Establishment of a method for evaluating intracellular antibiotic efficacy in *Brucella abortus*-infected Mono Mac 6 monocytes

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Background: Brucellae produce chronic and often lifelong infections in natural hosts. The persistent nature of these infections is predominantly due to the capacity of these bacteria to maintain intracellular residence in host macrophages. Successful antimicrobial therapy requires eradication of brucellae from this intracellular niche. It is important to seek new and improved antimicrobials for brucellosis therapy as well as a method to efficiently evaluate their intracellular efficacy.

Objectives: For that reason, we have developed a method to evaluate intracellular drug efficacy for new and improved antimicrobials that show initial *in vitro* activity against *Brucella* species during drug screening.

Methods: Mono Mac 6 monocytes (MM6) were used because they are the only human cell line that constitutively expresses the phenotypic and functional characteristics of mature monocytes. This cell line has not previously been used with *Brucella*, therefore parallel studies were performed with J774 murine macrophages. Both cell lines were infected with *Brucella abortus* 2308 and antibiotics used clinically for treatment of brucellosis were used to determine intracellular efficacy.

Results: Significant differences in bacterial burden were observed at or above the MIC in both cell lines. Drug concentrations that fell below the MIC were found to significantly reduce intracellular brucellae only in MM6.

Conclusions: The MM6 intracellular efficacy model will provide a useful method to examine the effect of novel antimicrobials for the treatment of human brucellosis.

Keywords: *Brucella*, macrophage, antibiotic, drug screening

Introduction

The *Brucella* species are Gram-negative bacteria that cause disease in humans and other mammals such as sheep, goats and cattle. Infection in food animals results in abortion and infertility, whereas human infection produces a disease known as undulant or Malta fever. Humans generally contract brucellosis directly from infected animals or their products, by accidental self-inoculation with animal vaccine strains or as a result of laboratory accidents. Human infection presents as a protracted debilitating febrile illness. Brucellae produce chronic and often lifelong infections in natural hosts. The persistent nature of these infections is predominantly due to the capacity of these bacteria to maintain intracellular residence in host macrophages. Successful antimicrobial therapy requires eradication of brucellae from this intracellular niche and thus prolonged antibiotic therapy.

Treatment of brucellosis with a single antibiotic is not recommended due to high rates of relapse, however a clear optimal dual therapy has not been agreed upon. The World Health Organization recommends treatment with doxycycline for 6 weeks and streptomycin for 14–21 days or gentamicin for 7–10 days. However, streptomycin and gentamicin must be administered parenterally (i.e. intramuscularly or intravenously) making outpatient treatment difficult. Consequently, doxycycline in combination with rifampicin (which both can be taken orally) is considered to be the principal alternative therapy for treatment of human brucellosis and must be taken for at least 6 weeks. Fluoroquinolones (e.g. ciprofloxacin) or trimethoprim/sulfamethoxazole in combination with doxycycline or rifampicin are recommended as secondary alternative therapies. Complicated brucellosis, where the patient develops meningitis or endocarditis, necessitates triple antibiotic therapy (doxycycline, co-trimoxazole and rifampicin). In children, a 3 week treatment...
of doxycycline or trimethoprim/sulphamethoxazole is recommended along with a 5 day course of gentamicin.\textsuperscript{3,9–11} Despite therapeutic compliance along with the patient, relapse rates of 5\% to 10\% are not uncommon.\textsuperscript{9–11}

Brucellae are listed as class B Select Agents according to the Centers for Disease Control and Prevention because of their potential to be used as agents of biological warfare and bioterrorism. This stems not only from the debilitating nature of brucellosis and the lack of a human vaccine, but also from the ease with which the organism can be aerosolized and the low infectious dose of $\sim$10–100 organisms.\textsuperscript{3,6,11} Our institution is a part of the NIAID Biodefense Research Program entitled ‘In vitro and Animal Models for Emerging Infectious Diseases and Biodefense’ whose primary objective is to ‘provide targeted screening and evaluation of potential therapeutic and prevention modalities for emerging infectious agents and bioterrorism pathogens’. As a part of that programme, we previously developed a broth dilution assay with Alamar Blue\textsuperscript{36} for the brucellae, allowing us to more narrowly define the MIC of a variety of drugs against \textit{B. abortus} 2308.\textsuperscript{12} Because of the intracellular nature of brucellae, it is equally important to determine the efficacy of individual drugs within the infected macrophages with relation to their MIC. The purpose of this study was to develop a method to evaluate single drug intracellular efficacy for treatment of human brucellosis. We chose to use the Mono Mac 6 (MM6) human monocytic cell line because it is the only human cell line that constitutively expresses the phenotypic and functional characteristics of mature monocytes and therefore may be more representative of macrophage infection in human brucellosis.\textsuperscript{13} Though this cell line has been used in the study of other intracellular bacteria such as \textit{Mycobacterium tuberculosis} and \textit{Legionella pneumophila}, we are aware of no prior studies with \textit{Brucella} and MM6.\textsuperscript{14–20} For this reason, we included the murine J774 monocytic cell line in our efficacy testing as it is commonly used to examine the pathogenesis of a wide variety of genetic \textit{Brucella} mutants and because trafficking of these bacteria in J774 cells has been studied.\textsuperscript{21} Significant differences in bacterial burden were observed at or above the MIC in both cell lines. Drug concentrations that fell below the MIC were found to significantly reduce intracellular brucellae only in MM6. The MM6 intracellular efficacy model will provide a useful initial screening method to examine the effect of novel antimicrobials for the treatment of human brucellosis.

