Plague Bacillus: Survival within Host Phagocytes

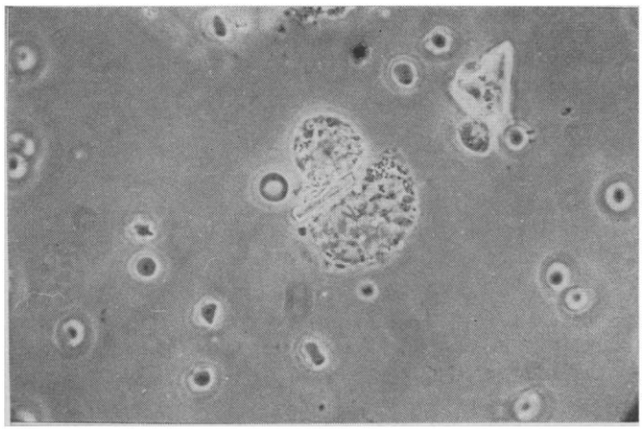
Abstract. Pasteurella pestis *within neutrophiles and macrophages removed from the peritoneal cavity of guinea pigs during experimental plague were shown to be viable by direct microscopic observation of the infected phagocytes incubating in suitable bacteriologic media. The time-honored hypothesis that the major determinant of the virulence of the plague bacillus is its ability to resist ingestion by phagocytes must be reevaluated.*

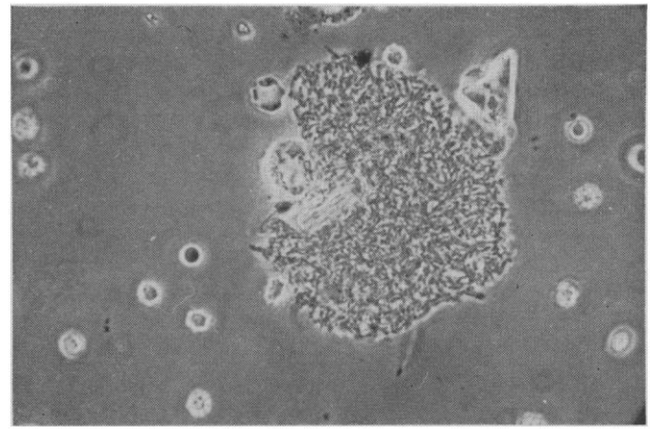
Classical plague is a highly fatal, acute infectious disease of man and other mammals caused by the bacterium *Pasteurella pestis*. When grown under certain conditions, this organism is susceptible to phagocytosis after inoculation into a suitable host; however, it becomes highly resistant to ingestion by free phagocytes after a few hours within the host (*1*). This ability to resist phagocytosis has long been thought to be the major factor determining the virulence of the plague bacillus, and much circumstantial evidence in support of this hypothesis has been presented (*2*).

On the other hand, in 1944 Jawetz and Meyer (*3*) reported that *P. pestis* ingested in vitro by phagocytes in whole blood or in induced exudates from a variety of nonimmune experimental animals were not destroyed. Furthermore, we have obtained evidence that (i) "phagocytosis resistance" of *P. pestis* applies only to free phagocytes and not to the fixed macrophages of the reticuloendothelial system in the guinea pig (*4*); (ii) there is no correlation between ability to resist phagocytosis and virulence in different strains of *P. pestis* (*5*); and (iii) the phagocytic defense system of the nonimmune guinea pig is apparently ineffective against plague (*6*).

Evidence that virulent *P. pestis* are killed after phagocytosis by host neutrophiles, but survive within free macrophages, has been presented by other workers (*7*). Their experiments included systems in which phagocytes were permitted to ingest susceptible *P. pestis* in vitro, and any bacteria escaping phagocytosis were killed by antibiotics in the media. The phagocytes were either observed directly or disrupted by various means, and the number of phagocytized organisms that survived after various intervals was determined by direct observation or by growth on bacteriologic media. Since these findings were inconsistent with the earlier work of Jawetz and Meyer (*3*) and with our overall impression that phagocytosis of the plague bacillus is ineffective in preventing plague, we decided to assay the survival of *P. pestis* at various intervals after phagocytosis by neutrophiles and free macrophages in experimentally infected guinea pigs. We felt that (i) the use of antibiotics to eliminate extracellular organisms in any test system might also kill some within phagocytes (*8*) or damage the bacteriocidal capacity of the phagocytes, and (ii) any attempt to release phagocytized organisms might cause fatal damage to bacteria merely weakened by the digestive processes of the phagocytes. Therefore, we simply suspended infected phagocytes from guinea pigs in media believed to be capable of initiating growth of even the most fragile individual *P. pestis* and observed them microscopically for evidence of growth of the intracellular organisms.

