STUDIES ON RESPIRATORY INFECTION

I. THE INFLUENCE OF PARTICLE SIZE ON RESPIRATORY INFECTION WITH ANTHRAX SPORES

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(With 9 Figures in the Text)

INTRODUCTION

Opinion is divided on how naturally occurring respiratory disease most commonly arises and spreads. Is infection by contact via the hands, for example, or by the direct inhalation of airborne particles? 'Field' studies in air hygiene are notoriously difficult, and the results have been disheartening, so that experiments must be made under controlled conditions in the laboratory with experimental animals. Unfortunately, few common human diseases can be studied satisfactorily in animals, especially in relation to methods of spread. However, some human pathogens can initiate disease in animals by the respiratory route and can therefore be used in studies of initiation of disease.

All previous work on this subject has been done with clouds of heterogeneous particle size. The disadvantage of this is that the site of deposition of particles (qualitatively and quantitatively) cannot be known. Our first task, therefore, was to develop apparatus which would produce clouds of homogeneous particle size ranging from single organisms to (dried) clusters of about 15μ diameter (Henderson, 1952; Druett & May, 1952). With this accomplished we believed that three lines of approach were necessary, namely, to develop technique for the visual detection of organisms and early pathological change in the respiratory tract; to devise methods for locating the site of deposition of bacterial particles of different size within the tract; and to investigate the influence of particle size on the initiation of disease.

For many reasons, the first pathogen to choose was *Bacillus anthracis*; *B. anthracis* was probably the first pathogen to be tested for infectivity by the respiratory route; also its spores are hardy and less likely than any other pathogen to suffer from the physical strains involved in conversion into airborne dried particles.

Experimental infection of animals with airborne anthrax spores was first studied by Buchner (1888). He exposed animals in a small chamber to clouds, either of atomized aqueous spore suspension, or of dried suspension blown up with a draught. In this way he infected mice, guinea-pigs and rabbits. As a result, he put forward the theory that infection occurred by the direct passage of the organisms through the alveolar wall into the blood stream. Others worked on similar lines, but none established the site and mode of initiation of infection.

More recently, Barnes (1947) studied the pathogenesis of infection in guinea-pigs exposed to clouds of single spores. He obtained evidence that in such animals the disease does not primarily involve lung tissue, but that the spores first germinate and multiply in associated lymphatic glands. He pointed out, however, that this experimental disease may not truly simulate the now rare woolsorter's disease, in most cases of which a pustule has been found at the bifurcation of the bronchus.

With colleagues in this Department we decided to reinvestigate the whole problem on the lines noted above. Studies on technique for the visual detection of spores and germinating forms in the respiratory tract have given encouraging results but are still incomplete. Success has attended the investigation of the site of deposition of spore clusters within the respiratory tract; the results are reported by Harper & Morton elsewhere in this issue of the *Journal*. The present paper presents the results of the investigation of the influence of particle size on infectivity.

METHODS

(a) *Strain used* (1) *Anthrax spore suspension*

The spore suspensions were prepared from a highly pathogenic strain of *B. anthracis* (M36) isolated by Henderson & Venzke (1944) from a monkey exposed by the respiratory route to spores of an original strain *Vollum*.

(b) *Spore production*

A dried spore preparation was suspended in distilled water and plated on peptidase-thiamine-marmite agar (Brewer, McCullough, Mills, Roessler & Herbst, 1946). After incubation at 37° C. selected colonies were allowed to spore; then suspended in distilled water, heated at 60° C. for 1½ hr., and inoculated on to Brewer agar contained in 8 oz. flat bottles. These were incubated for 2 days at 37° C., the growth was washed off in 25 ml. of distilled water, heated at 60° C. for 1½ hr., and used to inoculate Brewer agar contained in trays 15 × 10 in. After incubation at 37° C for

Table 1. *Guinea-pig response to single-spore clouds*

Spore batch	Experiment	<i>LNt</i> 50 × 10 ⁻⁶
M 36 B 2 18. vii. 50	11. x. 50	0.24-0.34-0.43
M 36 B 8 6. ii. 51	13. iii. 51	0.15-0.31-0.44
M 36 B 10 22. v. 51	22. vi. 51	0.25-0.36-0.44

