STUDIES ON RESPIRATORY INFECTION

III. EXPERIMENTS WITH *BRUCELLA SUIS*

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(With 2 Figures in the Text)

In an earlier paper (Elberg & Henderson, 1948) it was shown that the guinea-pig is highly susceptible to infection with *Brucella suis* by the respiratory route. However, the experiments were defective to the extent that intercurrent infection took a toll of the exposed animals, and the influence of this on the initiation and course of Brucellosis could not be known. It was decided to re-investigate the problem using animals from a proven healthy stock and also to use newer techniques for aerosol production which would allow examination of the influence of cloud particle size on infectivity. This paper describes the experiments made, and discusses the results in the light of what is known concerning the pathogenesis of disease initiated in the respiratory tract.

METHODS

(*a*) *Strain of* Brucella suis. This was a mutant of the strain used by Elberg & Henderson (1948). It was reputed to have a lower 'dissociation rate'. It gave highly reproducible results in *in vitro* culture, and its infectivity remained unaltered throughout the course of the experiments. The 50 % infective dose (ID_{50}) by the subcutaneous route was about five organisms. It was maintained as a dried stock culture on Stamp's (1947) medium.

(*b*) *Bacterial suspensions for cloud production.* These were made as described by Harper (1955). Preparations remained stable over many weeks, but they were not used after more than 10 days' storage at 0–4° C. The suspending fluid (Zobell) retarded drying of droplets dispersed from the sprays, therefore, immediately prior to use, the required amount of suspension was centrifuged and resuspended in freshly prepared glass-distilled water. Where large particles were used as a cloud they were 'bulked' with an inert chemical such as dextrin. By this means the number of organisms per particle was reduced to one or a very few.

(*c*) *Animals.* Guinea-pigs weighing 350–400 g. at the time of exposure were used. They were allocated to their groups by random sampling. After exposure they were housed five in a cage, and held for 4 weeks before examination. In early experiments normal animals were housed with the experimental ones to check the possibility of cross-infection; this occurred only when exposed animals had received extremely high concentrations of organisms as large particles. Clearly, cross-infection played no part in determining final results.

(*d*) *Diagnosis of infection.* Animals were killed by chloroform anaesthesia. The macroscopic appearance of spleen, liver, cervical and bronchial lymph glands were

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recorded. Cultures were made by rubbing the cut surfaces of spleen and glands on fortified tryptose agar (Harper, 1955) and incubating at 37° C. for 3 days. If doubts as to the identity of developing colonies arose, recourse was made to slide-agglutination technique using specific *B. suis* anti-serum.

(*e*) *Apparatus for producing infective aerosols.* As previously described (Henderson, 1952; Druett & May, 1952).

(*f*) *Cloud sampling, assessment, measurement of particle size and definition of terms used in expressing results.* As previously described (Druett, Henderson, Packman & Peacock, 1953; Druett, Robinson, Henderson, Packman & Peacock, 1956). Since animals rarely die of brucella infection, the term *INt* is used to define infecting dosage, rather than *LNt* used previously to express lethality.

RESULTS

Regression line data. Plans for experiments and assessment of results were made on similar lines to those used previously (Druett *et al.* 1953, 1956). The results are summarized in Tables 1–5 and shown graphically in Fig. 1. The data obtained

Table 1. *Response of guinea-pigs to clouds of single organisms* (Brucella suis)

