Short note

Brucella abortus siderophore 2,3-dihydroxybenzoic acid protects brucellae from killing by macrophages

BA Leonard [1], I Lopez-Goni [2], CL Baldwin [1*]

[1] *Department of Microbiology, The Ohio State University, 484 West 12th Avenue, Columbus, OH 43210, USA;*
[2] *Departamento de Microbiologia, Universidad de Navarra, Aptdo 273, 31080 Pamplona, Spain*

Summary — Addition of 2,3-dihydroxybenzoic acid, a siderophore produced by *Brucella abortus*, to macrophage cultures prevented intracellular killing of brucellae during the first 12 h after infection and increased the number of intracellular brucellae recovered at 48 h after infection. The protective effect could be demonstrated with inflammatory macrophages, interferon-γ-activated macrophages and with macrophages supplemented with iron, shown elsewhere to facilitate killing of *B abortus*.

Brucella / macrophage / iron / siderophore / intracellular bacteria

Résumé — L'acide 2,3-dihydroxybenzoïque prévient la destruction de *Brucella abortus* par les macrophages. L'addition d'acide 2,3-dihydroxybenzoïque, un sidérophore produit par *Brucella abortus*, à des cultures de macrophages infectés par cette bactérie, a empêché la destruction de celle-ci dans les 12 premières heures consécutives à l'infection et a augmenté le nombre de *Brucella* intracellulaires récupérées après 48 heures. L'effet protecteur de l'acide 2,3-dihydroxybenzoïque a été mis en évidence dans des macrophages inflammatoires, dans des macrophages activés par l'interféron-γ et dans des macrophages cultivés dans des milieux supplémentés en fer, lequel augmente l'activité bactéricide des macrophages.

Brucella / macrophage / fer / sidérophore / bactérie / intracellulaire

---

* Correspondence and reprints.
Present address: Department of Veterinary and Animal Sciences, Paige Laboratory, University of Massachusetts, Amherst, MA 01003, USA.
Tel: (1) 413 545 3167; fax: (1) 413 545 6326; e-mail: Cbaldwin@VASCI.Umass.edu

INTRODUCTION

Brucella spp are facultative intracellular bacterica that cause chronic infections in agricultural animals as well as in humans. Humans are infected zoonotically from direct contact with animals or unpasteurized dairy products and the resultant disease is characterized by fluctuating body temperature, malaise, headache, arthritis, endocarditis and occasionally death (Young, 1983). Chronic infections are thought to be related to the ability of brucellae to infect and survive in professional phagocytes as well as in nonprofessional phagocytes such as trophoblasts (for review see Baldwin and Winter, 1994).

Recent studies in our laboratory have evaluated the control of intracellular *Brucella abortus* by macrophages (Jiang and Baldwin, 1993a, b; Jiang et al, 1993). We found that the reactive oxygen intermediates superoxide anion and hydrogen peroxide are important in controlling brucellae in both inflammatory and interferon-γ (IFN-γ) activated macrophages, while reactive nitrogen intermediates are involved to a much lesser extent (Jiang et al, 1993). Moreover, we showed that supplementing macrophage cultures with iron further enhanced their brucellacidal activities. The increased control of brucellae was attributable to the effects of hydroxyl radicals (Jiang and Baldwin, 1993b), presumably generated in the iron-supplemented macrophages via the Haber–Weiss reaction which uses iron as a catalyst (Babior, 1984). Thus, while brucellae may be cleared from IFN-γ-activated macrophages 'artifically' supplemented with high levels of exogenous iron, they otherwise persist intracellularly.

