OBSERVATIONS ON THE BEHAVIOR IN VITRO OF PASTEURIELLA TULARENSIS AFTER PHAGOCYTOSIS

DAVID STEFANYE, HUGH B. TRESSELT, AND LEONARD SPERO

U. S. Army Chemical Corps, Fort Detrick, Frederick, Maryland

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The phagocytic leucocyte has been implicated as an initial receptor site in many diseases involving gram-negative bacteria. Thus Cavanaugh and Randall (1959) have shown that Pasteurella pestis will not multiply in granulocytes but proliferates abundantly in monocytes. Pomales-Lebron and Stinebring (1957) and Freeman and Vana (1958) have demonstrated that Brucella abortus proliferates in a similar manner in normal but not in immune phagocytes from guinea pigs and rats. On the other hand, Hopps, Showacre and Smadel (1960) observed rapid death of Salmonella typhosa present in L cells maintained in tissue culture with extracellular antibiotic. Recently, Shepard (1959) observed that Pasteurella tularensis is able to multiply in HeLa cells. This worker did not investigate its survival in leukocytes.

These findings and others pertaining to the intracellular residence of microorganisms encouraged us to investigate the effect of an intraphagocytic environment on P. tularensis in an attempt to determine if this pathogen were capable of intracellular proliferation.

MATERIALS AND METHODS

Peritoneal exudates rich in either neutrophils or mononuclear leukocytes were obtained from guinea pigs after intraperitoneal injection of physiological saline. In those experiments using neutrophils, the exudate was harvested 4 to 6 hr after saline injection. Otherwise, exudates were obtained 48 hr after saline injection, when the neutrophil response had subsided. Tissue culture techniques were similar to those described by Pomales-Lebron and Stinebring (1957) and Braun, Pomales-Lebron, and Stinebring (1958). Their recommended medium was supplemented with 40% by volume of TC199 tissue culture solution (Difco) as proposed by Osgood and Brooke (1958). Autologous serum was added to promote phagocytosis and avoid incompatibility between serum and cells from different animals.

Leukocytes thus established in Porter flasks containing flying cover slips were then treated with 48-hr-old cultures of strains of P. tularensis of a particular degree of virulence. The cultures were prepared by suspending the microorganism grown on glucose-cysteine-blood agar in Hanks solution and adding this suspension to the Porter flask in the proportion of 50 to 200 bacteria per leukocyte. After the prescribed 2-hr period of incubation (Braun et al., 1958), the suspending medium was removed with a pipette and replaced with fresh serum-free medium containing 10 μg of dihydrostreptomycin per ml and the culture was incubated for another 2 hr. At the end of this time, the number of viable bacteria per tissue culture flask was determined. This point was taken as a zero-time reference and the results of subsequent determinations were expressed relative to it. The determinations were carried out by removing the antibiotic medium from the flask, and after washing with Hanks solution to remove residual antibiotic, the flying cover slips were removed, and the leukocytes were disrupted in 0.1% gelatin-physiological saline by shaking vigorously with fine glass beads for 1 min to liberate the ingested bacteria. These were then serially diluted, plated on glucose-cysteine-blood agar, and incubated to ascertain the number of viable bacteria per culture flask. For several of the incubation times, the flying cover slips were stained with Macchiavello’s stain or with specific fluorescent antibody and examined to determine whether bacteria actually had been phagocytized. Each flask provided information for only one determination. During the investigation it became necessary to observe the effect of the intraphagocytic bactericidal substance, phagocytin, on the proliferation of P. tularensis. This material was prepared according to Hirsch (1956) and its potency determined by assay, using Escherichia coli as the test microorganism. Cultures of P. tularensis were then incubated with the phagocytic extract containing phago-
cytin for 4 hr at 37 C and the number of viable organisms was determined as noted above.

The subcutaneous Ld50 for guinea pigs of each strain used in this work was approximately as follows: Schu S-4, 1; Chur, 100; Jap, 106; Max, 106; 38A, avirulent.

EXPERIMENTAL RESULTS

The results of a typical experiment wherein mononuclear leukocytes were allowed to ingest a particular strain of *P. tularensis* are shown in Fig. 1a. It is seen that the number of viable cells of the 38A strain decreased much faster than was the case with strains possessing any degree of virulence. No significant differences were apparent in the death rates of the virulent strains.

Some difficulty in preserving the structure of polymorphonuclear leukocytes in tissue culture has been reported (Wilson, 1957). In preliminary experiments we also experienced this problem; however, the addition of the tissue culture solution described by Osgood and Brooke (1958) made it possible to maintain these cells without any great loss. The results of one experiment are shown in Fig. 1b. It is seen that all strains of *P. tularensis* were killed rapidly after ingestion by polymorphonuclear leukocytes. The initial death rate was considerably more rapid than in the mononuclear cells.

Under the experimental conditions, after the initial 4-hr incubation time had elapsed, it was found that there were always about 104 to 104 viable cells of *P. tularensis* per tissue culture flask. Consequently, the points depicted at the zero-time ordinate are an average of close points and are plotted as a single point for convenience.

