J. Hyg., Camb. (1983), 90, 81–89
Printed in Great Britain

Aerosol infection of animals with strains of *Legionella pneumophila* of different virulence: comparison with intraperitoneal and intranasal routes of infection

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(*Received* 10 *July* 1982; *accepted* 20 *September* 1982)

SUMMARY

Infection of guinea-pigs by intranasal (i.n.) instillation of 10^9 viable organisms of two newly isolated strains of *Legionella pneumophila* (74/81, serogroup 1; 166/81, serogroup 3) did not induce disease, but 10^4 organisms administered as a small particle aerosol (< 5 μm diameter) produced a fatal widespread bronchopneumonia within 3 days. Milder illness and less extensive bronchopneumonia were also produced in rhesus monkeys and marmosets by one of these two strains (74/81). Mice were resistant to induction of disease by aerosols of both these two strains, though organisms did persist in the lungs for at least 4 days. Both of these *L. pneumophila* strains were pathogenic for guinea-pigs by aerosol infection over a wide range of doses but the serogroup 1 type strain (NCTC 11192) was not. There was no mortality after infection of guinea-pigs by intranasal instillation of any of these strains but all proved to be fatal after intraperitoneal (i.p.) injection of large doses. Guinea-pigs, rhesus monkeys and marmosets exposed to aerosol infection with *L. pneumophila* provide relevant models for studying the pathogenesis of Legionnaires' disease.

INTRODUCTION

Improvements in isolation procedures have led to an increasing awareness of the widespread distribution of *Legionella pneumophila* in water supplies (Tobin, Bartlett & Waitkins, 1981; Tobin, Beare & Dunhill, 1980; Fliermans *et al.* 1979; Tobin, Swann & Bartlett, 1981) and of the frequency of occurrence of Legionnaires' disease (LD) as a form of pneumonia (Lattimer & Ormsbee, 1981). However, until recently there has been little progress in understanding the disease processes owing to the lack of an experimental animal model. The recognition of a non-pneumonic form of LD, Pontiac fever (Glick *et al.* 1978) (in addition to the pneumonia form) has stimulated speculation as to whether this reflects strain differences in pathogenic determinants of the organism, the route of infection or dose received, or whether it reflects the state of resistance of the host. Effective antibiotic therapy and the development and evaluation of possible vaccines against LD also present urgent problems which a relevant animal model could help resolve.

L. pneumophila is a fastidious, slow-growing organism which is difficult to isolate, especially when associated with faster-growing, less-fastidious contaminating organisms. Guinea-pigs have been used over a number of years for its detection and isolation from water samples and clinical specimens, since this animal is sensitive to *L. pneumophila* and is able to resist infection by most of the contaminating bacteria. Intraperitoneal injection of material containing *L. pneumophila* causes a fibrino-purulent peritonitis (Chandler *et al.* 1979). The organism can usually be isolated from the spleen, liver and peritoneal fluid and sometimes from blood, lung and pleural fluid. Surviving animals show evidence of infection by the development of antibody (Chandler *et al.* 1979; Ormsbee *et al.* 1981). However, this route of infection and disease are not comparable to the situation in man, where infection is now generally considered to be caused by inhalation of airborne *L. pneumophila* (Lattimer & Ormsbee, 1981).

The development of animal models for the study of LD using guinea-pigs, marmosets and rhesus monkeys has been reported previously (Baskerville *et al.* 1981; Baskerville *et al.* in press). This paper compares the pathogenicity for guinea-pigs of a type culture strain and two naturally occurring newly isolated strains of *L. pneumophila* administered by different routes and also compares the response of guinea-pigs, marmosets, rhesus monkeys and mice to aerosol infection with a newly isolated strain of *L. pneumophila*.

MATERIALS AND METHODS

Organisms

Three strains of *L. pneumophila* were used. Strains 74/81 and 166/81 (serogroups 1 and 3 respectively) were isolated in our laboratory from naturally contaminated water supplies and were sub-cultured only 3 times before administration to animals. The other was the serogroup type 1 strain obtained from the National Collection of Type Culture, Colindale, London. The fatty acid profile, together with the morphological, cultural and biochemical features exhibited by these strains, were characteristic of the species.

