Effect of Nutrient Depletion on Sensitivity of *Pseudomonas cepacia* to Phagocytosis and Serum Bactericidal Activity at Different Temperatures

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Pseudomonas cepacia grown under different specific nutrient depletions in batch culture showed varying degrees of sensitivity to engulfment and killing by human polymorphonuclear leukocytes (PMN) and to killing by human serum. Resistance to killing by the combined action of PMN and serum in whole blood was found to increase in the following order of depletions: glucose < iron < sulphate < phosphate or ammonium < magnesium. There was also an increase in resistance to killing by whole blood with decrease in temperature, except that carbon-depleted cells remained very sensitive irrespective of temperature. Cells in the exponential phase of growth also showed a consistent increase in resistance as the temperature was decreased. Similar, but smaller effects were observed with oxygen-depleted cells. The increase in killing by whole blood as the phagocytic temperature was raised correlated with an increase in the number of bacteria ingested per PMN. A pattern of serum sensitivity was observed with cells grown under different nutrient depletions similar to that for whole blood. But in all cases whole blood was 6 to 10 times more effective than serum alone in killing the cells at 37 °C.

INTRODUCTION

Pseudomonas cepacia, originally described as a plant pathogen (Ballard *et al.*, 1970), is appearing more frequently in the medical literature as a causative agent of human disease (Phillips & Eykyn, 1971; Speller *et al.*, 1971; Garvey & Neu, 1975; Weinstein *et al.*, 1976).

We have studied phenotypic factors affecting the susceptibility of the organism to killing by whole human blood, presumed to result from the combined action of phagocytes and serum. In infection there is often an elevated body temperature and host resistance is increased by enhancement of the bactericidal action of blood against certain organisms (Roberts, 1979; Mackowiak *et al.*, 1982). An increased bactericidal capacity of polymorphonuclear leukocytes (PMN) at 40 °C relative to 37 °C was observed with *Escherichia coli*, *Salmonella typhimurium* and *Listeria monocytogenes* but not with *Staphylococcus aureus* (Mandell, 1975; Roberts & Steigbigel, 1977). Therefore we investigated the effect of temperature upon the phagocytosis of *P. cepacia* in whole blood.

The cell envelope of bacteria plays a crucial role in drug resistance (Brown, 1975, 1977) and currently it is suggested to play a significant role in human infection (Beveridge, 1980; Costerton *et al.*, 1979; Smith, 1977). Van Oss and his associates (Van Oss *et al.*, 1975; Van Oss, 1978), by contact angle measurements, and Stendahl *et al.* (1979), by hydrophobic interaction chromatography, concluded that the hydrophobicity of the bacterial surface relative to that of the PMN plays a crucial role in the ingestion of the bacteria by PMN.

Finch & Brown (1978) reported the effect of nutrient limitation of *Pseudomonas aeruginosa*

Abbreviation: PMN, polymorphonuclear leukocyte(s).

grown in the chemostat on the killing by rabbit PMN and cationic proteins derived from the PMN. They found that slow-growing ($D = 0.05 \text{ h}^{-1}$) magnesium-limited cells were significantly more resistant to the lethal effects of PMN than were fast-growing magnesium-limited cells ($D = 0.5 \text{ h}^{-1}$) and slow-growing carbon-limited cells ($D = 0.05 \text{ h}^{-1}$). There is now much evidence that lack of free iron plays an important role in infection (Weinberg, 1978; Bullen, 1981). In this paper, we report the effects of various nutrient depletions in batch culture on the subsequent killing of *P. cepacia* both by serum alone and by PMN in whole blood at different temperatures.