Expt.							
1	$Nt \times 10^{-2}$	0·82	1·36	2·19	3·64	5·73	
	Infected/40	10	13	29	31	35	
	% infected	25	32·5	72·5	77·5	87·5	
		$INt\,50 \times 10^{-2}$ 1·3–1·6–1·9 orgs. min./l.					
		Slope 2·13	Variance 0·11	χ^2/n 1·1.			
2	$Nt \times 10^{-2}$	0·94	1·41	2·27	4·13	5·93	
	Infected/20	4	6	7	13	18	
	% infected	20	30	35	65	90	
		$INt\,50 \times 10^{-2}$ 1·9–2·5–3·3 orgs. min./l.					
		Slope 2·38	Variance 0·24	χ^2/n 0·91.			
3	$Nt \times 10^{-2}$	1·4	3·63	3·90	4·12	5·19	9·24
	Infected/30	15	17	23	25	23	30
	% infected	50	56·7	76·7	83·3	76·7	100
		$INt\,50 \times 10^{-2}$ 0·9–1·8–2·3 orgs. min./l.					
		Slope 1·98	Variance 0·18	χ^2/n 1·16.			
4	$Nt \times 10^{-2}$	0·87	1·62	2·8	5·25	6·33	
	Infected/20	6	8	11	13	17	
	% infected	30	40	55	65	85	
		$INt\,50 \times 10^{-2}$ 1·25–2·2–3·2 orgs. min./l.					
		Slope 1·5	Variance 0·17	χ^2/n 0·5.			
	Combined data	$INt\,50 \times 10^{-2}$ 1·67–1·9–2·2 orgs. min./l.					
		Slope 2·07	Variance 0·04	χ^2/n 0·9.			

with single organism clouds were sufficiently constant to allow the use of 'one point assay' (Peto, 1953) with single organism clouds to check the constancy of the suspensions in comparative experiments with large particles. It was found that none of these control experiments gave significantly different values for the *INt*50 from the mean value for single organisms given in Table 1. Therefore, in making

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Table 2. *Response of guinea-pigs to 2·5 μ diameter particles* (Brucella suis)

(Combined data from two experiments.)

$Nt \times 10^{-2}$	1·15	1·78	2·05	2·64	3·46	3·56	5·81	7·29	12·9	17·9
Infected/exposed	8/30	21/30	14/40	23/30	32/50	23/30	36/40	30/30	35/40	30/30
% infected	27	70	35	77	80	77	90	100	87·5	100

$INt50 \times 10^{-2}$ 1·0–1·5–2·0 orgs. min./l.
Slope 2·1 Variance 0·09 χ^2/n 2·4.
Single organism control data: $INt50 \times 10^{-2}$ 1·1–1·6–3·0 orgs. min./l.

$$\text{Ratio: } \frac{INt50 \text{ for } 2{\cdot}5\,\mu \text{ particles}}{\text{Mean } INt50 \text{ for single organisms}} = 0{\cdot}55\text{–}0{\cdot}8\text{–}1{\cdot}15.$$

Table 3. *Response of guinea-pigs to 5 μ diameter 'bulked' particles* (Brucella suis)

(Combined data from two experiments.)

$Nt \times 10^{-3}$	0·38	0·45	0·98	1·16	1·17	1·46	1·88	2·22	3·42	7·47
Infected/40	3	8	13	15	21	17	26	27	29	40
% infected	7·5	20	33	38	53	43	65	68	73	100

$INt50 \times 10^{-3}$ 1·18–1·35–1·55 orgs. min./l.
Slope 2·09 Variance 0·035 χ^2/n 0·9.
Single organism control data: $INt50 \times 10^{-2}$ 0·8–1·3–3·9 orgs. min./l.

$$\text{Ratio: } \frac{INt50\ 5\,\mu \text{ 'bulked' particles}}{\text{Mean } INt50 \text{ single organisms}} = 6{\cdot}0\text{–}7{\cdot}0\text{–}8{\cdot}5.$$

Table 4. *Response of guinea-pigs to 7·6 μ diameter 'bulked' particles* (Brucella suis)

$Nt \times 10^{-3}$	0·67	1·16	3·25	4·73	17·5	22·9
Infected/30	11	8	14	20	22	26
% infected	37	27	47	68	73	87

$INt50 \times 10^{-3}$ 1·5–2·6–4·2 orgs. min./l.
Slope 0·96 Variance 0·03 $\chi^2/n = 1{\cdot}11$.
Single organism control data: $INt50 \times 10^{-2}$ 1·1–1·7–3·8 orgs. min./l.

$$\text{Ratio: } \frac{INt50 \text{ for } 7{\cdot}6\,\mu \text{ particles}}{\text{Mean } INt50 \text{ for single organisms}} = 8\text{–}14\text{–}23.$$

Table 5. *Response of guinea-pigs to 12 μ diameter particles* (Brucella suis), *each containing from 7–70 organisms/l.*

(Combined data from two experiments.)

