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A comparison of virulence of two strains of *Legionella pneumophila* based on experimental aerosol infection of guinea-pigs

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SUMMARY

Two strains of *Legionella pneumophila* (LP) serogroup I, of differing virulence, were examined in terms of numbers of viable organisms in tissues, pyrexia and mortality following aerosol infection. The Corby strain was the more virulent, with pyrexia and deaths of guinea-pigs 3 to 6 days after infection. This strain multiplied very rapidly in the lungs to reach a peak of 5×10^{11} viable organisms/lung. Organisms were present in the blood, liver, spleen and kidney. The Philadelphia-1 strain (NCTC 11192) was unable to replicate in the lung and was cleared between 14 and 21 days after infection. Pyrexia was not observed. No guinea-pigs died and viable LP was not found in any organ other than the lung.

Lung lavages on aerosol infected animals were performed and the virulent Corby strain was found to be mainly intracellular. The avirulent Philadelphia-1 strain was found predominantly in the extracellular location. There were approximately 10 times the number of viable virulent LP in the lung macrophage fraction than in the lung PMNL fraction. In comparison, there were approximately equal numbers of the viable avirulent strain in the macrophages and the PMNL. Experimental evidence suggests that the macrophage preferentially supports the growth of the virulent Corby strain compared with the PMNL. The avirulent strain on the other hand appears to be destroyed by both the macrophages and the PMNL.

INTRODUCTION

Virulence is the result of factors which include the properties of the infecting agent and the response of the host to infection by a given route. The outcome, whether it results in disease, death or survival, is due to a delicate balance between these factors.

Legionnaires' disease (LD) presents several interesting features. Person to person transmission of the causative bacterium probably does not occur so that the possibility of enhancement of virulence due to passage is unlikely. Predisposing factors such as increasing age, underlying illness and immunosuppressive treatment (Lattimer & Ormsbee, 1981) may modify the eventual form of the disease within the spectrum pneumonic, non-pneumonic, lethal and non-lethal.

Organisms of the *Legionella* species are abundant in the environment and are

found in water from such diverse sources as rivers, household supplies and air-conditioning systems. Isolation of this ubiquitous, opportunistic pathogen by intraperitoneal inoculation of guinea-pigs is not an indication of its virulence for man (Fitzgeorge & Dennis, 1983).

LD results from infection with an aerosol of *L. pneumophila* (Lattimer & Ormsbee, 1981) and this has been reproduced experimentally in animals (Baskerville *et al.* 1981). Experimental evidence also indicates that the size of the particle is of great importance for the establishment of infection (Fitzgeorge *et al.* 1983).

Fitzgeorge *et al.* (1983) have shown that whereas *L. pneumophila* at a dose of 10^3 viable organisms induces a lethal infection in guinea-pigs when administered as a small particle aerosol, ($< 5\ \mu$m diameter), organisms of the same strain are not lethal at a dose of 10^9 viable organisms given intranasally. Infection by the i.p. route does not differentiate between virulent and avirulent strains, since the LD_{50} (10^8 viable organisms) for both virulent and avirulent strains is similar. However, infection by aerosol does differentiate between strains. The virulent strains we have studied have LD_{50} values of approximately 10^3 viable organisms whereas avirulent strains were not lethal under similar conditions. The aerosol guinea-pig model of LD developed in this laboratory has allowed investigation of the pathogenesis of the disease (Baskerville *et al.* 1981) and this paper reports its application in the evaluation of factors which may influence virulence. Guinea-pigs were infected by aerosols of *L. pneumophila* strains of varied virulence and differences in the pathogenesis of the resulting disease studied.

MATERIALS AND METHODS

Bacterial strains and cultivation methods

Two 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, and the Philadelphia-1 strain (NCTC 11192) was obtained from the National Collection of Type Cultures, Colindale, London. Both strains were of serogroup 1.

These strains of *L. pneumophila* have been shown to differ in pathogenicity for guinea-pigs when administered as a fine particle aerosol (Fitzgeorge *et al.* 1983). The Corby strain was shown to have an LD_{50} of $10^{2\cdot2}$ viable organisms and the Philadelphia-1 strain was not lethal under similar conditions. Stocks of these defined strains were kept frozen at -70 °C in 0·5 ml aliquots. Sub-culture was avoided by reconstitution of an aliquot for each experiment and the organisms were grown for 4 days at 37 °C on BCYEα medium (Edelstein, 1981). The organisms were harvested from the plates and suspended in distilled water. Details of growth and assay procedures were described by Fitzgeorge *et al.* (1983).