METHODS

Bacteria and culture conditions. The organism used throughout this study was *Pseudomonas cepacia* NCTC 10661. Cells were grown in chemically defined medium containing: glucose, 20 mM; NaCl, 3 mM; KCl, 3 mM; $(\text{NH}_4)_2\text{SO}_4$, 12 mM; MgSO_4 , 3.2 mM; K_2HPO_4 , 1.2 mM; FeSO_4 , 0.02 mM; 3-(*N*-morpholino)propanesulphonic acid (MOPS), 50 mM; pH adjusted to 7.4 with NaOH. All medium components were of Analar grade and were sterilized separately by autoclaving. To obtain the various nutrient depletions, concentrations were reduced as follows to allow growth to an optical density of only 1.0 at 470 nm: Mg^{2+} to 0.019 mM, Fe^{2+} to 4×10^{-5} mM, $(\text{NH}_4)^+$ to 2.2 mM, glucose to 4 mM, $(\text{PO}_4)^{3-}$ to 0.06 mM, $(\text{SO}_4)^{2-}$ to 0.15 mM. For sulphate depletion MgSO_4 was replaced by MgCl_2 (0.16 mM), FeSO_4 was replaced by FeCl_2 (0.27 mM), and MOPS buffer was replaced by phosphate buffer $(\text{NH}_4)_2\text{HPO}_4$, 18 mM and $\text{NH}_4\text{H}_2\text{PO}_4$, 0.48 mM. Growth was in an orbital shaker at 37 °C for 24 h (stationary phase) or for exponential-phase cells to an optical density of 0.5 at 470 nm. Cells grown to stationary phase in complete chemically defined medium were designated oxygen-depleted. Bacteria were harvested by centrifugation at 8000 *g* for 10 min, washed once with normal saline and resuspended in sterile normal saline to an OD_{470} of 0.2.

Glassware. Glassware was washed in Extran 300 (BDH), rinsed in distilled water, then in 1% (v/v) HCl, and finally rinsed thoroughly in double distilled water.

Serum bactericidal assays. Fresh venous blood was obtained from a single volunteer. Serum was separated by centrifugation at 3000 *g* for 5 min. To 0.5 ml of human serum in a capped polypropylene tube, 0.5 ml of the *P. cepacia* suspension was added. The mixture was incubated at 37 °C in a water bath shaking at 120 r.p.m., and at intervals 0.1 ml samples were taken, diluted with nutrient broth and the viable counts determined by the method of Miles & Misra (1938). Each experiment was done in triplicate.

Interaction of bacteria with PMN in whole blood. The method was that described by Jones *et al.* (1979) with the following modifications. Whole blood from a single volunteer was collected into a heparinized sterile plastic tube (Searle & Co., Bucks., U.K.) and was used within 1 h of collection. The blood (0.25 ml) was mixed with the bacterial suspension (0.25 ml) in a sterile plastic tube and incubated in a shaking water bath at 41 °C, 37 °C or 33 °C. At time zero, 0.1 ml samples were transferred into 0.9 ml sterile distilled water, mixed well and left for 10 min at room temperature, to allow lysis of blood cells without killing the bacteria. Further samples were removed at timed intervals, and treated similarly. Samples were further diluted to 10^{-5} in nutrient broth and the viable count was determined. The percentage of surviving bacteria was calculated.

A sample (0.05 ml) was removed from the phagocytic mixture after 30 min incubation, dried on a slide and stained with Giemsa stain. An estimate of the phagocytic index was made by counting the number of bacteria in 50 randomly dispersed PMN and also the number of PMN containing no bacteria. PMN which were actively involved in the ingestion of bacteria as judged by microscopy were designated active PMN. The results from a single experiment are presented (Table 1); replicate experiments carried out on separate blood samples gave similar results.

RESULTS

Effect of temperature on the phagocytosis of exponential-phase and oxygen-depleted cells

Exponential-phase cells showed a decrease in survival as the phagocytic temperature was increased from 33 °C to 41 °C (Fig. 1). Incubation of bacteria in normal saline at 33 °C to 41 °C did not affect the viability (data not shown). Decrease in survival was also consistently observed in the oxygen-depleted *P. cepacia* as the temperature was increased from 37 °C to 41 °C, but there was only a slight difference between 33 °C and 37 °C. Comparison of the killing of the exponential-phase and oxygen-depleted cells by whole blood at the same incubation temperature indicated that the latter were more resistant to the bactericidal action of the phagocytes. However, it was found that the oxygen-depleted cells were more easily engulfed than the exponential-phase cells by PMN (Table 1). Temperature seemed to have little effect on the

Fig. 1. Effect of temperature on killing of *P. cepacia* by whole blood. ■, Exponential-phase cells; ●, oxygen-depleted cells. Survival values indicate viable counts relative to time zero (= 100).