Siderophores are iron-chelating molecules produced by bacteria in low iron environments to facilitate iron acquisition (Payne, 1993) and which function as virulence factors. They have been shown to increase the survival of *Vibrio cholerae* (Sigel and Payne, 1982) in the host. The lethality of *Pseudomonas aeruginosa* can be enhanced by an exogenous supply of the siderophore pyochelin (Weinber, 1984), and the virulence of *Salmonella typhi*, which is normally

nonpathogenic for mice, is greatly enhanced by addition of exogenous iron and 2,3-dihydroxybenzoic acid (Weinber, 1984). *B abortus* has recently been shown to produce the siderophore 2,3-dihydroxybenzoic acid (Lopez-Goni et al, 1992). Since most iron in macrophages is not readily available but rather bound to macrophage iron-chelating molecules (Payne, 1993), siderophore production by brucellae would be expected to occur intracellularly. The ability of brucellae to produce siderophores for iron acquisition under such conditions is suggested by the observation that addition of the iron chelator deferoxamine to macrophage cultures does not inhibit intracellular growth of *B abortus* (Jiang and Baldwin, 1993b) while it does inhibit *Legionella pneumophila* (Bryd and Horwitz, 1989) an intracellular bacteria which is deficient in siderophore production. In addition to facilitating iron acquisition, the strong chelating potential of 2,3-dihydroxybenzoic acid may also promote survival of intracellular brucellae by chelating iron required by macrophages for generation of the potently antibrucellae hydroxyl radicals (Babior, 1984) as well as by scavenging reactive oxygen intermediates, a function ascribed to 2,3-dihydroxybenzoic acid by others (Feistner and Beaman, 1987). The experiments described here evaluated the ability of 2,3-dihydroxybenzoic acid to promote the intracellular survival of a virulent field strain of *B abortus*.

MATERIALS AND METHODS

Extracellular cultures of brucellae

Standard cultures of *B abortus* strain 2308 (kindly provided by AJ Winter, Cornell University) were conducted in *Brucella* broth (Difco, Detroit, MI, USA) as described previously (Jiang and Baldwin, 1993a; Jiang et al, 1993). Where indicated 500 µM of the iron chelators deferoxamine and/or 2,3-dihydroxybenzoic acid (Sigma, Saint Louis, MO, USA) were added. After culture in the presence of chelators as indicated in *Results,* bacteria were washed twice in phosphate-buffered saline and recultured in *Brucella*

broth without additives to assess bacterial viability. All bacterial cultures were incubated at 37 °C and bacterial growth assessed over time by optical density at 550 nm.

Intracellular cultures of brucellae

Peritoneal macrophages from BALB/c mice were infected and evaluated for CFU after various periods of infection as described previously (Jiang and Baldwin, 1993a; Payne, 1993). Where indicated macrophage cultures were supplemented with 100 units/mL IFN-γ (Boehringer Mannheim, Indianapolis, IN, USA), iron-saturated transferrin (Fe-Tf, Miles Inc, Kankakee, IL, USA) at 6 mg/mL (Jiang and Baldwin, 1993b) and/or 2,3-dihydroxybenzoic acid at concentrations indicated in *Results*.

RESULTS

Ability of 2,3-dihydroxybenzoic acid to facilitate iron acquisition for brucellae

To determine the ability of 2,3-dihydroxybenzoic acid to compete with deferoxamine for iron acquisition, extracellular cultures of *B abortus* were monitored for their growth in the presence of these two iron chelators. The premise was that if 2,3-dihydroxybenzoic acid could compete with deferoxamine to chelate iron, it would be able to facilitate iron acquisition by the bacteria and subsequently replication. Extracellular growth of brucellae was inhibited by approximately 40% after 30 h of culture in the presence of deferoxamine while brucellae cultured in *Brucella* broth supplemented with 2,3-dihydroxybenzoic acid grew at a rate more similar to those in cultures with free iron available (ie, in *Brucella* broth) (fig 1). The inhibition of growth by the presence of deferoxamine was not due to bacterial death since the growth rate of organisms pre-cultured with deferoxamine was normal when the bacteria were subsequently cultured in *Brucella* broth without deferoxamine (data not shown). Addition of 2,3-dihydroxybenzoic

acid to cultures containing deferoxamine resulted in replication rates similar to those with that 2,3-dihydroxybenzoic acid only, indicating 2,3-dihydroxybenzoic acid successfully competed with deferoxamine for acquiring iron (fig 1). It is unclear why growth is slower in cultures with 2,3-dihydroxybenzoic acid than in those with *Brucella* broth but it may indicate that iron acquisition is slower or more energy-requiring by the siderophore-dependent pathway.

