INFECTION AND IMMUNITY, July 1992, p. 3011–3014
0019-9567/92/073011-04$02.00/0

Vol. 60, No. 7

Survival of Virulent and Attenuated Strains of *Brucella abortus* in Normal and Gamma Interferon-Activated Murine Peritoneal Macrophages

SYLVIA M. JONES AND ALEXANDER J. WINTER*

Department of Microbiology, Immunology & Parasitology, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

Received 18 February 1992/Accepted 17 April 1992

Virulent *Brucella abortus* 2308 was phagocytized more readily than attenuated strain 19 following opsonization and survived at significantly higher levels in normal murine peritoneal macrophages and in macrophages treated with gamma interferon. Activation of macrophages with gamma interferon greatly inhibited intracellular replication of strain 2308 but did not result in its elimination. These data support the hypothesis that persistent infection of the host requires the ability of antibody-opsonized *B. abortus* to survive in activated macrophages.

Virulent strains of the facultative intracellular parasite *Brucella abortus*, exemplified by strain 2308, produce chronic infections, whereas the attenuated vaccine strain 19 is cleared relatively rapidly from the tissues of cattle (23) and BALB/c mice (19). On the basis of comparative analyses of infections in BALB/c mice with strains 19 and 2308, we hypothesized that chronic infection resulted from the ability of some portion of immunoglobulin G (IgG) antibody-opsonized strain 2308, but not strain 19, to survive in activated macrophages (4, 12). We report here the results of in vitro experiments designed to test that hypothesis.

(A preliminary report of these findings has been presented previously [16].)

Experiments were first performed to compare phagocytosis of the two strains. Resident peritoneal cells were recovered by lavage from BALB/cByJ female mice, 10 to 20 weeks old (Jackson Laboratory, Bar Harbor, Maine). The cells in Dulbecco modified Eagle medium plus 5% fetal bovine serum (FBS) were plated onto glass coverslips (12-mm diameter) at a concentration of 5×10^5 cells per coverslip and incubated at 37°C under 5% CO_2. Nonadherent cells were removed 2 to 3 h later. The number of macrophages per coverslip was estimated by microscopic counts to be approximately 1.8×10^5. For phagocytosis, macrophage monolayers were incubated for 30 min with opsonized brucellae at estimated concentrations of 10×10^7, 7.5×10^7, and 5×10^7 organisms per ml. Actual concentrations were obtained retrospectively (19). Fresh subcultures of strains 19 and 2308 were prepared each week from the same lots of frozen stocks and used after incubation for 3 days at 37°C under 5% CO_2. Stock cultures used in these experiments were 100% smooth in colony morphology and were derived from a single in vitro passage of strains that had produced typical patterns of infection in mice. Opsonization was performed for 30 min at 37°C under 5% CO_2 by incubation of bacteria with subagglutinating concentrations of antiserum pools from three to five mice infected 17 weeks earlier with strain 19 or strain 2308.

After phagocytosis, extracellular bacteria were removed by five washes with phosphate-buffered saline (PBS)–0.5% FBS followed by incubation for 1 h in Dulbecco modified Eagle medium-FBS containing 50 μg of gentamicin per ml (Sigma Chemical Co., St. Louis, Mo.) (10). Preliminary experiments had shown that extracellular bacteria could not be removed by washing alone without using a force that would dislodge the macrophage monolayer. Coverslips were washed three times in PBS-FBS and transferred individually to tubes containing 1 ml of PBS. After three cycles of rapid freezing and thawing in an ethanol-dry ice bath and a 37°C water bath to disrupt macrophages (25), samples were diluted and plated for colony counts (19). All treatments were done in quintuplicate.

Phagocytosis required the presence of specific antibodies (data not shown), in accordance with prior findings by us (25) and others (14, 26). Strain 19 was consistently internalized less well than strain 2308, regardless of the source of opsonizing antiserum (Fig. 1). Internalization of strain 19 was significantly less ($P \leq 0.05$ to $P < 0.01$) than that of strain 2308 even when concentrations of strain 19 added to the monolayer were greater (Fig. 1). Detilleux and coworkers (10) observed that strain 19 was also taken up by Vero cells less efficiently than strain 2308.

