SOME PHYSIOLOGICAL AND IMMUNOLOGICAL PROPERTIES OF *PASTEURELLA PESTIS* RECOVERED FROM AEROSOLS

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When bacteria are disseminated into air, the concentration of viable cells in the aerosol decreases with time. It has been shown that part of this loss is due to impingement of cells on the surfaces of the container and to settling, but there is also considerable evidence for biological inactivation (Lester, 1948; Dunklin and Puck, 1948; Boyd and Forstat, 1953). This study has been undertaken to determine the extent of physiological alteration associated with aerosolization, and if possible, to correlate these alterations with changes in virulence.

This report presents data which were obtained from a number of physiological and immunological experiments with organisms recovered from aerosols.

METHODS AND MATERIALS

The organism selected for study was the Alexander strain of *Pasteurella pestis*. The isolation of this strain has been described by Link (1950). Cultures to be used for the dissemination of aerosols were prepared by inoculating 100 ml of heart infusion broth (Difco), contained in a 1 L flask, with cultures from slants, and then incubating the flasks for 24 hr on a shaker at 26 C. The cultures were harvested by centrifugation, washed three times with sterile 0.01 M phosphate buffer, pH 7.0, and finally resuspended in a fresh solution of the same diluent.

Aerosols were disseminated in a modified Reyniers chamber by means of a University of Chicago Toxicity Laboratories type of atomizer in a manner similar to that described by Rosebury (1947). After the atomizer had been in operation for 15 min, samples were taken by means of 4 ten-liter-per-minute capillary impingers. Dissemination and sampling then were continued for 60 min. In every case the relative humidity of the chamber was held at 30 per cent (± 5 per cent), and the temperature maintained at 20 to 22 C. The bacteria which were recovered from the aerosol were estimated to have a mean aerosol age of 1.7 min.

The impinger samples then were pooled, centrifuged and resuspended in buffer. The turbidity of the samples was determined with a colorimeter, and a suspension of control cells (organisms obtained from the suspension from which the aerosol had been disseminated) prepared at the same turbidity. In certain cases, where greater accuracy was desired, the samples were adjusted to contain equal total cell concentrations on a basis of nitrogen content as determined by the Kjeldahl nitrogen method. In every case the proportion of viable to nonviable cells was determined by conventional plating methods.

EXPERIMENTAL PROCEDURES AND RESULTS

Virulence studies. The object of this study was to survey physiological alterations for their effect on infectivity. The initial experiments, therefore, dealt with the effect of dissemination into air upon the virulence of *P. pestis*. For this purpose, the LD_{50} values of organisms recovered from aerosols as well as control preparations were determined by intrapleural inoculation of guinea pigs. Calculations were performed by the method of Litchfield and Wilcoxon (1949). The results (table 1) indicated that aerosolization caused a significant decrease in the virulence of the organism.

Metabolic studies. In an initial effort to determine the physiological basis for the change in virulence, conventional Warburg techniques (Umbreit *et al.*, 1949) were employed to determine the oxygen uptake of both kinds of cells in the presence of a number of substrates. The findings in every case indicated that the reduction of the oxidation rate which resulted from aerosolization was not as extensive as the reduction of apparent (colony count) viability. In table 2 the data from several experiments of this type are given. The data are presented as the per cent of viable cells and the per cent of metabolic activity remaining after aerosolization. As

TABLE 1

Effect of aerosolization on the intrapleural LD_{50} of Pasteurella pestis

Type of Preparation	LD_{50}*	95 Per Cent Confidence Limits
Aerosolized	500	142–1750
Nonaerosolized (control)	10.3	1.1–95.2

* Determinations were run on 20 guinea pigs at each of five dose levels.

TABLE 2

Effect of aerosolization upon plate-count viability and rate of oxygen uptake of Pasteurella pestis

Substrate (20 μm/Flask)	Effect of Aerosolization	
	Per cent viable cells remaining	Per cent of initial oxidation rate
Water	1.4	25.4
Glucose	1.3	15.9
Pyruvate	2.5	48.2
L-Alanine	0.3	21.4
Citrate	2.4	10.7
Succinate	0.3	12.2
Mean	1.4	22.3

previously stated, both types of samples had been adjusted to the same total cell concentration.

These results suggested that either the metabolic rate of the surviving cells had increased, or that the nonmultiplying cells contributed to the oxidation of substrate, or most probably, that both factors contributed to the results.

Immunological studies. The metabolic behavior of cells recovered from aerosols provided little information as to the site of injury. Since the surface of the organism is most intimately associated with the environment, several tests were made to determine the extent of surface alteration by means of the agglutination reaction.

