TECHNICAL MANUSCRIPT 48

THE RELATIONSHIP BETWEEN THE PLAGUE BACILLUS AND THE PHAGOCYTIC DEFENSE SYSTEM OF THE HOST

JULY 1963

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

ABSTRACT

The hypothesis that the ability of Pasteurella pestis to resist phagocytosis is the major determinant of its virulence has been re-evaluated in the light of recent evidence. Lethality titrations of virulent phagocytosis-susceptible plague bacilli were carried out in guinea pigs under the following conditions: (a) the organisms were injected intraperitoneally into untreated animals; (b) the organisms were injected intraperitoneally into animals that had been pretreated 24 hours earlier with 7.6 per cent sodium caseinate solution in order to mobilize massive number of phagocytes in the peritoneal cavity; (c) organisms contained within phagocytes from mice were injected into the peritoneal cavity of untreated animals; (d) the organisms were introduced directly into the circulatory system of untreated animals in order to expose the bacilli to rapid ingestion by reticuloendothelial phagocytes. All of these titrations revealed that the defense systems of the experimental animals were ineffective against plague. Furthermore, photographs revealed that P. pestis survived within free phagocytes and were viable when released from these cells. Suggestive evidence was obtained that neutrophiles containing plague bacilli were ingested by macrophages, and following digestion of the neutrophiles, the bacilli were able to multiply within the macrophages. It is postulated that the major determinant of the virulence of P. pestis is its ability to survive and multiply within the phagocytic cells of the host.

THE RELATIONSHIP BETWEEN THE PLAGUE BACILLUS AND THE PHAGOCYTIC DEFENSE SYSTEM OF THE HOST

After the discovery of the etiologic agent of plague, the earliest workers in the field observed that the exudates from plague victims were teeming with bacilli and leucocytes, yet few, if any, of the phagocytic cells contained bacilli.[1] Figure 1 is an impression smear of the spleen of a guinea pig that died of plague. This ability of Pasteurella pestis to resist phagocytosis has long been thought to be the major determinant of its virulence.

The phagocytosis resistance of P. pestis grown in vivo can be demonstrated in standardized test systems in vitro. Phagocytes from blood or exudates are mixed with a known ratio of bacilli and incubated under carefully controlled conditions. As you can see in Figure 2, there is no evidence of phagocytosis.

When P. pestis is grown in vitro at 26°C or less the organisms are highly susceptible to phagocytosis (Figure 3).

As shown in Table I, the relative resistance or susceptibility of a virulent strain can be measured in the in vitro test system by counting the number of organisms ingested by 100 neutrophiles and 100 macrophages and/or determining the percentage of these phagocytes that contain bacilli, Either index shows the striking difference in phagocytosis resistance between cultures grown in vivo and in vitro.

TABLE I. A COMPARISON OF THE PHAGOCYTOSIS RESISTANCE OF P. PESTIS (ALEXANDER STRAIN) GROWN IN VITRO AND IN VIVO

Growth Condition[a]	Leucocyte Source Guinea Pig No.	Bacteria/ Leucocyte	Phagocytic Activity[b] Neutrophile	Macrophage
In vitro	1	10	34/27	268/78
In vivo	1	21	1/1	10/7
In vitro	2	14	45/30	256/74
In vivo	2	32	3/3	6/4
In vitro	3	16	70/42	512/86
In vivo	3	15	13/11	94/38

a. In vitro - Grown in Difco Heart Infusion Broth at 26°C.
In vivo - Grown in the peritoneal cavity of guinea pigs.

b. Numerator - Number of intracellular bacteria in 100 neutrophiles or macrophages.
Denominator - Percentage of neutrophiles or macrophages containing bacteria.

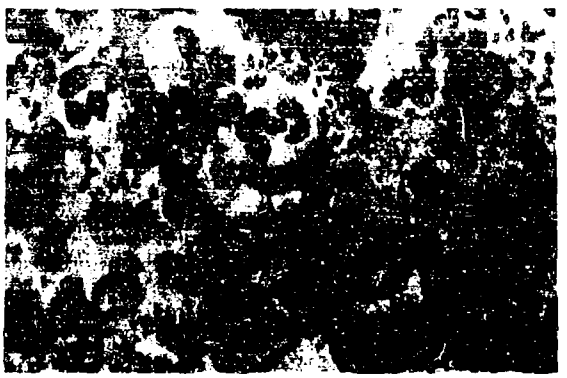

Figure 1. Impression Smear of Spleen of Guinea Pig Dead of Plague, Showing P. pestis and Leucocytes.

Figure 2. Demonstration of In Vitro Phagocytosis Resistance (P. pestis grown In Vivo).