2 days, the growth was collected into distilled water, passed through a glass-wool pad, ball milled, and heated at 60° C. for 1½ hr. A.R. phenol was added to give a concentration of 1 %. The viable count of such a suspension was about 4 × 10¹⁰ per ml. No loss of viability or virulence by the respiratory route was detected after storage for a year. Various batches were prepared and tested. Table 1 shows the *LNt* 50 values (defined below) for guinea-pigs, obtained with three suspensions over 9 months; the consistent values are satisfactory. Before use the suspension was passed through a sintered glass filter to remove particles of agar or other small aggregates.

(2) *Animals*

Guinea-pigs weighing 350–400 g. were used. In all experiments they were allocated to their group by random sampling. After exposure they were housed four in a cage in an isolation room, and held for 10 days.

Macacus rhesus monkeys varying in weight from 7 to 14 lb. were used. They were housed singly in cages, and held for 3 weeks after exposure.

Confirmation of death from anthrax infection was obtained in all instances by microscopic examination of heart's blood. Blood culture was carried out in all experiments with monkeys.

(3) *Apparatus for producing spore clouds*

For experiments with clouds of spore clusters the Druett & May (1952) apparatus was used. Most of the experiments with clouds of single organisms were made in the apparatus described by Henderson (1952); it is simpler to operate, and the cloud concentrations required were more conveniently produced in it.

(4) *Cloud sampling and assessment*

The impinger technique previously described (Henderson, 1952) was used for collecting the samples. In experiments with the Druett & May apparatus, a glass extension tube with a right-angle bend was fitted to the neck of the impinger. This tube was placed in the tunnel with the opening of the bent part facing the passing cloud. The impingers carried 10 ml. of 0.1 % Manucol in $M/10^4$ sodium carbonate buffer. A standard sampling time of 1 min. was used and the flow-rate was 1 l./min. When clouds of large particles were used, much of the sample was retained by impaction in the extension tube and in the neck of the impinger. Table 2 shows the

Table 2. *Distribution in the sampling apparatus of particles containing B. subtilis spores*

No. of experiments	Particle size (μ)	Percentage sample in	
		Bottle	Neck and adaptor
3	12.4	4.6	95.4
3	8	18.9	81.1
3	5	75.8	24.2
3	1	97.0	3.0

distribution of particles of *B. subtilis* spores in the sampling apparatus. The deposit in the neck and adaptor was collected by repeated washing with the impinger fluid. Dilutions of the fluid were then made and a viable count carried out by the method of Miles & Misra (1938). The spore clusters disintegrated into individual spores on contact with water. The cloud concentrations are, therefore, expressed in terms of single spores per unit volume.

(5) *Definition of terms used in expressing results*

(a) *Cloud concentration (N)* is the number of viable cells contained in unit volume of air at a given position and time. It is expressed in terms of the number of organisms per litre of air. In practice a continuous sample is taken at a constant

rate (S l./min.) for a given period (t_s min.) and the mean concentration (N) is determined by

$$N = \frac{\text{number of organisms collected}}{\text{volume (in litres) of air sampled}} = \frac{n}{St_s} \text{ organisms/l.}$$

(b) *Dosage* (Nt), a measure of the exposure, is defined as the product of mean cloud concentration (N) and period of exposure (t); thus

$$Nt = \frac{n}{St_s} t \text{ organisms-minutes/l.}$$

(c) *The $LNt50$* is the Nt estimated to produce 50 % deaths in exposed animals.

(d) *Dose* (D) is an approximate assessment of the number of organisms inhaled by an animal, and is defined as the total number of organisms contained in the volume of air inhaled during the exposure time. The volume of air breathed per minute by animals of known weight was taken from data given by Gaddum (1944).

To calculate the number of organisms retained in the respiratory system with precision, one would need to know: (i) the volume inhaled by the animal during exposure, (ii) the sampling intake efficiency of the animal's nose, or nose and mouth, and (iii) the percentage of particles retained in the lung; but none of these can be measured in actual experiments. The estimate of dose defined as above has a large error, perhaps fourfold or more (Harper & Morton, 1953).