We believed that the most meaningful comparisons of intracellular survival would be obtained by equalizing starting numbers of bacteria. Strain 19 was therefore phagocytized at a concentration of 10×10^7 organisms per ml while strain 2308 was used at concentrations of 7.5×10^7 and 5.0×10^7 organisms per ml. The data used for calculations were taken from whichever concentration of strain 2308 yielded numbers of internalized organisms closer to those of strain 19.

In bactericidal assays, numbers of brucellae on coverslips harvested after 1 h of incubation with 50 μg of gentamicin per ml provided baseline values in calculating percent survival. Remaining coverslips were washed and reincubated in Dulbecco modified Eagle medium-FBS plus 12.5 μg of gentamicin per ml, with a change in medium every 24 h. Coverslips in quintuplicate were harvested at 4, 24, and 48 h and in some experiments at 72 h after phagocytosis. Additional harvesting times, at 8 and 12 h, were added in selected experiments. Intracellular counts were obtained as described above, and samples of supernatants were cultured to count extracellular bacteria. Preliminary experiments had, in

* Corresponding author.

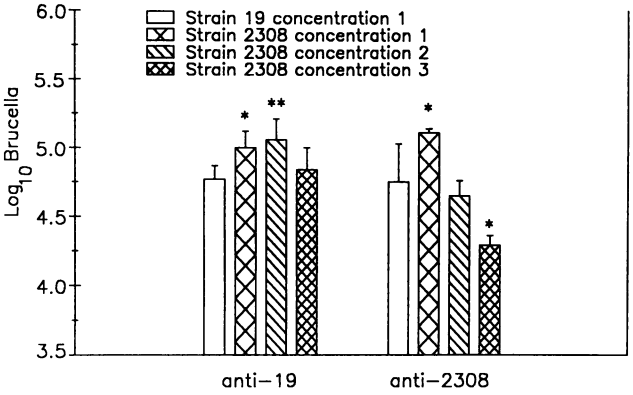

FIG. 1. Phagocytosis of *B. abortus* strains 19 and 2308 opsonized with homologous and heterologous antisera. Intracellular bacterial numbers were determined after 30 min of phagocytosis and 1 h of incubation with gentamicin to kill extracellular brucellae. Opsonized *B. abortus* was added to the macrophage monolayers at the following concentrations (per milliliter) for the anti-strain 19 experiment: the strain 19 concentration 1 was 13.6×10^7, the strain 2308 concentration 1 was 10.5×10^7, the strain 2308 concentration 2 was 8.7×10^7, and the strain 2308 concentration 3 was 4.5×10^7. For the anti-strain 2308 experiment, the strain 19 concentration 1 was 12.5×10^7, the strain 2308 concentration 1 was 10.1×10^7, the strain 2308 concentration 2 was 6.5×10^7, and the strain 2308 concentration 3 was 5.1×10^7. All treatments were done in quintuplicate. Bars indicate standard deviations. * and **, $P < 0.05$ and 0.01, respectively, in comparison with intracellular numbers of strain 19. These experiments were repeated five times with the same results.

agreement with prior reports (7, 21), demonstrated that 12.5 μg of gentamicin per ml was sufficient to control extracellular growth but was low enough not to affect intracellular numbers (data not shown). In the principal experiments (see Fig. 2 and 3), extracellular bacteria constituted <10% of intracellular numbers in 96% of the assays and <1% in more than 70% of the assays. Additional coverslips from each treatment were used to enumerate macrophages. Trypan blue was added to the coverslips, and counts of 10 representative oil immersion fields were made. Macrophage viability consistently exceeded 95%. There was a gradual loss of macrophages with time, but even at 48 and 72 h, numbers averaged >70% of those at the outset. Within experiments there were no differences at individual time points in numbers of macrophages in strain 19- and strain 2308-treated monolayers.