Animals

Female Dunkin–Hartley guinea-pigs of Category 4 health status (MRC, 1974) weighing 300–500 g were used and housed in groups of 4. Blood samples from normal guinea-pigs showed these animals to be free from antibody to *L. pneumophila*.

Immunofluorescent procedures

Organisms in air-dried smears were acetone fixed and stained with rabbit antiserum to serogroup 1 *L. pneumophila* and goat anti-rabbit antiserum conjugated with fluorescein isothiocyanate, for indirect immunofluorescence.

Clinical observations

Rectal temperatures were recorded at daily intervals following infection. Temperatures were considered elevated when they were $\geqslant 40$°C.

Aerosol generation and respiratory infection

Bacterial aerosols containing particles < 5 μm diameter were generated using a three jet Collison spray at a survival optimum of 65% relative humidity in a Henderson-type apparatus (Druett, 1969; Henderson, 1952; Hambleton *et al.* 1983). Guinea-pigs were infected by inhalation of the aerosol over a period of 5 min. Evaluation of dose and descriptions of procedures have been reported by Baskerville *et al.* 1981) and Fitzgeorge *et al.* (1983).

Experimental procedures

For evaluation of bacterial numbers, guinea-pigs were killed with ether and blood (obtained by cardiac puncture), lung, liver, spleen and kidneys were removed. Whole blood and macerated tissue (MSE Homogeniser) were diluted and plated out onto BCYEα agar and colonies counted after 4 days incubation at 37 °C. Tissues were stored at -20 °C prior to maceration.

Lung lavage procedures

Guinea-pigs were killed by the injection of sodium pentobarbitone. Using aseptic techniques a sterile polyethylene catheter was inserted and tied into the exposed trachea; lavage was performed by the instillation (using an attached 10 ml syringe) of 3×10 ml quantities of MEM Eagles medium containing sodium bicarbonate (10 mM), Hepes buffer (20 mM), L-glutamine (2 mM) and heparin (5 u/ml). Before withdrawal of the lavage, the chest area was massaged for approximately 1 min.

The number of cells was determined by counting in a haemocytometer using dark ground illumination. Cells were separated from the lavage supernatent by centrifugation at 200 *g* for 10 min. Samples of the cell pellet in an equal volume of 10% foetal calf serum were spread onto clean slides, air dried, and stained by the Leishman technique for differential counting of alveolar macrophages, polymorphonuclear leucocytes (PMNL) and other cells.

Density gradient cell separation

Cell pellets were suspended in 2 ml of fresh lavage medium and layered onto 3 ml of Ficoll–Paque (Pharmacia Fine Chemicals) in 10 ml plastic centrifuge tubes (Sterilin). The tubes were centrifuged at 400 *g* for 30 min at 18–20 °C in a swinging bucket rotor centrifuge. Cell layers were collected and cell and organism numbers were estimated as described previously. Release of viable intracellular *L. pneumophila* was by lysis with 0·8 mg/ml digitonin in distilled water for 5 min. This treatment was found to have no adverse effect on the viability of *L. pneumophila* over a period of 2 h at 37 °C.

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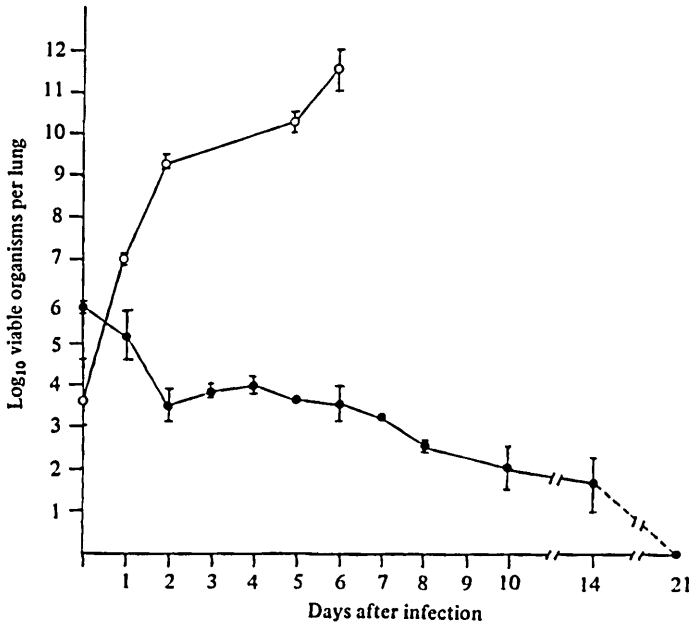

