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The effect of oxygen-dependent antimicrobial systems on strains of *Legionella pneumophila* of different virulence

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SUMMARY

Four strains of *Legionella pneumophila* of different virulence as identified by ability to produce pneumonia and death in guinea-pigs infected by a fine-particle aerosol were examined for factors which may intracellularly influence virulence.

Possible bactericidal mechanisms possessed by alveolar phagocytes were examined. A relationship could be established between resistance to H_2O_2, catalase activity and virulence amongst the strains.

Virulent strains resisted the bactericidal activity generated by the xanthine oxidase system; avirulent strains did not. Incorporation of various specific inhibitors of the xanthine oxidase system indicated that the main bactericidal activities were associated with the production of H_2O_2 and hydroxyl radicals ($^{\cdot}OH$).

All strains of *L. pneumophila* were susceptible to the bactericidal activity generated by the myeloperoxidase–H_2O_2–halide system, confirming earlier observations that polymorphonuclear neutrophil leucocytes (PMNLS) are able to kill both virulent and avirulent strains of *L. pneumophila*.

INTRODUCTION

Legionnaires' disease (LD) results from the inhalation of an infectious aerosol containing *Legionella pneumophila* (Lattimer & Ormsbee, 1981) and this has been reproduced experimentally in animals (Baskerville *et al.* 1981). Aerosol infection differentiates between virulent and avirulent strains of *L. pneumophila* in guinea-pig animal models (Baskerville *et al.* 1981; Fitzgeorge *et al.* 1983). Virulent strains produce a widespread bronchopneumonia similar to the human disease.

L. pneumophila has been shown to replicate within phagocytes both *in vivo* (Blackmon *et al.* 1978; Winn *et al.* 1978; Winn & Myerowitz, 1981) and *in vitro* (Horwitz & Silverstein, 1981*a*, *b*) and intracellular growth is an important feature of the pathogenesis of Legionnaires' disease (Baskerville *et al.* 1981). The lung contains a heterogeneous population of phagocytic cells. For *L. pneumophila* to survive, it must be able to resist the antimicrobial mechanisms possessed by these cells. These antimicrobial properties can be divided into oxygen dependent and oxygen independent mechanisms. Among the oxygen independent mechanisms are low pH (Jensen & Bainton, 1973), lactoferrin (Baggiolini *et al.* 1970; Leffell & Spitznagel, 1972), cationic proteins (Spitznagel & Chi, 1963) and lysozyme (Cohn

& Hirsh, 1960; Baggiolini *et al.* 1969) and other degrading enzymes (Elsbach, 1980). Oxygen-dependent mechanisms include toxic oxygen metabolites (Johnson *et al.* 1975) and the enzymatic myeloperoxidase (MPO)–H_2O_2–halide system (Klebanoff, 1967; Klebanoff & Hamon, 1972).

The MPO mediated system is extremely potent for microorganisms (Klebanoff, 1980). Information currently available suggests that this system is associated with polymorphonuclear neutrophil leucocytes (PMNLs) and blood monocytes. However, MPO-mediated antimicrobial activity is found predominantly in PMNLs rather than macrophages which rely mainly on MPO independent antimicrobial systems. (Klebanoff, 1980; Badwey & Karnovsky, 1980). Lochner *et al.* (1983) have shown that both virulent and avirulent *L. pneumophila* are sensitive to the MPO–H_2O_2–halide system. Davis *et al.* (1983) have shown that *L. pneumophila* proliferates mainly in the macrophage fraction of guinea-pig lung phagocytes, and it has been shown in this laboratory (Jepras, Fitzgeorge & Baskerville, 1985) that guinea-pig alveolar macrophages preferentially support the growth of virulent *L. pneumophila* when compared with PMNLs whereas avirulent *L. pneumophila* appear to be destroyed by both macrophages and PMNLs. This paper reports a study which compares the effects of H_2O_2, toxic oxygen metabolites produced by the xanthine oxidase systems and the MPO–H_2O_2–halide system as models for some bactericidal mechanisms within alveolar phagocytes, on strains of *L. pneumophila* of varied virulence.