Effect of 2,3-dihydroxybenzoic acid on recovery of brucellae from macrophage cultures after 48 h of culture

We exploited the culture system described above to compare brucellae pre-cultured in *Brucella* broth with iron available with that grown in the presence of deferoxamine for their abilities to survive and replicate intracellularly. We did this in an attempt to directly assess the role of endogenously produced 2,3-dihydroxybenzoic acid for intracellular survival of brucellae. While in some experiments the brucellae pre-cultured in the presence of deferoxamine had a higher survival and/or growth rate in macrophage cul-
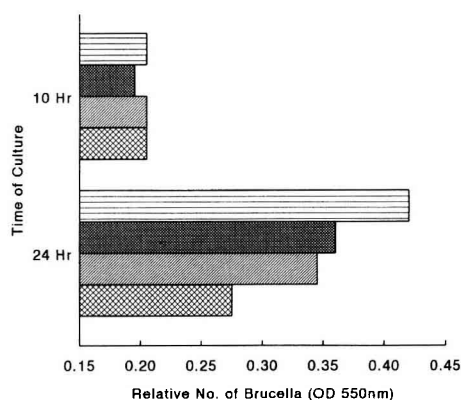

Fig 1. Typical growth patterns of extracellular cultures of *B abortus* with deferoxamine (DFA) and/or 2,3-dihydroxybenzoic acid (DHBA). Relative concentrations of brucellae were determined by reading OD at 550 nm.▤: broth;▨: DHBA; ▧: DHBA + DFA ;▧ : DFA.

tures over the 48 h culture period the effect was not consistently observed under all culture conditions in the three experiments performed (data not shown). Thus, because of the inherent variability in that assay system (perhaps due to unpredictable outcomes of stress due to the low iron environment), further experiments evaluated the effect of exogenous 2,3-dihydroxybenzoic acid on altering intracellular survival rates of brucellae. Initial experiments indicated that the addition of 2,3-dihydroxybenzoic acid to macrophage cultures resulted in a dose-dependent increase in recovery of brucellae: $100~\mu M$ resulted in a mean increase of $\log_{10} 0.24$ colony forming units (CFU) at 48 h after infection

whereas $500~\mu M$ resulted in an increase of $\log_{10} 0.65$ CFU, in two experiments performed with four replicate cultures per experiment. Thus in further experiments, 2,3-dihydroxybenzoic acid was added at $500~\mu M$ to macrophage cultures. Addition resulted in a mean increase in recovery of CFU of $\log_{10} 0.50 \pm 0.66$ from inflammatory macrophages, of $\log_{10} 1.18 \pm 0.64$ in cultures of macrophages activated with IFN-γ, and of $\log_{10} 0.72 \pm 0.44$ in macrophage cultures activated with IFN-γ as well as supplemented with iron (fig 2).

Ability of 2,3-dihydroxybenzoic acid to protect brucellae from killing by oxidative burst during the first 24 h after infection

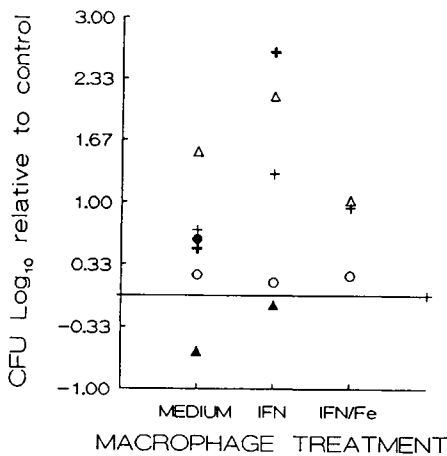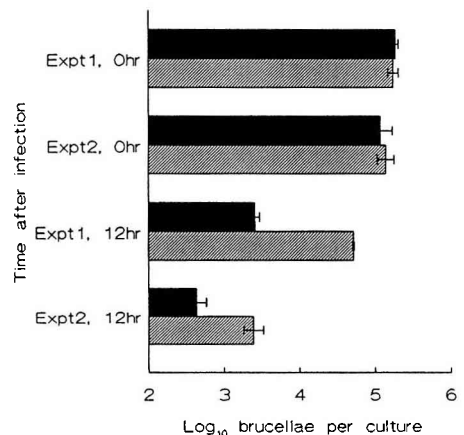By 12 h post-infection the mean increase in number of brucellae recovered from