$Nt \times 10^{-3}$	9·26	10·2	18·4	19·3	33·9	34·4	43·3	87·9	88·5	207	515
Infected/40	1	6	6	6	8	10	18	14	15	29	31
% infected	2·5	15	15	15	20	25	45	35	37·5	73	78

$INt50 \times 10^{-3}$ 87·5–118–1600 orgs. min./l.
Slope 1·45 Variance 0·03 $\chi^2/n = 0{\cdot}88$.
Single organism control $INt50 \times 10^{-2}$ 1·8–2·4–3·3 orgs. min./l.

$$\text{Ratio: } \frac{INt50 \text{ for } 12\,\mu \text{ particles}}{\text{Mean } INt50 \text{ for single organisms}} = 440\text{–}620\text{–}870.$$

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comparisons of the $INt50$ values at various particle sizes, the mean value of $INt50$ for single organisms was used as the reference standard.

The slope of the log-dose *v.* probit mortality regression line obtained with single organism, 2·5 and 5μ diameter clouds follows closely that predicted (1·9) on the basis of random chance infection (Druett, 1952). The slopes for 7·6 and 12μ diameter particles are significantly different from the others.

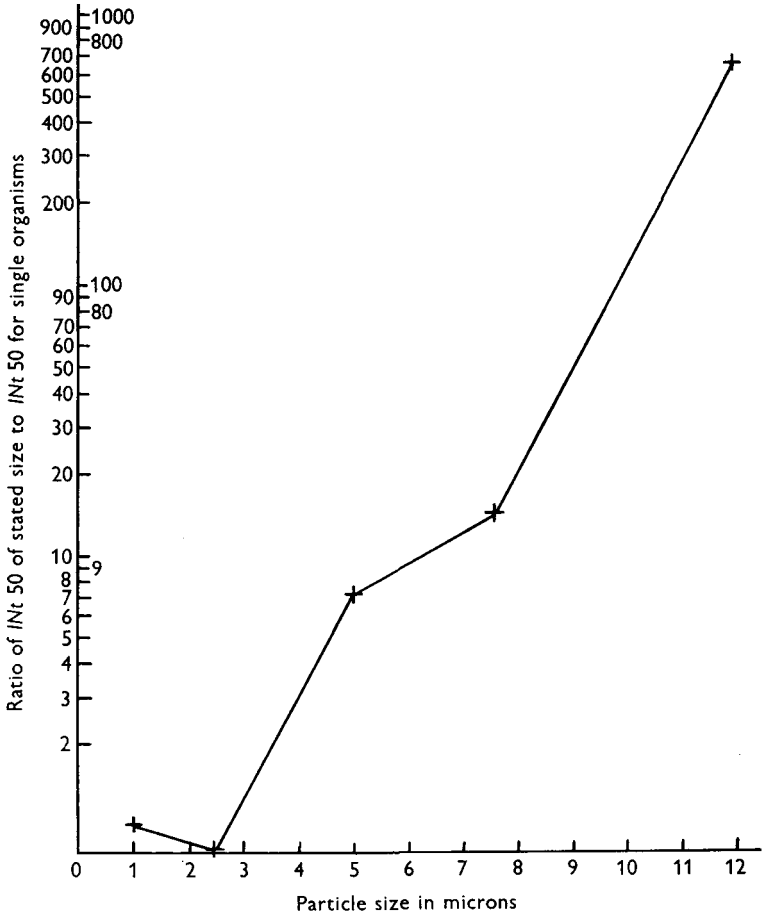

Fig. 1.

An outstanding feature of the results is the increase in $INt50$ dosage in passing from clouds of 2·5μ diameter to 5μ and thence to 12μ diameter particles where the dosage is about 600-fold greater than with single organism clouds.

