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CYTOPATHOGENIC EFFECT OF BRUCELLA SPHEROPLASTS ON MONOCYTES IN TISSUE CULTURE

BOB A. FREEMAN* AND BARRY H. RUMACK

Department of Microbiology, University of Chicago, Chicago, Illinois

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ABSTRACT

Freeman, Bob A. (The University of Chicago, Chicago, Ill.), and Barry H. Rumack. Cytopathogenic effect of Brucella spheroplasts on monocytes in tissue culture. J. Bacteriol. 88:1310–1315. 1964.—Mononuclear phagocytes from guinea pig peritoneal exudates were shown to ingest both normal Brucella suis and spheroplasts prepared from B. suis by treatment with glycine and with penicillin. Quantitative ingestion studies with 32P-labeled Brucella showed that rough normal Brucella are ingested at a greater rate than are smooth normal Brucella. Spheroplasts prepared from smooth cells were phagocytized at a greater rate than were the normal smooth cells, and spheroplasts prepared from rough Brucella were phagocytized well, although apparently to a lesser extent than from the normal rough Brucella. The degree of phagocytosis of all spheroplasts appeared to reach a peak and then decrease, indicating a release of ingested bacteria; this release of intracellular bacteria is believed to be due to the cytopathogenic effect exerted by the spheroplasts. Direct microscopic observations showed that infection with living spheroplasts prepared from either smooth or rough Brucella destroyed a major portion of the host cells within 4 hr, but that formalin-killed spheroplasts were no more destructive than were normal Brucella. When host cell destruction was assayed by the release of cellular constituents into the medium, it was apparent that host-cell destruction by spheroplasts reaches a significant level within 0.5 hr after ingestion begins, and is almost complete by 4 hr. The implications of these findings in studies on the nature of intracellular Brucella are discussed.

1 Present address: Department of Microbiology, University of Tennessee Medical Units, Memphis.

tention in this regard, and the growth of virulent Brucella in guinea pig monocytes in tissue culture has been studied from the viewpoint of cellular immunity (Elberg, 1960; Braun, Kessel, and Pomales-Lebron, 1962), sensitivity of monocytes from immunized animals to the cytotoxic action of Brucella antigens (Heilman, Howard, and Carpenter, 1957; Heilman et al., 1960; Hinadil and Berman, 1962), and other factors affecting intracellular residence.

The recognition that intracellular residence and growth may lead to changes in the parasite has led to several interesting observations. Stonebringer (1962) demonstrated that B. abortus grown in guinea pig mononuclear phagocytes maintained in vitro shows an enhanced resistance to the bactericidal action of bovine serum and an increased susceptibility to phagocytosis. These observations suggest that the cell surface of such intracellularly grown organisms has been altered in some manner.

Ralston and Elberg (1960, 1961) and Ralston Baer, and Elberg (1961) showed that glycine, along with a lysozymelike material from rabbit mononocytes, will cause in vitro lysis of B. melitensis, and that the addition of glycine to cultures of Brucella-parasitized monocytes causes suppression of intracellular bacterial growth. Since both glycine and lysozyme are known to induce bacterial spheroplast or protoplast formation, it is probable that, under these circumstances, the bacterial cell surface is altered and that this affects the host-parasite relationship.

In an earlier report (Hines, Freeman, and Pearson, 1964), we demonstrated that spheroplasts of B. suis may be induced by a variety of methods, and that the nature of the bacterial surface changes is, to some extent, dependent upon the nature of the agent used. This paper reports our efforts to infect guinea pig mononuclear phagocytes with a variety of Brucella spheroplasts and the cytopathogenic effects exerted by these altered parasites.

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Materials and Methods

A description of the Brucella cultures used in this investigation was previously published, and the induction and isolation of rough variants were described in detail (Freeman, Kross, and Circo, 1961).

_B. suis_ strain 32P and its rough variant were used exclusively in this study. Spheroplasts were prepared from these cultures by treatment with either glycine or penicillin by use of methods described by Hines et al. (1964).