Fig 2. Relative differences in CFU recovered after 48 h of culture from *B abortus*-infected macrophage cultures supplemented with 2,3-dihydroxybenzoic acid compared to the CFU recovered from control macrophage cultures that did not receive $500~\mu M$ 2,3-dihydroxybenzoic acid. The solid line indicates the zero level or no difference between CFU recovered from 2,3-dihydroxybenzoic acid-supplemented cultures and the unsupplemented control cultures. Macrophages were either cultured with medium only, activated with IFN-γ (IFN) or activated with IFN-γ and supplemented with iron (IFN/Fe) as indicated on the abscissa. The increase in CFU in the presence of 2,3-dihydroxybenzoic acid was significant as determined by the Mann–Whitney U-test for all three types of cultures ($P < 0.05$). Each point represents the mean CFU of replicate cultures from individual treatment groups and experiments.

Fig 3. Recovery of *B abortus* organisms, expressed as $\log_{10}$ CFU per culture, from macrophages cultured in the presence (speckled bars) or absence (solid bars) of 2,3-dihydroxybenzoic acid ($500~\mu M$). Macropages were cultured for 1 h with *B abortus* and then lysed to evaluate initial infection rates (0 h) and additional cultures were lysed after 12 h. Each bar represents the mean ± SD of CFU from replicate cultures for each treatment and time point. Addition of 2,3-dihydroxybenzoic acid resulted in a significant increase in CFU recovered at 12 h after infection in both experiments (Exp 1 and Exp 2) as assessed by the Mann–Whitney U-test ($P < 0.05$). ■: no DHBA; ▨ + DHBA.

macrophages treated with 2,3-dihydroxybenzoic acid compared to the control cultures was $\log_{10}$ 0.76 ± 0.44 CFU ($P < 0.01$; see fig 3). There was no significant difference in the number of bacteria phagocytosed initially in macrophage cultures with or without 2,3-dihydroxybenzoic acid (fig 3).

DISCUSSION

Although the results of experiments reported elsewhere suggested that production of 2,3-dihydroxybenzoic acid in the presence of the iron chelator deferoxamine facilitated iron acquisition (Jiang and Baldwin, 1993b), it had not been formally demonstrated that 2,3-dihydroxybenzoic acid can compete with deferoxamine to promote growth of brucellae. The experiments performed here indicated that 2,3-dihydroxybenzoic acid can effectively compete with deferoxamine and thereby facilitate growth of *B abortus*. This result concurred with the results of previous experiments with indicated that growth of brucellae is not retarded in macrophages supplemented with deferoxamine (Jiang and Baldwin, 1993b). Similarly, the inability of deferoxamine to entirely inhibit growth of brucellae in extracellular cultures as reported here is likely to have reflected the production of endogenous 2,3-dihydroxybenzoic acid by brucellae as a result of the low iron environment.

Since brucellae produce siderophores in low iron environments but discontinue production when iron is readily available (Lopez-Goni et al, 1992), we tried to exploit this culture system (ie, that with deferoxamine) to evaluate the role of endogenously produced siderophores in promoting survival of brucellae in macrophages. However, we were unable to consistently show that bacteria pre-cultured in the presence of deferoxamine had an advantage for intracellular growth over controls. The induction of siderophore production may have been so rapid after infection of macrophages in some experiments that any difference in siderophore production prior to infection did not influence sub-

sequent intracellular survival and/or growth. Unfortunately, due to the limited number of brucellae recoverable from macrophage cultures, it was not possible to quantitate intracellular production of 2,3-dihydroxybenzoic acid directly at various times following macrophage infection to directly assess this. The reasons for the variability among experiments thus remain unresolved.