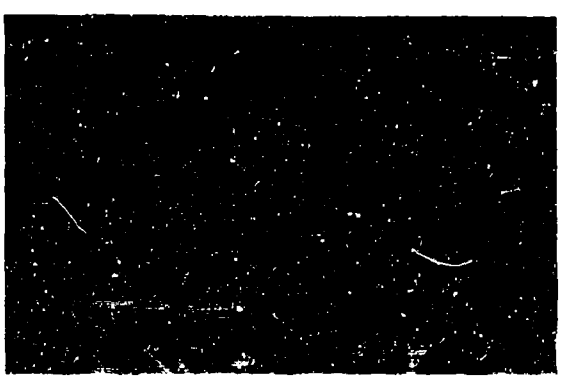

Figure 3. Demonstration of In Vitro Phagocytosis Susceptibility. (P. pestis grown at 26°C)

Many workers have contributed circumstantial evidence in favor of the hypothesis that phagocytosis resistance is the major virulence determinant of P. pestis.[2-10] Our data in Figure 4 show that when virulent P. pestis grown in vivo were injected intratracheally into guinea pigs,* the number of viable organisms recovered from the lungs at various time intervals continued to increase from the time of injection, and there was relatively little indication of phagocytosis by leucocytes observed in the impression smears of the lungs; however, injection of the same strain grown in vitro at 26°C was followed by a rapid decrease in viable organisms in the lungs during the first 16 hours, and many of the organisms were taken up by phagocytes.

When the fate of phagocytosis-resistant and -susceptible organisms was followed after intracardial injection into guinea pigs, a rather puzzling observation was made. Both types of P. pestis disappeared from the blood at the same rate; however, the resistant type increased in number much earlier than the susceptible type (Figure 5).

Assay of the number of viable organisms in the liver, spleen, lungs, and kidneys revealed that most of the organisms disappearing from the blood were taken up in the liver and spleen (Figure 6). The phagocytosis-susceptible type were apparently initially killed in large numbers; the resistant type were able to increase continually from the start.

As may be seen in Figure 7, histologic sections of the organs showed that the so-called phagocytosis-resistant type were taken up by the fixed macrophages of the reticulo-endothelial system of the liver and spleen just as the susceptible types were. The fixed phagocytes in the lungs and kidneys do not have access to bacteria in the blood, so these organs had little blood filtering effect.

Sections of the lungs following intratracheal injection of P. pestis revealed essentially the same thing (Figure 8). Fixed alveolar macrophages often contained large numbers of the resistant as well as phagocytosis-susceptible types. This indicated that the ability of P. pestis to resist phagocytosis was limited to the system of free phagocytes in the host, and did not apply to the fixed phagocyte system.

Burrows et al,[5] and Cavanaugh and Randall[10] have presented evidence that virulent P. pestis, when ingested by neutrophiles, are subsequently killed, but that the organisms multiply within macrophages. These experiments involved systems in which phagocytes were permitted to ingest susceptible P. pestis and any uningested bacteria were killed by antibiotics in the media surrounding the phagocytes in vitro. The phagocytes were either observed directly or disrupted by various means, and the number of

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* In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

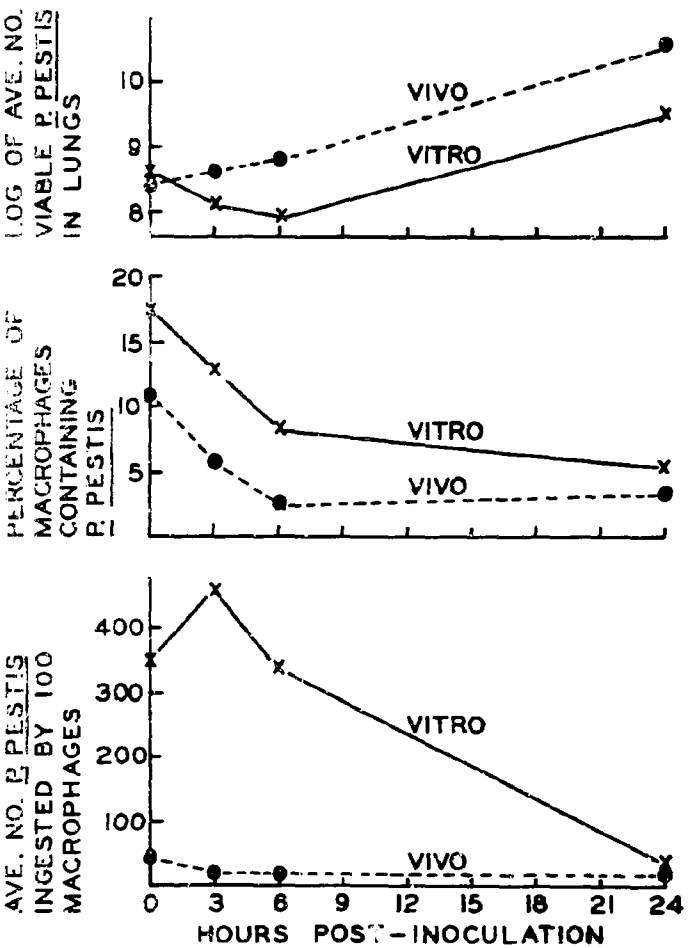

Figure 4. Correlation Between the Lung Clearance Phenomenon and Phagocytic Activity of Macrophages in Guinea Pig Lungs.