(6) *Estimation of particle size*

The bacterial clouds were homogeneous in particle size (Druett & May, 1952). Clouds with a mean particle size of 8μ or over were sampled by direct deposition on a glass slide placed in the exposure arm of the tunnel. For smaller particles a one-stage impactor (May, 1945) was used, care being taken to employ the lowest velocity jet which would give satisfactory impaction, so that flattening of the fragile particles against the glass slide was kept to a minimum. The size of the particles was measured under a microscope fitted with an eyepiece and graticule. For safety in operation, and to prevent the collapse of particles due to the deposition of water from the operator's breath, the microscope was placed in a box from which only the eyepiece protruded. Adjustments were made through rubber gauntlets attached to the box.

RESULTS

General

The first experiments were designed to determine the $LNt50$ for animals with clouds of different particle size. To obtain an accurate value with fiducial limits ($P=0.95$) the probit regression technique (Finney, 1947) was used. Regression lines were obtained with guinea-pigs exposed to clouds of single organisms and to particles of 3.5, 4.5, 8 and 12μ diameter respectively. Monkeys were exposed to clouds of single spores and to particles of 12μ diameter.

In all experiments the animals were exposed to the cloud for 1 min. Guinea-pigs were presented in pairs or groups of four, and monkeys were exposed singly. At least three cloud samples were taken for assessment at each level of concentration:

one at the commencement of the run, an intermediate sample, and one after the last animal had been exposed. In each experiment the lowest cloud concentration was produced first. The apparatus was then flushed out with clean air before proceeding to the next higher concentration.

The main experiments with guinea-pigs were done after preliminary sighting experiments had established the working range of concentrations. Four or five concentration levels were chosen in geometrical sequence covering the estimated range of Nt 25–75. In calculating the regression lines, results of sighting experiments were included where they fell within the working range. The number of animals exposed at each level was 20–40, according to the supplies available. The experiments on each particle size were completed within a period of 2–3 weeks. In experiments with monkeys, the practical considerations of housing and supply limited the number that could be used at any concentration to eight. For the same reasons, experiments had to be widely spaced in time; the data were collected over many months.

The possibility of cross-infection among exposed guinea-pigs was examined. In many experiments normal animals were placed in the same cage as those under test. The only evidence of cross-infection was found in experiments in which high concentrations of 12μ particles were used; for example, at Nt 12×10^6 the death-rate among control guinea-pigs was 12%. Correction was made, where relevant, in analysing the results, as is shown in Fig. 5.

Influence of time of exposure on response

In all the experiments the exposure time was 1 min. It seems logical to assume that an animal exposed to a cloud of given particle size and concentration N for a time t will inhale the same number of spores as a similar animal exposed to a cloud

Table 3. *Influence of time of exposure on response*

$N \times 10^{-6}$	t (min.)	$Nt \times 10^{-6}$	Mortality
1.13	$\frac{1}{4}$	0.28	16/40
0.33	1	0.33	14/40
0.68	$\frac{1}{2}$	0.34	15/39
3.06	$\frac{1}{12}$	0.26	12/40
0.41	1	0.41	15/40
2.21	$\frac{1}{8}$	0.37	16/40
0.22	2	0.44	11/40
0.37	1	0.37	15/40
0.11	4	0.43	16/40

concentration N/m for a time mt . This point was verified by experiments with guinea-pigs in which times of exposure above and below 1 min. were used.

Three experiments were made with clouds of single spores. In each, one group of animals was exposed for 1 min. to a cloud concentration of about 0.34×10^6 organisms/l. (Nt). The other two groups were exposed for different times to a cloud concentration designed to keep $Nt = \text{const.}$ The value of t ranged from 5 sec. to 4 min. Forty animals were exposed at each time level. The results are given in Table 3. There is no significant difference in mortality between the groups and the

Nt values are not widely scattered. A statistical examination of these results leads to the conclusion that Nt was constant.

Guinea-pig response is shown in Tables 4–8, and the regression lines in Figs. 1–5.

Monkey response is shown in Tables 9 and 10, and the regression lines in Figs. 6 and 7.

