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Infectivity of *Pasteurella tularensis* clouds

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INTRODUCTION

Schlamm (1960) showed with mice that the respiratory infectivity of *Pasteurella tularensis* held in clouds for at least 6 hr. was lower than that in clouds held for 3 sec. Schlamm, Goldberg & Dimmick (1959) reported similar results with guinea-pigs. Pirsch, Day, Bailey & Griffith (1959) showed correlation of this loss with age of culture, but failed to demonstrate infectivity loss in guinea-pigs challenged peritoneally with cloud cells held $5\frac{1}{2}$ hr. This paper describes an investigation of *P. tularensis* infectivity in clouds of various ages using mice and guinea-pigs as test animals, and respiratory and intra-peritoneal injection as challenge routes.

MATERIALS AND METHODS

The highly virulent Schu D strain of *P. tularensis* was used throughout the investigation. Suspensions were prepared initially by shake culture at 37° C. in a partially hydrolysed casein medium 'MCPH', similar to that described by Mills, Berthelsen, Donaldson & Wilhelm (1949). This contained casein hydrolysate, yeast extract, cysteine, glucose, thiamine, sodium chloride and sodium phosphates. The casein was hydrolysed with sulphuric acid and neutralized with sodium hydroxide, and the medium therefore also contained sodium sulphate.

Subsequently growth media based on the medium described by Hood (1961) were introduced. These were 'CBPC': cysteine (0·1 %), glucose (2·5 %), peptone (2 %), catalase (0·03 %), plasma (5 %) and sodium chloride (0·5 %); and 'CBC', in which the plasma was omitted. All suspensions were stored at 0 to +4° C. and pH 6·6–6·8, and spray fluids were adjusted if necessary to a similar pH.

Clouds were generated in a Henderson apparatus (1952) and stored in a 75 l. stainless steel drum rotating at 3 r.p.m. to reduce physical loss by sedimentation (similar to that described by Goldberg, Watkins, Boerke & Chatigny, 1958). An exposure tube to accommodate eight guinea-pigs was attached to the apparatus. Provision was made for passing the cloud produced either directly to the exposure tube, if a young cloud was to be tested, or to the drum if the cloud was to be held for some time. The aged cloud could then be drawn from the drum into the exposure tube and then into an impinger ('raised impinger': May & Harper, 1957), which acted as a critical orifice to meter the air flow and also provided a sample for assessing the concentration of viable cells to which the guinea-pigs had been exposed. The impingers had a flow rate of about 11 l./min. and contained 10 ml. of sampling fluid. The withdrawn cloud was replaced in the drum by air conditioned to the same temperature and humidity.

Guinea-pigs *ca.* 370 g. in weight were used for respiratory exposure, twenty-four being exposed for each test in groups of six or eight. Challenge by the respiratory route was made by exposure for periods varying from 20 sec. to 6 min. according to the expected viable cell concentration of the cloud. Guinea-pigs and mice (*ca.* 20 g.) were challenged intra-peritoneally with 0·2 ml. of the sample (diluted as necessary) obtained during exposure of guinea-pigs. The animals were held for 10 days and deaths recorded.

The sampling and diluting fluid used was cysteine broth (1 % peptone, 2·5 % glucose, 0·1 % cysteine HCl, and 0·01 % Dow Corning antifoam, at pH 6·8). This fluid allowed fragile cells of *P. tularensis* to survive in it after impinger collection for long periods at various temperatures without multiplication.

Spray fluids used were either the spent mother liquors (ML), centrifuged to remove nearly all of the cells, or the unused media with various additions.

In calculating the respiratory dose for the guinea-pig a breathing rate of 129 ml./min. (Guyton, 1947) coupled with a retention factor of 0·55 was used (Harper & Morton, 1953). Thus the calculated retained dose was taken as one-fourteenth of the measured *Nt* (cells/l. × time in min.). For assessment of LD 50's and fiducial limits the method of Peto (1953) was used.