Strains were grown for 4 days at 37 °C in 5 % CO_2 in air on charcoal–yeast extract agar (CYE) (Edelstein, 1981). Colonies were washed off the plate and resuspended in distilled water. Standardization of inoculum was obtained by a total count using a Helber slide under a dark background illumination. Colony counts on the inoculum using CYE agar were used to confirm numbers of viable organisms administered. Under these conditions the viable count was consistently found to be approximately 20 % of the total count. For culture from tissue, macerates were inoculated onto CYE and incubated as above. The doses indicated in the text refer to numbers of viable organisms inhaled or injected.

Immunofluorescent procedures

Antibody titres were evaluated and the presence of organisms in smears and tissue macerates were demonstrated by indirect immunofluorescence techniques recommended by the Division of Microbial Reagents and Quality Control (DMRQC), PHLS, Colindale, London.

Aerosol generation and respiratory infection

Bacterial aerosols containing particles 5 μm in diameter were generated using a three jet Collison spray at 65% relative humidity in a Henderson-type apparatus (Druett, 1969; Henderson, 1952). Viable organisms in a known volume of aerosol were estimated by collection in an impinger over a known period of time (Henderson, 1952). Guinea-pigs, mice and marmosets were allowed to breathe the aerosol for 5 min and rhesus monkeys for 10 min, details of these procedures have been reported by Baskerville *et al.*(in press). The respiratory/minute volume was estimated according to the method of Guyton (1947) and a 50% retention of inhaled bacteria was assumed, based on the findings of Harper & Morton (1953). Calculated inhaled and retained doses were found to correlate closely with estimates made by viable counts on macerates of lung carried out immediately after exposure of guinea-pigs to the aerosol.

Animals

Female Dunkin–Hartley guinea-pigs of category 4 health status (MRC, 1974), weighing 300–350 g were used and housed in groups of 4–6. Common marmosets (*Callithrix jacchus*) weighing approximately 300 g were kept individually. Rhesus and cynomolgous monkeys of either sex weighing 3–5 kg were anaesthetized by i.m. injection of ketamine hydrochloride (Vetalar, Parke-Davis) prior to infection with aerosols. Random-bred Porton mice were housed in groups of 10 throughout the experimental period. Blood samples from normal guinea-pigs and mice and pre-infection samples taken from monkeys and marmosets showed these animals to be free of antibody to *L. pneumophila*. Histological examination of normal guinea-pig and marmoset lungs revealed no abnormalities.

Clinical observations

Rectal temperatures were recorded at intervals following infection. Monkeys were anaesthetized prior to temperature measurement. Temperatures were considered elevated when they were $\geqslant$ 104 °F ($\geqslant$ 40 °C).

Average survival time (AST)

The average survival time in days was calculated, excluding survivors to the 10th day or beyond, by dividing the total number of days animals survived before dying by the total number of animals which died.

RESULTS

Response of guinea-pigs to infection with three strains of L. pneumophila *administered by different routes*

As Table 1 shows, all three strains of *L. pneumophila* killed guinea-pigs when injected i.p. at a dose of approximately 5×10^9 viable organisms. Deaths occurred within 24 h. At necropsy the pathological findings were typical of an i.p. infection with this organism (Chandler *et al.* 1979). The diffuse fibrino-purulent peritonitis particularly involved the liver and spleen. *L. pneumophila* was detected by the fluorescent antibody technique (FAT) and by cultures from spleen and liver.

https://doi.org/10.1017/S0022172400063877 Published online by Cambridge University Press

Table 1. *Response of guinea-pigs to infection with three strains of* L. pneumophila

