INFECTION AND IMMUNITY, May 1978, p. 340-346
0019-9567/78/0020-0340$02.00/0

Vol. 20, No. 2
Printed in U.S.A.

Effect of Growth Environment on *Pseudomonas aeruginosa* Killing by Rabbit Polymorphonuclear Leukocytes and Cationic Proteins

J. E. FINCH* AND M. R. W. BROWN†

Department of Pharmacy, University of Aston, Birmingham, England

Received for publication 9 November 1977

Pseudomonas aeruginosa grown in a chemostat under carbon- and magnesium-limited conditions showed varying resistance to killing by rabbit peritoneal exudate polymorphonuclear leukocytes. Slow-growing ($D = 0.05\ h^{-1}$), magnesium-limited cells were significantly more resistant to the lethal effects of the phagocytes than were fast-growing magnesium-limited cells and carbon-limited cells ($D = 0.05\ h^{-1}$ and $D = 0.5\ h^{-1}$, respectively). The resistance of magnesium-limited cells to killing by cationic proteins isolated from the leukocytes was shown to be growth-rate dependent, the slowest-growing ($D = 0.05\ h^{-1}$) cells being the most resistant. Carbon-limited cells were sensitive to killing by the cationic proteins at all growth rates tested. Antisera raised in rabbits to all types of cells and commercial anti-*Pseudomonas* serum rapidly agglutinated magnesium-limited cells but failed to agglutinate carbon-limited cells. There was some indication that slow-growing ($D = 0.05\ h^{-1}$), magnesium-limited cells agglutinated most readily with both types of antisera. No difference was detected in the mouse toxicity of heat-killed cells grown under the various conditions.

The relationship between the phagocytosis of bacteria and serum factors has been studied in detail for relatively few microorganisms. Young and Armstrong (38) and Young (37) deduced that, since most strains of *Pseudomonas aeruginosa* are resistant to the bactericidal action of human serum, the principal role of serum antibody would appear to be opsonization prior to phagocytosis and that the antibody present in normal human serum appears to have an obligatory requirement for complement and other heat-labile factors. Immunoglobulin G antibodies, however, are potentiated by complement but can function without it (4). Bjornson and Michael (4) showed that, in the presence of natural antibodies, properdin and the proactivator of factor C3 of the complement system were essential for the successful opsonophagocytosis of *P. aeruginosa* by human polymorphonuclear (PMN) leukocytes.

Several workers have shown that avirulent organsisms are ingested and killed more efficiently by phagocytes than are virulent ones (5, 11, 21). Because a well documented range of virulent and avirulent cell wall mutants of *Salmonella typhimurium* exists, this difference in resistance to phagocytosis by rabbit PMN leukocytes has been shown to be principally a property of the cell wall envelope lipopolysaccharide (14). The structure of the lipopolysaccharide of this organism has also been shown to influence markedly the virulence and degree of phagocytic killing (32), the response to three bactericidal mechanisms operating within rabbit PMN leukocytes (34) and the physicochemical properties of the cell envelope (33).

Although it has been known for a number of years that phenotypic change caused by variation in growth environment can alter the antigenicity of bacteria (22, 23), this does not appear to have been considered in relation to phagocytosis. Tempest and Ellwood (35) studied the cell wall chemistry of glycerol- and magnesium-limited *Klebsiella aerogenes* and found that both the amount and the ratio of cell wall 2-keto-3-deoxy-octonate to heptose were highly variable with growth rate. Marked differences were also reported between slow-grown, magnesium-limited and glycerol-limited organisms in their ability to agglutinate with O-specific rabbit antibodies, although no further details were given.

Finch and Brown (13) showed that *P. aeruginosa* grown in magnesium-limited and in carbon-limited conditions in a chemostat exhibited marked changes in sensitivity to ethylenediaminetetraacetic acid or polymyxin, both with the limiting nutrient and with the growth rate, supporting the idea of profound changes in the outer membrane structure (6). In the present report we have extended the above study to

† Present address: Reckitt & Colman Ltd., Hull, England.

determine whether these chemostat-grown cells differ in their sensitivity to phagocytic killing and in their susceptibility to killing by cationic proteins isolated from similar PMN leukocytes. These cultural conditions did not favor slime formation.

MATERIALS AND METHODS

Bacteria and culture conditions. The organism used throughout this study was *P. aeruginosa* NCTC 6750 grown in a 350-ml-working-volume chemostat (Bioflow model C30, New Brunswick Scientific Co., New Brunswick, N.J.) under carbon- and magnesium-limited growth conditions as described previously (13). In such a continuous culture, the dilution rate (D) = specific growth rate (μ) = $\log_e$ 2/doubling time. The carbon source was glucose, which is unfavorable to the production of extracellular slime (6).