Antisera against cells recovered from aerosols as well as control organisms were prepared by intraperitoneal inoculation of rabbits with formalin-killed suspensions of the two kinds of organisms. Three rabbits were employed for each antigen, and each animal was given a series of 6 immunizing inoculations at 3 to 4 day intervals. One week later the animals were bled, and the

TABLE 3

Effect of aerosolization on the antigenicity of Pasteurella pestis

Antigen	Antiserum Titer*	
	Anti-aerosolized	Anticontrol
Control (nonaerosolized)	1:275	1:11,200
Aerosolized	1:6400	1:1400

* Each figure represents the mean of eight experiments.

serum from each group of 3 was pooled. Conventional agglutination tests using both types of antigens with homologous and heterologous antisera were then done. The data (table 3) suggested that dissemination in an aerosol induced a marked change in the antigenic characteristics of the surface of the cells.

Cell growth and multiplication. Since the virulence of cells which have been suspended in an aerosol depends to a great extent upon the activity of the progeny of these organisms, a study of the growth and multiplication characteristics of *P. pestis* recovered from aerosols was carried out.

The effect of dissemination into air upon cell growth, i. e., increase in cell dimensions, was determined by inoculating duplicate flasks of medium with organisms recovered from impingers and control cells. At various times throughout the incubation period samples were removed, streaked on microscope slides and stained with crystal violet. Viable counts were made concurrently. The cells were examined microscopically and the length determined by means of an ocular micrometer. The median cell length was calculated from the measurement of approximately 500 cells. The data (figure 1) showed a distinct difference between the median lengths of the two types of cells after incubation for 24 hr. The median length of the cells which had been recovered from the aerosol was approximately twice that of the controls.

In order to confirm these findings, a series of experiments were performed in which the change in mean cell volume was measured by centrifuging the samples in calibrated tubes. The mean volume was calculated by dividing the packed-cell volume by the total number of cells as determined turbidimetrically and by nitrogen analysis. The results of these experiments (figure 2) con-

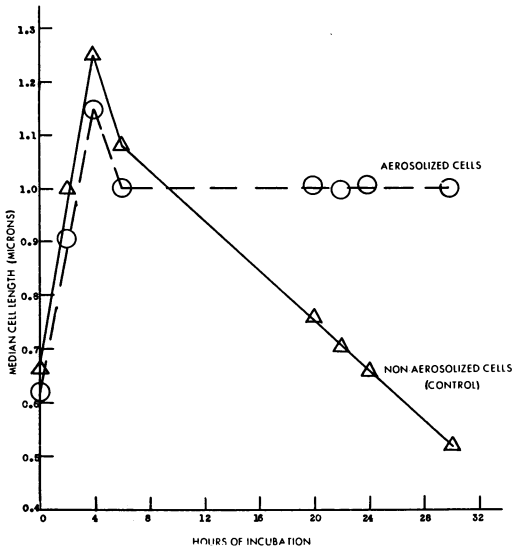

Figure 1. The effect of aerosolization upon the median length of *Pasteurella pestis* during a 24 hr growth period.

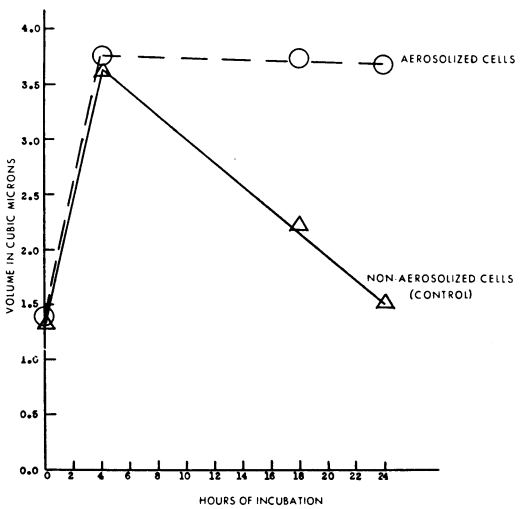

Figure 2. The effect of aerosolization upon the mean cell volume of *Pasteurella pestis* during a 24 hr growth period.

firmed those obtained by microscopic measurement.