Dose:Route	Elevated temperature: No./Total	Elevated temperature: Day	AST (days)	Mortality: dead/total	Antibody titre† (reciprocal of dilution)
		NCTC 11192 (serogroup 1)			
2×10^6:A	−ve	—	ND	0/12	64–128
5×10^5:A	2/12	1	ND	0/12	< 8
4×10^4:A	3/13	1	ND	0/12	< 8
2×10^3:A	2/12	1	ND	0/12	< 8
5×10^9:i.n.	−ve	—	ND	0/8	< 8
5×10^9:i.p.	*	—	< 1	8/8	*
		74/81 (serogroup 1)			
4×10^5:A	9/16	1	2	16/16	NA
	5/9	2	—	—	—
5×10^4:A	1/6	1	2	12/12	NA
	4/6	2	—	—	—
4×10^3:A	2/10	1	4	1/10	< 8
	9/10	2	—	—	—
	10/10	3	—	—	—
2×10^2:A	10/10	3 and 4	ND	0/10	< 8
	4/10	5	—	—	—
5×10^9:i.n.	10/10	1 and 2	ND	0/10	< 8
	3/10	3 and 4	—	—	—
5×10^9:i.p.	*	—	< 1	8/8	NA
		166/81 (serogroup 3)			
3×10^5:A	2/8	1 and 2	2·8	8/8	NA
5×10^9:i.n.	3/8	1	ND	0/6	< 8
	2/8	2	—	—	—
	1/8	3	—	—	—
4×10^9:i.p.	*	—	< 1	8/8	NA

AST, Average survival time; ND, none dead; A, aerosol; i.n., intranasal (0·1 ml each nostril); i.p., intraperitoneal; NA, not assayed.

* Died too early for temperature and antibody estimation.

† Estimated 10 days following infection (< 8 considered negative).

No strain of *L. pneumophila* killed guinea-pigs when administered intranasally (i.n.). The NCTC strain was the only one that failed to induce a temperature when given by this route. No circulating antibody was detected up to 10 days post-infection in guinea-pigs infected with up to 5×10^9 viable organisms of either of the two pyrexia-inducing strains of *L. pneumophila* (Table 1).

Infection by the aerosol route differentiated these strains; the two fresh isolates (74/81, 166/81) caused mortality at inhaled retained doses of 10^3 and 10^4 organisms (both strains had an LD50 by the aerosol route of 10^4), whereas much higher retained doses (approx. 10^6 viable organisms) of the other strain (NCTC 11192) did not kill (Table 1). Nevertheless, this last combination of strain, dose and route produced the only antibody response among the combinations listed in Table 1.

L. pneumophila was detected by culture and FAT in the lungs of all animals which died as a result of aerosol infection. Animals in the terminal stages of disease exhibited ruffled fur, dyspnoea, anorexia and were emaciated. Generally, the disease was peracute, giving an average survival time (AST) after aerosol infection of 2·8 days.

https://doi.org/10.1017/S0022172400063877 Published online by Cambridge University Press

Table 2. *Response of monkeys to aerosol infection with* L. pneumophila *strain 74/81 (serogroup 1)*

Monkey species	Dose	Temperature rise	/ Day	Day killed	Antibody titre† (reciprocal of dilution)	/ Day	*L. pneumophila* detected in lung‡
Rhesus	6×10^6	+ve	3–4	4	< 8	4	+ve
Rhesus	1×10^6	+ve	1–3	4	< 8	4	−ve
Rhesus	2×10^6	+ve	5	5	< 8	5	+ve
Rhesus	6×10^6	+ve	3–6	6	128	6	+ve
Rhesus	1×10^6	+ve	1–2	7	128	7	−ve
Rhesus	6×10^6	+ve	3	10	256	10	+ve
Cynomolgous	2×10^6	−ve	—	36	128	36	−ve
Rhesus	6×10^6	−ve	—	*	256	10	NA

NA, not assayed.
* Monkey survived infection and retained for long term observation and antiserum production
† Titre < 8 considered negative.
‡ Culture and FAT on day killed.

Table 3. *Response of marmosets to aerosol infection with* 4×10^5 L. pneumophila *strain 74/81 (serogroup 1)*

Marmoset	Temperature rise	/ Day	Day killed	*L. pneumophila* detected in lung†
D5	+ve	2 and 3	17	+
113	+ve	2 and 3	10	+
22	−ve	—	4*	+
K	+ve	1 and 2	6	+

* Animal moribund on day killed.
† Culture and FAT on day killed.