Approximately 20 to 30 ml of bacteria grown at a specified growth rate in a specified medium was removed from the chemostat, collected by centrifugation at 10,000 × g for 1 min, washed once in gel-Hanks medium (29), and resuspended in the same medium to give a concentration of 10^8 cells per ml.

Collection and treatment of phagocytes. Peritoneal leukocytes were obtained from male New Zealand white rabbits by a method similar to that described by Hirsch (18). Briefly, 250 ml of sterile 0.9% (wt/vol) saline containing 0.1% (wt/vol) oyster glycogen was drained into the peritoneal cavity by gravity flow via a no. 17 Keith cannula. In 18 h an additional 250 ml of sterile 0.9% (wt/vol) saline was injected in the same manner. By careful manipulation of the cannula and gentle kneading of the animal, about 250 ml of exudate was collected in siliconized flasks over a period of 15 to 20 min. Microscopical examination of the exudate showed >95% PMN cells. When gross contamination by erythrocytes was present, the exudate was discarded. The PMN leukocytes were sedimented by centifugation at 200 × g in plastic tubes, washed once in gel-Hanks medium, and resuspended in the same medium to a concentration of 10^7 cells per ml.

Preparation of cationic proteins from rabbit PMN leukocytes. Exudates containing PMN leukocytes from several rabbits were pooled and centrifuged at 0°C for 5 min at 300 × g. The cells were resuspended in ice-cold 0.32 M sucrose and washed once in the same medium. The cells were resuspended in a small volume of 0.32 M sucrose and disrupted in a hand-held Teflon-glass tissue homogenizer for about 5 min and examined microscopically for 100% cell breakage. The homogenate was centrifuged at 1,000 × g for 5 min, the pellet discarded, and the supernatant was centrifuged at 8,000 × g for 30 min. The pellet (granular fraction) was resuspended in ice-cold 0.25 M sucrose, and an equal volume of 0.36 N H_2SO_4 was added. This was left at 4°C for 30 min. The mixture was then centrifuged at 10,000 × g for 10 min to remove debris. The clear supernatant was transferred to an ice-chilled glass centrifuge tube, and the temperature was brought to −4°C. Absolute ethyl alcohol at the same temperature was added very slowly with constant, gentle stirring to a final concentration of 20% (vol/vol). The mixture was allowed to stand for 4 h at −4°C for precipitate formation. The precipitate was then collected by centrifugation at 300 × g for 15 min at −4°C, immediately redissolved in ice-cold distilled water, and dialyzed overnight in a large excess of distilled water at the same temperature. The dialysate was lyophilized and stored in a tightly stoppered bottle at −20°C. Fractions of cationic proteins prepared in this way from the granular fraction of PMN leukocytes have been shown to be free from contaminating acid hydrolase enzymes (20) and histones (39). Before use a weighed amount of the cationic proteins was dissolved in phosphate-buffered saline (pH 7.2), suitable dilutions were made, and known amounts were added to incubation mixtures.

Interaction of bacteria with phagocytes. The incubation mixture for phagocytosis contained 1 ml of rabbit peritoneal exudate PMN leukocytes (final concentration, 5 × 10^6 cells per ml), 0.2 ml of bacterial suspension (final concentration, 1 × 10^7 cells per ml), and 0.8 ml of normal (preimmune) rabbit serum (final concentration, 40%). The mixture was incubated in a siliconized test tube at 37°C. The tubes were rapidly, but not violently, shaken. Control tubes were prepared which contained (i) normal serum replaced with heated (56°C for 30 min) normal rabbit serum, (ii) serum replaced with gel-Hanks medium, and (iii) PMN leukocytes replaced with gel-Hanks medium. At appropriate time intervals after the addition of the bacteria to the incubation mixture, a 0.1-ml sample was removed and pipetted into 9.9 ml of 0.01% (wt/vol) bovine plasma albumin in water to release any viable bacteria from the leukocytes (29). Portions of this, diluted in bovine plasma albumin-water, were plated onto nutrient agar and incubated overnight at 37°C, and the colonies were counted.

Interaction of bacteria with cationic proteins. The incubation mixture contained 0.2 ml of bacteria (final concentration, 10^7 cells per ml), 0.4 ml of diluted cationic protein solution (final concentration, 20 μg of protein per ml), and 1.4 ml of phosphate-buffered saline. Control tubes contained (i) phosphate-buffered saline replacing the protein solution and (ii) nutrient broth containing 0.5 mM $FeSO_4$ and 0.7 mM sodium citrate instead of the phosphate-buffered saline. Samples (0.1 ml) were removed from the incubation mixtures at appropriate time intervals and diluted in Fe^{2+}-citrate-nutrient broth to inactivate the action of the cationic proteins (16). Viable counts were carried out on this mixture as above.