It was observed with all strains that the intracellular population of *P. tularensis* decreased at least 2 logs in viable count within 36 to 48 hr after ingestion. When phagocytes containing bacteria were disrupted before the expiration of this period and the liberated bacteria were plated on agar, strains possessing even a low level of virulence grew on incubation as well as microorganisms which had never been phagocytized. In marked contrast, after phagocytosis and liberation, the avirulent 38A strain exhibited unusually slow growth on agar, as compared with both phagocytized virulent strains and nonphagocytized organisms of 38A that were either grown in proteose-peptone media or obtained from slants. The apparent injury to 38A following phagocytosis was not attributable to enzymatic adaptation to intracellular nutrients because exposure to ruptured leukocytes failed to alter the growth characteristics of this strain. Furthermore, if the fully developed colonies were transferred to either liquid or solid medium, the organism grew as rapidly as the original strain. The change was therefore not permanent and presumably did not involve a genetic alteration. The phenomenon of altered growth after phagocytosis is not peculiar to this organism. Wilson (1957) observed that some strains of streptococci which survived phagocytosis grew with difficulty. Typical results are shown in Table 1.

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**Fig. 1.** Number of viable cells of *Pasteurella tularensis* in leukocytes after phagocytosis and in the presence of extracellular dihydrostreptomycin used to prevent extracellular proliferation. a) Left, mononuclear phagocytes; b) right, polymorphonuclear phagocytes.

**TABLE 1**

Colonial growth of *Pasteurella tularensis* strain 38A liberated from polymorphonuclear leukocytes

<table>
<thead>
<tr>
<th>Ingestion Time (hr)</th>
<th>Number of Days of Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>0 (control)</td>
<td>6.2 X 10^4</td>
</tr>
<tr>
<td>2.5</td>
<td>9.4 X 10^4</td>
</tr>
<tr>
<td>4</td>
<td>1.6 X 10^4</td>
</tr>
<tr>
<td>19.5</td>
<td>2.8 X 10^1</td>
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<tr>
<td>33</td>
<td>20</td>
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<tr>
<td>45</td>
<td>10</td>
</tr>
</tbody>
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* Results are expressed as total number of viable organisms per tissue culture flask.
DISCUSSION

The finding that a single organism of some strains of *P. tularensis* can cause fatal tularemia in guinea pigs has suggested the possibility that the invading microorganism lodges in a motile cell of the host. In such an environment, conditions conceivably could be proper for proliferation and the establishment of an infection. The evidence presented here argues against such an hypothesis so far as the granulocytic or monocytic cell series are concerned. The very rapid death rate of the completely avirulent strain 38A in the mononuclear leukocytes does, however, imply some relation between virulence and intraphagocytic death.

The possibility that the inability of the microorganism to survive for more than 48 hr in appreciable numbers in the phagocyte was caused by antibiotic penetrating the leukocyte wall and attaining a concentration sufficient to inhibit bacterial growth has been considered. This does not seem likely, however, because it has been demonstrated that *P. tularensis* and *B. abortus* have approximately the same susceptibility in vitro to streptomycin (0.75 µg/ml), yet Brucellae multiply within monocytes in tissue cultures containing more than 100 times the antibiotic concentration used in the present studies (Murat and Stinebring, 1969).

Because streptomycin was presumably ineffective in the intracellular death, other bactericidal possibilities were considered. Phagocytin, a basic protein isolated by Hirsch (1956) from neutrophils, was investigated. This material was reported to be active against gram-negative organisms. In our hands, phagocytin prepared from rabbit neutrophils, although exhibiting high activity against *E. coli*, showed no activity against any strain of *P. tularensis*.

It must be pointed out that these experiments do not eliminate either the mononuclear or polymorphonuclear leukocyte as primary sites of infection in vivo. The cells used were obtained from peritoneal exudates and may not be representative of cells of the same series found in other tissues. Furthermore, the tissue culture conditions, although apparently adequate for the survival and maintenance of structural integrity, may not represent the true physiological state of cells in situ. This factor could be especially important for a fastidious organism such as *P. tularensis*, whose requirement of suitable environ-

mental conditions for multiplication poses difficult problems. It has been pointed out by Traub, Mager, and Grossowicz (1955) that the need for a low oxidation-reduction potential on the one hand and a copious oxygen supply on the other create a paradoxical problem when the cultivation of *P. tularensis* from small inocula is attempted.

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SUMMARY

Experiments in vitro demonstrated that the number of viable cells of *Pasteurella tularensis* recoverable after phagocytosis by either mononuclear or polymorphonuclear leukocytes decreased very rapidly. Strains possessing virulence persisted in low but significant numbers in the phagocytes over a period of 72 hr, as contrasted with an avirulent strain which did not. A more rapid decrease in viable count was observed in polymorphonuclear cells than in mononuclear cells. Cells of the avirulent strain 38A which survived after liberation from leukocytes showed evidence of damage as indicated by their inability to grow as readily as nonphagocytized organisms. All strains resisted the bactericidal action of phagocytin obtained from polymorphonuclear leukocytes.

REFERENCES