Fig. 1. Numbers of viable organisms in guinea-pig lung following aerosol infection with:—●—●—, Philadelphia-1; and —○—○—, Corby strains of *L. pneumophila*. Each point is the mean of three values, range bars indicate spread.

Table 1. *Viable* L. pneumophila (*Corby strain*) *in infected guinea-pig organs* (Log_{10})

Day	Blood/ml	Spleen	Kidney	Liver
0	0	0	0	0
1	0·7	1·5	0·8	1·7
2	2·0	5·4	1·2	3·7
5	3·0	3·0	0·9	0
6	5·2	2·9	0	0

Results are the mean of three values; variations within each group $< 0{\cdot}9$ $\log_{10}$.

RESULTS

Guinea-pig responses to aerosol infection with two strains of L. pneumophila *of differing virulence*

Two strains of *L. pneumophila* were examined in terms of numbers of organisms in tissues, pyrexia and mortality following aerosol infection with the Philadelphia-1 strain at 1×10^6 and Corby at 4×10^3 viable organisms per lung.

The two strains showed marked differences in their behaviour. The Corby strain was the more virulent, guinea-pigs dying 3–6 days after infection. This strain multiplied very rapidly in the lungs to reach a peak of 5×10^{11} viable organisms per lung (Fig. 1). Organisms were present in the blood from day 1 (Table 1) following infection and persisted until all animals were dead at day 6. As a result of the bacteraemia, the liver, spleen and kidney also contained viable *L. pneumophila*

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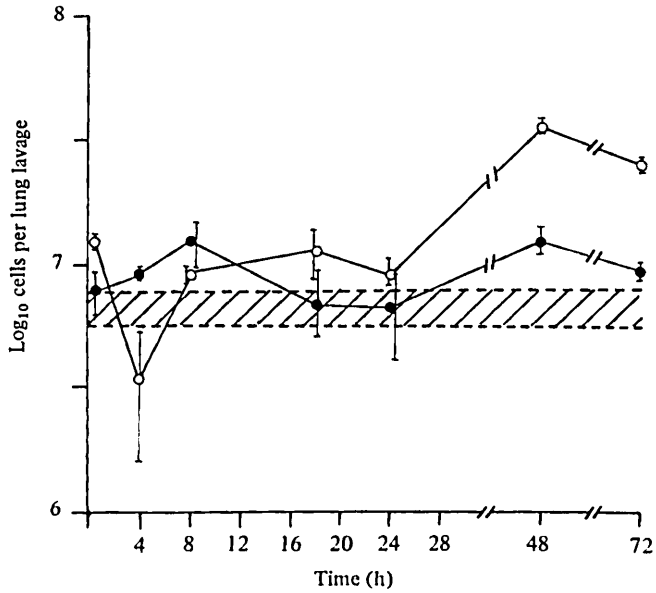

Fig. 2. Cells in guinea-pig lung lavage as a sequel to aerosol infection with *L. pneumophila*. Guinea-pig levels; —○—○—, Corby strain; —●—●—, Philadelphia-1 strain. Each point on graph is the mean of 2 values.

