Plague Bacillus: Survival within Host Phagocytes

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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 (4) or damage the bactericidal 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).](image1)

![Fig. 2 (upper right). The same preparation as shown in Fig. 1, but 24 hours later.](image2)

![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).](image3)
This induced 24 hours later the production of 20 to 30 ml of sterile exudate containing approximately $2 \times 10^7$ phagocytes per milliliter, of which 90 percent were neutrophiles and 10 percent macrophages. At this time, $1 \times 10^7$ phagocytosis-susceptible \textit{P. pestis} (either the virulent Alexander strain or the avirulent A-1224 strain grown in Difco heart infusion broth for 24 hours at $26^\circ C$) 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-mm 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 0.3M 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 0.004M CaCl$_2$. All media were sterilized by filtration.

The percentage of neutrophiles and macrophages containing \textit{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 \textit{P. pestis} was determined by microscopic observation of the microcultures under phase illumination after 24 hours incubation at $23^\circ C$; 100 neutrophiles and 100 macrophages were counted in each preparation. Under phase illumination very few phagocytes containing any \textit{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 \textit{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.

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 \textit{P. pestis}, respectively. In general there was considerable variation in phagocytic capacity between individual phagocytes, types of phagocytes, and phagocytes in individual guinea pigs; however, in all the samples the addition of bacteriologic media to infected exudate promoted growth of phagocytized \textit{P. pestis}. Difco heart infusion broth with 0.3M sucrose seemed to be the most effective (see Table 1).

<table>
<thead>
<tr>
<th>Strain of \textit{P. pestis}</th>
<th>Percentage of cells containing \textit{Pasteurella pestis}</th>
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<tbody>
<tr>
<td></td>
<td>Initial smear</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td>Alex</td>
<td>58</td>
</tr>
<tr>
<td>A-1224</td>
<td>78</td>
</tr>
<tr>
<td>Macrophages</td>
<td>30</td>
</tr>
<tr>
<td>A-1224</td>
<td>57</td>
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Table 1. Survival of \textit{Pasteurella pestis} in guinea pig exudate neutrophiles and macrophages. Exudate was sampled 6 hours after infection and was diluted with Difco HIB, HIB plus 0.3M sucrose, mouse HI plus Dubos' oleic acid-albumin complex and coconut water, or undiluted. Microcultures were incubated for 24 hours; MHI, mouse heart infusion.
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.

References and Notes
10. "In conducting the research reported herein, the investigators adhered to 'Guide for Laboratory Animal Facilities and Care' established by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, NAS-NRC."

14 November 1966