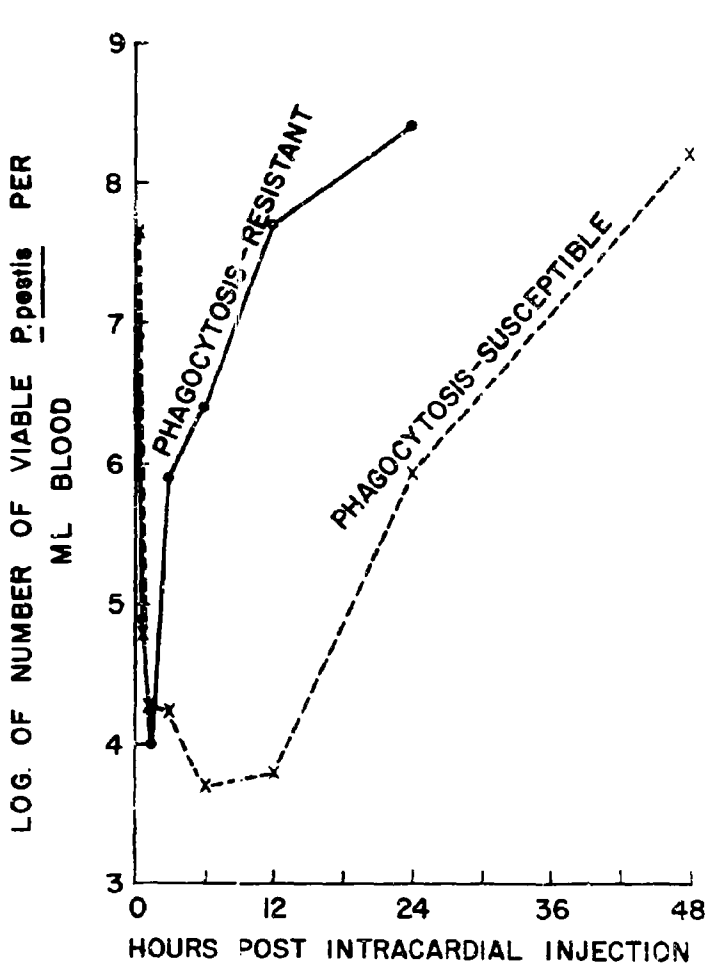

Figure 5. P. pestis in Guinea Pig Blood.

organisms remaining after various intervals was determined by direct and/or viable counts. We repeated this type of study, but instead of killing the extracellular bacilli with antibiotics, we attempted to wash away the uningested organisms by several washing cycles followed by light centrifugation. Control studies indicated that this was very effective. Our results tended to confirm the results of these other workers. However, in our studies, as in theirs, direct evidence of killing was based on morphologic evidence of degeneration of the intracellular bacteria, and assays of viability following rather rigorous mechanical or chemical disruption of the leucocytes (Table II).

TABLE II. THE EFFECT OF PHAGOCYTOSIS BY GUINEA PIG LEUCOCYTES ON *P. PESTIS* IN AN *IN VITRO* SYSTEM[a]

Length of Incubation, hours	No. Viable *P. pestis*, 10^7 per ml of system	No. Intracellular *P. pestis* per 100		Phagocytes Containing *P. pestis*, per cent	
		Neutrophiles	Macrophages	Neutrophiles	Macrophages
0	3.8	468	1654	85	100
1	2.6	284	1504	69	100
3	1.7	196	1144	54	98

a. Mixture of phagocytes and bacteria was incubated for 15 minutes, then extracellular bacteria were removed by washing before first sample was taken.

Morphologic evidence of killing included in large part the change from the bacillary form to a spherical form, as seen in this neutrophile in Figure 9. Direct observation of these "spheroplasts" within neutrophiles for 24 hours failed to detect any further degenerative or necrotic changes in the bacilli.

