Osmotically Sensitive Brucella in Infected Normal and Immune Macrophages

JERRY R. MCGHEE AND BOB A. FREEMAN

Department of Microbiology, University of Tennessee Medical Units, Memphis, Tennessee 38103

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When Brucella suis is grown in tissue cultures of normal guinea pig macrophages, the Brucella multiplies significantly without the induction of osmotically sensitive forms. In immune macrophages in the presence of normal guinea pig serum, there is a reduction in the number of intracellular Brucella and no multiplication is seen over a 72-hr period. After 6 hr of exposure to immune macrophages, however, approximately 50% of the surviving intracellular Brucella are osmotically sensitive, i.e., they will survive and grow only on medium containing 0.2 M sucrose. Brucella grown in immune macrophages, in the presence of rabbit antiserum against whole guinea pig serum, show the presence of osmotically sensitive forms, although at a reduced level compared to the number seen with immune macrophages in normal guinea pig serum.

Although the exact mechanism by which Brucella infects and continues to grow in tissues of immune individuals has never been clearly demonstrated, several investigators have approached this problem with tissue culture techniques. Smooth, virulent Brucella species survive and multiply in monocytes derived from normal guinea pigs (5, 10, 13). Stinebring and Kessel (13) were able to pass Brucella cells through 27 tissue culture transfers in macrophages without intervening growth on artificial media. Under these conditions, the Brucella showed a 100-fold increase in numbers.

Several workers have reported that rough Brucella exhibits a much greater cytopathogenic effect on normal macrophages than smooth, virulent Brucella (1, 2, 7, 8). Whether rough Brucella species actually multiply within macrophages before destruction is a subject of some controversy. Since rough Brucella species in high numbers are cytopathogenic, early intracellular growth may be observed only if the infectivity ratio is kept low.

In contrast, infection of macrophages from previously immunized animals with both smooth and rough Brucella leads to rapid bacterial and cellular destruction (1, 10). In fact, these and other workers (1, 8, 12) found little, if any, multiplication of the Brucella. There was also some indication that smooth, virulent Brucella species destroy immune monocytes more rapidly than rough Brucella, which is opposed to the observations with rough and smooth Brucella in normal macrophages (8, 13).

To gain insight into the sequence of events in infected cells, we searched for a possible alteration of the bacterial cell wall after residence in normal and immune macrophages. It has been established that antibody and complement can induce spheroplast formation of Vibrio cholerae (3), and that other gram-negative organisms can undergo such alteration by antibody, provided that lysozyme is present (9). It is possible that alteration of these surface structures might account for the marked destruction of both parasite and host cell. It may be that in macrophages derived from immune animals, there is an uncovering or synthesis of antigens, or other surface components of the bacterium, which are not compatible with continued coexistence of parasite and host. It is also possible that these intracellular Brucella may have lost some cell wall rigidity because of host lysosomal enzymes, but may be protected osmotically as long as they are within host cells (McGhee and Freeman, in press). Rupture of the host macrophage would be expected to lead to dissolution of the sensitive bacteria. This report describes the differences observed when Brucella were grown either in normal or immune macrophages and subsequently cultured in osmotically protected media.

MATERIALS AND METHODS

Cultures. Smooth B. suis 1330 was used in this study. Stock cultures were grown on 2% Tryptose-agar slants, and 18-hr logarithmic cultures were harvested in tryptose broth and stored at −78 C. Cultures for
regular use were maintained on 2% Tryptose-agar slants and stored at 5°C.

**Immunizing antigens.** Brucella immunizing antigens were prepared by growing smooth \( B. suis \) on 2% Tryptose-agar slants for 18 hr. The cells were harvested and washed three times in sterile 0.85% sodium chloride solution (saline) and were finally suspended in saline with 1% Formalin for sterilization. When sterile, the vaccine was centrifuged, and the packed cells were suspended in Freund's complete adjuvant (6), to a final concentration of 10⁶ cells/ml.

**Immune serum.** Hartley strain guinea pigs weighing 300 to 400 g were immunized by subcutaneous injection of 0.5 ml of Brucella vaccine, followed by two intraperitoneal doses of 1.0 ml at weekly intervals. One week after the last dose, the animals were exsanguinated by cardiac puncture, and peritoneal macrophages were collected as described below. Antiserum against guinea pig serum was prepared by immunizing rabbits with four intraperitoneal doses of 2.0 ml of whole guinea pig serum at weekly intervals. The rabbits were bled by cardiac puncture 1 week after the last injection.

Collected blood was allowed to clot, and the serum was removed, sterilized by membrane filtration, and stored at −20°C.