Table 2. *Phagocytic cellular responses to* L. pneumophila *infection in guinea-pig lungs*

Time after exposure (hours)	macrophages (%)		polymorphs (%)	
Normal guinea-pigs 0	75 (77–73)		25 (29–21)	
L. pneumophila-infected guinea-pigs	Philadelphia-1 strain	Corby strain	Philadelphia-1 strain	Corby strain
4	84 (83–85)	63·5 (70–57)	16 (17–15)	36·5 (30–43)
8	70 (68–72)	75 (76–74)	30 (28–32)	25 (24–26)
18	40 (32–48)	37·5 (33–42)	60 (68–52)	62·5 (58–67)
24	50 (48–52)	23·5 (27–20)	50 (52–48)	76·5 (73–80)
48	52·5 (49–56)	38 (34–42)	47·5 (51–44)	62 (66–58)
72	50·5 (51–50)	20·5 (38–33)	49·5 (49–50)	63·5 (63–64)

Figures in body of table are the mean of values in parentheses.

(Table 1). The liver and kidney contained no viable *L. pneumophila* by days 5 and 6 respectively, even though viable organisms were present in blood (10^3–$10^{5\cdot2}$/ml) at these times. Pyrexia occurred (≥ 40 °C) in all animals between days 2–6 following infection.

In contrast the other *L. pneumophila* strain, Philadelphia-1, appeared unable to replicate in the lung and was slowly cleared from that organ between days 14 and 21 after infection (Fig. 1). Pyrexia was not observed at any time. No guinea-pigs died and viable *L. pneumophila* was not found in any organ other than the lung.

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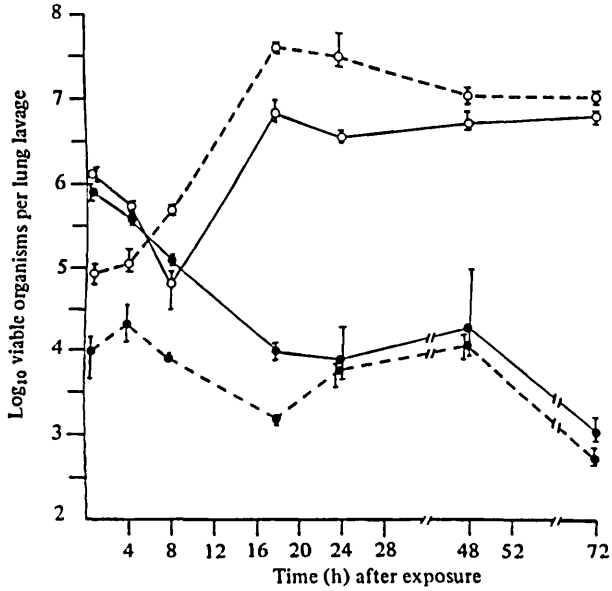

Fig. 3. Intracellular counts of *L. pneumophila* strain: ···○···○··· Corby, ···●···●···, Philadelphia-1; and supernatant count of: —○—○—, Corby; —●—●—, Philadelphia-1. The values expressed are a mean of two guinea-pigs.

Although no replication of this strain occurred in the lung, viable organisms were demonstrated up to 14 days post infection and for a period over 2–7 days following infection the numbers were approximately constant.

Histopathology

Examination of the lungs and other organs from guinea-pigs after 6 days' infection with an aerosol of the Corby strain showed areas of fibrinopurulent bronchopneumonia, intra-alveolar oedema, fibrin exudation and heavy PMNL and macrophage infiltration with lysis. In the splenic red pulp there were moderate numbers of PMNL and macrophages, and many of these were also lysed. Lesions were not found in the brain, liver, kidneys or myocardium.

Aerosol infection with the Philadelphia-1 strain produced at 7 and 10 days numerous small foci of thickening of interalveolar septa throughout the lung. These foci consisted of small groups of macrophages and occasional lymphocytes in the interstitium. PMNL were rare, as were inflammatory cells in the alveoli.

Cellular responses in the lung to infection

Lung lavages were carried out on guinea-pigs infected with an aerosol of *L. pneumophila* strains Philadelphia-1 and Corby at $2{\cdot}9 \times 10^6$ and $7{\cdot}8 \times 10^6$ organisms per guinea-pig respectively. Samples were taken at intervals between 0–72 h. Total cell numbers and types were investigated. Fig. 2 shows that lung lavage of four normal guinea-pigs yielded a mean total of $6{\cdot}7 \times 10^6$ free cells per lavage (excluding red blood cells and epithelial cells). 75% of these were alveolar macrophages (Table 2).