Guinea pigs were each injected intraperitoneally with 25 ml of sterile 7.6 percent solution of sodium caseinate.





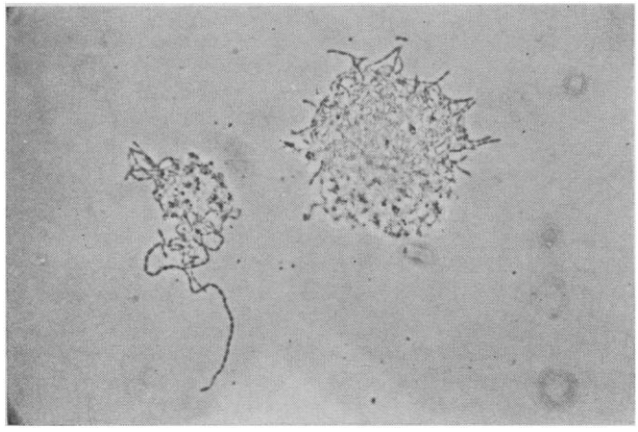


Fig. 1 (upper left). Macrophage containing *Pasteurella pestis* in a microculture. A 24-hour, caseinate-induced exudate was infected with *P. pestis* 6 hours before sampling (phase illumination; × 880).

Fig. 2 (upper right). The same preparation as shown in Fig. 1, but 24 hours later.

Fig. 3 (left). Photomicrograph of the same culture shown in Fig. 2. Note the difference in appearance of the macrophage (right) as compared to that of the neutrophile (left).

This induced 24 hours later the production of 20 to 30 ml of sterile exudate containing approximately 2×10^7 phagocytes per milliliter, of which 90 percent were neutrophiles and 10 percent macrophages. At this time, 1×10^{10} phagocytosis-susceptible *P. pestis* (either the virulent Alexander strain or the avirulent A-1224 strain grown in Difco heart infusion broth for 24 hours at 26°C) were inoculated into the peritoneal cavity of each animal, and thereafter at various time intervals, from 2 to 48 hours, samples of the infected exudate were removed. A portion of each sample was smeared immediately onto a microscope slide and another portion was used immediately to prepare a microculture. Then 0.2-ml portions were withdrawn from the animal directly into tuberculin syringes, each containing 0.8 ml of the desired bacteriologic medium; they were mixed thoroughly and microcultures were prepared. The microcultures were made by placing a small drop of sample on a microscope slide and carefully overlaying it with a 25-mm² cover slip in such a manner as to exclude all air bubbles and obtain the thinnest preparation possible without disrupting the phagocytes. Edges of the cover slip were sealed with vaspar. Smear preparations were stained with Giemsa's blood stain in the usual way. All glassware had been chemically cleaned, meticulously rinsed, and sterilized. We used the following media: (i) Difco heart infusion broth (HIB); (ii) HIB and 0.3*M* sucrose; and (iii) mouse heart infusion to which had been added 20 percent coconut water, 6 percent Difco oleic acid–albumin complex (Dubos), 0.5 percent xylose, and 0.004*M* $CaCl_2$ (*9*). All media were sterilized by filtration.

The percentage of neutrophiles and macrophages containing *P. pestis* in the infected exudate at the moment of sampling was determined by microscopic observation of the stained smears, and the percentage of these cells containing viable *P. pestis* was determined by microscopic observation of the microcultures under phase illumination after 24 hours incubation at 23°C; 100 neutrophiles and 100 macrophages were counted in each preparation. Under phase illumination very few phagocytes containing any *P. pestis* could be detected immediately after removal of the exudate; however, in the stained preparations, visualization of infected phagocytes was easy and frequent, although they rarely contained more than a few organisms. After 24 hours' incubation of the microcultures, many phagocytes were bursting with organisms, and visualization under phase illumination was easy; thus evidence of the viability of the phagocytized organisms was obvious. Figure 1 shows a macrophage containing a few *P. pestis* in a microculture, under phase illumination. The infected exudate had been diluted with mouse heart infusion broth containing Dubos' oleic–albumin complex and coconut water; Fig. 2 shows the same macrophage 24 hours later. Note the conspicuous growth of the intracellular organisms. Figure 3 is a photomicrograph of the same culture shown in Fig. 2, but shows a neutrophile in addition to the macrophage. The difference in appearance of intracellular growth in the neutrophile compared with the macrophage is typical. Chains of bacteria apparently penetrate directly through the cell wall of neutrophiles quite easily, but in macrophages the growing bacteria are confined until they burst the host cell.