These data suggested that many of the cells recovered from the aerosol were able to grow but not multiply. If this explanation were valid, the results which were demonstrated in metabolic experiments could be attributed to the presence of a considerable number of metabolizing, nonmultiplying cells. When the viable count data from the previous experiments were plotted, however, it was shown that the relatively higher metabolic rate of these cells did not result solely from nonmultiplying organisms. In this case the aerosolized cells multiplied at a significantly greater rate than did the controls (figure 3). Subsequent experiments indicated that the difference in multiplication rates persisted when the initial inoculum concentration was varied between limits of 10^4 to 10^9 cells per ml. Thus, the increased metabolic rate also could have been due to increased metabolic activity by the surviving bacteria. Furthermore, the increased multiplication rate suggested that the larger mean dimensions of the aerosolized cells, after 24 hr incubation, could not have resulted solely from an increase in the size on nonmultiplying organisms since this type of cell accounted for somewhat less than 10 per cent of the total population at the end of the incubation period. Therefore, it can be suggested that cells were present in the suspen-

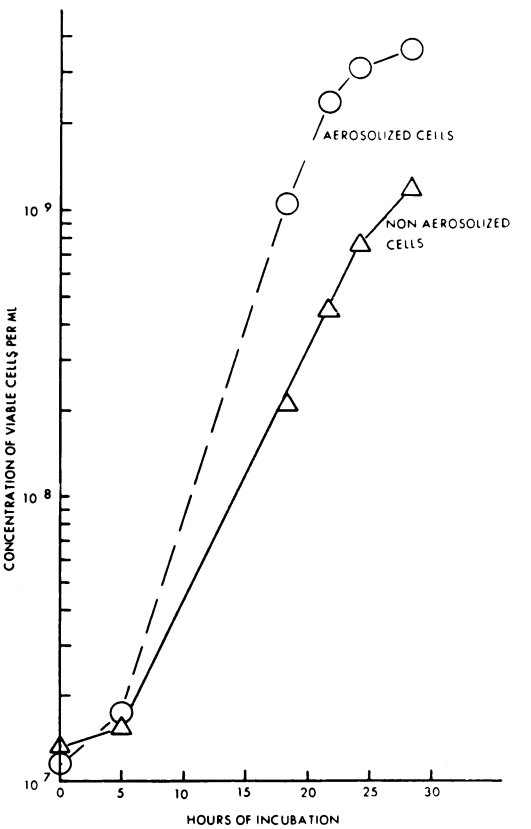

Figure 3. The effect of aerosolization on the multiplication of *Pasteurella pestis*.

sion recovered from the aerosol which could both multiply more rapidly and grow to greater dimensions than those in the control suspension.

Mechanism of transmission. As previously stated, the virulence of *P. pestis* depends upon events which take place as the organisms multiply in host tissue. It was necessary, therefore, to determine whether the alterations which had been observed persisted as multiplication progressed. The question here was whether the reactions described were the result of the selection of naturally occurring segments of the population or of physiological changes directly induced by aerosolization.

The persistence of immunological change was measured by inoculating cells recovered from aerosols as well as control cells into culture media, incubating for 48 hr, and at various times removing samples for the preparation of antigens.

Titrations were made with both homologous and heterologous antisera. The data (figure 4) showed that the cells recovered from aerosols revert to the control type of antigenicity within 48 hr. From these data it can be concluded that the antigenic alteration is caused by aerosolization and is not a selective change.

Persistence of altered multiplication and growth rates was determined by inoculating media as above, and after 24 hr incubation inoculating two fresh flasks with material from the 24 hr cultures. These flasks then were incubated for another 24 hr period. Samples were taken for dimension and multiplication rate determinations throughout both incubation periods. The persistence of differences indicated that the increased multiplication rates (figure 5) and the greater size (figure 6) both resulted from

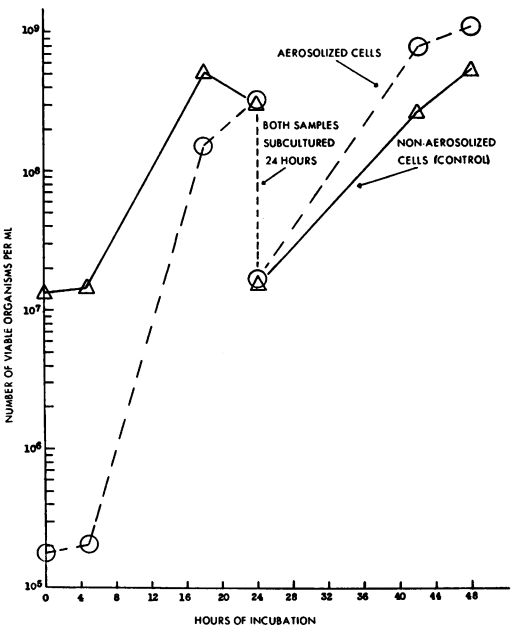

Figure 5. The effect of subculture on the multiplication rate of aerosolized *Pasteurella pestis.*