We were able to show that both virulent and avirulent *P. pestis* can be readily converted into spheroplast-like forms by egg white lysozyme[11] (Figure 10) (Table III). These "spheroplasts" are osmotically stable, viable, and retain the surface antigens that render them resistant to phagocytosis. Since the lysozyme in leucocytes is thought to be an important enzyme involved in the destruction of bacteria following phagocytosis, we suggest that the morphologic changes in *P. pestis* occasionally observed in

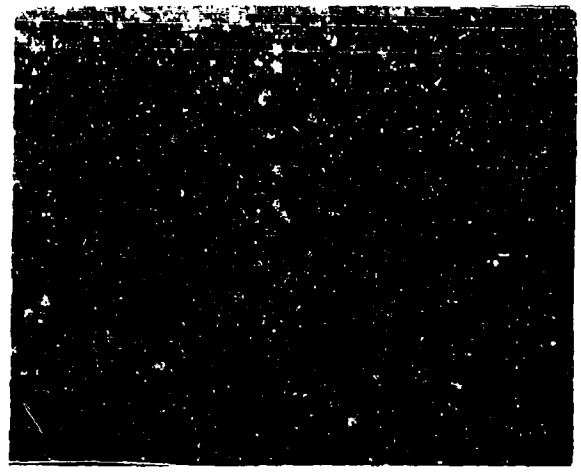

Figure 9. Guinea Pig Neutrophile Containing "Spheroplast-Like" Forms of P. pestis. (Giemsa)

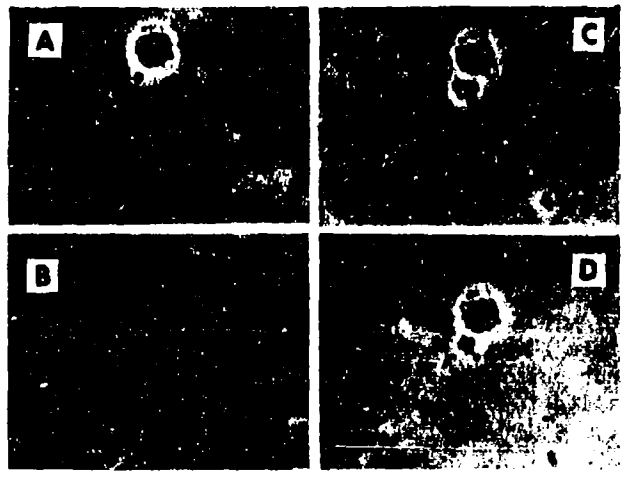

Figure 10. Growth of "Spheroplast-Like" Forms of P. pestis. (Time lapse with phase contrast illumination)

phagocytes may be due to lysozyme, but these changes do not necessarily indicate that the bacteria are dead. It is conceivable that the weakened cell walls of the ingested bacteria may render them more susceptible to lethal damage during mechanical or chemical disruption of the phagocytes in the various test procedures mentioned above. This might give a false impression that the phagocytes were killing the bacteria.

TABLE III. EFFECT OF LYSOZYME ON VIRULENT PASTEURELLA PESTIS

Culture Grown	Lysozyme Treatment	Per Cent Protoplasts in Culture	Viable Organisms x 10^9/ml	Phagocytic Index[a]	
				Neutrophile	Macrophage
In vivo	-	0	2.3	4/4	4/4
In vivo	+	67	2.2	2/2	4/4
In vitro 26°C	-	0	1.8	551/88	978/100
In vitro 26°C	+	2	1.4	545/81	1051/100

a. $\frac{\text{No. Organisms ingested by 100 Phagocytes}}{\text{\% Phagocytes containing Organisms}}$

More recent circumstantial evidence from our laboratory argues strongly against the hypothesis that the ability of P. pestis to resist phagocytosis is the major determinant of its virulence.

Fukui found that when virulent P. pestis organisms were grown at 5°C they were attenuated, but rapidly regained virulence upon incubation at 37°C for as little as six hours.[12] There was no concurrent increase in phagocytosis resistance associated with this recovery of virulence for guinea pigs (Figure 11).

When the fully toxigenic avirulent strain A-4, and the virulent non-encapsulated strain M-23 were grown under conditions that induced phagocytosis resistance in the virulent strain Alexander, the avirulent strain also became resistant while the virulent nonencapsulated strain did not (Table IV).