In one series of experiments, survival was examined in untreated macrophages at intervals up to 48 h postphagocytosis. Numbers of both strains generally decreased at 4 h postphagocytosis, reached a low point at 24 h, and increased at 48 h (Fig. 2). Survival of both strains at 8 h was similar to survival at 4 h, and survival at 12 h was intermediate between that at 4 h and that at 24 h (data not shown). Although the percent survival of each strain varied between experiments, within an individual experiment the percent survival of strain 19 was uniformly lower than that of 2308 at each time point (Fig. 2). This held true whether the opsonizing antiserum was specific for strain 19 or strain 2308 (Fig. 2). Differences in percent survival between strains 19 and 2308 were statistically significant ($P < 0.05$ to $P < 0.001$) in 15 of 17 comparisons made at corresponding time points within experiments. At 48 h postphagocytosis, numbers of strain 2308 exceeded baseline values, whereas with one exception the survival of strain 19 was below 100% (Fig. 2). The lower percent survival of both strains following opsonization with 2308 antiserum may have been related to the unusually high concentrations of *O*-polysaccharide-specific antibodies of IgG2a and IgG2b isotypes in this serum pool (data not shown).

The decreased rate of intracellular survival of strain 19 compared with that of strain 2308 is similar to that observed recently by others (10, 22). Price et al. (22) used bovine mammary macrophages and compared survival between 12 and 26 h postphagocytosis. Strain 19 survived less well than strain 2308 in macrophages derived from both genetically resistant and genetically susceptible cattle. Detilleux et al.

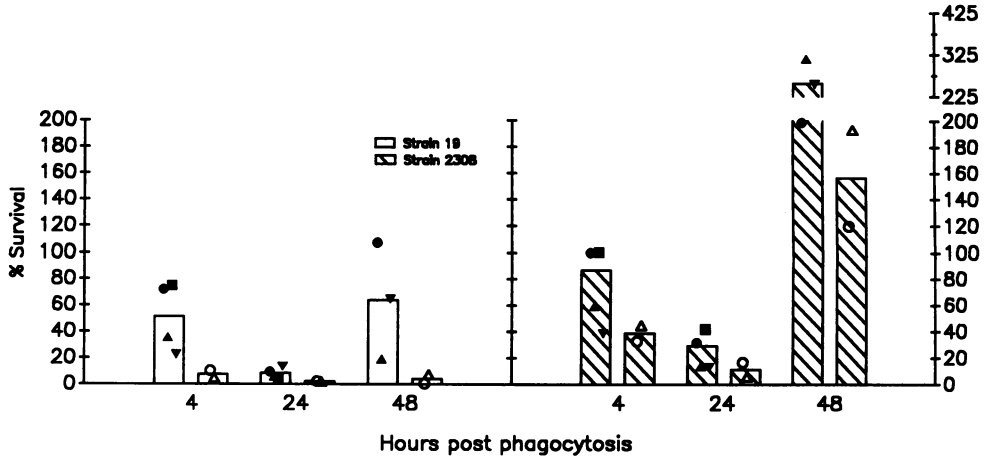

FIG. 2. Survival of *B. abortus* strains 19 and 2308 in murine peritoneal macrophages. Bacterial survival was determined 4, 24, and 48 h postphagocytosis. Percent survival was calculated by dividing the number of bacteria on the coverslip by the number of bacteria at 1 h postphagocytosis and multiplying by 100. All treatments were done in quintuplicate, and in 98% of the observations, standard deviations of bacterial counts did not exceed 0.30. Both strains of *B. abortus* were used in each experiment. Each symbol represents a separate experiment. Closed and open symbols, experiments in which anti-strain 19 and anti-strain 2308, respectively, were used as the opsonin. Bars represent the mean percent survival from all experiments. In one experiment (closed squares), the mean percent survival of strain 2308 at 48 h postphagocytosis was 160%, but the value was omitted from the calculations because the corresponding value for strain 19 was not obtained.