Using exogenous 2,3-dihydroxybenzoic acid, however, we were able to show that it promoted survival of brucellae in macrophages over a 48 h culture period. Since the protective effect was observed regardless of whether or not the macrophages were supplemented with iron, it is possible that 2,3-dihydroxybenzoic acid is protecting brucellae by mechanisms in addition to chelating iron needed as a catalyst for generation of hydroxyl radicals. This speculation is based upon the fact that hydroxyl radicals are apparently not involved in killing brucellae in macrophages that are not supplemented with iron (Jiang et al, 1993). We have shown elsewhere that during the first 12 h after infection reactive oxygen intermediates superoxide anion and hydrogen peroxide reduced the number of CFU of brucellae phagocytosed by macrophages approximately 100-fold (Jiang et al, 1993). Further experiments evaluated the ability of 2,3-dihydroxybenzoic acid to protect brucellae from oxidative killing during this time. The results indicated that the addition of 2,3-dihydroxybenzoic acid decreased killing, suggesting that it may scavenge reactive oxygen intermediates. The magnitude of protection in these experiments was similar to that which occurred following addition of other reactive oxygen inhibitors, superoxide dismutase and catalase, reported by us elsewhere (Jiang et al, 1993).

In conclusion, the fact that addition of exogenous 2,3-dihydroxybenzoic acid had a significant impact on intracellular survival of *Brucella* provides support for the hypothesis that brucellae use 2,3-dihydroxybenzoic acid to survive intracellularly by mechanisms which extend beyond iron acquisition. Moreover, clearance of brucellae from iron-supplemented/IFN-γ-acti-

vated macrophages may not be purely a consequence of the generation of hydroxyl radicals but may also reflect the absence of protective siderophores whose production is suspended in the iron-rich environment. Based upon these observations, we propose that siderophore-negative mutants of *Brucella* spp may be valuable as vaccine strains. Such mutants are predicted to be incapable of surviving for prolonged periods intracellularly and thus establishing chronic infections. Work has been initiated to produce both siderophore-negative mutants (*entC* mutants) and constitutively expressing *(fur)* mutants of *B abortus* to evaluate their abilities to survive in macrophages as well as persist in vivo.

ACKNOWLEDGMENTS

REFERENCES

Babior BM (1984) The respiratory burst of phago-cytes. *J Clin Invest* 73, 599-601

Baldwin CL, Winter AJ (1994) Macrophages and *Brucella*. In: *Macrophage Pathogen Interactions* (B Zwilling, T Eisenstein, eds), Marcel Dekker, NY, USA, 363-380

Bryd TF, Horwitz MA (1989) Interferon-gamma-activated human monocytes downregulate transferrin receptors and inhibit intracellular multiplication of *Legionella pneumophila* by limiting the availability of iron. *J Clin Invest* 83, 1457-1465

Feistner GJ, Beaman BL (1987) Characterization of 2,3-dihydroxybenzoic acid from *Nocardia asteroides* GUH-2. *J Bacteriol* 169, 3983-3987

Jiang X, Baldwin CL (1993a) Effects of cytokines on the intracellular growth of *Brucella abortus*. *Infect Immun* 61, 124-134

Jiang X, Baldwin CL (1993b) Iron augments macrophage-mediated killing of *Brucella abortus* alone and in conjunction with interferon-gamma. *Cell Immunol* 48, 397-407

Jiang X, Leonard B, Benson R, Baldwin CL (1993) Macrophage control of *Brucella abortus*: role of reactive oxygen intermediates and nitric oxide. *Cell Immunol* 151, 1-11

Lopez-Goni I, Moriyon I, Neilands JB (1992) Identification of 2,3-dihydroxybenzoic acid as a *Brucella abortus* siderophore. *Infect Immun* 60, 4496-4503

Payne SM (1993) Iron acquisition in microbial pathogenesis. *Trends Microbiol* 1, 66-69

Sigel SP, Payne SM (1982) Effect of iron limitation on growth, siderophore production, and expression of outer membrane proteins of *Vibrio choleerae*. *J Bacteriol* 150, 148-155

Weinber ED (1984) Iron withholding: a defense against infection and neoplasia. *Physiol Rev* 64, 65-102

Young EJ (1983) Human brucellosis. *Rev Infect Dis* 5, 821-842