\section*{Materials and methods}

\subsection*{Bacterial growth conditions}

\textit{B. abortus} 2308 was grown from frozen stock by streaking onto Tryptic soy agar containing 5\% sheep blood (TSAB, Hardy Diagnostics, Santa Maria, CA, USA) and incubating for 48 h at 37\(^\circ\)C and 5\% CO\textsubscript{2}. Subcultures from this initial plate were used for a maximum of one month for streaking plates used for infection. These bacterial passages were incubated as above and used once. We confirmed that 2308 maintained its smooth lipopolysaccharide using crystal violet dye exclusion.\textsuperscript{22}

\subsection*{Growth of monocytic cell lines}

The MM6 monocytic cell line was obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The MM6 line was originally established by H. W. L. Ziegler-Heitbrock, Institute of Immunology, University of Munich, Munich, Germany, and is a human acute monocytic leukaemia cell line.\textsuperscript{13} MM6 cells were maintained in RPMI 1640 (Sigma Chemical Co., St Louis, MO, USA) containing 2 mM l-glutamine (Sigma), 1 mM sodium pyruvate (Sigma), 9 \mu g of bovine insulin (Sigma) and a final concentration of 1 x non-essential amino acids (Sigma) (referred to hereafter as RPMIsup) and 10\% (v/v) fetal calf serum (FCS; Sigma). J774 cells were maintained in Cellgro DMEM (Mediatech, Herndon, VA, USA), 2 mM l-glutamine (Sigma) (referred to hereafter as DMEMsup) and 10\% (v/v) FCS. Both cell lines were tested for contamination with \textit{Mycoplasma} prior to infection.

\subsection*{Drug toxicity testing}

The toxicity of the highest drug concentration was evaluated in uninfected cells using the MTT assay\textsuperscript{16} treated in the same manner as infected cells. Monocytes were treated with 8 \times\ the respective MIC of rifampicin (1 mg/mL suspended in sterile DMSO; Sigma), ciprofloxacin (1 mg/mL suspended in sterile water, Millipore, Billerica, MA, USA), tetracycline (1 mg/mL suspended in sterile water, Sigma), doxycycline (1 mg/mL suspended in sterile water; Sigma), gentamycin sulphate (4 mg/mL suspended in sterile water; Sigma) or streptomycin sulphate salt (8 mg/mL suspended in sterile water; Sigma) for 24 h before determination of viability.

\subsection*{Oxidative burst testing of cell lines}

Each cell type was monitored for the ability to mount an oxidative burst upon stimulation using the nitroblue tetrazolium reduction assay.\textsuperscript{23} Cells were evaluated under a microscope for progressive darkening as purple formazan is deposited intracellularly in cells where an oxidative burst is taking place.