Table 1. Survival of *Pasteurella pestis* in guinea pig exudate neutrophiles and macrophages. Exudate was sampled 6 hours after infection and was diluted with Difco HIB, HIB plus 0.3*M* sucrose, mouse HI plus Dubos' oleic acid–albumin complex and coconut water, or undiluted. Microcultures were incubated for 24 hours; MHI, mouse heart infusion.

Strain of *P. pestis*	Percentage of cells containing *Pasteurella pestis*				
	Initial smear	Viable organisms in microcultures			
		Undiluted	HIB	HIB + sucrose	MHI complex
		Neutrophiles			
Alex	58	13	21	30	12
A-1224	78	13	22	22	38
		Macrophages			
Alex	30	28	60	90	50
A-1224	57	25	51	72	50

A total of 30 guinea pigs were used in the studies; eight were uninoculated controls and two groups of 11 each were inoculated with the virulent and avirulent strains of *P. pestis*, respectively. In general there was considerable variation in phagocytic capacity between individual phagocytes, types of phagocytes, and phagocytes from individual guinea pigs; however, in all the samples the addition of bacteriologic media to infected exudate promoted growth of phagocytized *P. pestis*. Difco heart infusion broth with 0.3*M* sucrose seemed to be the most effective (see Table 1).

Virulent and avirulent *P. pestis* ingested in vivo by macrophages and many neutrophiles in caseinate-induced peritoneal exudates of guinea pigs were found to be viable, provided the infected phagocytes were incubated in suitable bacteriologic media. This was true even when the infected phagocytes were removed 48 hours after inoculating the animals, in which case it is assumed that the bacteria had resided within the phagocytes for at least 42 hours, since both strains of *P. pestis* become highly resistant to phagocytosis within 3 to 6 hours in vivo (*1*, *5*).

The number of neutrophiles containing viable *P. pestis* was always less than the total number containing ingested organisms. However, we believe that this is due to the inadequacy of our test media rather than to a lethal effect of the neutrophiles, since the effectiveness of media in initiating growth of the phagocytized organisms differed greatly. Furthermore, we often detected viable *P. pestis* in macrophages that had become infected by ingesting and digesting infected neutrophiles. In contrast, the number of macrophages observed to contain viable *P. pestis* in the microcultures was often greater than the number of infected macrophages detected initially in stained smears, which suggests that organisms lying underneath nuclei and other organelles may go undetected in stained preparations or that *P. pestis* may exist within phagocytes in some unrecognized morphologic state. Preparations from uninoculated control exudates remained sterile and could not have been confused with the inoculated preparations in any way. Neutrophiles and macrophages in control and infected microcultures continued very sluggish amoeboid movements for several hours, but phagocytosis was never observed during the period of in vitro incubation; extracellular bacteria were rarely observed, so that the apparent growth of intracellular *P. pestis* could not have been an illusion due to continuing phagocytosis during the period of incubation. Brownian movement stopped and nuclear swelling occurred within the 24-hour incubation period, but the cells were

otherwise morphologically intact for several days. We believe that the phagocytes degenerated rapidly when suspended in the media employed and became permeable to the surrounding medium, thus providing the intracellular bacteria with a favorable environment for growth.

It is suggested that the method outlined may be more useful generally in evaluating the effectiveness of phagocytosis against pathogenic bacteria than conventional techniques since, in our hands, the use of mechanical, immunological, osmotic, and electrical methods for releasing ingested *P. pestis* often indicated falsely that these bacteria had been killed by the phagocytes, as was the case with other workers using antibiotics to control extracellular organisms.

These data add further evidence that the ability of *P. pestis* to resist phagocytosis in the host is not important in determining its virulence since virulent, and even some avirulent strains, are able to survive within free phagocytes. We believe that a major determinant of the virulence of the plague bacillus may be its ability to multiply within the fixed macrophages of the host's reticuloendothelial system, and this possibility is under investigation.