Since the glycine spheroplasts employed here are osmotically sensitive, it was necessary to carry out all experiments in a medium containing sucrose as a stabilizing agent. Preliminary experiments had shown that guinea pig monocytes were not visibly affected by the concentration of sucrose employed, and that phagocytosis of normal _Brucella_ in the presence of sucrose, as determined by viable counts, was equivalent to phagocytosis in plain Hanks' balanced salt solution (BSS) with autologous serum.

Mononuclear phagocytes were collected from the peritoneal cavity of young male guinea pigs 4 days after mineral oil induction. The monocytes were washed twice in Hanks' BSS containing 0.2 M sucrose and were counted microscopically. These monocytes were then infected with _Brucella_, as described in detail below, and were maintained in Hanks' BSS with 25% fresh autologous serum and 0.2 M sucrose. These procedures were previously described (Freeman et al., 1961).

Because not all spheroplasts revert, even in the presence of an osmotic stabilizer, it was not possible to assay phagocytosis by the usual techniques which employ viable plate counts. To determine the degree of phagocytosis, smooth and rough _B. suis_ 32P were grown for 24 hr in Tryptose broth containing inorganic phosphorus52. The bacteria were washed thoroughly, and portions of both smooth and rough were used to prepare glycine and penicillin spheroplasts by the methods described earlier (Hines et al., 1964).

The labeled spheroplasts and normal _Brucella_ were washed thoroughly in sucrose-Hanks' BSS to eliminate traces of soluble, labeled material. Monocytes in suspension were then infected with these _Brucella_ at a multiplicity of 10 by adding 10^8 _Brucella_ to siliconized tubes containing 10^8 washed monocytes in 1.0 ml of Hanks' BSS with 50% autologous serum and 0.2 M sucrose. At 0.5, 1.0, and 2.0 hr, a series of cultures was removed, and the phagocytes were sedimented by light centrifugation. The supernatant fluid was removed and the cells were washed with sucrose-Hanks' BSS. The sedimented cells were killed with formalin and were brought to a known volume. The supernatant fluid and washings were combined, formalin was added, and the volume was measured.

Portions of each preparation were distributed to planchetts, and the activity was determined with a decade scaler (Nuclear-Chicago Corp., Des Plaines, Ill.) with a windowless gas-flow detector. The counts were corrected for background, decay, and dilution, and the degree of phagocytosis was determined by the formula:

\[
\text{per cent phagocytized} = \frac{\text{counts per min of sediment}}{\text{counts per min of sediment and supernatant fluid}} \times 100
\]

Two methods were employed to study the effects of ingestion of normal _Brucella_ and spheroplasts on monocytes. After washing in sucrose-Hanks' BSS, 10^9 monocytes were transferred to Leighton tubes with cover slips. _Brucella_ (10^9) were added, and 1.0 ml of sucrose-Hanks' BSS with 25% autologous serum was added. Phagocytosis was allowed to proceed for 4 hr; the cover slips were removed and were stained with Giemsa, and the monocytes remaining attached to the cover slip were counted microscopically. In parallel cultures, the release of cellular constituents into the culture supernatant fluid was measured by a method described in detail by Freeman et al. (1961). At intervals up to 4 hr, the supernatant fluid from infected cultures was removed and was centrifuged at high speed to remove all cellular particles. Trichloroacetic acid was added to the supernatant fluid to a final concentration of 5% (w/v), and it was heated at 100°C for 20 min. The precipitate was removed by centrifugation and was discarded. The optical density of the supernatant fluid was determined in a Beckman DU spectrophotometer at 268 m\(\mu\). The blank prepared by extraction of the maintenance medium alone. By this procedure, the degree of damage to the cells is measured by the release of nucleic acids (and possibly other cellular constituents) into the culture supernatant fluid, and is expressed as optical density at 268 m\(\mu\).
TABLE 1. Phagocytosis of phosphorus$^{32}$-labeled normal and spheroplast cells of Brucella suis $^{32}P$

<table>
<thead>
<tr>
<th>Cell surface</th>
<th>Treatment</th>
<th>Per cent phagocytosis at 0.5 hr</th>
<th>1.0 hr</th>
<th>2.0 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth</td>
<td>None</td>
<td>39</td>
<td>38</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Penicillin</td>
<td>51</td>
<td>85</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>60</td>
<td>40</td>
<td>37</td>
</tr>
<tr>
<td>Rough</td>
<td>None</td>
<td>52</td>
<td>97</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Penicillin</td>
<td>30</td>
<td>78</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>43</td>
<td>40</td>
<td>44</td>
</tr>
</tbody>
</table>