Response of monkeys to aerosol infection with L. pneumophila *strain 74/81*

Under the conditions used here the highest dose of organisms administered by aerosol route was approximately 10^6. This dose produced pyrexia in 7 of 8 monkeys over 1–5 days following exposure to the aerosol and an antibody response in 5 of 8 monkeys. At necrospy 4 of 7 monkeys were shown to have *L. pneumophila* in the lungs by culture and FAT (Table 2). No animal died as a result of aerosol exposure and it was apparent that the infection produced a milder form of LD in monkeys than in the guinea-pig.

Response of marmosets to aerosol infection with L. pneumophila *strain 74/81*

Although no marmoset died during the periods indicated (Table 3), after aerosol infection 3 of 4 animals had pyrexia and all were dull and anorexic. The most severely affected animal showed respiratory distress and was moribund on day 4 when it was killed. *L. pneumophila* was detected in lung macerates from all four animals by culture and by FAT (Table 3).

The disease induced in the marmosets was thus intermediate in severity between the fulminating, peracute pneumonia in the guinea-pigs and the mild illness produced in rhesus monkeys.

Table 4. *Numbers of viable* L. pneumophila* *in mouse lungs following aerosol infection with strain 166 (serogroup 3)*

Day 0†	Day 2·	Day 4	Day 7
3×10^4	2×10^3	4×10^1	< 10
$2{\cdot}5 \times 10^4$	1×10^4	1×10^2	< 10
5×10^4	5×10^3	5×10^1	< 10

* Detected by viable counts on lung macerate (all mice exposed as a group to same aerosol concentration).

† Day 0 lung samples taken and macerated immediately following animal exposure to aerosol. Similar results were obtained with *L. pneumophila* strain 166/81 (serogroup 3).

Response of mice to aerosol infection with L. pneumophila

As shown in Table 4, retained infective doses of $2{\cdot}5–4 \times 10^4$ of *L. pneumophila* were progressively cleared from the lungs of Porton mice and by day 7 could no longer be detected. Other mice similarly infected appeared healthy throughout the period of the experiment (10 days), exhibiting no illness or deaths, and when bled at the end of the experiment an antibody response could not be detected. This was not a progressive infection and the concentration of *L. pneumophila* in the lungs was insufficient to stimulate an antibody response.

Histopathology

At 24 h guinea-pigs exposed to aerosol infection exhibited numerous pneumonic foci with accumulation of neutrophils and macrophages in alveoli in all regions of the lungs. By 48 h there was oedema, fibrin exudation, haemorrhage, collapse and infiltration of alveoli and interalveolar septa by macrophages. The epithelium of bronchi was not affected and only occasionally was there necrosis of terminal bronchiolar epithelium. Rhesus monkeys and marmosets displayed similar changes, those in marmosets being more severe and widespread.

In infected mice, pathological lesions were limited to small areas of thickening of interalveolar septa caused by infiltration of macrophages and capillary congestion and these persisted to the 7th day. In the early stages of infection small numbers of macrophages and occasional polymorphs were present in alveoli.

DISCUSSION·

Whole-body exposure to *L. pneumophila* aerosols has been attempted by Berendt *et al.* (1980), but these experiments were confined to the use of guinea-pigs. Fever and death were observed in some of these animals, but pathological changes were not reported. Whole-body exposure may be a less precise method of infection than that used in this work and may lead to difficulties in interpretation and dose estimation, since the organism may also contaminate the conjunctiva and body surface. Also in Berendt's infections there was apparently no control of the relative humidity within the system, although preliminary studies (Broster & Hambleton, personal communication) on aerosols indicate that at least one of the virulent strains of *L. pneumophila* survives well at a wide range of relative humidity values.