MATERIALS AND METHODS

Bacterial strains and cultivation methods

Four strains of *L. pneumophila* were used. The Corby strain was a human isolate kindly supplied by Dr R. A. Swann of John Radcliffe Hospital, Oxford; the Philadelphia-1 strain (NCTC 11192) was obtained from the National Collection of Type Cultures, Colindale, London; strain 74/81 was isolated in this laboratory from a naturally contaminated water supply; and Corby A was produced in this laboratory by a continuous subculture of the Corby strain on buffered charcoal yeast extract plus α-ketoglutarate agar (BCYE α agar) (Edelstein, 1981) at 37 °C. All strains were of serogroup 1. Each strain differed in pathogenicity for guinea-pigs when administered as an aerosol. The Corby and 74/81 strains had an LD 50 of $10^{2.2}$ and 10^4 respectively. The Philadelphia-1 and Corby A strains were not lethal under similar conditions (Fitzgeorge *et al.* 1983).

L. pneumophila strains Corby A and 74/81 had only previously been subcultured twice on artificial media; Philadelphia-1 (NCTC 11192) has an unrecorded history, and Corby A had been subcultured 32 times on BCYE α agar. All were kept at −70 °C. Frozen stocks of BCYE α agar-grown *L. pneumophila* were used to inoculate yeast extract broth (YEB) (Ristroph, Hedlund & Allen, 1980) or BCYE α agar plates. For procedures requiring broth grown organisms, batches (100 ml) of YEB contained in 500 ml conical flasks, were inoculated with *L. pneumophila* and were incubated in air at 37 °C for 24 h on an orbital shaker (80–100 rev/min). These cultures were used as inocular for 500 ml volume cultures contained in 2 L flasks. These cells were harvested when still in the exponential phase of growth (18–20 h) by centrifugation at 2000 rev./min at 4 °C for 1 h using an M.S.E. Mistral

6 L centrifuge. Pellets from 4 × 500 ml volumes were washed by resuspending in a total volume of 100 ml of 50 mM potassium phosphate buffer pH 7·0, and centrifuging at 4 °C, at 10000 rev./min for 15 min, using a Sorval RC-5B centrifuge. The final pellet was suspended in 20 ml of the appropriate buffer (see section on catalase and superoxide dismutase assays).

Effect of H_2O_2 and the xanthine oxidase system on the viability of L. pneumophila

Colonies of *L. pneumophila* were washed off BCYE α agar plates and resuspended in distilled water. Aliquots (10 ml) of the suspension containing 1×10^6 viable organisms per ml were held for 45 min at room temperature in the presence of 20 mM H_2O_2 (BDH chemicals). Samples were taken at various times and viable counts were made on BCYE α agar. Controls consisted of bacterial suspensions without H_2O_2 and with H_2O_2 + catalase (100 μg/ml, Sigma Chemicals Ltd). In the xanthine oxidase system 1×10^6 viable organisms per ml in phosphate buffered saline pH 7·4 were incubated for 60 min at 37 °C in final volumes of 5 ml, containing $1{\cdot}5 \times 10^{-4}$ M xanthine and 100 μg/ml xanthine oxidase with or without catalase (100 μg/ml), superoxide dismutase (SOD) (200 μg/ml), mannitol (10 mM) or histidine (10 mM). Samples were removed at various times and viable counts were made on BCYE α agar. Plates were incubated at 37 °C and 3–4 days later colonies were counted.

Catalase and superoxide dismutase

Pellets of broth grown *L. pneumophila* were washed and suspended in either 50 mM disodium-potassium phosphate buffer at pH 7·0 for catalase assays or 50 mM potassium phosphate buffer containing 0·1 mM EDTA at pH 7·8 for SOD.

Catalase was assayed using a Clark oxygen electrode (Rank Brothers, High Street, Bottisham, Cambridge) as described by Del Rio *et al.* (1977) and the activity was expressed as μmol oxygen produced/min/mg protein. The concentration of H_2O_2 was determined by measuring the absorbance at 240 nm $E_{240} = 39{\cdot}4\ M^{-1}\ cm^{-1}$ (Nelson & Kiesow, 1972). SOD was assayed spectrophotometrically by the method of Crapo *et al.* (1978). Protein was determined by the method of Bradford (1976) using bovine serum albumen as the reference standard.