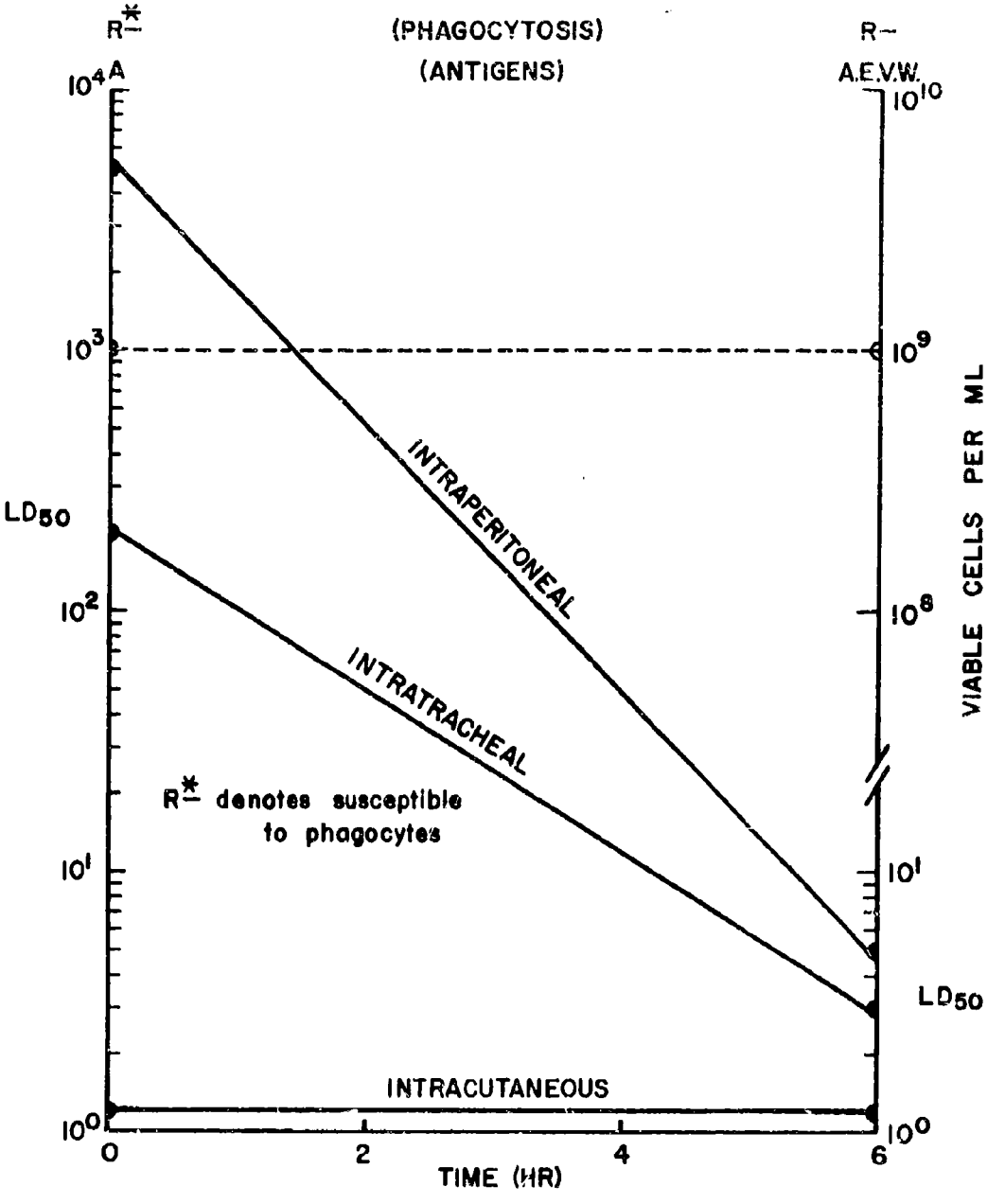

Figure 11. Enhancement of Virulence for Guinea Pigs in Nonmultiplying Cells.

TABLE IV. A COMPARISON OF THE PHAGOCYTOSIS RESISTANCE OF A NUMBER OF P. PESTIS STRAINS

P. PESTIS STRAIN	MEDIUM	INCUBATION TEMPERATURE, °C	BACTERIA/ LEUCOCYTE	PHAGOCYTIC ACTIVITY[a] Neutrophile	Macrophage	DEGREE OF RESISTANCE
Sterile Control	HIB[b]	-	0.0	0/0	0/0	-
Alexander	HIB	26	6.8	90/35	373/77	0
Alexander	HIB + 5% Blood	37	6.9	38/25	92/44	+++
A-4 Avirulent	HIB + 5% Blood	37	5.8	32/26	64/40	+++
A-12 Avirulent	HIB + 5% Blood	37	9.7	194/54	444/82	0
M-23	HIB + 5% Blood	37	9.2	95/51	252/84	0
M-23	in vivo[c]	>37	14.5	96/67	897/100	0
Alexander	in vivo	>37	18.8	10/8	139/58	++++

a. Numerator - Number of intracellular bacteria in 100 neutrophiles or macrophages.
Denominator - Percentage of neutrophiles or macrophages containing bacteria.
b. HIB - Difco Heart Infusion Broth.
c. In vivo - Grown in the peritoneal cavity of a guinea pig.

The degree of virulence of these strains did not change, however. It is also interesting to note that toxigenic avirulent strains grow equally as well as virulent strains in guinea pig blood maintained under simulated in vivo conditions of temperature and oxygenation. This led us to suspect that the major determinant of P. pestis' virulence is its ability to survive and multiply within the free and fixed phagocytic systems of the host. Evidence in support of this hypothesis follows.