Table 4. *Guinea-pig response to single-spore clouds*

$Nt \times 10^{-6}$	0.168	0.346	0.646	1.000
Mortality/32	8	18	21	28
Mortality (%)	25	56.3	65.6	87.5

$$LNt 50 = 0.24-0.34-0.43 \times 10^6.$$

Slope 2.13 (variance 0.19) and $\chi^2/n = 0.73$.

Table 5. *Guinea-pig response to 3.5 μ particles*

$Nt \times 10^{-6}$	0.26	0.44	0.17	0.29	0.44	0.52	0.69
Mortality	13/40	23/40	7/20	7/20	9/20	13/20	18/20
Mortality (%)	32.5	57.5	35	35	45	65	90

$$LNt 50 = 0.29-0.36-0.44 \times 10^6.$$

Slope 2.44 (variance 0.32) and $\chi^2/n = 1.3$.

Table 6. *Guinea-pig response to 4.5 μ particles*

$Nt \times 10^{-6}$	0.597	0.269	0.374	0.125	1.025	1.34	0.385	0.231
Mortality/40	22	17	13	6	28	26	22	18
Mortality (%)	55	42.5	32.5	15	70	65	55	45

$$LNt 50 = 0.33-0.49-0.78 \times 10^6.$$

Slope 1.26 (variance 0.11) and $\chi^2/n = 1.9$.

Table 7. *Guinea-pig response to 8 μ particles*

$Nt \times 10^{-6}$	7.32	2.28	3.39	4.78	1.72
Mortality/40	31	14	21	20	5
Mortality (%)	77.5	35	52.5	50	12.5

$$LNt 50 = 3.25-3.8-4.65 \times 10^6.$$

Slope 2.54 (variance 0.19) and $\chi^2/n = 1.46$.

Table 8. *Guinea-pig response to 12 μ particles*

$Nt \times 10^{-6}$	3.16	12.19	2.84	1.87	5.8	12.8	7.65
Mortality/40	16	28	13	11	22	27	23
Mortality (%)	40	70	32.5	27.5	55	67.5	57.5

$$LNt 50 = 4.0-5.3-7.1 \times 10^6.$$

Slope 1.31 (variance 0.066) and $\chi^2/n = 0.1$.

These results were recalculated in the light of information from cross-infection experiments to give:

$$LNt 50 = 4.2-5.7-7.7 \times 10^6.$$

Slope 1.25 (variance 0.65) and $\chi^2/n = 0.1$.

Table 9. *Monkey response to single-spore clouds*

$Nt \times 10^{-6}$	0.0293	0.0321	0.0453	0.0573	0.0648	0.0670	0.1000	0.1250	0.1660
Mortality	1/8	4/8	5/8	6/8	5/8	3/8	8/8	7/8	8/8
Mortality (%)	12.5	50.0	62.5	75	62.5	37.5	100	87.5	100

$$LNt 50 = 0.030-0.045-0.052 \times 10^6.$$

Slope 3.19 (variance = 0.75) and $\chi^2/n = 1.4$.

Table 10. *Monkey response to 12 μ particles*

$Nt \times 10^{-6}$	0.251	0.320	0.422	0.615	0.682	1.760	3.310	3.74
Mortality	1/8	5/8	4/8	3/8	4/8	4/7	6/6	6/8
Mortality (%)	12.5	62.5	50.0	37.5	50.0	57.1	100	75

$LNt\ 50 = 0.2-0.64-1.39 \times 10^6$.

Slope 1.17 (variance = 0.18) and $\chi^2/n = 1.11$.

Fig. 1. Guinea-pig response to single-spore clouds. $y = 2.13x + 3.88$.

Fig. 2. Guinea-pig response to 3.5 μ particles. $y = 2.44x + 3.63$.

Fig. 3. Guinea-pig response to 4.5 μ particles. $y = 1.26x + 4.13$.

Fig. 4. Guinea-pig response to 8 μ particles. $y = 2.54x + 3.51$.

Fig. 5. Guinea-pig response to 12 μ particles. + Uncorrected for cross-infection, $y = 1.31x + 4.05$. ⊙ Corrected for cross-infection, $y = 1.25x + 4.05$.