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Direct Pathway to the Brain

Abstract. *Whole-body autoradiographic studies demonstrated that, when isotopically labeled glucose is placed in the ligated oropharynx, there is a rapid movement of the isotope directly to the intracranial cavity. This passage involves nonspecific diffusion, bypassing all recognized routes to the brain.*

It has been reported (*1*) that the introduction of isotopically labeled glucose and sodium chloride into the ligated oropharyngeal cavity of the rat produced a greater concentration of radioactivity in the brain than did direct gastric or intestinal administration of these substances. These results raise questions concerning the pathway for the movement of these compounds into the intracranial cavity. Whole-body autoradiography was utilized to investigate this phenomenon.

Twelve adult male rats (200 ± 20 g) were anesthetized with chloral hydrate (400 mg/kg intraperitoneally); ten of these rats were in the experimental group (group 1) and two were used as controls (group 2). In the experimental animals, the esophagus and trachea

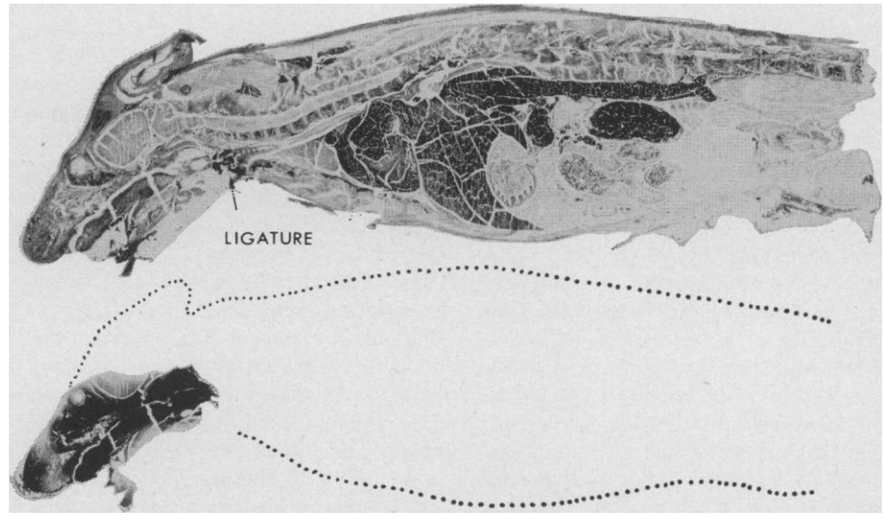

Fig. 1. The massive activity in the head region after labeled glucose was placed in the mouth. Above the autoradiograph is a photograph of the actual section from which it was made. There is no activity beyond the ligature, but it is present throughout the face region and is evident in the brain.

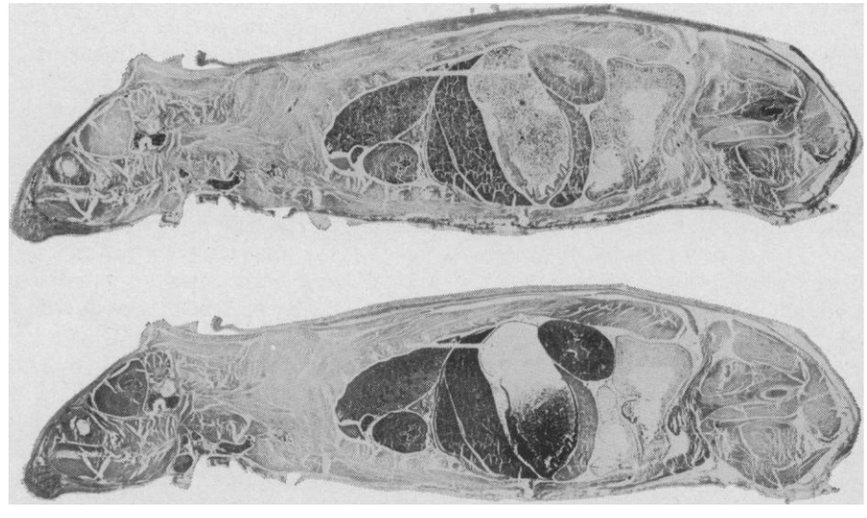

Fig. 2. Autoradiogram of a control animal after duodenal administration of the isotope. A photograph of the same tissue section is above it. The distribution of activity throughout the body is apparent.