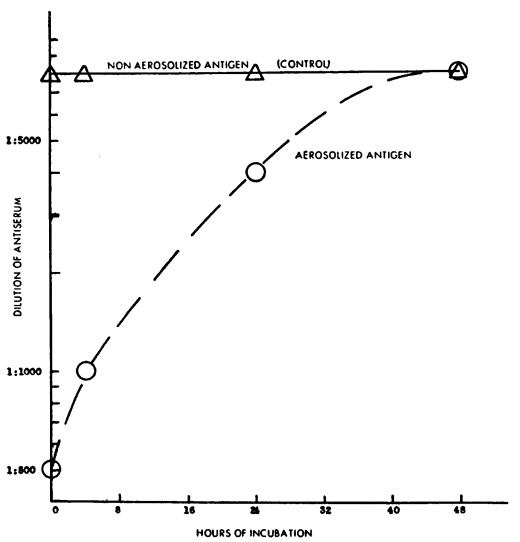

Figure 4. Antiserum titer of subcultured aerosolized and nonaerosolized cells titrated with antinonaerosolized serum.

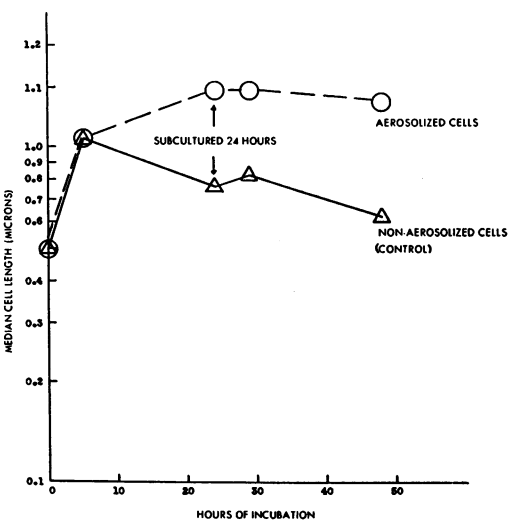

Figure 6. The effect of subculture on the growth of aerosolized *Pasteurella pestis.*

the selection of naturally occurring segments of the parent (control) population.

The question of persistence of altered virulence was of primary importance. This determination was made by inoculating guinea pigs with samples taken at various times from the flasks used in the multiplication and growth experiments described above. The persistence of altered virulence following subculture showed that this phenomenon was also the result of selection by some environmental factor in the aerosol and was not the result of an aerosol-induced alteration.

The experiments which have just been described suggest that the cells in the population which are more resistant to the deleterious effects of suspension in aerosols have the properties of lessened virulence, greater rate of multiplication and greater dimensions than do the cells which are more sensitive to these effects. The question arose, therefore, as to whether these properties, particularly aerosol-resistance and lessened virulence, were causally related, i. e., whether all of these properties have some common physiological basis, or whether they have been selected by chance.

An analogous situation is the well-known correlation of virulence with S-R variation which has been observed in brucella by Henry (1928, 1933) and in other organisms by a number of workers.

In order to study this question, a sample of cells recovered from an aerosol was spread on agar and incubated for 48 hr. Five colonies were selected for further study and subcultured to liquid media. At the same time, the contents of an entire plate of control cells as well as one of organisms recovered from an aerosol were also inoculated into broth. The flasks were then incubated for 24 hr. Samples were removed at various intervals for LD_{50}, cell dimension and multiplication rate determinations. The results of this study, which are given in table 5, showed that the lowered virulence of the organisms after suspension in an aerosol appeared to correlate directly with the degree of alteration of dimension and multiplication rate. Although an insufficient number of clones were tested to provide statistical reliability, the data suggests that the alterations studied are causally related.

All of the foregoing experiments have indicated that a considerable degree of heterogeneity exists in the parent control culture. This heterogeneity is especially important with regard to virulence and aerosol stability. An additional experiment to demonstrate heterogeneity was performed by selecting five clones from an agar plate which had been streaked with a control suspension and inoculating liquid media with each of the isolates. In addition, the contents of a slant of an avirulent culture as well as a slant of the parent culture were inoculated into broth. After 24 hr incubation, the cultures were used for virulence and aerosol stability determinations. Aerosol stability in this case is expressed as per cent recovery, i. e., the concentration of cells per liter of aerosol at 1.7 min relative to the concentration of those initially disseminated.