Bacterial agglutination studies. Sera prepared from heated bacterial suspensions and classified by the scheme of Habs (17) completed by Veron (36) were purchased from A.P.I. Laboratory Products Ltd., Rayleigh, Essex.

Anti-*P. aeruginosa* sera against various cell types were prepared in the following manner. Carbon-limited and magnesium-limited cultures of *P. aeruginosa* grown in the chemostat at dilution rates of 0.5 h^{-1} and 0.05 h^{-1} were collected and centrifuged at 15,000 × g for 1 min, washed once in water, and resuspended in water. The cells were then heated in a boiling-water bath for 2.5 h, freeze-dried, and stored over P_2O_5 in vacuo. After samples of all four cell types had been collected, 4 mg of each type was resuspended in 1 ml of water. A 0.5-ml portion of this suspension was injected intradermally into both flanks of a rabbit on

each of days 0, 14, and 28. Seven days later, ca. 25 ml of blood was collected from an ear vein. The blood was stored at 4°C overnight, the clot was rimmed, and the serum was divided into several small portions and stored at −20°C. Sera that had been frozen and thawed once only were used for experiments. Serum from a second untreated rabbit was collected in a similar manner and used as a control.

Two drops of a suspension of live *P. aeruginosa* in saline (ca. 10^9 cells per ml) were added to two drops of serially diluted serum in saline on microscope slides and mixed with a wire loop for 1 min (3). The agglutination was examined by eye, and the end point was estimated.

Mouse toxicity studies. The toxicity of magnesium- and carbon-limited cells grown at $D = 0.5\ h^{-1}$ and $D = 0.05\ h^{-1}$ was tested by a method similar to that described by Pearson and Ellwood (28). Each batch of cells was freeze-dried immediately after collection and stored at −20°C. The freeze-dried cells were reconstituted in pyrogen-free 0.9% (wt/vol) saline and heated at 60°C for 30 min to kill the cells and to destroy heat-labile toxic proteins. A 0.5-ml portion of saline containing known amounts of each batch of cells was injected into the peritoneum of three 20-g mice. The mice were observed for 7 days, and the mortalities were recorded.

RESULTS

Killing of chemostat-grown *P. aeruginosa* by rabbit PMN leukocytes. It can be seen from Fig. 1 that, in the presence of heated normal rabbit serum, fast- and slow-grown, magnesium-limited and carbon-limited cells were killed to the same extent at all time intervals, between 46 and 62% surviving ($P' = 0.05$) after 120 min. In normal rabbit serum in the absence of PMN leukocytes, all four types of cells showed a small significant increase in viable count over the 120-min incubation period.

In normal rabbit serum, magnesium-limited cells grown at $D = 0.05\ h^{-1}$ showed between 14.6 and 27% survival ($P' = 0.05$) after 120 min. All other cell types showed greater sensitivity to the killing action of phagocytes in normal rabbit serum, between 0.31 and 2.3% surviving ($P' = 0.05$) after 120 min. The survival of bacteria