Fig. 6. Monkey response to single-spore clouds. $y = 3.19x + 2.91$.

Fig. 7. Monkey response to 12μ particles. $y = 1.17x + 4.05$.

From these data the following values are obtained:

	Guinea-pigs		Monkeys	
Particle size of cloud	$LNt50 \times 10^{-6}$	$D \times 10^{-5}$	$LNt50 \times 10^{-6}$	$D \times 10^{-5}$
Single spore	0.34	0.5	0.045	0.53
3.5μ	0.36	0.55	—	—
4.5μ	0.49	0.61	—	—
8μ	3.8	5.7	—	—
12μ	5.7	8.6	0.64	7.6

Total air breathed per minute: guinea-pigs 150 ml.; monkeys 1200 ml.

These results show, first, that the two species are equally susceptible; secondly, that, with guinea-pigs, D for 8μ particles is much bigger than with 4.5μ particles, and, thirdly, that with the 12μ particles the dose is 14–17 times greater than with single spores. The significance of these findings is discussed later in relation to the site of deposition of particles of different size in the respiratory tract.

Influence on infectivity of the size of the particle in relation to the number of spores it contains

The experiments described above were made with particles consisting wholly of spores, the estimated number of spores in the various clusters being: 3.5μ , 18; 4.5μ , 36; 8μ , 235; 12μ , 680. It was of interest to find if the size of the particle determined the difference in infectivity, or whether the total number of spores carried in it was also of importance. To this end an experiment was made to compare the infectivity for guinea-pigs of

(a) 3.5μ particles, each composed wholly of spores and estimated to contain about eighteen of them;

(b) 8μ particles, each containing approximately eighteen spores and made up to size with dextrin added to the suspending fluid;

(c) 12μ particles, each containing approximately eighteen spores per particle and made up to size with dextrin.

The results are given in Table 11. For comparison there is included in the table the calculated performance of 8 and 12μ particles consisting wholly of spores, the value being taken from the regression line data.

The relative ineffectiveness of the 8 and 12μ clouds is again clearly demonstrated. At equal Nt values the calculated performance of 8 and 12μ particles consisting wholly of spores is similar to the mortality observed with particles of the same size each carrying about eighteen spores. However, this simply means that to achieve the same mortality the total number of spores presented to the animal, in particles of a given size, must be the same, irrespective of the number of particles carrying them. These findings are discussed later in relation to the site of deposition of particles.

Table 11. *Influence on infectivity of the size of the particle in relation to the number of spores it contains*

Particle diameter (μ)	No. spores/part.	$Nt \times 10^{-6}$	No. of particles per litre cloud $\times 10^{-4}$	G.P.	
				D/E	M %
3.6	18	1.66	9.2	40/40	100
8.4*	19	1.39	7.3	11/40	27.5
8.4†	235	1.39	0.59	†	22
11.6*	18	1.1	6.1	10/40	25
12.0†	680	1.1	0.16	†	18

D = number died. E = number tested. M % = percentage mortality.

* Particles made to size by addition of dextrin to spray fluid.

† Calculated performance from regression-line data.

DISCUSSION

Evidently the size of the inhaled particle is important in determining infection of the host with anthrax. The greatest danger comes from the inhalation of clouds of single spores. Particles of diameter up to 5μ are only slightly less effective, but the infectivity of particles larger than this falls sharply. This phenomenon is shown graphically in Fig. 8. Harper & Morton (1953) and others have demonstrated that few particles greater than 4μ diameter reach the lung of the guinea-pig; they are retained in the upper respiratory tract. It is concluded, therefore, that the most dangerous area for the initiation of infection is the lung proper. How the spores deposited there set up disease is another matter, and the question is not relevant to the present experiments.