Table 1. Engulfment of *P. cepacia* NCTC 10661 by PMN at three different temperatures

Depleting nutrient	Factors investigated†	Temperature		
		41 °C	37 °C	33 °C
Iron	Percentage of active PMN	94	90	74
	No. bacteria per active PMN	7.8 ± 1.3	5.5 ± 0.9	4.1 ± 0.7
Carbon	Percentage of active PMN	98	90	98
	No. bacteria per active PMN	10.3 ± 2.4	9.8 ± 1.7	8.0 ± 1.3
Nitrogen	Percentage of active PMN	96	92	90
	No. bacteria per active PMN	7.0 ± 1.6	4.7 ± 1.1	4.8 ± 0.9
Phosphate	Percentage of active PMN	90	76	74
	No. bacteria per active PMN	6.3 ± 1.4	4.7 ± 0.9	4.1 ± 1.1
Sulphate	Percentage of active PMN	95	94	92
	No. bacteria per active PMN	8.5 ± 1.6	7.7 ± 1.2	5.9 ± 1.1
Magnesium	Percentage of active PMN	86	68	55
	No. bacteria per active PMN	3.7 ± 0.8	2.8 ± 0.8	2.2 ± 0.7
Oxygen	Percentage of active PMN	100	98	100
	No. bacteria per active PMN	10.6 ± 2.7	9.8 ± 2.1	9.3 ± 1.8
Exponential phase*	Percentage of active PMN	92	90	90
	No. bacteria per active PMN	7.5 ± 1.4	5.6 ± 1.0	5.0 ± 0.9

* Exponential-phase cells: no specific nutrient was depleted.

† The data are given ± S.D. (50 PMN counted).

engulfment of oxygen-depleted cells, possibly because they were readily engulfed even at the lowest temperature (33 °C). However, an increase in the number of bacteria engulfed per PMN was observed with exponential-phase cells as the phagocytic temperature was increased. It was also found that exponential-phase cells were much more susceptible to serum killing than oxygen-depleted cells (Fig. 3).

Fig. 2. Killing of *P. cepacia* by whole blood at 41 °C (a), 37 °C (b) and 33 °C (c). ■, Mg^{2+} -depleted; □, $(\text{PO}_4)^{3-}$ or $(\text{NH}_4)^{+}$ -depleted (results not significantly different); ●, $(\text{SO}_4)^{2-}$ -depleted; ○, Fe^{2+} -depleted; and ▲, carbon-depleted cells. Survival values expressed as for Fig. 1.

Effect of nutrient depletion on phagocytosis of P. cepacia at different temperatures

Figure 2 shows the killing by whole blood of *P. cepacia* grown under six different nutrient depletions at three different temperatures. Resistance to killing by whole blood at any temperature was found to be in the following order: carbon- < iron- < sulphate- < nitrogen- or phosphate- < magnesium-depleted cells. Carbon-depleted cells were also found to be most easily engulfed by PMN (Table 1). There was only a slight effect of temperature upon the number of carbon-depleted cells engulfed per PMN. Magnesium-depleted cells were not only the most resistant to killing but were also the least susceptible to engulfment of all the nutrient depletions studied. The other nutrient-depleted cells were intermediate in sensitivity between carbon- and magnesium-depleted cells.