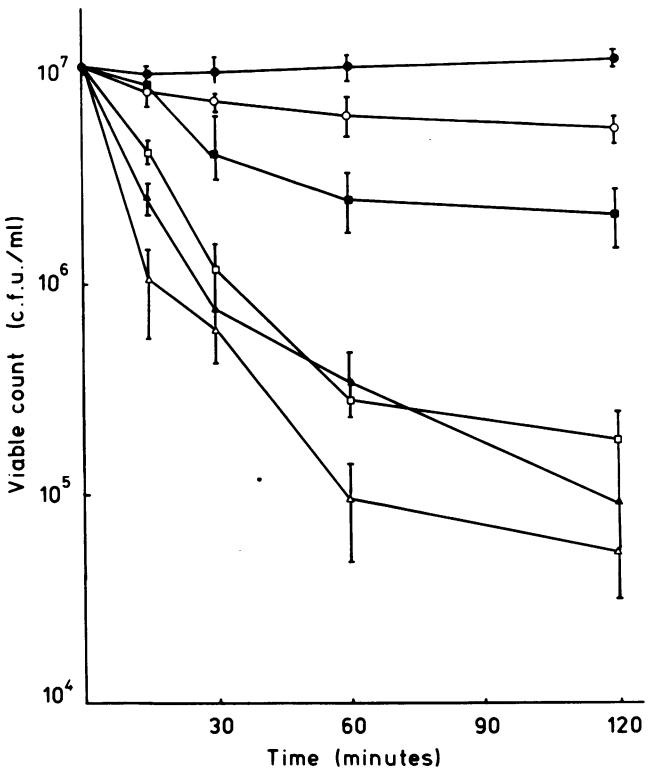

FIG. 1. *Killing by rabbit PMN leukocytes of P. aeruginosa grown under various conditions in a chemostat. The incubation mixtures contained 5×10^6 phagocytes per ml together with 40% normal rabbit serum. Absence of PMN leukocytes (●). Heated rabbit serum replacing normal serum (○); the results shown are the mean for all cell types. P. aeruginosa grown in magnesium-limited, $D = 0.05\ h^{-1}$ (■); carbon-limited, $D = 0.05\ h^{-1}$ (□); carbon-limited, $D = 0.5\ h^{-1}$ (▲); and magnesium-limited, $D = 0.5\ h^{-1}$ (△) conditions. The bars indicate the standard error of the mean $\left(\frac{SD}{\sqrt{n}}\right)$ for at least three determinations. c.f.u., Colony-forming units.*

without any serum in the incubation mixture was identical to that of bacteria without added PMN leukocytes (not shown).

Killing of chemostat-grown *P. aeruginosa* by isolated cationic proteins. Figure 2 shows that the inclusion of Fe^{2+}-citrate-nutrient broth in the incubation mixture completely neutralizes the effect of the cationic protein, all cell types showing an increase in viable count over the 120-min incubation period. Carbon-limited cells grown at $D = 0.05\ h^{-1}$ and $D = 0.5\ h^{-1}$ and magnesium-limited cells grown at $D = 0.5\ h^{-1}$ were all sensitive to the effects of cationic proteins, between 2.4 and 0.6% surviving after a 120-min incubation ($P' = 0.05$). Magnesium-limited cells grown at $D = 0.05\ h^{-1}$ were very resistant to the killing effects of the protein, between 130 and 53% surviving ($P' = 0.05$) after 120 min. Magnesium-limited cells grown at $D = 0.3\ h^{-1}$ showed between 38 and 22% survival ($P' = 0.05$) after 120 min. Control tubes containing no cationic proteins showed no change in bacterial count throughout the 120-min incubation period (not shown).

Agglutination of chemostat-grown *P. aeruginosa* with specific rabbit antisera. Carbon-limited cells grown at $D = 0.05\ h^{-1}$ and $D = 0.5\ h^{-1}$ did not agglutinate with any of the commercially prepared sera. Magnesium-limited cells grown at $D = 0.05\ h^{-1}$ agglutinated immediately with serum 1 and showed no reaction with sera 2 to 15. Magnesium-limited cells grown at $D = 0.5\ h^{-1}$ also agglutinated only with serum 1. With serum 1, magnesium-limited cells grown at $D = 0.5\ h^{-1}$ had a titer of 1:4, and magnesium-limited cells grown at $D = 0.05\ h^{-1}$ had a titer of 1:128.