**Macrophage collections.** Normal and immune macrophages were collected from exsanguinated 300- to 400-g Hartley strain guinea pigs by injecting 30 ml of Hanks balanced salt solution (HBSS) with 20 units of heparin per ml into the peritoneal cavity, followed by gentle kneading. The fluid, which contained predominantly mononuclear cells, was withdrawn through an incision in the abdominal wall. On occasion, glycerol was used as a macrophage stimulant; a 2-ml amount was injected intraperitoneally, and the cells were collected 4 days later as outlined above. Although these cells were predominantly mononuclear, they were not all the same type. For convenience, therefore, we refer to these cells as macrophages. Glycerol was removed from the cells by washing with HBSS before proceeding with tissue culture; cells from normal and immune animals not stimulated with glycerol were washed only once. The mononuclear cells were suspended in HBSS and counted microscopically.

**Tissue culture.** Cultures were prepared by pipetting 2 × 10⁶ macrophages to each of several Leighton tubes. These were infected by adding 2 × 10⁶ cells of \( B. suis \) from an 18-hr slant culture suspended in 0.5 ml of inactivated serum. The mixture was incubated for 2 hr at 37°C to allow phagocytosis. The supernatant fluid was then removed, and the cells were washed free of extracellular bacteria with HBSS before final addition of maintenance medium. This was composed of HBSS with 25% guinea pig serum adjusted to pH 7.4 with sodium bicarbonate. Antibiotics were not added to the maintenance medium because their presence might lead to the production of osmotically fragile Brucella. Unphagocytized bacteria were removed, by washing, to control extracellular growth. The low numbers of extracellular bacteria remaining after washing do not grow significantly in the tissue culture medium (5). The serum type used in the maintenance medium, whether normal or immune, was always the same as used for phagocytosis. After 6 hr of incubation at 37°C and at 24-hr intervals after infection, one of the Leighton tubes was removed, the medium was withdrawn, and the cells were washed once with HBSS. The volume was then replaced with HBSS containing 0.2 m sucrose. The macrophages were suspended by gentle agitation of small, sterile glass beads in the tube. The intracellular bacteria were released from the cell by electrocution with a direct current of 150 v for 1 to 2 sec. Controls showed that smooth Brucella cells and spheroplasts could withstand this treatment without loss of viability. The released bacteria were then diluted in saline with 0.2 m sucrose, and differential plate counts were performed by using plain Tryptose-agar and the same medium with 0.2 m sucrose (sucrose-trypotose agar) for added osmotic protection.

**RESULTS**

**Growth of Brucella in immune and normal macrophages.** Figures 1 and 2 show the viability and growth of \( B. suis \) in cultures of normal and immune guinea pig macrophages in the presence of normal guinea pig serum. It can be seen that Brucella grows readily in normal macrophage culture; over a period of 72 hr there was a 200-fold increase in viable Brucella cells. It can also be noted that, initially, considerably fewer bacteria are phagocytized by normal than immune macrophages. There is, however, a significant reduction of viable intracellular bacteria in the immune macrophage culture within the first 6 hr. In immune macrophages in normal serum, the number of Brucella cells after 6 hr of infection remains constant; there is no evidence of intracellular growth. In these infected immune cells, microscopic examination revealed that most host cells contained one to five Brucella cells at 6 hr.
and that host cell morphology was normal at this time. At 24 hr, however, the cytoplasm of the host cells was foamy, and cell fragments and free nuclei were frequently seen. No cytopathogenicity was noted in macrophages from nonimmune animals cultured in normal serum at intervals up to 72 hr postinfection. In fact, it was often necessary to replace media in these cultures after 24 to 48 hr, indicating more metabolically active cells.

Figure 3 shows the viability of Brucella in normal macrophages maintained with homologous immune serum. In this system, the phagocytosis of Brucella cells is most efficient. No appreciable change of viability occurred until 48 hr, when a 10-fold decrease was noted. These cells appeared healthy at 24 hr, but by 48 hr a marked cytopathogenicity was noted. Normal macrophages in the presence of normal serum showed no cytopathogenicity attributable to Brucella even after 72 hr of infection. When immune macrophages in immune serum were employed, massive disintegration of host cells occurred almost immediately upon addition of immune serum to the infected macrophage cultures. Phagocytosis and subsequent growth of Brucella were, therefore, not possible with this combination.

Figure 4 shows the effect of infected immune macrophages when rabbit anti-guinea pig serum was substituted for normal serum in the maintenance medium. Presumably this antiserum would neutralize the effect of Brucella antibody in these immune cells. The viability curve of Brucella resembles that of normal cells with immune serum, although survival is significantly reduced. Microscopically, these host cells were healthy at 48 hr, but cytopathogenicity was well developed by 72 hr. Control preparations without Brucella showed that the rabbit anti-guinea pig serum has no discernible effect on the macrophage culture. Further experiments must be directed toward preparing antiserum to purified immunoglobulin fractions to establish firmly that cytophilic antibody is not responsible for spheroplast production in these immune cells.