Effect of nutrient depletions on serum killing of P. cepacia at 37 °C

The bactericidal action of human serum on *P. cepacia* grown under different nutrient depletions is shown in Fig. 3. Carbon-depleted and exponential-phase cells were much more sensitive than iron- or oxygen-depleted cells. Comparison of killing in whole blood (Figs 1 and 2) and in serum (Fig. 3) shows that whole blood was at least six- to tenfold more effective than serum in eliminating the organism after 30 min incubation.

DISCUSSION

The rate of killing of *P. cepacia* by the combined action of PMN and serum factors in whole blood increased with increasing temperature from 33 °C to 41 °C. The nutrient conditions under which the cells were grown had a dramatic effect upon the susceptibility to blood killing. The order of resistance of cells grown under different nutrient depletions was carbon < iron <

Fig. 3. Killing of *P. cepacia* by human serum at 37 °C. □, oxygen-depleted; ■, Fe²⁺-depleted; △, exponential-phase; and ▲, carbon-depleted cells. Survival values expressed as for Fig. 1.

nitrogen or phosphate < magnesium. In the case of sulphate-depleted cells the possibility of excess phosphate or chloride contributing to phenotypic changes cannot be ruled out. Engulfment and killing should be considered as two separate events. Cells might be easily engulfed and easily killed by PMN. On the other hand, they might also be engulfed easily but be resistant to killing inside the PMN. Possibly the physicochemical surface properties of oxygen-depleted cells render them susceptible to engulfment but resistant to chemical and enzymic attack inside the PMN or serum. Differences in the composition and structure of the outer membrane might possibly explain their difference in susceptibility to the bactericidal action of PMN or serum alone (Costerton *et al.*, 1979). Temperature had little effect upon the rapid rate of killing by blood of the very sensitive carbon-depleted cells. Engulfment of the different nutrient-depleted cells reflected the same pattern of sensitivity found for killing by blood. Carbon-depleted cells showed the greatest engulfment and magnesium-depleted cells were engulfed the least. In all cases engulfment increased with increasing temperature. The results with iron-depleted cultures have special significance in view of the role of iron *in vivo* (Bullen, 1981; Weinberg, 1978).

The results confirm the observations by Finch & Brown (1978) that the growth environment has a significant effect on the killing of micro-organisms by PMN plus serum or by serum alone. Depending on the nutrient depletion, *P. cepacia* can be highly sensitive to blood killing (e.g. carbon-depleted cells) or highly resistant (e.g. magnesium-depleted cells at 33 °C). Presumably the wide range of sensitivity shown by the organism reflects the phenotypic variation in cell envelope composition which has been shown with many organisms to depend upon growth rate and specific nutrient depletion (Brown, 1977; Costerton *et al.*, 1979; Finch & Brown, 1978). The protein and phospholipid composition of the outer membrane of *P. cepacia* varied considerably with nutrient depletion (Anwar *et al.*, 1983). Variation in the ability of PMN to engulf these different nutrient-depleted cells might be due to changes in the surface free energy of the cells

resulting from these alterations in the chemical composition of the envelope. Surface free energy is thought to be an important factor in the engulfment process (Van Oss *et al.*, 1975; Van Oss, 1978; Stendahl *et al.*, 1979). Altered sensitivity to opsonization is another possibility. Cells grown under different nutrient depletions might possibly have altered surface antigens which would be opsonized to different extents. For example, the heterogeneity of the lipopolysaccharide as suggested by several workers (Goldman & Leive, 1980; Palva & Makela, 1980; Day & Marceau-Day, 1982) in other Gram-negative bacteria might also occur in *P. cepacia* as a result of phenotypic variation. This alteration would possibly affect the degree of opsonization.

We believe that future studies should use cells grown under defined conditions which closely mimic those occurring *in vivo* (Brown, 1977). For example, the amount of free iron available for bacterial growth in body fluids is very low, of the order of 10^{-18} M (Weinberg, 1978). Under these conditions cells are likely to be growing slowly under iron limitation (Bullen, 1981; Weinberg, 1978). Undoubtedly similar considerations apply in the area of antigen and vaccine production.

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