Of the four animal species exposed to aerosols of *L. pneumophila* the mouse was

the only one in which infection did not become established and cause lung lesions and death. Although in mice lung infection persisted until approximately 4 days following exposure to an aerosol, no increase in viable bacterial numbers was observed, nor did these organisms induce an antibody response. This suggests that the Porton mouse strains may be resistant to *L. pneumophila* infection. However, mouse strain difference may influence susceptibility to infection and future investigations using other strains of mice are proposed. Mice were also resistant to i.p. infection, but have been shown to be susceptible to i.c. infection with virulent strains, a purulent ependymitis resulting (Fitzgeorge, unpublished observations).

Although numbers of monkeys used in these experiments were necessarily small, it is evident that these animals can be infected with aerosols of *L. pneumophila* and subsequently mount an immune response. This, together with the detection of the causative organism in the lungs of over half the animals examined, indicates that rhesus monkeys are susceptible to infection.

All marmosets killed at varying times after exposure had viable organisms in the lungs. However, since anti-marmoset serum fluorescein conjugate was not available it was not possible to carry out an immunofluorescence test to determine antibody response. Nevertheless, as for the monkey, marmosets may be infected with *L. pneumophila* aerosols without dying, although the lung lesions in these animals were more extensive than those in the monkey. Both these species are of value in representing a milder form of LD which is clinically similar to the majority of human cases and which could be modified by immunosuppression and used as a model to study the role of the immune response to LD.

The design of the Henderson apparatus did not allow a higher dose of organisms than approximately 10^6 being given to monkeys by the aerosol route. Calculations based on weight and lung surface area (Baskerville *et al.*, in press) show that this dose is, by comparison, approximately 30-fold less than that administered as a lethal dose (approximately 10^4 organisms) to guinea-pigs. A higher dose, if this could be administered, might kill in monkeys. However, similar calculations indicate that marmosets and guinea-pigs used in these experiments have comparable weight and lung surface areas and responses to doses administered as aerosols can be directly compared.

The responses of guinea-pigs to aerosol infection with *L. pneumophila* closely resemble pneumonic LD in man in terms of pulmonary lesions and bacteriological findings. Death generally occurred in guinea-pigs within 4 days of exposure to the aerosol at inhaled and retained doses of approximately 10^3–10^5 organisms, and even 10^2 organisms were sufficient to induce an elevated temperature up to 5 days after exposure (Table 1). In spite of this, these doses were apparently insufficient to give rise to an antibody response in surviving animals and indeed 10^9 organisms given intranasally did not produce seroconversion. This may be because the inoculum was not retained for a sufficient period in the respiratory tract and findings in both instances may depend on method and time of sampling.

It is of interest that a laboratory-adapted strain (NCTC 11192) that caused a fatal disease when given i.p. to guinea-pigs, did not when administered by the aerosol route (an LD 50 of approximately 10^8 organisms for i.p. infection is common to all strains). This appears to add weight to other workers' observations (Ormsbee

et al. 1981; McDade & Shephard, 1979) that virulence may be lost by *in vitro* cultivation. It also appears to confirm that i.p. inoculation is of use in the isolation of strains of differing virulence which may be present in samples from environmental sources. Further evidence that human LD results from airborne infection is provided by the observation that the guinea-pig is most sensitive to infection (in relation to the two freshly isolated strains) when this is administered as a fine particle aerosol.

Probably the most significant finding is that while approximately 10^3–10^4 *L. pneumophila* caused a fatal infection in guinea-pigs when given as an aerosol, 10^9 organisms of the same preparation given intranasally did not. The most obvious difference between these methods is the size of the infective particles and their site of deposition in the respiratory tract. The aerosols generated with a Collison spray in the Henderson-type apparatus contain a majority of particles of 5 μm in diameter (Henderson, 1952) which would penetrate to and be deposited on the surface of alveoli and terminal bronchioles. Intranasal instillation on the other hand results in massive flooding of the upper respiratory tract only and produces relatively few particles small enough to be deposited in the terminal respiratory bronchioles and alveoli. The pneumonic lesions produced by aerosol infection in guinea-pigs, rhesus monkeys and marmosets were very similar to that seen in LD in man and have been compared in detail previously (Baskerville *et al.*, in press).

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