Cultivation of osmotically sensitive Brucella. The results of experiments to demonstrate production of osmotically fragile bacteria within macrophages under the individual cultural conditions are shown in Fig. 1–4. Figure 1 shows the production of osmotically fragile forms expressed as relative viability on Tryptose and sucrose-Tryptose-agar in cultures of normal macrophages in the presence of normal guinea pig serum. Although the number of viable Brucella cells in-
creased considerably during the 72-hr period, no significant differences could be seen in counts on osmotically protected media over that of ordinary media, thus indicating the presence of few, if any, osmotically sensitive forms. In contrast, the bacterial count within immune macrophages (Fig. 2) showed a rapid production of osmotically fragile cells; total numbers of cells present remained relatively constant during the experimental period.

The possible role of humoral antibody in the production of osmotically sensitive cells is indicated by the data shown in Fig. 3, in which infected normal macrophages were maintained in serum derived from Brucella-immune guinea pigs. There was greater survival of intracellular bacteria at the end of 6 hr, but intracellular multiplication of Brucella did not occur. Under these conditions, there was slight production of osmotically sensitive bacteria.

These findings suggest that "cellular" factors are more important in the production of spheroplasts than circulating antibody. To confirm this observation and to determine the effects of any cytophilic antibody in immune macrophages, rabbit antiserum to normal guinea pig serum was substituted for guinea pig serum in the tissue culture medium. In this system, production of osmotically sensitive bacteria should be primarily due to cellular factors. There was a marked reduction in viable Brucella at 6 hr and, as shown in Fig. 4, the macrophages were still capable of inducing spheroplasts, although at a reduced level as compared to immune macrophages in normal guinea pig serum.

**DISCUSSION**

The intracellular growth of Brucella in normal guinea pig macrophages has been confirmed in this study. An increase of up to 200-fold was noted in the number of intracellular Brucella cells within 72 hr. There is no doubt that smooth Brucella can multiply within these cells and that normal macrophages have no observable inhibitory effect on the bacteria. Instead, they provide a suitable environment for growth without obvious alteration of the bacterial cell surface.

In addition, it appears that Brucella does not multiply in immune macrophages, but is seen to decrease in viability with increasing exposure to the intracellular position. Immune macrophages are apparently more efficient in phagocytosis of Brucella than normal macrophages. After phagocytosis, Brucella reaches a steady state of approximately 10^8 bacteria per immune macrophage culture as detected on ordinary medium.

It appears that macrophages from immune animals are better able to cope with the Brucella, perhaps by increased surface alterations of the bacteria, leading to death of a number of intracellular bacteria. It may be that the remaining Brucella, many of which are spheroplasts, are also important in the pathogenesis of infection. Late in the disease, when antibody has appeared, the hardier and more resistant bacteria may have adapted to a physiological steady state. This might explain why humoral and bactericidal responses as well as antibiotic therapy are often ineffective in chronic brucellosis.

These data show a direct correlation between host cell damage, the appearance of osmotically fragile forms, and the disappearance of viable, osmotically stable Brucella cells from the culture. The marked cytopathogenic effect observed in immune macrophages might be explained by the fact that such a high percentage of the infecting cells are spheroplasts. It has been shown that infection of macrophages with spheroplasts leads to an increased cytopathogenicity (4), whereas other investigators (11) found that agents which convert Brucella into osmotically sensitive forms in tissue culture often lead to a 90% degeneration of macrophage cultures after 3 days.

The role of cellular factors is again emphasized by the finding that normal serum does not promote the formation of Brucella spheroplasts. It does, however, allow growth of Brucella in normal host cells. It should be noted that immune serum inhibits intracellular growth of Brucella in normal macrophages and that the greatest rate of phagocytosis occurs with this system. It should again be stressed that even these presumably more desirable host benefits did not result in a significant decrease in the viability of the Brucella or to a fragility change in the bacterial cell surface. The immediate disintegration of immune host cells in the presence of Brucella and immune serum suggests the occurrence of a possible hypersensitivity reaction. Whatever the mechanism involved, it was impossible to evaluate this system by using the methods employed in other experiments.

The use of rabbit anti-guinea pig antibody further emphasized the importance of the cellular elements in this host-parasite system. Although spheroplast production was somewhat reduced in these cultures, significant levels did occur. There was also a marked decrease in viable Brucella in this system which was similar to immune macrophages and normal serum. In fact, the slight decrease in spheroplast production in this system might be due simply to the substitution of rabbit serum for normal guinea pig serum in the tissue culture.
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LITERATURE CITED