Viable cell assays of the organism were made on glucose–cysteine agar with human blood, or with plasma and catalase (Hood, 1961). Viability of the clouds was assessed by the ^{32}P-tagged cell method of Harper, Hood & Morton (1958) using killed tagged *P. tularensis* cells as tracers.

All clouds were held at 21–24° C., and relative humidities of 55 or 85 %.

RESULTS

MCPH-grown cultures

Infectivity, unless otherwise stated, refers to respiratory challenge in the guinea-pig. No significant loss of infectivity was found in clouds aged 20 min. at 55 % R.H. or 8 hr. at 85 % R.H. using cultures less than 2 weeks old; the viability of the clouds was *ca.* 1·0 and 50 % respectively. Significant infectivity loss (respiratory and peritoneal) for guinea-pigs was found in clouds held 20 hr. at 85 % R.H. (Table 1). Where 3 sec. clouds gave a respiratory and peritoneal LD 50 of 1–4 cells, 20 hr. clouds gave a LD 50 of 4–360 cells respiratory and 26–114 cells peritoneally, the LD 50 increasing with suspension age. (With a 50-day-old suspension, even 3 sec. clouds showed loss of infectivity.) Loss of infectivity by the respiratory route was always accompanied by a loss of infectivity by the peritoneal route.

The results obtained from peritoneal challenge in mice confirmed those reported by Schlamm (1960): i.e. no significant loss of infectivity, although cells from the same 20 hr. cloud samples showed both peritoneal and respiratory infectivity loss in the guinea-pig. Further investigation of cloud infectivity was therefore confined to 20 hr. clouds and guinea-pigs challenged by the respiratory route.

Attempts to overcome loss of infectivity were made by spraying the *P. tularensis* from resuspensions in fluids other than ML. Two of the fluids used were cysteine broth and the liquid medium CBPC without sodium chloride. There was no significant difference between the LD 50's obtained with cysteine broth and ML.

With CBPC there was an apparently significantly greater infectivity in that 64 cells were required for an LD 50 as compared with 410 cells (Table 2).

CBPC and CBC media and influence of sodium chloride

The cysteine-catalase media with plasma (CBPC) or without plasma (CBC), unlike MCPH medium, promote growth of *P. tularensis* from small inocula (< 10 cells/ml.). It was therefore likely that these cells would have different

Table 1. *LD* 50 (*guinea-pig*) *of* Pasteurella tularensis *clouds aged* 3 *sec. and* 20 *hr.*

(MCPH cultures sprayed from ML.)

Batch	Age (days)	3 sec. cloud		20 hr. cloud	
		IP	RESP	IP	RESP
		LD 50, cells			
57	6	1·4 (0·86–3·3)	1·4 (0·9–3·4)	27 (8·4–∞)	4·2 (2·1–350)
	12	1·5 (0·87–5·0)	1·2 (0·6–11)	26 (15–83)	145 (50–∞)
	22	3·1 (1·5–∞)	2·2 (1·4–5·1)	92 (52–380)	72 (24–∞)
	37	1·1 (0·68–2·5)	4·2 (2·5–13)	114 (62–630)	340 (144–∞)
	50	12 (4·7–∞)	46 (23–2800)	57 (25–∞)	350 (240–1100)
56	6	—	2·2 (1·4–5·3)	—	59 (37–144)
	21	—	3·9 (1·8–∞)	—	170 (110–490)
58	7	—	0·5 (0·3–1·2)	—	59 (34–200)
	28	—	1·6 (0·9–6·2)	—	360 (150–∞)
60	19	—	—	—	82 (52–180)
	41	—	—	—	200 (110–1100)

MCPH = hydrolysed casein medium; ML = mother liquor; Figures in parenthesis are 95 % confidence limits.

Table 2. *Respiratory* (*guinea-pig*) *LD* 50 *of* Pasteurella tularensis *clouds aged* 20 *hr.*

(MCPH cultures sprayed from ML and other fluids.)