It has been noted that the 50% infecting dose by the subcutaneous route with the strain of *Br. suis* used in these experiments contained about five organisms. Accepting a respiratory minute volume for the guinea-pigs used of 150 ml., and an immediate lung retention of single organism clouds of 44% (Harper & Morton, 1953) the data in Table 1 show that approximately twelve retained organisms

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form an ID_{50}. Thus, there is no very marked difference in infectivity by the two routes.

The importance of the number of organisms per particle. From data given by Harper & Morton (1953) on lung penetration and retention of particles it can be deduced by interpolation and extrapolation that the order of 0·5% of 12μ diameter particles and 6% of 5μ diameter particles inhaled penetrate beyond the trachea. The volume of one brucella organism is about 0·6μ^3, then allowing for a packing fraction of 0·75, a 5 and 12μ diameter particle consisting solely of brucella organisms would contain about 80 and 1000 organisms respectively. Now, it can be readily demonstrated that such particles disintegrate into single cell units on impingement on a moist surface. There is reason, therefore, to consider that each cell will act as if it had been deposited originally as a single particle. Since the ID_{50} of single organisms retained in the lung is about 12 cells, it is obvious that in comparative studies of infectivity with particles of different size, care had to be taken to reduce the number of cells per particle to one or a very few. This was done as described under the section on Methods.

Influence of satellites in large particle clouds. The spinning top sprayer used in the Druett & May (1952) apparatus produces clouds of large particles which are closely distributed about their mean size, together with some satellites of smaller size, but most of the latter are withdrawn from the emerging cloud into the system which removes spent air from the turbine (May, 1949). A few still escape, but the

Table 6. *Effect of satellite concentration in* 12μ *cloud experiments*

(The cloud was sampled with an impinger fitted with a pre-impinger and assays made of the two fractions.)

Concentration of organisms/litre in particles greater than 4μ (12μ nominal)	9,260	19,300	34,400	87,900	207,000	515,000
Concentration of organisms/litre in particles less than 4μ (satellite)	0	0	15	18	18	4
Observed infectivity, %	2·5	15	25	35	73	78
Infectivity % attributable to satellites	0	0	0·2	0·3	0·3	0

total mass of material carried by them is so small as to be wholly unimportant in most forms of experiment. However, in view of the very high infectivity of small particles of *Br. suis* it seemed important to eliminate any possible role of satellites in experiments with larger particles. In one of the two experiments summarized in Table 5 a detailed study was made of particle size distribution in the cloud. The results are given in Table 6, and the analysis shows the influence of satellites to be negligible.

DISCUSSION

An outstanding finding of the present series of experiments is the 600-fold increase in dosage necessary to produce 50% infection with 12μ diameter particles over that needed for clouds of single organisms. It is in sharp contrast to the other studies we have made, where a 2·8-fold difference was observed with *Pasteurella*

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pestis and a 17-fold difference with *Bacillus anthracis* (Druett *et al.* 1953, 1956). Fig. 1 shows that the dose response decreases rapidly with particles greater than 2·5μ diameter. Reasons for this are probably twofold. First, it is known that a high proportion of particles deposited in the head region and down the respiratory tract to the limit of ciliated epithelium are rapidly removed. Secondly, and more important, is that the bronchiolar and alveolar sites are probably more suited to support the multiplication of Brucella. Harper's (1955) experiments have already demonstrated very rapid multiplication of Brucella on these surfaces after inhalation of a cloud of single organisms.