Serum raised in rabbits against carbon-limited

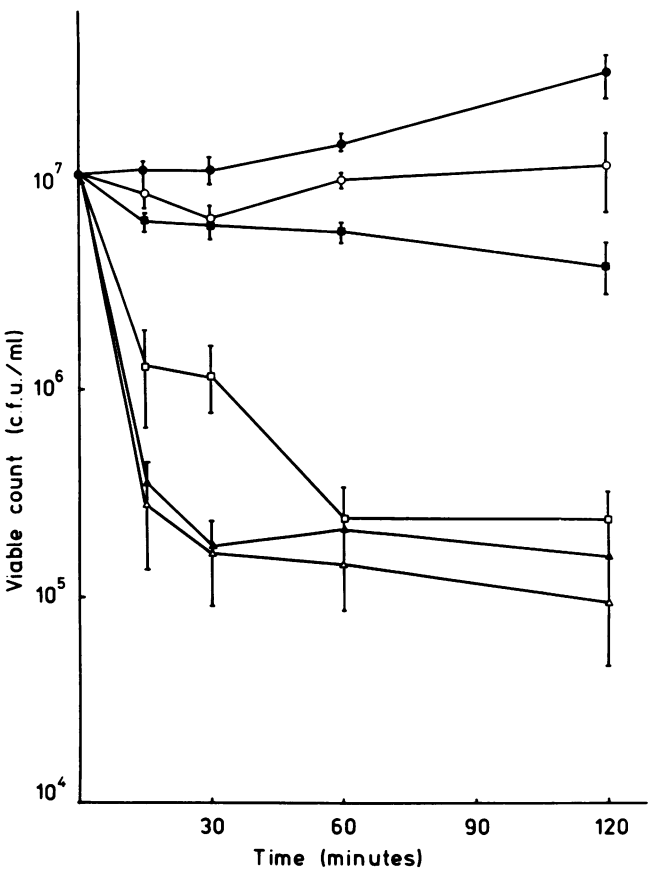

FIG. 2. *Killing by a 20-μg/ml amount of cationic protein of Pseudomonas aeruginosa grown under various conditions in a chemostat. Fe^{2+}-citrate-nutrient broth replacing phosphate-buffered saline (●); the results shown are the mean for all cell types. P. aeruginosa grown in magnesium-limited, $D = 0.05\ h^{-1}$ (○); magnesium-limited, $D = 0.3\ h^{-1}$ (■); carbon-limited, $D = 0.05\ h^{-1}$ (□); magnesium-limited, $D = 0.5\ h^{-1}$ (▲); and carbon-limited, $D = 0.5\ h^{-1}$ (△) conditions. The bars indicate the standard error of the mean $\left(\frac{SD}{\sqrt{n}}\right)$ for at least three determinations. c.f.u., Colony-forming units.*

and magnesium-limited cells grown at $D = 0.05$ h^{-1} and $D = 0.5$ h^{-1} failed to agglutinate carbon-limited cells. Magnesium-limited cells grown at $D = 0.05$ h^{-1} had a titer of 1:512, and magnesium-limited cells grown at $D = 0.5$ h^{-1} had a titer of 1:256. Control normal rabbit serum failed to agglutinate any of the cell types.

Mouse toxicity of chemostat-grown *P. aeruginosa*. The maximum dose possible, 10 mg (dry wt) of cells in 0.5 ml of 0.9% (wt/vol) saline, of all cell types tested failed to cause any deaths in the mice.

DISCUSSION

Normally functioning PMN leukocytes are essential to host defenses against invasion by *P. aeruginosa*, since most invasive strains of this organism are resistant to the bactericidal action of humoral factors (38). The strain of *P. aeruginosa* used in these studies appeared to be resistant to normal rabbit serum, since there occurred a small increase in viable count in the absence of PMN leukocytes (Fig. 1). Incubation of bacteria with the supernatant from a preparation of PMN leukocytes in phagocytosis medium also failed to produce a reduction in viable bacterial count, and it was therefore concluded that bacterial cell death was due to ingestion and killing by whole phagocytes.

Attempts to determine whether the difference in degree of killing of different bacterial phenotypes was due to differences in engulfment or intracellular survival were frustrated by the difficulty in distinguishing adsorbed from engulfed microorganisms by using light microscopy. This difficulty has been reported by other workers (8, 31, 32). The problem is usually resolved by using a method whereby extracellular bacteria are killed by an added antibiotic and the protected, surviving intracellular bacteria are subsequently counted (19). However, it was considered that, since the growth conditions described in this study have been used previously to produce bacteria that varied markedly in their sensitivity to ethylenediaminetetraacetic acid and polymyxin (13), it was likely that the cells' sensitivity to an antibiotic would also vary, thus confusing the results of this method.

The killing of the four types of cells, fast- and slow-growing, magnesium-limited and carbon-limited, by rabbit PMN leukocytes showed significant differences after 2 h of incubation. In the presence of heated normal serum, however, the degree of killing of all four cell types was significantly reduced, and the differences between the four cell types were removed. This agrees with previous findings that heat-labile factors are necessary for the rapid phagocytosis and killing of *P. aeruginosa* (38).

Figure 2 shows that the inclusion of Fe^{2+}-citrate-nutrient broth in the incubation mixture completely neutralizes the effect of the cationic proteins with all cell types showing an increase in numbers over the 120-min incubation period and verifies the inclusion of Fe^{2+}-citrate in the medium used for dilution of the incubation mixture before viable counting. Carbon-limited cells at both growth rates were sensitive to the bactericidal effects of the cationic protein, whereas magnesium-limited cells grown at $D = 0.05$ h^{-1} showed 100% survival after 2 h of incubation. Magnesium-limited cells grown at $D = 0.3$ h^{-1} showed a degree of survival of 30%, intermediate between the two, suggesting that sensitivity to cationic proteins of *P. aeruginosa* grown under these conditions is growth-rate dependent. The pattern of killing of the cell types by intact PMN leukocytes therefore closely follows that of the bactericidal effect of the isolated cationic proteins. It has been shown that changes in the lipopolysaccharide of mutants of *S. typhimurium* conferred sufficient surface change on the bacteria to influence the engulfment by rabbit phagocytes and also the bactericidal effect of three different systems operating within the PMN leukocyte (32, 34). The pattern of the response in both cases could be related to distinct changes in the structure of the lipopolysaccharide molecule.