Batch	Age (days)	Spray fluid	LD 50, cells
59	38	Cysteine broth	140 (88–340)
	40	ML	410 (260–950)
	45	Cysteine broth	280 (96–950)
	48	CBPC	64 (40–170)

CBPC = cysteine glucose catalase plasma medium; ML = mother liquor; MCPH = hydrolysed casein medium.

characteristics from those grown in MCPH. This proved to be so (Table 3). Cells grown in CBPC or CBC and sprayed from ML or fresh medium with NaCl showed a reduction in infectivity. For example, a 20 hr. cloud from an old (43-day) suspension gave an LD 50 of 196 cells. The reduction in infectivity increased with salt concentration and suspension age. When, however, sodium chloride was omitted from the spray fluid (whether CBPC or CBC) or the growth medium CBC (when used as spray fluid), no infectivity was lost in 20 hr. old clouds formed from suspensions stored for 7–10 weeks.

Growth of *P. tularensis* in CBPC and CBC was comparable with that in MCPH (*ca.* 4×10^{10} cells/ml.) in shake culture at 37° C. When sodium chloride was omitted from CBPC or CBC only about one-quarter of this growth was obtained, except when one batch of peptone was used which was unusual in having a chloride content of 13·5 %, w/w (expressed as NaCl). Batches of peptone used in subsequent tests contained 0·7 %, w/w, or less so that as used (2 %) they contributed, at the most, 0·014 % salt to the medium. Cells grown without added sodium chloride and with three different brands of peptone had similar infectivity characteristics to those grown with it.

Table 3. *Respiratory LD* 50 (*guinea-pig of* Pasteurella tularensis *clouds aged* 20 *hr.*

(Cultured other than in MCPH, and various spray fluids.)

Batch	Growth medium	Age (days)	Spray fluid	LD 50, cells
61 D	CBPC with NaCl	2	ML	59 (20–∞)
		4	CBPC	4·1 (2·5–10)
		16	ML	> 49 (No deaths)
		23	CBPC	4·9 (2·8–20)
		43	CBPC with NaCl*	196 (120–460)
		44	CBPC	4 (2·4–13)
H	CBC with NaCl	4	CBC with NaCl*	12 (7–36)
		5	CBC	2·8 (1·6–8·7)
		12	CBC with NaCl	27 (13–∞)
		13	ML	24 (12–1780)
		18	CBC	3 (1·9–7·2)
		19	CBC with NaCl	14 (7·9–47)
E	CBC	4	ML	8·3 (5·2–21)
		5	ML	3·4 (2·1–8·4)
		38	CBC with NaCl	5·8 (3·5–19)
		39	CBC with 1·0 % NaCl	43 (19–∞)
		70	CBC	4·2 (2·5–13)

ML = mother liquor; CBPC = cysteine glucose catalase plasma medium; CBC = cysteine glucose catalase medium.

* NaCl = concentration 0·5 % unless otherwise stated.

Influence of anions and cations in spray fluids

Sodium chloride was replaced in CBC by sodium sulphate or sodium phosphates (equal parts di-sodium hydrogen phosphate and sodium di-hydrogen phosphate) to give the same sodium concentration; potassium chloride to give the same chloride concentration; potassium sulphate to give potassium concentration equal to that in the potassium chloride. Each of these fluids, and CBC with and without sodium chloride, were used as spray fluids to form *P. tularensis* clouds. It was found that only clouds formed from CBC containing sodium or potassium chloride lost infectivity—clearly implicating the chloride ion as the responsible factor (Table 4).

MCPH medium without sodium chloride

Cells were grown in MCPH medium without added sodium chloride (chloride 0·04 % as NaCl) and about one-half of the usual concentration of viable cells was obtained. 20 hr. clouds sprayed from this ML lost infectivity progressively with the age of the culture like those grown previously in MCPH with 0·5 % NaCl. Similar loss occurred when the cells were sprayed from the MCPH and CBC media without sodium chloride (Table 5).

Table 4. *Respiratory LD* 50 (*guinea-pig*) *of* Pasteurella tularensis *clouds aged* 20 *hr.*

(Influence of anions and cations.)