When the intraperitoneal LD_{50} of virulent phagocytosis-susceptible P. pestis in normal guinea pigs was compared with the dose in guinea pigs that had a pre-existing casein-induced peritoneal exudate, no significant

difference was noted (Table V). Control studies showed that the ratio of phagocytic cells to bacteria in the peritoneal exudates was approximately 40,000,000 to 1, so that the chance of the bacteria escaping phagocytosis must have been very small. Therefore, it appeared that phagocytosis had no protective effect.

In these experiments, guinea pig phagocytes were incubated with virulent P. pestis in vitro for six hours; the uningested organisms were removed by washing, and the number of ingested cells was determined by direct and viable counting methods. Comparative LD_{50} titrations in mice were carried out using dilutions of suspensions of leucocytes containing a known number of P. pestis and dilutions of a sample of the same culture used in the test system, but that had not been so treated. The LD_{50} of P. pestis contained within free phagocytes from guinea pigs was not significantly different from the LD_{50} of nonphagocytosed organisms, even though the bacteria may have been within the phagocytes for as long as six hours. In the closed in vitro systems used to study phagocytosis, as a rule the maximum number of bacteria will be taken up within 30 minutes. So, again, it appeared that ingestion of P. pestis by guinea pig phagocytes had no effect on their viability or virulence (Table VI).

Since previous experiments revealed that P. pestis, like most bacteria, is removed from the blood by the reticulo-endothelial system, and that large numbers of the phagocytosis-susceptible type are apparently killed, it seemed logical that an LD_{50} by the intracardial route should be relatively high, especially when compared with an LD_{50} by the intradermal route which was known to present the least resistance to lethal infection with P. pestis. A comparison of the LD_{50}'s of phagocytosis-susceptible P. pestis injected into guinea pigs via the intracardial and intradermal routes did not show significant differences (Table VII). This indicated that the reticulo-endothelial system is not an effective defense against plague either, at least in guinea pigs.

Direct evidence that phagocytosis is not effective in killing virulent P. pestis has also been obtained. Time-lapse motion pictures have been taken in which a neutrophile was observed to ingest a number of P. pestis cells; no change in the morphology of the bacteria resulted during a two-hour period of continuous observation under the phase microscope. Figure 12 represents a typical example of studies in which guinea pig phagocytes were observed to ingest P. pestis; after varying lengths of intracellular residence, the bacteria were released by lysing of the phagocyte. As shown, the bacteria were able to multiply. This was true whether the phagocyte was a neutrophile or a macrophage. We intend to exploit this technique further in order to study the fate of avirulent as well as virulent P. pestis in phagocytes.

When an inflammatory exudate is induced in a guinea pig by intraperitoneal injection of sodium caseinate solution, the predominant type of phagocyte during the initial phase is the neutrophile. By the 24th hour, many of the neutrophiles have been ingested by the increasing number of macrophages that are mobilized; this is the apparent fate of all senile or damaged neutrophiles.

TABLE V. EFFECT OF PRE-MOBILIZED PHAGOCYTES ON THE INTRAPERITONEAL LD_{50} OF VIRULENT P. PESTIS IN GUINEA PIGS

EXP. NO.	UNTREATED CONTROL GUINEA PIGS		GUINEA PIGS WITH INDUCED PERITONEAL EXUDATE[a]	
	LD_{50}	95% Conf. Limits	LD_{50}	95% Conf. Limits
1	9.2	4.8 - 17.5	7.2	4.2 - 12.2
2	42.0	22.0 - 79.0	19.0	7.2 - 47.5
3	19.0	9.5 - 38.0	15.0	4.5 - 49.5

a. Peritoneal exudate induced by intraperitoneal injection of 25 ml of 7.6 per cent sodium caseinate solution 24 hours preceding challenge.

TABLE VI. COMPARATIVE LD_{50} IN MICE OF NON-PHAGOCYTOSED P. PESTIS AND P. PESTIS CONTAINED WITHIN GUINEA PIG PHAGOCYTES

EXP. NO.	LD_{50} OF NON-PHAGOCYTOSED P. PESTIS[a]		LD_{50} OF PHAGOCYTOSED P. PESTIS	
	Viable Count	Direct Count	Viable Count	Direct Count
1	23 (15.5 - 34)	110 (73 - 165)	15.4 (7.3 - 31.3)	42 (20 - 88.2)
2	7.2 (2.4 - 21.2)		35 (11.2 - 108.5)	110 (29 - 407)

a. 95 per cent confidence limits shown in parentheses.