The serology of *P. aeruginosa* is not as yet fully understood, although, unlike the *Enterobacteriaceae*, the chemical differences in lipopolysaccharide extracted from different serotypes of the Habs scheme (17) appear to be due to amino compounds located in the side chains (10). Studies on the relationship between this chemical structure and virulence have yet to be published, although the lipopolysaccharide of *P. aeruginosa* appears to be relatively nontoxic (25). Heat-killed extracts of the cells described in this paper also appeared to be relatively nontoxic to mice; an intraperitoneal injection of 10 mg (dry wt) of heat-killed cells in 0.5 ml of 0.9% saline per 20-g mouse was not fatal. Live cells of *P. aeruginosa* NCTC 6750 used in these studies had a 100% lethal dose of 2.1×10^8 cells per 25 g for mice, which is highly virulent compared with other strains of the same organism (R. J. Jones, personal communication). The lipopolysaccharide of *P. aeruginosa* therefore remains nontoxic under a variety of growth conditions, unlike that of *Escherichia coli* (28) or *K. aerogenes* (35) for which both the growth rate and the growth-limiting nutrient were shown to have marked effects on the toxicity of heat-killed cells.

Differences were observed in the degree of agglutination of chemostat-grown cells with specific O-antisera, suggesting that the presence or

degree of exposure of the O-antigenic side chains also varied with growth conditions. Gilbert and Brown (15) reported that the lipopolysaccharide content of *P. aeruginosa* NCTC 6750 grown in a chemostat was higher in magnesium-limited than in carbon-limited cultures and that it decreased with increasing growth rate in both cases. Chemical and agglutination studies of this bacterium therefore suggest that magnesium-limited cells grown at $D = 0.05\ h^{-1}$ would have the maximum amount of exposed lipopolysaccharide, followed by magnesium-limited cells grown at $D = 0.5\ h^{-1}$, carbon-limited cells grown at $D = 0.05\ h^{-1}$, and carbon-limited cells grown at $D = 0.5\ h^{-1}$, in that order. Magnesium-limited cells grown at $D = 0.05\ h^{-1}$ were also the most resistant to killing by PMN leukocytes and to the bactericidal effects of the cationic proteins, suggesting that the presence of an exposed O-antigenic side chain had a major influence.

Similarities have previously been noted between the activity of cationic proteins and other basic polypeptides (27). Phenotypic changes induced by varying the divalent cation content of the growth medium have also been observed to alter the sensitivity of *P. aeruginosa* to a polypeptide antibiotic (13), and the changes in the degree of exposure of the O-antigenic side chain of the lipopolysaccharide reported here would therefore appear to be an important factor in the exclusion mechanism postulated for the induced resistance of *P. aeruginosa* to polymyxin (6). The presence of excess Ca^{2+} and Mg^{2+} has been shown to inhibit the action of polymyxin (26) and also the action of cationic proteins (2, 27). It has also been shown that cationic proteins, like the polymyxins, are inactive against the *Proteus* group of bacteria (18). Bader and Teuber (1) found that lipid A was the binding site for polymyxin in *S. typhimurium* and that the greater the degree of exposure of this structure in the cell envelope, the greater the capacity to bind polymyxin. The work presented here has suggested that the greater the degree of exposure of the antigenic side chain of the lipopolysaccharide, the greater the resistance to the action of cationic proteins, and it may indicate that polymyxin and cationic proteins have a similar initial target site capable of being protected by divalent cations.

Divalent cations themselves also appear to affect the phagocytosis of bacteria independently of the lipopolysaccharide structure. Ethylenediaminetetraacetic acid alters the engulfment rate of *Neisseria gonorrhoeae* by human or rabbit PMN leukocytes (30). This appears to occur without the release of material from the bacterial cell surface, which occurs with other gram-negative bacteria (24, 30), and therefore the divalent cations appear to be responsible for some physical arrangement of the cell surface involved in phagocytosis.

Both lipopolysaccharides and divalent cations have been shown to have a marked effect on both the engulfment stage of phagocytosis (14, 31) and the resistance to the action of cationic proteins acting within the phagocyte (2, 25, 26, 27). Growth in conditions capable of changing the cation and lipopolysaccharide composition of the outer membrane of *P. aeruginosa* is therefore highly likely to affect the bacterial response to phagocytosis. It is impossible at present to state with certainty whether the induced phenotypic changes resulting in differing responses to phagocytic killing were the result of variation in resistance to engulfment or variation in the degree of resistance to bactericidal systems operating within the PMN leukocytes.