TABLE 2. Direct microscopic observations on monocyte culture infected with normal and spheroplast cells of Brucella suis $^{32}P$

<table>
<thead>
<tr>
<th>Cell surface</th>
<th>Treatment</th>
<th>Monocyte culture infected with</th>
<th>Living</th>
<th>Killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth</td>
<td>None</td>
<td>50*</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>20</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Penicillin</td>
<td>13</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>Rough</td>
<td>None</td>
<td>54</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>8</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Penicillin</td>
<td>6</td>
<td>47</td>
<td>47</td>
</tr>
</tbody>
</table>

* Figures indicate number of monocytes per microscopic field.

RESULTS

Under certain conditions, spheroplasts of Brucella are capable of reversion to rods and may be cultivated on agar media. Since reversion does not occur with all types of spheroplasts, however, it was not possible to estimate degree of phagocytosis by viable counts of ingested bacteria and spheroplasts. Additionally, spheroplasts of Brucella do not stain well, and direct microscopic counts of intracellular parasites were not feasible.

To show phagocytosis of spheroplasts and to compare quantitatively phagocytosis of normal B. suis and spheroplasts, radioactive-labeling techniques were employed. Table 1 shows the degree of phagocytosis of $^{32}P$-labeled B. suis normal cells and spheroplasts. The data shown represent the average of duplicate experiments. Spheroplasts of smooth B. suis are phagocytized more rapidly than are normal cells, as seen by comparison at 0.5 hr after addition of the bacteria to the monocyte suspension. In the case of smooth, virulent, normal cells, maximal phagocytosis occurred at the 2.0-hr interval. In the case of the spheroplasts, maximal phagocytosis occurred at the 1.0-hr interval with penicillin spheroplasts and at 0.5 hr with the glycine spheroplasts. It will be noted that, in these latter cases, apparent phagocytosis was reduced at the later intervals.

Spheroplasts prepared from rough, avirulent B. suis were phagocytized well, although apparently to a lesser extent than were the normal rough Brucella. Again, the degree of phagocytosis appears to reach a peak and then decrease, indicating a release of the ingested bacteria.

Our experience with large inocula of normal Brucella in monocytes indicated that rough avirulent and, to some extent, smooth virulent Brucella tend to be cytopathogenic for guinea pig mononuclear cells. Preliminary microscopic observations had shown that monocytes exposed to spheroplasts disintegrate and slough from the glass in culture tubes. To estimate quantitatively this destruction, we exposed $10^7$ monocytes to normal Brucella and to spheroplasts at a multiplicity of 10, and allowed phagocytosis to proceed for 4 hr. Cover slips were removed and stained, and the average number of monocytes per microscopic field was determined by counting several hundred fields. All slides were counted in a similar pattern to assure accuracy. A similar series of slides was prepared with the use of normal Brucella and spheroplasts which had been killed by brief exposure to formalin and then thoroughly washed before ingestion. Table 2 shows the results of this experiment. It is apparent that living spheroplasts prepared with either glycine or penicillin are markedly cytopathogenic for host cells under these conditions, and that spheroplasts prepared from rough Brucella are most destructive. Killed spheroplasts, however, are not cytopathogenic and have no discernible effect on the host cell.

To determine the rate of destruction by spheroplasts, $10^7$ monocytes were exposed to normal cells and spheroplasts of B. suis at a multiplicity of 10. At intervals, appropriate cultures were tested for the release into the supernatant fluid of cellular constituents. The results are shown in Fig. 1 and 2. Figure 1 shows the effect of normal cells and spheroplasts derived from smooth B. suis. During the 4-hr period, there was some destruction of the host cell by normal B. suis, but it was much more marked in the case of the

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spheroplasts. The rate of destruction appears to be much greater in the case of the spheroplasts, since the release of cellular constituents is almost complete by 2 hr. The release of cellular constituents from the uninfected monocyte control culture is negligible.