Batch	Growth medium	Age (days)	Spray fluid = CBC Additive:	LD 50, cells
R 4	CBC	57	0·5 % NaCl	76 (45–262)
		59	None	19 (8·4–∞)
		64	0·6 % Na_2SO_4	30 (13–∞)
		65	None	24 (13–122)
1	CBC + 0·6 % Na_2SO_4 and no NaCl	21	0·5 % NaCl	2·9 (1·7–8·6)
		23	0·6 % Na_2SO_4	1·8 (1·1–4·8)
		28	1·0 % NaCl	25 (13–206)
		29	1·2 % Na_2SO_4	2·4 (1·5–7·7)
		30	Spent growth medium	5·3 (3·3–12)
		36	1·5 % Na phosphates	3·1 (2–7·2)
		37	1·0 % NaCl	21 (13–54)
		44	0·75 % K_2SO_4	1·6 (1–3·6)
		49	1·3 % KCl	6·3 (3·9–15)
		51	1·5 % K_2SO_4	4·2 (2·5–13)
		68	None	2·8 (1·7–7·2)
		71	0·5 % NaCl	15 (9·6–36)
		72	1·3 % KCl	> 50 (No deaths)
		87	1·5 % K_2SO_4	14 (15–77)
		90	None	15 (8·2–78)

CBC = cysteine glucose catalase medium.

Table 5. *Respiratory LD* 50 (*guinea-pig*) *of* Pasteurella tularensis *clouds aged* 20 *hr.*

(MCPH without sodium chloride cultures.)

Batch	Age (days)	Spray fluid	LD 50, cells
M	5	ML	1·1 (0·66–3·5)
	13	MCPH (no NaCl)	3·7 (2·3–8·7)
	28	MCPH (no NaCl)	45 (28–112)
	33	ML	> 32 (No deaths)
	41	CBC	44 (28–99)

ML = mother liquor; MCPH = hydrolysed casein medium; CBC = cysteine glucose catalase medium.

It was apparent that *P. tularensis* grown in MCPH with or without salt had an inherent characteristic of losing infectivity independent of the effect of chloride ion. To test the remaining ingredients of MCPH it was therefore necessary to use

CBC-grown organisms. They were found to lose infectivity when sprayed from MCPH (without salt) or from CBC to which was added MCPH ingredients—yeast extract (0·4 %) or casein hydrolysate (10 %). Although very old suspensions (10–13 weeks) were used for these tests, the control cloud (sprayed from CBC) before and after the tests, had LD 50's of 2·8–15 cells, whereas test clouds had LD 50's of 33–65 cells (Table 6). These were the results obtained with the organism grown in CBC with Na_2SO_4 replacing NaCl. A culture in CBC with sodium phosphates replacing NaCl behaved similarly.

Table 6. *Influence of MCPH medium* (*omitting* NaCl) *and ingredients on* Pasteurella tularensis *grown in CBC*

Batch	Growth medium	Age (days)	Spray fluid	20 hr. cloud LD 50, cells
1	CBC + 0·6 % Na_2SO_4 and no NaCl	68	CBC	2·8 (1·7–7·2)
		71	MCPH	33 (18–154)
		73	MCPH	65 (40–166)
		85	CBC + 10 % cas. hyd.	52 (24–∞)
		86	CBC + 0·4 % yeast	33 (21–81)
		90	CBC	15 (8·2–78)

CBC = cysteine glucose catalase medium; cas. hyd. = casein hydrolysate; MCPH = hydrolysed casein medium.

Sodium chloride alternatives for growth in CBC

It was found that sodium sulphate or sodium phosphate adequately replaced sodium chloride in CBC for growth of the organisms. The cells produced lost no infectivity after 20 hr. cloud ageing when sprayed from their ML after at least 10 weeks storage of cultures. The results obtained with suspensions grown with sodium sulphate instead of sodium chloride are shown in Tables 4 and 6.