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Table 3. *Distribution of* L. pneumophila *in lung lavage samples* 24 *h after guinea-pig exposure to infective aerosols*

Strain and number of organisms at 0 h	Organisms: cells in lavage	Organisms in supernatant	PMNL: macrophages in lavage	Organisms in PMNLs from lung	Organisms in macrophages from lung
Corby $1{\cdot}3 \times 10^6$ viable organisms/lung	$1{\cdot}2 \times 10^9 : 4{\cdot}9 \times 10^7$	$3{\cdot}7 \times 10^7$	$3 \times 10^7 : 2 \times 10^7$	$1{\cdot}4 \times 10^8$	1×10^9
Philadelphia-1 $8{\cdot}5 \times 10^5$ viable organisms/lung	$2{\cdot}4 \times 10^4 : 4 \times 10^6$	$6{\cdot}8 \times 10^3$	$2{\cdot}6 \times 10^6 : 1{\cdot}4 \times 10^6$	$1{\cdot}4 \times 10^4$	$9{\cdot}6 \times 10^3$

Results are the mean of two values; variation within each pair was $< 0{\cdot}8$ $\log_{10}$.

Over the 72 h period of infection with strain Philadelphia-1 the total cell numbers remained fairly constant, only rising above the normal guinea-pig level slightly, to a mean of $9{\cdot}2 \times 10^6$ cells/lung lavage at 48 h (Fig. 2). The number of PMNL increased steadily from 25% at 0 h to 50% at 72 h (Table 2). The total number of cells in guinea-pigs infected with the Corby strain remained constantly above the normal guinea-pig number from 8 h onwards, rising to a maximum of $4{\cdot}7 \times 10^7$ cells/lung lavage after 48 h, and decreasing slightly just prior to death (Fig. 2). The number of PMNL increased steadily from 25% to a maximum of 77% at 24 h and decreased slightly just before death (Table 2).

Comparison of intracellular behaviour of L. pneumophila *strains Philadelphia-1 and Corby*

Lung lavages on aerosol infected animals were performed and after separation by centrifugation, viable counts were made on supernatants and cells disrupted by digitonin treatment. Fig. 3 shows that, from 4 to 24 h the virulent strain was predominantly intracellular. At 24 h there were 10 times more organisms in the cellular fraction than in the supernatant. The avirulent strain was found predominantly in the extracellular location up to 24 h after infection and then became approximately equal to the intracellular number over the remaining course of the infection. This differentiation occurred between 4–8 h; before this time, both strains were similar in being mainly extracellular. This was confirmed by immunofluorescent studies on lavage smears.

L. pneumophila *intracellular survival and growth within lung lavage macrophage and PMNL*

The first part of the study demonstrated that the virulent strain of *L. pneumophila* replicates intracellularly but the avirulent does not. Horwitz & Silverstein (1980) have shown that *L. pneumophila* proliferates within blood monocytes. However, the phagocytic cell population of the lung is a mixture of alveolar macrophages and PMNL, the latter cell increasing in numbers in response to infection. The possibility that virulent and avirulent strains of *L. pneumophila* differentially grow or are killed within macrophages or PMNL was explored by

separating lung lavage cells from infected guinea-pig lungs, on a density gradient Cells were disrupted by digitonin treatment and released organisms assayed by BCYEα medium.

Table 3 shows that 24 h after infection the virulent Corby strain increased in numbers and the avirulent Philadelphia-1 strain decreased. The total viable count of the virulent strain was approximately 10 times higher in the lung macrophage fraction than in the lung PMNL fraction, thus suggesting preferential survival and growth in the macrophage. There were approximately equal numbers of the viable avirulent strain in the macrophages and PMNL. The total viable count of the avirulent Philadelphia-1 strain in the extracellular fraction was approximately the same as the intracellular counts. However the number of viable virulent strain organisms (Corby) was much lower in the extracellular fraction than in the intracellular fraction.