Myeloperoxidase demonstration

Air-dried smears of alveolar phagocytes from normal and 24 h-infected guinea-pigs were stained for MPO using the Sigma leukocyte peroxidase kit, based on the method of Hanker *et al.* (1977).

Effect of myeloperoxidase–H_2O_2–halide system on the viability of L. pneumophila

Colonies of *L. pneumophila* were washed off BCYE α agar plates and resuspended in 50 mM potassium phosphate buffer pH 5·0, containing 2 mM sodium chloride and 10^{-4} M H_2O_2. Aliquots of the suspension (2 ml) containing 1×10^7 viable organisms per ml were incubated at 37 °C with and without MPO (8 mU). Samples were removed at 0 and 60 min and viable counts were made on BCYE α agar.

Table 1. *Effect of 20 mM H_2O_2 on the survival of* L. pneumophila *strains of different virulence*

L. pneumophila strain	Decrease in viability (log_{10}) over 45 min at room temperature
Corby	1·3
74/81	2·0
Philadelphia-1	3·75
Corby A	4·2

Figures in the body of the table are the mean of three values, variation within each group was $\leqslant 0{\cdot}25$ log_{10}. Control samples organisms held at room temperature in distilled water or in 20 mM H_2O_2 + catalase (100 μg/ml) showed $\leqslant 0{\cdot}1$ log_{10} decrease in viability.

Table 2. *Toxic effect of xanthine oxidase system on* L. pneumophila *strains of different virulence*

	L. pneumophila (log_{10})			
	Corby V	74/81	Corby A	Philadelphia-1
Control (distilled water)	6·3	6·4	6·1	6·2
Additive				
Xanthine-xanthine oxidase + no additive	6·1	6·2	4·76	4·14
Xanthine-xanthine oxidase + mannitol	6·1	6·2	5·8	5·5
Xanthine-xanthine oxidase + histidine	6·1	6·1	5·3	4·0
Xanthine-xanthine oxidase + catalase	6·3	6·2	6·1	5·9
Xanthine-xanthine oxidase + SOD	6·2	6·2	5·9	5·6

Figures in the body of the table are the mean of three values. Variation within each group was $\leqslant 0{\cdot}2$ log_{10}.

RESULTS

Effects of H_2O_2 and the xanthine oxidase system on L. pneumophila

Table 1 shows that the Corby strain is the most resistant to H_2O_2 followed by strains 74/81, Philadelphia-1 and Corby A. The killing by H_2O_2 is prevented by the addition of catalase. The Corby and 74/81 strains are also the most resistant to the oxygen metabolites produced by the xanthine oxidase system, followed by Corby A and Philadelphia-1 (Table 2). Bactericidal activity is significantly reduced by the addition of catalase and to a lesser extent by SOD and mannitol. This suggests that primarily resistance to H_2O_2 is a major factor governing intracellular survival and growth within the alveolar macrophage, but that superoxide anions (O_2^-) and hydroxyl radicals ($^{\cdot}OH$) also exert an effect. The addition of mannitol and histidine suggests that hydroxyl ions ($^{\cdot}OH$) have a greater effect than singlet oxygen ($^1O_2$) on the Corby A and Philadelphia-1 strains, but the Philadelphia-1 strain is differentiated by being more sensitive to the action of singlet oxygen ($^1O_2$) as shown by incorporation of histidine (a quencher of singlet oxygen activity) into the xanthine oxidase system.

Table 3. *Catalase activity of* L. pneumophila *strains of different virulence*

L. pneumophila strain	Catalase activity μmol O_2/min/mg protein
Corby	0·63
74/81	0·48
Corby A	0·28
Philadelphia-1	0·11

Figures in the body of the table are the mean of three values with a variation within each group of $\leqslant 5\%$.