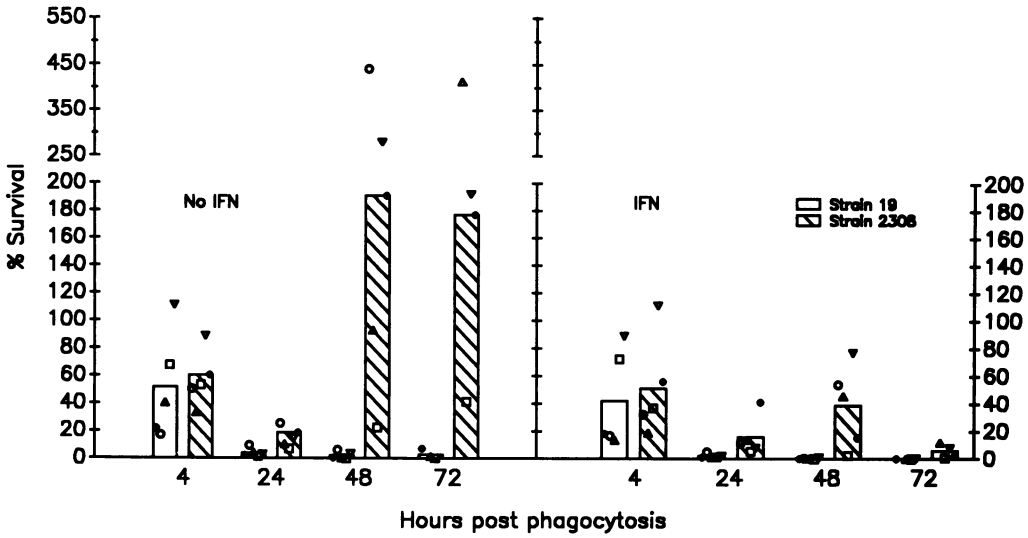

FIG. 3. Survival of *B. abortus* strains 19 and 2308 in murine peritoneal macrophages with and without IFN-γ. IFN-γ was added to macrophage monolayers 1 h postphagocytosis. All treatments were done in quintuplicate, and in 87% of the observations, standard deviations of bacterial counts did not exceed 0.30. Both strains of *B. abortus* were used in each experiment. Each symbol represents a separate experiment. Closed and open symbols, experiments in which anti-strain 19 and anti-strain 2308, respectively, were used as the opsonin. Bars represent the mean percent survival from all experiments.

(10) demonstrated that in Vero cells the growth profiles of strains 19 and 2308 were similar but that numbers of strain 19 remained about 10-fold lower.

In another set of experiments, we examined the effect of macrophage activation by gamma interferon (IFN-γ). In order to maintain uniformity in the phagocytosis step, 100 U of recombinant murine IFN-γ (Genentech, Inc., South San Francisco, Calif.) per ml was added to the macrophage monolayers at 1 h postphagocytosis, when coverslips were switched to a medium containing 12.5 μg of gentamicin per ml. Murray et al. (20) had demonstrated that IFN-γ effectively enhanced the killing of a slowly replicating pathogen even when added to macrophages after infection. All but one of the experiments were extended to 72 h. Medium was replaced every 24 h in order to minimize extracellular bacteria and to supply fresh IFN-γ. Again, in the absence of IFN-γ (Fig. 3, left panel) the percent survival of strain 2308 was significantly greater than that of strain 19 ($P < 0.05$ to $P < 0.001$) in every comparison at 24, 48, and 72 h. Treatment with IFN-γ decreased numbers of both strains. The decrease was most apparent for strain 2308 at 48 and 72 h postphagocytosis (Fig. 3), when the percent survival in the presence of IFN-γ was in all but one instance significantly lower ($P < 0.01$ to $P < 0.001$) than in its absence. At 72 h, the percent survival of strain 19 in cultures containing IFN-γ averaged 0.12%, with a range of 0.04 to 0.41%, and in two of four instances numbers were below detection (<13 bacteria per coverslip). In contrast, strain 2308 was detectable at 72 h in every experiment, with a mean survival rate of 5.8% and a range of 0.74 to 11.9%. The percent survival of strain 2308 at 72 h in IFN-γ-treated cultures was in every instance significantly higher ($P < 0.01$ to $P < 0.001$) than that of strain 19 in corresponding cultures. The effectiveness of IFN-γ in reducing replication of *B. abortus* within murine macrophages (Fig. 3) has recently been demonstrated in two other laboratories (5, 15). These results are consistent with our hypothesis (4, 12) that the capacity of virulent strains of *B. abortus* to produce persistent infection requires their ability to survive in activated macrophages despite being opsonized by IgG antibodies. Experiments are in progress to determine whether the relatively low survival rate of strain 2308 at 72 h in IFN-γ-treated cultures is attributable to the potentially more protective IgG2a antibodies (25) that are present in sera of chronically infected mice (11).

