic phagocytosis. Opsonised bacteria (5×10^6 cells/ml) were added to boiled PBSC containing dithionite $100 \mu\text{g/ml}$. The vials were closed hermetically, and 10^7 PMN/ml were injected. At different intervals of anaerobic phagocytosis, vials with PMN (solid symbols) or without PMN (open symbols) were exposed to air (as indicated by arrows), and the luminescence was determined for 60–90 min. Standard test: ■—■ = aerobic decrease in luminescence.

that were incubated in anaerobic conditions is shown in figs. 1 and 2, respectively. Neither the luminescence nor the viability was affected by the PMN in anaerobic conditions. The exposure to air after different periods of anaerobic phagocytosis resulted in a prompt burst of luminescence, followed by a rapid decrease in the emitted light. This drop in luminescence occurred only in the presence of PMN; in their absence, the light emission remained high and steady throughout the experiment.

Microscopic examination revealed that after 1 h most of the bacteria had been ingested during the anaerobic phase. This observation was further strengthened by the fact that most of the bacteria disappeared from the supernate of both the anaerobic and the aerobic assay systems (fig. 2). However, while the anaerobically-phagocytised bacteria accumulated within the PMN, the aerobically-phagocytised bacteria disappeared with time. It should be noted that the method employed for achieving anaerobiosis by addition of dithionite $100 \mu\text{g/ml}$ did not interfere with the potential of PMN to act in aerobic conditions.

As we have already shown (Barak *et al.*, 1983b), the fall in the luminescence of *V. cholerae* cells upon the addition of PMN in aerobic conditions began after only a short

FIG. 2.—Viability of bacteria during aerobic and anaerobic phagocytosis. Opsonised bacteria (2.3×10^5 cells/ml) were incubated aerobically or anaerobically, as in fig. 1, with 10^6 PMN/ml. After incubation for 30, 60 or 90 min, 2-ml samples were centrifuged at 150 *g* for 10 min at 4°C and the supernate (●—● or ○—○) and sediment (■—■ or □—□) sampled for viable counts. Open symbols represent controls without PMN and solid symbols represent test mixtures with PMN.

time lag that was required for the adherence and engulfment process. This pre-ingestion stage did not require the presence of oxygen. Upon shifting from anaerobic to aerobic conditions, the PMN killing apparatus was immediately potentiated, as could be judged from the prompt decrease in luminescence (fig. 1). Using the data of fig. 2, the extent of the intracellular and extracellular killing of bacteria in the phagocytosis process was estimated. The table shows that a difference of 8–10% existed between the intracellular and the total killing of the bacteria in the phagocytosis assay.

In order to obtain a more direct estimation of the extracellular killing, we performed the following experiments: *V. cholerae* cells were filtered through a 3- μ m membrane filter, and the non-entrapped bacteria were washed out by repeated washings; the entrapped bacteria could not be engulfed because they were trapped in the filter. Fig. 3 shows that *c.* 60% decrease in luminescence occurred when such filter-bound bacteria were incubated with PMN for 30 min. This decrease in luminescence could not be accounted for by bacteria that escaped from the filter,

FIG. 3.—Decrease in luminescence of entrapped bacteria incubated with PMN. Opsonised bacteria (10^6) were trapped on 3- μ m membrane filters by filtration under vacuum. The filters were placed in vials containing 2×10^6 PMN in 1 ml of PBSC (▲—▲). The standard test consisted of 10^6 opsonised bacteria in 1 ml of PBSC containing 2×10^6 PMN (●—●). Open symbols represent bacteria without PMN, either free (O—O) or entrapped on the filter (Δ—Δ). The vials were incubated with shaking at 37°C, and the luminescence was determined at 0, 10, 20, 30, 45 and 60 min.

because only 0.08% of the bacteria were released from the filter during incubation for 1 h in the absence of PMN (data not shown). Fig. 4 shows that supernates from active PMN were able to reduce the luminescence of opsonised bacteria. It can be seen that the effect of the supernate was proportional to the length of the previous phagocytosis period.

DISCUSSION

The effect of anaerobiosis on phagocytosis by PMN has been studied in detail (Selvaraj and Sbarra, 1966; McRipley and Sbarra, 1967; Mandell, 1974; Ingham *et al.*, 1981) but the conclusions of these studies do not agree with each other. While some authors showed that anaerobiosis interfered with the intracellular killing of *Escherichia coli* (Selvaraj and Sbarra, 1966), *Pseudomonas aeruginosa* and *Staphylococcus albus* (McRipley and Sbarra, 1967), other investigators claimed that other species were killed by PMN in anaerobic conditions (Mandell, 1974). Ingham *et al.* (1981) showed that only dilute bacterial suspensions ($\leq 10^7$ cells/ml) were killed in anaerobic conditions and that anaerobic bacteria interfered with the killing of the aerobic ones by depleting the serum of essential opsonic factors. The discrepancy between these results can be explained by the different techniques employed and by different susceptibilities of bacterial genera to the oxygen-dependent bactericidal mechanism. The methods employed for achieving anaerobiosis (Mandell, 1974; Ingham *et al.*,

FIG. 4.—Effect of supernates from phagocytosis assays on luminescence of *V. cholerae*. The aerobic phagocytosis assays were performed as described in the legend to fig. 1 (standard test: $\square$ — $\square$). At intervals of 10, 30 and 60 min (solid symbols), 2-ml samples were centrifuged for 10 min at 10 000 *g* at 4°C, and 1 ml of the supernate was added to 10^8 luminous bacteria. Supernate from PMN incubated alone for 60 min (Δ — Δ) was the control; $\circ$ — $\circ$ = bacteria alone. The vials were incubated with shaking at 37°C and the luminescence was determined at 0, 10, 20, 30, 45 and 60 min.

TABLE
Comparison of intracellular and total bacterial killing

Time (min)	Number of bacteria in aerobic PMN (X) ($\times 10^4$)	Number of bacteria in anaerobic PMN (Y) ($\times 10^5$)	Intracellular killing (Z)* (%)	Decrease in viable count (direct test) (%)
30	2.5	1.4	82.14	92.50
60	2.0	2.2	90.91	99.00
90	1.9	2.3	91.74	99.63

$$* Z = (Y - X) / Y \times 100.$$

1981), such as nitrogen washout or the use of the anaerobic jar, cannot provide the strict anaerobic environment that was obtained with the use of dithionite. The pre-opsonisation procedure that was employed in the present study further improved the test by eliminating the possibility of improper opsonisation during the anaerobic period.

The technique that we have described has the advantage of being a non-destructive test in which the presence of even trace amounts of O_2 in the system can be detected continuously. Luminous bacteria emit light at O_2 concentrations as low as $10^{-8}M$

(Oshino *et al.*, 1972). The shift from anaerobiosis to aerobiosis revealed instantly the degree of bacterial killing and allowed determination of the oxygen-dependent killing. The killing of the luminous bacteria almost exclusively in aerobic conditions is obvious if we consider their high susceptibility to oxygen-derived metabolites with regard to viability (Barak *et al.*, 1983*b*) and mutagenesis (Barak *et al.*, 1983*a*).

With the aid of immobilised luminous bacteria we were also able to evaluate directly the extent of extracellular killing of bacteria during phagocytosis. Other authors (Koch, 1978; Gabig and Babior, 1981) could not assess extracellular killing by employing the direct bactericidal test. The experiments performed to assess the extracellular killing showed that there was almost no further decrease in luminescence after the first 30 min of the test. The factor(s) responsible for this phenomenon must be investigated further.

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