DISCUSSION

The use of an aerosol model for the study of LD has enabled distinct differences between virulent and avirulent strains of *L. pneumophila* to be observed. The avirulent strain Philadelphia-1 (NCTC 11192) used in this investigation gave rise to a non-pyrexic, non-lethal, self-limiting infection with the organism being confined to the lung and producing minimal lesions of interstitial macrophage accumulation. The long and ill defined subculture of this strain, originally isolated from human fatalities in the 1976 Philadelphia outbreak of LD has presumably led to loss of virulence as demonstrated for this strain by Bornstein *et al.* (1984). In contrast the virulent Corby strain, a human isolate cultured only twice *in vitro* before use, gave rise to a progressive infection leading to extensive fibrinopurulent bronchopneumonia and death. Extrapulmonary infection occurred as a result of a bacteraemia which was present from day 1 until death, but the overwhelming focus of infection was in the lung and histopathological lesions were not found in any organs other than the spleen. Baskerville *et al.* (1983) also found no histopathological evidence for extrapulmonary damage in this model for LD with the 74/81 strain. Hicklin *et al.* (1980) and Watts *et al.* (1980) have demonstrated *L. pneumophila* in the human extrathoracic organs by immunofluorescent staining.

Lung infectivity in guinea-pigs infected with the avirulent strain remains at a constant level over 2 to 7 days following infection but is cleared between 14 and 21 days. This suggests that even the avirulent *L. pneumophila* strain, causing a self-limiting benign infection is cleared with some difficulty by defence mechanisms operating within the lung, which is confirmed by the presence of foci of macrophages in the interstitium. In contrast the virulent strain seems to replicate without restraint and when animals die, lungs contain between 10^{11} and 10^{12} viable organisms.

L. pneumophila has been shown to be a facultative intracellular pathogen (Horwitz & Silverstein, 1980) and the bacterium multiplies intracellularly in human monocytes, but the effect of phagocytosis by PMNL's was not evaluated. In man (Chandler *et al.* 1979) and animal models (Baskerville *et al.* (1983) phagocytosis has been seen to occur mainly in macrophages, although Davis *et al.* (1983) and Baskerville *et al.* (1983) have observed some involvement of PMNLS.

In this study, evidence of *in vivo* intracellular infection by *L. pneumophila* is shown by direct isolation from phagocytic cells of both types prevalent in the lung.

Over the 72-h period following infection with virulent and avirulent *L. pneumophila* the phagocytic cell population within the lung changed. The phagocytic cell population of PMNLs and macrophages of the avirulent *L. pneumophila* infected animals remained only slightly above the uninfected control guinea-pigs. However, there was an influx of PMNLs at 18 h infection from 24 % at 0 h to 60 % of the total number of cells. The phagocytic cell population of the virulent *L. pneumophila* infected animals increased at 48 h to about seven times the number of cells in the uninfected control, also there was an influx of PMNL s at 24 h so that this formed $> 70\%$ of the total phagocytic cells. For the first 72 h of infection, the majority of avirulent organisms were extracellular, whereas the virulent strain was predominantly intracellular from 8 h over the whole period of infection. Davis *et al.* (1983) also found an influx of PMNLs in the early events of infection with *L. pneumophila*.

This may be interpreted as indicating that the virulent strain is efficiently phagocytosed but replicates within phagocytic cells. In contrast the avirulent strain appears so resist phagocytosis and does not replicate within the phagocytic cells. An alternative explanation is that after phagocytosis the avirulent strain is more efficiently killed intracellularly than the virulent strain.

Separation of infected macrophages at 24 h following infection gave ratios of PMNL-viable virulent *L. pneumophila* and macrophage-viable virulent *L. pneumophila* of 1:4·7 and 1:51·7 respectively. In contrast, ratios of PMNL-viable avirulent *L. pneumophila* and macrophage-viable avirulent *L. pneumophila* were 1:0·05 and 1:0·075 respectively. These results appear to indicate that the macrophage preferentially supports the growth of the virulent Corby strain compared with the PMNL. The avirulent strain on the other hand appears to be destroyed by both the macrophage and the PMNL.

The difference in virulence between the Philadelphia-1 and Corby strains of *L. pneumophila* may relate in part to their ability to resist the defence mechanisms of the alveolar macrophages as well as the possible production of tissue damaging substances. It appears that the antimicrobial systems of the PMNL are potent for both strains and phagocytosis by the macrophage of the virulent strain appears to protect it and allows its replication.

An examination of the different defence systems of the alveolar phagocytes and the abilities of the avirulent and the virulent strains to cope with these is the basis of current work.

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