The virulent *B. abortus* strain 2308 can inhibit phagolysosomal fusion (6, 13), whereas attenuated strain 19 does not (13). *B. abortus* may also inhibit the metabolic burst associated with phagocytosis (17). There is no evidence that *B. abortus* can, like *Listeria monocytogenes* (24), disrupt the phagosomal membrane, but it is noteworthy that large numbers of *B. abortus* have been found in the rough endoplasmic reticulum (RER) of several cell types (2, 3, 8–10, 18). There is evidence that in Vero cells, transfer of brucellae to the cisternae of the RER results in bacterial replication, whereas transfer to phagolysosomes leads to bacterial death (9). Although *B. abortus* has not to date been demonstrated in the RER of macrophages (1, 3, 18), it may be hypothesized in parallel with the findings of Detilleux et al. (9, 10) that under natural conditions of infection, strain 19 opsonized with IgG antibody is destroyed within phagolysosomes, whereas a portion of similarly opsonized strain 2308 gains access to the RER and there establishes chronic infection. However, in macrophages activated by IFN-γ before strain 2308 reaches the RER, the combined effects of IFN-γ and IgG opsonins would facilitate phagosome-lysosome fusion and the killing of the bacterium within phagolysosomes, thereby preventing or minimizing penetration into the RER.

We thank Kelly Clark for technical assistance, Sandy Holsten for preparation of the manuscript, and Cynthia Baldwin and Fred Enright for helpful discussions.

REFERENCES

1. **Ackermann, M. R., N. F. Cheville, and B. L. Deyoe.** 1988. Bovine ileal dome lymphoepithelial cells: endocytosis and transport of *Brucella abortus* strain 19. Vet. Pathol. **25**:28–35.
2. **Anderson, T. D., and N. F. Cheville.** 1986. Ultrastructural morphometric analysis of *Brucella abortus*-infected trophoblasts in experimental placentitis. Am. J. Pathol. **124**:226–237.