TABLE 4

Effect of subculture on the intrapleural LD_{50} of Pasteurella pestis in an aerosol

Hours of Incubation	Nonaerosolized Cells		Aerosolized Cells	
	LD_{50}	95% Confidence Limits	LD_{50}	95% Confidence Limits
0	10.3	1.1–95.2	500	142–1750
18	10.0	1.6–63.4	700	65.4–5502
24	8.0	0.9–74.4	890	130–6052
48	22.0	5.1–94.0	745	133–4172

TABLE 5

Growth, multiplication and virulence characteristics of five selected colonies of Pasteurella pestis

Culture Tested	LD_{50}	95 Per Cent Confidence Limits	Generation Time*	Slope of Median† Cell Length Curve
			hr	$\times 10^3$
Nonaerosolized	10.0	1.1–99.0	4.4	−22.0
Combined aerosolized	500	142–1750	3.0	0
Selected aerosolized clone				
1	110	17.7–682	4.2	−16.0
2	9.8	1.8–52.9	4.1	−14.0
3	$>10^5$		2.8	+12.0
4	$>10^5$		2.9	+32.0
5	$>10^5$		3.0	+6.0

* These figures are derived from the logarithmic portion of the multiplication rate curves.

† These figures are derived from the 4 to 24 hr segment of the cell length curves. A positive slope indicates continued increase in size, a negative slope a decrease.

TABLE 6

Aerosol stability and intrapleural LD_{50} of five single cell isolates of Pasteurella pestis

Type of Suspension	LD_{50}	Per Cent Recovery
Clone		
1	1100	0.18
2	$>10^5$	2.70
3	5.0	0.27
4	$>10^5$	7.90
5	9.0	0.85
Control cells	10.0	0.36
Avirulent cells	$>10^5$	8.30

The data (table 6) not only seem to confirm the hypothesis of heterogeneity, but also provide additional evidence for the inverse relationship between aerosol stability and virulence.

Effect of the sampling method. In any study of this kind, it is necessary to determine the extent to which the sampling method influenced the results. This determination was made by inoculating control cells into impingers and operating them for 60 min. At various times, samples were removed and viability, oxidation rates, antigenicity, virulence, cell growth and cell multiplication rates determined. Corrections for volume changes caused by evaporation of impinger fluid were made in every case. The results of this study indicated that most of the characteristics were unchanged. Only viability and oxidative metabolism rates were altered (60 per cent of the initial rate). However, these reactions differed from those observed with cells recovered from an aerosol in that both viability and oxidation rates were affected to the same extent.

DISCUSSION

A suspension of *Pasteurella pestis* apparently may be composed of a heterogeneous population. Present in this population are at least two kinds of living cells: some which multiply rapidly, grow to a large size, possess little or no infectivity for guinea pigs and are resistant to aerosolization, and others which multiply more slowly, do not attain the dimensions of the above-mentioned cells, are more virulent, and are affected to a marked degree by aerosolization. Undoubtedly, cells having intermediate characteristics are also present in the population. Since the evidence indicates that the characteristics of a suspension of cells recovered from an aerosol result from selection of naturally occurring segments of a heterogeneous population, it follows that the two kinds of cells are also present in the control culture. The experimental findings suggest that the population of a control culture demonstrates a wide spectrum of activity with respect to the characteristics which have been studied. The effect of aerosolization, therefore, might be explained in terms of a shift in the average activity of this wide spectrum of cellular activity. This shift results from the killing or inactivation of the more virulent, smaller and less rapidly multiplying organisms in the culture. Therefore, the experimentally determined activity of both kinds of cultures would depend upon the relative proportion of cells of each activity level in the culture.

The activity of the surviving bacteria apparently can be explained on a basis of selection, but very little evidence has been obtained concerning the cause of inactivation of those cells which do not survive. Also, no studies have been made as yet to explain why the slow-growing, virulent cells are the ones which are primarily affected by aerosolization, nor have the environmental factors which are responsible for inactivation been determined.

The only property which seems to be induced directly by some factor present in the aerosol is the alteration in the antigenic configuration. This change may be taken as an index of surface alteration and possibly of altered susceptibility to phagocytosis, but it is difficult to see how any other change in the patterns of infection or resistance can be attributed to it. It is possible, however, that the altered antigenic configuration may be of importance if an immunized host were exposed to an aerosol containing a high concentration of cells.

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SUMMARY

The dissemination of *Pasteurella pestis* into air significantly increases the intrapleural LD_{50} for guinea pigs, increases the oxidative metabolic

rate for several substrates, and alters the antigenic configuration of the cell surface (agglutination reaction). On subsequent cultivation, these cells were characterized by increased length, cell volume, and multiplication rate.

The antigenic configuration of cells recovered from aerosols reverts to the control type within 48 hr of cultivation, but the changes in virulence, cell size and multiplication rate persist for at least this period. It is suggested that the latter changes result from the selective action of factors present in aerosols on a heterogeneous cell population.

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