Clouds of single spores are about 17 times as infective for the guinea-pig as clouds of 12μ particles. Harper & Morton (1953) have related lung penetration and retention to particle size. We have compared our correlation of particle size and infectivity with their figures. Curve *A* in Fig. 9 is calculated from Harper & Morton's data on the supposition that, if infection was initiated only in the region of the lung, the $LNt50$ for particles of different sizes would vary inversely as their retention in the lung. In drawing curve *A*, ordinate values have been multiplied by an arbitrary constant to make the curve pass through the point *F*, corresponding to 0.34×10^6 organisms min./l., or the $LNt50$ for single spores. On this showing, the ratio of the $LNt50$ value for 12μ particles to that for single spores was calculated to have a most probable value of 80. Curve *B* in Fig. 9 was constructed from our results, where the most probable value of difference between 12μ particles and

single spores was 17-fold (11–17–28; $P=0.95$). The difference is marked, and it cannot be assumed that the lung is the only vulnerable site in the guinea-pig.

In experiments with monkeys we found 12μ particles to be about 14 times less efficient than clouds of single spores. This is in conformity with guinea-pig results. On the other hand, Harper & Morton's results with monkeys lend no support to the theory that the site of deposition would account for this difference. One would deduce from their data that there should be little difference between the $LNt50$ for single-spore clouds and for 12μ particles. We have no explanation for this except perhaps to note Harper & Morton's observation of nose- and mouth-breathing irregularities in monkeys. The number of animals examined was probably too small to allow an opinion to be formed.

Harper & Morton showed that many spores deposited in the upper respiratory tract rapidly found their way to the stomach and intestines. It might be argued, therefore, that in our experiments the disease was initiated in the intestinal tract.

Fig. 8. Change of $LNt50$ for guinea-pigs with particle size.

This is most improbable. We have repeatedly failed to induce infection in guinea-pigs or rabbits by feeding 10^8 spores. Young, Zelle & Lincoln (1946), in more extensive experiments with the same strain of *B. anthracis* that we used, failed to obtain more than an occasional infection by depositing 10^8 spores in the mouth, eye, vagina or rectum. In the present experiments the highest respiratory dosage was about 10^6 spores. Therefore, we conclude that anthrax developed by way of the respiratory tract and not to any significant extent through the digestive tract.

Evidence was obtained that infection could be initiated in the upper respiratory tract. Monkeys exposed to clouds of 12μ diameter often developed massive oedema of the face and head which persisted for a day or two before death, and indicated a localized infection beginning somewhere in the head. We did not observe this phenomenon in monkeys or guinea-pigs exposed to clouds of single spores.

Probably, therefore, if n spores deposited in the lung produce a given mortality, then nx spores will do the same when deposited elsewhere in the respiratory tract.

However, if an arbitrary value of $x=30$ is chosen, Harper & Morton's figures, treated as were the figures of curve *A* to make the curve pass through point *F*, yield curve *C*; which approximates closely to curve *B* for our own figures.

Further evidence was obtained to show that the change in LNt_{50} with particle size was directly associated with the site of deposition of the particle. By adjusting the number of spores in 8 and 12μ particles it was shown conclusively that mortality was determined by the total number of spores deposited in an area and was

Fig. 9. Change of LNt_{50} with particle size. *A*(Δ), curve predicted on basis of lung infection. *B*($\oplus$), experimental curve for guinea-pigs. *C*(∇), curve calculated on basis of lung and upper respiratory infection.

independent of the number of particles carrying them. The independent action, as it were, of each spore in a cluster may be explained by the ease and rapidity with which clusters disintegrate to become single spores on contact with water.

Examination of the results by probit analysis gives as parameters the LNt_{50} and the slope of the regression line. This paper is not concerned with interpreting the significance of the slope in relation to dosage response. This will form the subject of other investigations, one of which is published (Druett, 1952). In this connexion, however, it should be made clear that although we have referred to the importance of site of deposition in relation to infectivity there is no intention to suggest that invasion of the body takes place at the site.

SUMMARY

Experiments to determine the role of particle size in the infectivity of anthrax spores are described. Clouds of homogeneous particles were produced. The mortality-dosage curves for guinea-pigs and monkeys are given for clouds of various particle sizes. Data are given on the effect of time in the concentration-time relationship. The results are compared with those recorded by other workers on the relationship of particle size to respiratory retention.

Infectivity was highest with single-spore clouds, falling off as particle size increased. Reasons are given for attributing this effect to difference in site of deposition of different-sized particles.

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