3. **Anderson, T. D., N. F. Cheville, and V. P. Meador.** 1986. Pathogenesis of placentitis in the goat inoculated with *Brucella abortus*. II. Ultrastructural studies. Vet. Pathol. **23**:227–239.
4. **Araya, L. N., and A. J. Winter.** 1990. Comparative protection of mice against virulent and attenuated strains of *Brucella abortus* by passive transfer of immune T cells or serum. Infect. Immun. **58**:254–256.
5. **Bagchi, T.** 1990. Ph.D. thesis. Virginia Polytechnic Institute and State University, Blacksburg.
6. **Bertram, T. A., P. C. Canning, and J. A. Roth.** 1986. Preferential inhibition of primary granule release from bovine neutrophils by a *Brucella abortus* extract. Infect. Immun. **52**:285–292.
7. **Dees, C., M. W. Fountain, J. R. Taylor, and R. D. Schultz.** 1985. Enhanced intraphagocytic killing of *Brucella abortus* in bovine mononuclear cells by liposomes containing gentamicin. Vet. Immunol. Immunopathol. **8**:171–182.
8. **Detilleux, P. G., N. F. Cheville, and B. L. Deyoe.** 1988. Pathogenesis of *Brucella abortus* in chicken embryos. Vet. Pathol. **25**:138–146.
9. **Detilleux, P. G., B. L. Deyoe, and N. F. Cheville.** 1990. Entry and intracellular localization of *Brucella* spp. in Vero cells: fluorescence and electron microscopy. Vet. Pathol. **27**:317–328.
10. **Detilleux, P. G., B. L. Deyoe, and N. F. Cheville.** 1990. Penetration and intracellular growth of *Brucella abortus* in nonphagocytic cells in vitro. Infect. Immun. **58**:2320–2328.
11. **Elzer, P. H., R. H. Jacobson, and A. J. Winter.** 1991. Program Abstr. 72nd Conf. Res. Workers Anim. Dis., abstr. 96.
12. **Enright, F. M., L. N. Araya, P. H. Elzer, G. E. Rowe, and A. J. Winter.** 1990. Comparative histopathology in BALB/c mice infected with virulent and attenuated strains of *Brucella abortus*. Vet. Immunol. Immunopathol. **26**:171–182.
13. **Frenchick, P. J., J. F. Markham, and A. H. Cochrane.** 1985. Inhibition of phagosome-lysosome fusion in macrophages by soluble extracts of virulent *Brucella abortus*. Am. J. Vet. Res. **46**:332–335.
14. **Harmon, B. G., L. G. Adams, and M. Frey.** 1988. Survival of rough and smooth strains of *Brucella abortus* in bovine mammary gland macrophages. Am. J. Vet. Res. **49**:1092–1097.
15. **Jiang, X., and C. L. Baldwin.** Effects of cytokines on the intracellular growth of *Brucella abortus*: IL-2 and IFN-gamma independently mediate inhibitory effects. Submitted for publication.
16. **Jones, S. M., and A. J. Winter.** 1991. Program Abstr. 72nd Conf. Res. Workers Anim. Dis., abstr. 95.
17. **Kreutzer, D. L., L. A. Dreyfus, and D. C. Robertson.** 1979. Interaction of polymorphonuclear leukocytes with smooth and rough strains of *Brucella abortus*. Infect. Immun. **23**:737–742.
18. **Meador, V. P., and B. L. Deyoe.** 1989. Intracellular localization of *Brucella abortus* in bovine placenta. Vet. Pathol. **26**:513–515.
19. **Montaraz, J. A., and A. J. Winter.** 1986. Comparison of living and nonliving vaccines for *Brucella abortus* in BALB/c mice. Infect. Immun. **53**:245–251.
20. **Murray, H. W., G. L. Spitalny, and C. F. Nathan.** 1985. Activation of mouse peritoneal macrophages in vitro and in vivo by interferon-γ. J. Immunol. **134**:1619–1622.
21. **Nesthus, I., B. Haneberg, J. Glette, and C. O. Solberg.** 1985. The influence of antimicrobial agents on macrophage-associated *Staphylococcus aureus*. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **93**:189–194.
22. **Price, R. E., J. W. Templeton, and L. G. Adams.** 1990. Survival of smooth, rough and transposon mutant strains of *Brucella abortus* in bovine mammary macrophages. Vet. Immunol. Immunopathol. **26**:353–365.
23. **Subcommittee on Brucellosis Research.** 1977. Brucellosis research: an evaluation. Report of the Subcommittee on Brucellosis Research, National Academy of Sciences. National Academy Press, Washington, D.C.
24. **Tilney, L. G., and D. A. Portnoy.** 1989. Actin filaments and the growth, movement and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. J. Cell Biol. **109**:1597–1608.
25. **Winter, A. J., J. R. Duncan, C. G. Santisteban, J. T. Douglas, and L. G. Adams.** 1989. Capacity of passively administered antibody to prevent establishment of *Brucella abortus* infection in mice. Infect. Immun. **57**:3438–3444.
26. **Young, E. J., M. Borchert, F. L. Kretzer, and D. M. Musher.** 1985. Phagocytosis and killing of *Brucella* by human polymorphonuclear leukocytes. J. Infect. Dis. **151**:682–689.