Table 4. *Superoxide dismutase activity of* L. pneumophila *strains of different virulence*

L. pneumophila strain	SOD (units/mg protein)
Corby	117·3
74/81	79·4
Philadelphia-1	82·9
Corby A	64·8

Figures in the body of the table are the mean of three values, variation within each group was $\leqslant 3\%$.

Table 5. *Effect of the MPO–H_2O_2–halide system on* L. pneumophila *strains of different virulence over a period of 60 min at 37 °C*

Reaction mixture	Time (min)	*L. pneumophila* viable count (c.f.u./ml $\times 10^{-7}$)			
		Corby	74/81	Philadelphia-1	Corby A
MPO omitted	0	1·3	1·2	1·4	1·2
	60	1·0	1·1	1·1	1·0
MPO + H_2O_2 + Cl^-	0	1·3	1·2	1·2	1·1
	60	0·00003	0·000028	0·000012	0·00002

Figures in the body of the table are the mean of two values, variation within each pair was $\leqslant 7\%$.

Catalase and superoxide dismutase

Catalase is a haem protein which catalyses the decomposition of H_2O_2 to give H_2O and O_2. The virulent Corby strain had the highest activity of 0·63 μmol O_2/min/mg protein and Philadelphia-1 strain had the lowest activity which was 0·11 (Table 3). This correlates with resistance to toxic oxygen metabolites generated in the xanthine oxidase reaction and resistance to H_2O_2 (Table 1).

Superoxide dismutases constitute a defence against oxygen toxicity and are found in oxygen-utilizing organisms (Fridovich, 1974). These enzymes catalyze the dismutation of O_2 to give H_2O_2. When the four strains of *L. pneumophila* used in this study were assayed for SOD activity there was no clear correlation between virulence and activity (Table 4).

Effect of the myeloperoxidase–H_2O_2–halide system on L. pneumophila

Analysis of the data presented in Table 5 indicates that all strains of *L. pneumophila* were equally sensitive to the MPO-mediated killing process.

DISCUSSION

L. pneumophila strains of varied virulence, based on aerosol infection of guinea-pigs, were used in this study to investigate possible differences in their susceptibility to H_2O_2 and the toxic oxygen products of the xanthine oxidase systems. The avirulent strains of *L. pneumophila* used were derived from virulent strains; the Philadelphia-1 strain probably lost its virulence because of continuous subculture on artificial media (Bornstein *et al.* 1984) and the avirulent Corby A lost its virulence as a sequel to defined continuous subculture of the Corby strain on BCYE α agar (Fitzgeorge, unpublished). These strains differ in virulence in terms of their LD 50 as a result of aerosol challenge of guinea-pigs. The Corby being the most virulent followed by strain 74/81. The Philadelphia-1 and Corby A do not produce a pneumonia and do not kill when administered as an aerosol.

The presence of H_2O_2 and its antimicrobial properties in phagocytes has been well documented (Wilson & Dasinger, 1960; Fitzgeorge, Keppie & Smith, 1965; Klebanoff, 1975). We found that strains of *L. pneumophila* differ in their susceptibilities to 20 mM H_2O_2. This difference was reflected in the catalase activities possessed by these strains the Corby strain having approximately three times more activity than the Corby A derived strain. A correlation between virulence and resistance to H_2O_2 has been observed in the past (Wilson & Dasinger, 1960; Fitzgeorge *et al.* 1965) and this resistance has been attributed to catalase content (Amin & Olson, 1968; Mandell, 1975).

Xanthine oxidase systems have previously been used as a model (Klebanoff, 1975; Rosen & Klebanoff, 1979; Lochner *et al.* 1983) for the reactive oxygen metabolities such as those generated by the phagocytosis-induced respiratory burst of phagocytes (Rossi, Bellaville & Burton, 1982). Xanthine oxidase catalyzes the oxidation of xanthine to hypoxanthine with the resultant production of superoxide anion (O_2^-) and hydrogen peroxide (Fridovich, 1970). These compounds may subsequently interact to form hydroxyl radicals ($^{\cdot}OH$) Beauchamp & Fridovich, 1970) and singlet oxygen ($^1O_2$) (Kellogg & Fridovich, 1975). The susceptibility of *L. pneumophila* to the reactive oxygen metabolites produced by the xanthine oxidase system has been assessed by using specific inhibitors of the xanthine oxidase reaction mixture. A reduction in H_2O_2, superoxide anions (O_2^-), hydroxyl radicals ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) is achieved by the addition of catalase, SOD, mannitol and histidine respectively.