Essentially, the same results were obtained with rough B. suis and its spheroplasts. The rough, normal cells are somewhat more destructive than are the smooth, normal cells, yet are much less destructive than the spheroplasts. The spheroplasts derived from rough cells appear to be somewhat more destructive than those derived from smooth B. suis.

DISCUSSION

Although it is generally recognized that intracellular bacteria, as they exist in the host, may differ from those grown on artificial media in the laboratory, little is known of the actual differences that do exist.

It is possible that the therapeutic use of antibiotics, known to affect bacterial cell-wall synthesis, may lead to the production of spheroplasts or protoplasts in the infected animal. Guze and Kalmanson (1964) showed that such organisms do occur in rats with experimental enterococcal pyelonephritis after treatment with antibiotics, and may be cultured from the kidney when 0.3 M sucrose is present in the culture medium, but not in its absence. They also noted that "the disease appears to progress histologically despite the absence of bacterial infection as determined by . . . culture."

It seems equally possible that infecting organisms may be acted upon by bodily defenses in such a manner as to lead to spheroplast formation. Meloni and Bragadin (1962) reported the existence of "spontaneous spheroplasts" of Klebsiella isolated from a human infection. Freeman, Musteikis, and Burrows (1963) showed that "protoplast" production is the mechanism for lysis of Vibrio cholerae by immune serum and complement.

Ralston and Elberg (1960, 1961) and Ralston et al. (1961) found that rabbit monocytes produce a lysozyme-like substance which will lyse B. melitensis previously treated with glycine in low concentration. The authors believe that glycine or some other unknown substance present in phagocytes alters ingested bacteria so that intracellular enzymes may inhibit bacterial growth.

Stinebring (1962) showed that B. abortus grown within guinea pig mononuclear phagocytes...
appears to have undergone surface changes. Intracellularly grown \textit{B. abortus} was compared with slant-grown cells in susceptibility to the bactericidal effects of normal bovine serum. The monocyte-grown cells were more resistant to normal bactericidins, but, once killing had begun, both types of cells showed similar rates of killing. Monocyte-grown \textit{B. abortus} cells were also ingested better or survived better, or both, in guinea pig mononuclear cells maintained in vitro than were slant-grown organisms.

Insofar as we may compare our findings on infection of host cells with spheroplasts and the reports of others studying the nature of intracellular changes, there are areas of agreement. Spheroplasts induced with either glycine or penicillin resemble intracellularly grown \textit{Brucella} in their enhanced susceptibility to phagocytosis (Table 1). It is perhaps pertinent to note that the spheroplasts prepared from smooth \textit{B. suis} retain their antigenicity but have lost their endotoxins as measured by rabbit skin test (Hines et al., 1964).

When we attempted to cultivate spheroplasts within guinea pig monocytes maintained in vitro, it was apparent that cultivation was not possible because of the rapid cytopathogenic effect exerted by the bacteria on the host cell. As judged by microscopic counts of surviving monocytes on cover slips, destruction by spheroplasts is well advanced by 4 hr. In general, the spheroplasts prepared by penicillin treatment are more destructive than are those prepared by glycine treatment. Although this may represent a qualitative difference in the removal of surface components, penicillin spheroplasts, as judged by osmotic fragility, are less fragile than are glycine spheroplasts, and presumably retain more undegraded cell-wall material. That the cytopathogenic effect is an active rather than mechanical disruption caused by over-ingestion of particles is shown by the lack of cytopathogenic effect when killed \textit{Brucella} are employed.

From this and other investigations it appears, too, that the removal of surface components, in general, results in cells which are more destructive, and spheroplasts prepared from rough \textit{Brucella} are more destructive than are those prepared from smooth.

It is not possible, at this time, to offer an explanation of the increased cytopathogenic effect exerted by spheroplasts. It may be presumed that localized enzymes which act in a deleterious manner on the host cell are exposed. The exact mechanism awaits further study.

The results presented here suggest that the intracellular form of \textit{Brucella}, insofar as its surface is concerned, lies intermediate between those grown on ordinary media and those treated by the more-or-less drastic procedures involving penicillin or glycine. Additional studies are underway to compare intracellularly grown \textit{Brucella} with spheroplasts in their surface antigenicity, endotoxin activity, susceptibility to phage, and other biochemical properties.

\section*{Acknowledgments}
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\section*{Literature Cited}