From Harper & Morton's (1953) data on penetration of particles to the lung of the guinea-pig, it can be deduced that about 6 % of 5μ diameter particles inhaled are found in the bronchioles and alveolar spaces. If the remaining 94 % found in the upper tract were fully effective it would be expected that the infectivity at a given dosage level would be greater than for single organisms, of which proportionately many more are lost on exhalation. If, however, the organisms deposited in the upper tract are ineffective in initiating disease, either because of their rapid removal, or because the site is unsuitable, or both, then it could be predicted that a 5μ diameter cloud of 'bulked' particles would be about 7 times less effective in initiating disease than a cloud of single organisms, since only 6 % of the former, compared with 44 % of the latter, are to be found in the lung. Assuming the second alternative operative, and taking the $INt50$ for single organisms as 190

Table 7. *Response of guinea-pigs to 5μ diameter 'solid' particles* (Brucella suis) *each containing about* 80 *organisms*

$Nt \times 10^{-3}$	4·02	6·66	8·00	22·2	39·9
Infected/28	11	13	13	15	22
% infected	39	46	46	54	79

$INt50 \times 10^{-3}$ 4·1–9·3–17 orgs. min./l.
Slope 0·92 Variance 0·88 $\chi^2/n = 0{\cdot}6$.
Single organism control data $INt50 \times 10^{-3}$ 1·1–1·8–4·5 orgs. min./l.

$$\text{Ratio: } \frac{INt50 \text{ for } 5\mu \text{ 'solid' particles}}{\text{Mean } INt50 \text{ for single organisms}} = 25\text{–}50\text{–}100.$$

organisms min./l., then the $INt50$ for 'bulked' particles would be expected to be about 1360 organisms min./l. We found experimentally (Table 3) that it was 1350 organisms min./l. In view of the experimental error inherent in the data, this close agreement is no doubt somewhat fortuitous, but it does suggest that the hypothesis is correct.

An experiment was made with 5μ 'solid' particles (Table 7), and the results can be interpreted in a similar way. As noted earlier, a 5μ 'solid' particle will contain about 80 organisms, each cell potentially able to infect. The retention of one such particle in the lung would mean that about 7 ID_{50} had been deposited, since about 12 organisms in the lung are sufficient to initiate infection in 50 % of guinea-pigs exposed to single organisms. This is completely wasteful of organisms. For instance, 480 organisms distributed as 5μ bulked particles in the lungs of forty

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guinea-pigs (an average of 12 organisms per guinea-pig) might be expected to produce twenty infected animals. To achieve the same result with 5μ 'solid' particles, each containing 80 organisms ($7\ ID_{50} \approx ID_{95}$) then $80 \times 20 \times 100/95 \times 1{\cdot}37 = 2310$ organisms would be required. (The factor of 1·37, which can be deduced from probability theory, makes allowance for animals receiving more than one particle in a random distribution.) Thus 5μ 'solid' particles would be expected to be $2310/480 \approx 5$ times less effective at a given concentration level than 5μ 'bulked' particles. As shown in Table 8, we found experimentally a difference of sevenfold.

Harper & Morton's data for the penetration of 12μ particles to the lung led to the conclusion that if the upper respiratory tract is not vulnerable to infection by *Br. suis*, then 12μ particles would be expected to be about 200 times less effective than single organisms, and allowing for the removal of some organisms by ciliary action from such particles that do penetrate to the bronchus and bronchioles ratios in excess of 200 might be expected. Experimentally we found the ratio to be 600, as shown in Table 8. This figure clearly supports the hypothesis that the surfaces of the lower part of the lung form the most suitable route for the initiation of brucellosis by inhalation. There is no reason to suppose, however, that the

Table 8. *Ratio of the INt*50 *at various particle sizes to that obtained from the combined data obtained with clouds of single organisms*

Particle size	Approx. no. of organisms/particle	Ratio
$2{\cdot}5\mu$	1	0·55–0·80–1·15
5μ 'bulked'	1	6·0–7·0–8·5
5μ solid	80	25–50–100
$7{\cdot}6\mu$	1	8–14–23
12μ	7–70	440–620–870

results show that no infection takes place through the upper tract. The value of $1{\cdot}18 \times 10^5$ organisms min./l. obtained for 12μ particles is quite commensurate with the values obtained with *Past. pestis* and lower than those obtained with *B. anthracis*. It is the high vulnerability of the lung rather than the invulnerability of the upper tract as a route of infection which is so striking.