TABLE VII. COMPARATIVE LD_{50} OF P. PESTIS FOR GUINEA PIGS WHEN INJECTED INTRACARDIALLY AND INTRADERMALLY

EXP. NO.	INTRACARDIAL ROUTE		INTRADERMAL ROUTE	
	LD_{50}	Conf. Limits	LD_{50}	Conf. Limits
1	20.0	10 - 40	9.4	3 - 26
2	20.0	10 - 38	14.0	9 - 38
3	5.4	3.4 - 8.5	2.4	0.8 - 6.7

NOTE: Method of Litchfield and Wilcoxson[13] used to calculate LD_{50} and confidence limits.

The ingested neutrophile becomes surrounded by a refractile vacuole; the multilobulated nucleus becomes pyknotic, and the cytoplasmic constituents become progressively condensed into a homogeneous-appearing mass (Figure 13).

If virulent phagocytosis-susceptible P. pestis cells are injected into guinea pigs 24 hours after inducing a sterile peritoneal exudate, many neutrophiles containing the bacillus may be observed within macrophages. As seen in Figure 14, when a neutrophile containing P. pestis is ingested by a macrophage, the neutrophile shows all the signs of necrosis and digestion while the bacteria continue to appear normal.

Figure 15 under phase microscopy shows two macrophages containing neutrophiles. One neutrophile is in an early stage of digestion, and one is in a late stage.

Figure 16 is the same preparation at a slightly different focal plane showing normal-appearing P. pestis in the vacuole occupied by the neutrophile remnant.

Many macrophages in exudates infected with P. pestis 24 hours or more before sampling contained remnants of neutrophile cytoplasm and nuclei in large vacuoles that also contained P. pestis. Many P. pestis cells were also observed within macrophages that were not encased in vacuoles. When P. pestis cells were contained in large vacuoles there was almost always some evidence of neutrophile debris in the same vacuole (Figure 17).

Many of the macrophages in old plague-infected exudates were filled with what appeared to be actively growing P. pestis with no evidence of any large digestive vacuoles (Figure 18). By this time any extracellular organisms would be resistant to further phagocytosis, so it may be assumed that the intracellular organisms were progeny of the original susceptible type ingested earlier. If the susceptible type were taken up directly by a macrophage they apparently multiplied quite rapidly and were not encased within vacuoles, or, if they were, the vacuole was not much larger than the individual bacterium. However, if the susceptible type were originally taken up by neutrophiles, and these phagocytes then were ultimately taken up by macrophages, it is our impression that the bacteria were released by digestion of the neutrophile and then were able to grow within the large vacuole containing the remnants of the neutrophile, since large numbers of normal-appearing P. pestis were seen within the large cytoplasmic vacuoles of macrophages that result from ingestion of neutrophiles.

In summary, circumstantial evidence indicates that the hypothesis that "the ability of P. pestis to resist phagocytosis is the major determinant of its virulence" must be re-evaluated. We suggest that the major determinant of the virulence of P. pestis is its ability to survive and multiply within the phagocytic cells of the host defense system.

Figure 12. Active Growth of Intracellular P. pestis Released by Electrical Lysis of Guinea Pig Neutrophile. (Time lapse with phase contrast illumination)

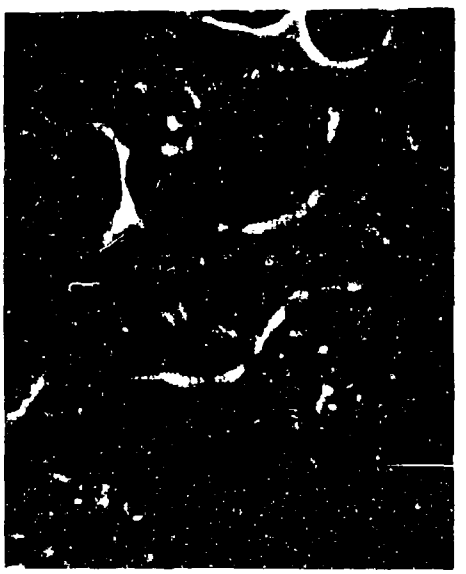

Figure 13. Phagocytosis of Neutrophiles by Macrophages in Peritoneal Exudate. (Phase contrast illumination)

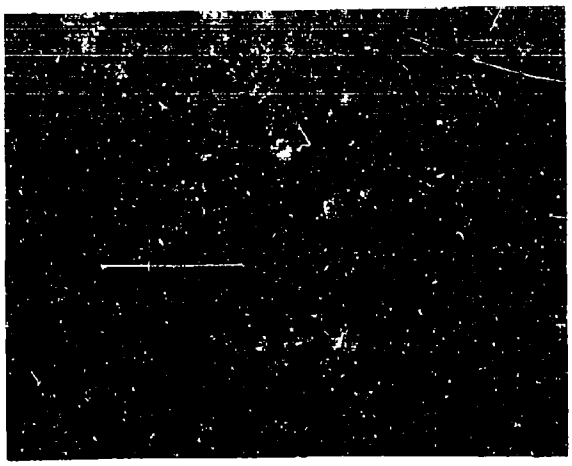

Figure 14. Macrophage Which Has Ingested a Neutrophile Containing P. pestis Organisms. (Giemsa)