Relationship between infectivity and viability

The viability of *P. tularensis* at 20 hr. in clouds held at 85 % R.H. at 21–24° C. varied from 0·5 to 40 % depending to some extent upon type of growth medium but mainly upon the age of the suspension. It is important to note that viability had no correlation with infectivity. Clouds of similar viability often had markedly different infectivity; some highly infective clouds were low in viability (about 1 %) and some highly viable (40 %) ones were greatly reduced in infectivity.

The cells grown in CBC with sodium sulphate (0·6 %) or sodium phosphate (0·7 %) replacing sodium chloride were of similar viability to those grown in MCPH in clouds held at 55 and 85 % R.H.

DISCUSSION

The information presented implicates chloride ion, yeast extract and casein hydrolysate as factors causing loss of infectivity of *P. tularensis* clouds. The magnitude of the loss depends upon the animal species, method of challenge, the concentration of the ion, and the age of the culture and cloud. Thus, in guinea-pigs

challenged by either the respiratory or peritoneal route, the loss is of a similar magnitude with a tendency towards greater loss by respiratory challenge. In mice no loss occurs by the peritoneal route. Conditions for survival and growth of *P. tularensis* are presumably better in the peritoneum of the mouse than in the lungs or peritoneum of the guinea-pig.

What happens to the organism to reduce its infectivity is not yet known. Experiments with droplets of suspension on a watch-glass showed that the salt concentration of the suspension increased 24-fold in coming to equilibrium at 85% R.H.; a similar increase can be expected in airborne particles at this R.H. After dilution in collecting fluid, growth takes place *in vitro* on solid media. Dilution would also be expected *in vivo* and the salt is therefore unlikely to affect growth. Yeast extract and casein hydrolysate would also not be likely to inhibit growth *in vivo* at the concentration present. It is probable that the organism loses infectivity because of a fundamental change, during cloud ageing, which reduces its capacity for growth *in vivo*.

Preliminary tests with stored suspensions (MCPH cultures) showed that their lag phase (as determined by successive viable counts of suspensions held in CBPC at 37° C.) increased with length of storage: e.g. 1-, 4- and 8-month-old cells had lag phases of 5, 9 and 10 hr. respectively. Furthermore, many (50–90%) of the organisms in the older suspensions died during the lag phase. It may well be that the less infective airborne cell behaves similarly and so has insufficient time to multiply before being overcome by the body defences. Little is known of the fate of such cells in the lungs of guinea-pigs and it is a subject to be investigated at some future date.

It was observed in some tests with less infective cells that some of the animals surviving displayed signs of sickness similar to animals that died of tularaemia. They recovered from these symptoms and appeared quite normal after 1–2 days. It was apparent that loss of infectivity could probably be associated with loss of virulence (defined as ability to kill). Whether loss of virulence was wholly responsible for what is called loss of infectivity in this paper was outside the scope of this work.

The different characteristics of the organism when grown in different media show that the ingredients influence its infectivity when airborne. *P. tularensis* grown in CBC retains infectivity for much longer periods than cultures in MCPH. The latter also have the characteristic of losing infectivity even when sprayed from CBC (without NaCl) which as far as is known contains no infectivity depressors.

The results presented emphasize the need to use more than one animal species and various challenge routes for determining the infectivity of clouds of *P. tularensis*, and also indicate the importance of culture and spray media. The effect of chloride ion is of particular interest because of its natural occurrence in the animal body and its presence in most culture media.

SUMMARY

The infectivity of *Pasteurella tularensis* clouds decreases with age, for guinea-pigs challenged by the peritoneal and respiratory routes. No decrease occurs in mice challenged peritoneally. The loss of infectivity depends to some extent on age of culture but more especially on culture medium used. Cultures grown in a partially hydrolysed casein medium and aged 2–3 weeks lose infectivity severely in clouds held 20 hr. at 85 % R.H., whereas if grown in cysteine broth with catalase (CBC) no loss occurs in this time until cultures are aged several months. Chloride ion, yeast extract and casein hydrolysate are shown to be infectivity depressors when used in culture media or when added to suspensions before spraying. Sodium chloride in CBC can be replaced adequately by sodium sulphate or sodium phosphate for growth of the organism.

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