In vivo there is evidence that iron in serum can be the growth-limiting nutrient (9), and it has been shown that the doubling time of bacteria present in infections can be between 20 and 24 h (12). The chemical composition of such cells, the presence and structure of their lipopolysaccharide component, and, therefore, the response to phagocytic killing are likely to be markedly different from those of broth-grown cultures, often oxygen depleted, which have long been regarded as normal for phagocytic studies. It would therefore seem worthwhile to consider carefully the environment and growth rate of the bacteria produced for in vitro phagocytosis studies when attempts are made to assess an in vivo phenomenon.

ACKNOWLEDGMENTS

We are grateful to R. J. Jones and E. A. Roe for helpful discussions.

J.E.F. was the recipient of a maintenance grant from the Science Research Council.

LITERATURE CITED

1. **Bader, J., and M. Teuber.** 1973. Action of polymyxin-B on bacterial membranes. I. Binding to the O-antigenic lipopolysaccharide of *Salmonella typhimurium*. Z. Naturforsch. **28**:422-430.
2. **Beckerdite, S., C. Mooney, J. Weiss, R. Franson, and P. Elsbach.** 1974. Early and discrete changes in permeability of *Escherichia coli* and certain other gram-negative bacteria during killing by granulocytes. J. Exp. Med. **140**:396-400.
3. **Bergan, T.** 1975. *In* M. R. W. Brown (ed.), Resistance of *Pseudomonas aeruginosa*. John Wiley & Sons, Inc., London.
4. **Bjornson, A. B., and J. G. Michael.** 1974. Factors in human serum promoting phagocytosis of *Pseudomonas aeruginosa*. I. Interaction of opsonins with the bacterium. J. Infect. Dis. **130**(Suppl.):S119-S126.
5. **Braun, W., A. Pomalis-Lebrun, and W. R. Stinebring.** 1958. Interactions between mononuclear phagocytes and *Brucella abortus* strains of different virulence. Proc. Soc. Exp. Biol. Med. **97**:393-397.
6. **Brown, M. R. W.** 1975. The role of the cell envelope in