Figure 15. Macrophages Containing Neutrophiles in Different Stages of Digestion. (Phase contrast illumination)

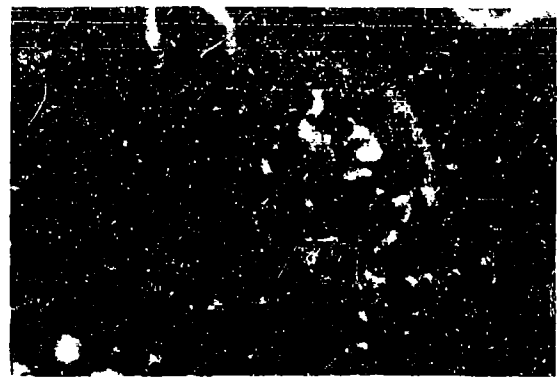

Figure 16. Same Macrophages as Figure 15, at Different Focal Plane, Showing P. pestis in Same Vacuole Containing Partially Digested Neutrophile.

Figure 17. Macrophage Containing P. pestis and Neutrophile Remnants in Same Vacuole.

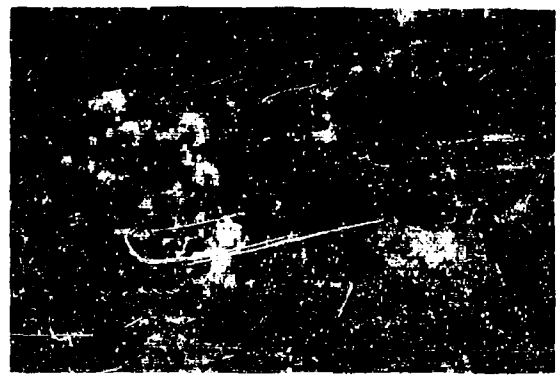

Figure 18. Macrophage Containing Actively Growing P. pestis. (Giemsa)

LITERATURE CITED

1. Pollitzer, R. "Plague," Monograph 22, Geneva, World Health Organization, 1954.

2. Englesberg, E.; Chen, T.H.; Levy, J.B.; Foster, L.E.; and Meyer, K.F. "Virulence in Pasteurella pestis," Science 119;413-414, 1954.

3. Burrows, T.W., and Bacon, G.A. "The basis of virulence in Pasteurella pestis: Comparative behavior of virulent and avirulent strains in vivo," Brit. J. Exptl. Pathol. 35:134-143, 1954.

4. Burrows, T.W. "The basis of virulence for mice of Pasteurella pestis," in "Mechanisms of microbial pathogenicity," Cambridge, England, Cambridge Univ. Press, 1955. pp 151-175.

5. Burrows, T.W., and Bacon, G.A. "The basis of virulence in Pasteurella pestis: An antigen determining virulence," Brit. J. Exptl. Pathol. 37:481-493, 1956.

6. Burrows, T.W., and Bacon, G.A. "The basis of virulence in Pasteurella pestis: The development of resistance of phagocytosis in vitro," Brit. J. Exptl. Pathol. 37:286-299, 1956.

7. Wessman, G.E.; Miller, D.J.; and Surgalla, M.J. "Toxic effect of glucose on virulent Pasteurella pestis in chemically defined media," J. Bacteriol. 76:368-375, 1958.

8. Fukui, G.M.; Lawton, W.D.; Janssen, W.A.; and Surgalla, M.J. "Response of guinea pig lungs to in vivo and in vitro cultures of Pasteurella pestis," J. Infect. Diseases 100:103-107, 1957.

9. Janssen, W.A.; Fukui, G.M.; and Surgalla, M.J. "A study of the fate of Pasteurella pestis following intracardial injection into guinea pigs," J. Infect. Diseases 103:183-187, 1958.

10. Cavanaugh, D.C., and Randall, R. "The role of multiplication of Pasteurella pestis in mononuclear phagocytes in the pathogenesis of flea-borne plague," J. Immunol. 83:348-363, 1959.

11. Janssen, W.A.; Beesley, E.D.; and Surgalla, M.J. "The effect of lysozyme on Pasteurella pestis," Bacteriol. Proc. 1960.

12. Fukui, G.M.; Lawton, W.D.; Ham, D.A.; Janssen, W.A.; and Surgalla, M.J. "The effect of temperature on the synthesis of virulence factors by Pasteurella pestis," Ann. N. Y. Acad. Sci. 88:1146-1151, 1960.

13. Litchfield, J.T., Jr., and Wilcoxson, F. "A simplified method of evaluating dose-effect experiments," J. Pharmacol. Exptl. Therap. 96:99-113, 1949.