In Fig. 2 are shown results obtained by examining the cervical and bronchial glands of several thousand animals, visually and by culture, about 1 month after the animals were exposed to infection. By this time the infection is generalized, gross enlargement of the spleen being almost invariably present. It was found that the cervical glands were always enlarged where particles over 4μ diameter had been used to initiate infection, and were mostly enlarged (80 %) below this size. This is not incompatible with the hypothesis of multiplication on the lung surface, since both the upward sweep of the ciliated epithelium and the generalization of the infection could account for it.

The interpretation of the curve showing the percentage of bronchial glands enlarged is not simple. The absolute magnitude of the ordinates will inevitably depend on the sensitivity of the techniques used in detection, but the general

downward sweep of the results at the large particle sizes is unmistakable. Any assumption that animals not showing bronchial enlargement became infected through deposition of particles in the upper tract is clearly wrong for if, as seen in Fig. 2, 58% of animals exposed to 5μ 'bulked' particles were infected in this way, then little change in the $INt50$ would be expected when the particle size was increased to 12μ, whereas experimentally we found that it increased nearly 90 times. Conversely, if it can be assumed that all observed infections resulting from inhalation of 12μ particles took place through primary deposition in the upper tract, then virtually no infection due to this cause could be induced at the very much lower concentrations needed to produce infection with 5μ 'bulked' particle experiments. Moreover, Fig. 2 shows that the greatest change in the percentage of bronchial glands enlarged occurs between the single organism and 2·5μ diameter

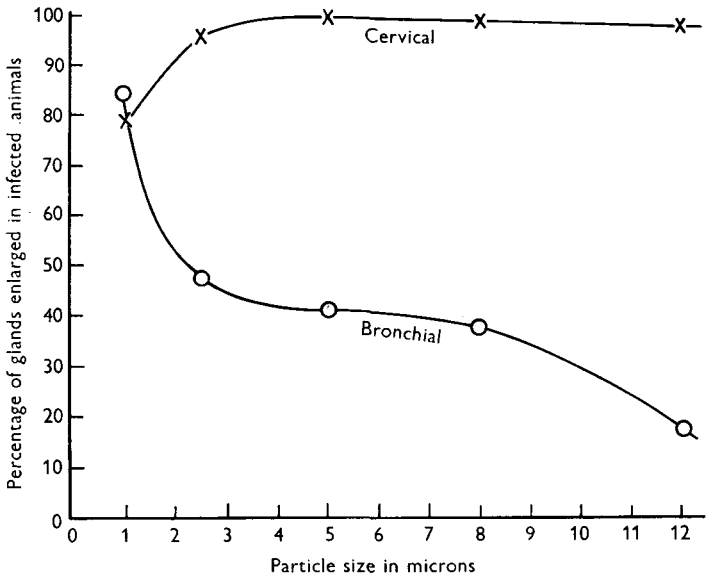

Fig. 2.

clouds of which the $INt50$ is virtually the same, but in which region of particle size the site of deposition may well be changed from the non-ciliated to the ciliated areas of the respiratory tract. It would seem reasonable to divide the curve (Fig. 2) depicting bronchial gland enlargement into three zones. First, for particles up to about 3μ, changes in the site of deposition of particles in the lung itself could cause an initial drop in the percentage of bronchial glands infected. Secondly, in the range of particle size from 3 to 8μ probably little change takes place in the type of surface on which the particles are deposited, and consequently in the mechanism of invasion, therefore the curve is flat. Thirdly, above 8μ the increasing importance of organisms in the upper respiratory tract at the high dosage level used, results in the downward trend of the curve. But in the absence of detailed histological investigation these observations must remain speculative.

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SUMMARY

The infectivity of *Brucella suis* for the guinea-pig by the respiratory route has been studied. *Br. suis* was dispersed in airborne particles of various sizes from single organisms to 12μ in diameter, and it was found that the infectivity decreased 600-fold with increasing particle size within this range. It is suggested that this is due to the ability of *Br. suis* to multiply rapidly on the surface of the lower reaches of the respiratory tract.

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