resistance. *In* M. R. W. Brown (ed.), Resistance of *Pseudomonas aeruginosa*. John Wiley & Sons, Inc., London.
7. **Brown, M. R. W.** 1977. Nutrient depletion and antibiotic susceptibility. J. Antimicrob. Chemother. **3**:198-201.
8. **Brumfitt, W., A. A. Glynn, and A. Percival.** 1964. Factors influencing the phagocytosis of *Escherichia coli*. Br. J. Exp. Pathol. **45**:215-226.
9. **Bullen, J. J., H. J. Rogers, and E. Griffiths.** 1974. Bacterial iron metabolism in infection and immunity. *In* J. B. Nieland (ed.), Microbial iron metabolism: a comprehensive treatise, p. 517. Academic Press Inc., London.
10. **Chester, I. R., P. M. Meadow, and T. L. Pitt.** 1973. The relationship between the O-antigenic lipopolysaccharides and serological specificity in strains of *Pseudomonas aeruginosa* of different O-serotypes. J. Gen. Microbiol. **78**:305-318.
11. **Cohn, Z. A., and S. I. Morse.** 1959. Interactions between rabbit polymorphonuclear leucocytes and staphylococci. J. Exp. Med. **100**:419-443.
12. **Eudy, W. W., and S. E. Burrous.** 1973. Generation times of *Proteus mirabilis* and *Escherichia coli* in experimental infections. Chemotherapy **19**:161-170.
13. **Finch, J. E., and M. R. W. Brown.** 1975. The influence of nutrient limitation in a chemostat on the sensitivity of *Pseudomonas aeruginosa* to polymyxin and to EDTA. J. Antimicrob. Chemother. **1**:379-386.
14. **Friedberg, D., and M. Shilo.** 1970. Role of cell wall structure of *Salmonella* in the interaction with phagocytes. Infect. Immun. **2**:279-285.
15. **Gilbert, P., and M. R. W. Brown.** 1977. The influence of growth rate and nutrient limitation upon cell composition and resistance to phenols in *Pseudomonas aeruginosa* NCTC 6750. Proc. Soc. Gen. Microbiol. **4**:79.
16. **Gladstone, G. P., and E. Walton.** 1970. Effect of iron on the bactericidal proteins from rabbit polymorphonuclear leucocytes. Nature (London) **227**:849-856.
17. **Habs, I.** 1957. Untersuchungen über die O-antigene von *Pseudomonas aeruginosa*. Z. Hyg. Infektionskr. **144**:218-228.
18. **Hirsch, J. G.** 1956. Phagocytin: a bactericidal substance from polymorphonuclear leucocytes. J. Exp. Med. **103**:589-611.
19. **Holmes, B., P. G. Quie, D. B. Windhorst, B. Pollara, and R. A. Good.** 1966. Protection of phagocytised bacteria from the killing action of antibiotics. Nature (London) **210**:1131-1132.
20. **Janoff, A., and B. W. Zwiefach.** 1964. Adhesion and emigration of leukocytes produced by cationic proteins of lysosomes. Science **144**:1456-1458.
21. **Jenkin, C., and B. Benacerray.** 1960. *In vitro* studies on the interaction between mouse peritoneal macrophages and strains of *Salmonella* and *Escherichia coli*. J. Exp. Med. **112**:403-417.
22. **Lacey, B. W.** 1954. Variation of the antigenic structure of certain bacteria with temperature of incubation and ionic composition of the medium. Biochem. J. **56**:xiv.
23. **Lacey, B. W.** 1961. Microbial reaction to environment. Symp. Soc. Gen. Microbiol. **11**:434.
24. **Leive, L.** 1965. Release of lipopolysaccharide by EDTA treatment of *E. coli*. Biochem. Biophys. Res. Commun. **21**:290-296.
25. **Liu, P. V.** 1974. Extracellular toxins of *Pseudomonas aeruginosa*. J. Infect. Dis. **130**(Suppl.):S94-S99.
26. **Newton, B. A.** 1956. The properties and mode of action of polymyxins. Bacteriol. Rev. **20**:14-27.
27. **Odeberg, H., and I. Olsson.** 1976. Mechanisms for the microbicidal activity of cationic proteins of human granulocytes. Infect. Immun. **14**:1269-1275.
28. **Pearson, A. D., and D. C. Ellwood.** 1974. Growth environment and bacterial toxicity. J. Med. Microbiol. **7**:391-393.
29. **Roberts, R. B.** 1967. The interaction *in vitro* between group B meningococci and rabbit polymorphonuclear leucocytes. Demonstration of type specific opsonins and bactericidins. J. Exp. Med. **126**:795-817.
30. **Rogers, S. W., H. E. Gilleland, Jr., and R. G. Eagon.** 1969. Characterization of a protein-lipopolysaccharide complex released from cell walls of *Pseudomonas aeruginosa* by ethylenediaminetetraacetic acid. Can. J. Microbiol. **15**:743-748.
31. **Rosenthal, R. S., R. S. Fulbright, M. E. Eads, and W. D. Sawyer.** 1977. Ethylenediaminetetraacetic acid-sensitive antiphagocytic activity of *Neisseria gonorrhoeae*. Infect. Immun. **15**:817-827.
32. **Stendahl, O., and L. Edebo.** 1972. Phagocytosis of mutants of *Salmonella typhimurium* by rabbit polymorphonuclear cells. Acta Pathol. Microbiol. Scand. **80**:481-488.
33. **Stendahl, O., C. Tagesson, and L. Edebo.** 1974. Influence of hyperimmune immunoglobulin G of the physicochemical properties of the surface of *Salmonella typhimurium* 395 MS in relation to interaction with phagocytic cells. Infect. Immun. **10**:316-319.
34. **Tagesson, C., and O. Stendahl.** 1973. The influence of the cell surface lipopolysaccharide structure of *Salmonella typhimurium* on resistance to intracellular bactericidal systems. Acta Pathol. Microbiol. Scand. **81**:473-480.
35. **Tempest, D. W., and D. C. Ellwood.** 1969. The influence of growth conditions on the composition of some cell wall components of *Aerobacter aerogenes*. Biotechnol. Bioeng. **11**:775.
36. **Veron, M.** 1961. Sur l'agglutination de *Pseudomonas aeruginosa*: subdivision des groupes antigeniques 0:2 et 0.5. Ann. Inst. Pasteur **101**:456-460.
37. **Young, L. S.** 1974. The role of antibody in infections due to *Pseudomonas aeruginosa*. J. Infect. Dis. **130** (Suppl.):S111-S118.
38. **Young, L. S., and D. Armstrong.** 1972. Human immunity to *Pseudomonas aeruginosa*. I. *In vitro* interaction of bacteria, polymorphonuclear leucocytes and serum factors. J. Infect. Dis. **126**:257-276.
39. **Zeya, H. I., and J. K. Spitznagel.** 1966. Cationic proteins of polymorphonuclear leukocyte lysosomes. I. Resolution of antibacterial and enzymatic activities. J. Bacteriol. **91**:750-762.