The susceptibilities of the *L. pneumophila* strains used in this study to the xanthine oxidase system differed considerably. The virulent Corby and 74/81 strains were resistant, the avirulent strains Corby A and Philadelphia-1 strains were susceptible.

Killing is significantly impaired by the inclusion of catalase, SOD and mannitol exert a lesser reduction in the bactericidal activity of the xanthine oxidase system. The addition of histidine appears to be moderately effective in reducing bactericidal activity in the case of one avirulent strain, Corby A, but not the other, Philadelphia-1.

These observations using the above selective scavenging agents suggest that the production of H_2O_2, superoxide anions (O_2^-) and hydroxyl ions (OH) are predominant factors responsible for the bactericidal activity of the xanthine

oxidase systems and that virulent *L. pneumophila* strains have the ability to resist their bactericidal properties. Susceptibility to singlet oxygen ($^1O_2$) appears to differentiate between the two avirulent strains.

Lochner *et al.* (1983) suggest that catalase and SOD influence the hydroxyl ion ($^{\cdot}OH$) concentration indirectly by decreasing the concentration of the substrates used in hydroxyl ion ($^{\cdot}OH$) formation. On this basis it would seem that the production of hydroxyl radicals ($^{\cdot}OH$) is predominantly responsible for the bactericidal activity of the xanthine oxidase system for *L. pneumophila*.

There appears to be little or no correlation between SOD content and virulence among the strains of *L. pneumophila* used in this work, the SOD activities being similar, i.e. 79·4, 82·4 and 64·8 units/mg protein for 74/81, Philadelphia-1 and Corby A respectively. However, the virulent Corby strain possessed activity of 117·3 units/mg of protein ($\times$ 1·8 that of its avirulent derived strain Corby A).

It has been suggested that the antimicrobial activity of H_2O_2 and the oxygen metabolites generated by the xanthine oxidase system is modest when compared with the microbiocidal activity of the MPO–H_2O_2–halide system (Rosen & Klebanoff, 1979; Klebanoff & Hamon, 1972; Klebanoff, 1975). However, this system has been shown to be associated primarily with PMNLs and blood monocytes and there is little or no MPO activity in normal unactivated macrophages (Klebanoff, 1980; Badwey & Karnovsky, 1980). We have not been able to demonstrate MPO in normal guinea-pig alveolar macrophages and in macrophages removed from infected animals. It has been shown that all four strains of *L. pneumophila* used are susceptible to the MPO–H_2O_2–halide system and it has also been demonstrated that all these strains were killed by PMNLs, but only the avirulent strains were killed by macrophages (Jepras *et al.* 1985). However, the organisms may be protected from this mainly PMNL system by the alveolar macrophage and it may be that the bactericidal mechanisms operating within this cell are the ones which are important with regard to virulence.

Locksley (1982) and Lochner *et al.* (1983) have reported that their virulent and avirulent strains of *L. pneumophila* were equally susceptible to model antimicrobial systems based on the xanthine oxidase reaction and the MPO–H_2O_2–halide systems. However, these workers used strains which had their virulence defined in relation to i.p. infection of guinea-pigs and effects on embyronated hens eggs respectively. In contrast the strains used in this study had their virulence assessed by their ability to cause a lethal bronchoneumonia in guinea-pigs as a result of aerosol infection, a relevant model system.

We report that virulence, based on aerosol infection, of strains of *L. pneumophila* may be related to their ability to resist the antimicrobial properties of the alveolar macrophages such as H_2O_2 and toxic reactive products generated by the phagocytosis-induced respiratory burst.

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