\subsection*{MM6 infection and antibiotic treatment}

Non-adherent MM6 cells were grown to $\sim$1 x 10\textsuperscript{6} cells/mL in RPMIsup + 10\% FCS. Viable monocytic cells were counted by staining with Trypan Blue (Sigma) and enumerating live cells on a haemocytometer. Once this cell density was achieved, the medium was changed to RPMIsup + 1% FCS and the cells were allowed to grow overnight at 37\(^\circ\)C and 5\% CO\textsubscript{2}. Sterile phosphate-buffered saline (PBS) was adjusted to a previously determined concentration of 5 x 10\textsuperscript{5} bacteria/mL by visually matching it to a no. 4 McFarland standard (Hardy Diagnostics), using a fresh bacterial cell scraping (see above). The bacterial suspension was serially diluted 10-fold in sterile PBS, plated on TSAB and incubated for 2–3 days at 37\(^\circ\)C and 5\% CO\textsubscript{2} to determine the number of viable cells. For a multiplicity of infection (MOI) of 50:1, aliquots of the 5 x 10\textsuperscript{5} suspension were added to each of 10, 25 cm\textsuperscript{2} flasks. Cells were mixed by gently swirling. Plates were incubated for 2 h at 37\(^\circ\)C and 5\% CO\textsubscript{2}. The contents of the flasks were distributed to individual 50 mL conical tubes, paralyzed and placed inside a gasketed centrifuge bucket cover to prevent aerosolization. The infected macrophages were washed three times in an equal volume of warm PBS + 1\% FCS by centrifugation (1000 rpm and room temperature for 10 min) to remove extracellular bacteria. The MM6 cells were then suspended in an equal volume of warm RPMIsup + 1\% FCS and 50 mg/L gentamicin sulphate and placed into new sterile 25 cm\textsuperscript{2} flasks. The flasks were incubated at 37\(^\circ\)C and 5\% CO\textsubscript{2} for 1 h. After that time, cells were washed again as above. The infected cells were combined and dispensed as 1 mL aliquots into 24-well sterile tissue culture plates. Rifampicin,
ciprofloxacin, tetracycline or doxycycline (in concentrations described above) was added to the wells at 8, 4, 1, 0.25 and 0.125× their MIC as well as the amount of sterile DMSO used for the highest rifampicin concentration to triplicate wells. Gentamicin sulphate or streptomycin sulphate salt (in concentrations described above) was added to the wells at 8× their MIC. Plates were incubated at 37°C and 5% CO₂ for 24 h. The cells were placed in separate 15 mL paraffilmed conical tubes, and were centrifuged as before. The supernatant was removed and plated on TSAB. MM6 cells were lysed in an equal volume of 0.5% Tween 20 diluted in sterile (EMD Biosciences, La Jolla, CA, USA) water for 2–3 min. Lysates were diluted immediately in sterile PBS and plated for intracellular bacterial enumeration. TSAB plates were incubated for 2–3 days at 37°C. No live bacteria were identified in undiluted monocyte supernatants. After 24 h of drug treatment, wells containing infected monocytes were treated as before and lysates plated in a similar fashion. The results are depicted in Figure 1.

J774 infection and antibiotic treatment

Adherent J774 macrophages were grown to 70% to 80% confluency in DMEMsup + 10% FBS in 75 cm² tissue culture flasks (Corning). At such time, cells were dislodged from the flask, and viable macrophages were counted as described previously. Macrophages were dispensed as 1 mL aliquots containing 1.5 × 10⁶ cells into 24-well sterile tissue culture plates and were incubated overnight at 37°C and 5% CO₂. After 24 h, the medium was replaced with DMEMsup + 1% FBS. The plates were then incubated overnight again at 37°C and 5% CO₂. The next morning, spent medium was again replaced. The average number of cells per well was determined by using a Reichert ocular micrometer according to the manufacturer's instructions. Sterile PBS was adjusted to a McFarland 4 volume of 0.5% Tween 20 diluted in sterile PBS and plated on TSAB to determine the number of viable cells. The tissue culture plates were covered with sterile plastic adhesive and centrifuged at 300 g for 5 min to infect the cells. The plates were incubated for 20 min at 37°C and 5% CO₂ to allow for phagocytosis of bacterial cells. The J774 cells were washed with sterile PBS + 1% FBS and 1 mL fresh medium containing 50 mg/L gentamicin sulphate was added to each well. The bacterial suspension was diluted in sterile PBS and plated on TSAB to determine the number of viable cells. The tissue culture plates were covered with sterile plastic adhesive and centrifuged at 300 g for 5 min to infect the cells. The plates were incubated for 20 min at 37°C and 5% CO₂ to allow for phagocytosis of bacterial cells. The J774 cells were washed with sterile PBS + 1% FBS and 1 mL fresh medium containing 50 mg/L gentamicin sulphate was added to each well. The plates were incubated for 1 h at 37°C and 5% CO₂ and wells were washed three times with PBS + 1% FBS. Fresh medium (1 mL of DMEMsup + 1% FBS) was added to each well. Rifampicin, ciprofloxacin, tetracycline or doxycycline (in concentrations described above) were added as described for MM6. The experimental protocol from this time on was performed as with MM6 with the exception that no centrifugation was required for the adherent J774 cells.

Statistical analysis of results

Each time a drug was evaluated, results are tabulated and analysed statistically using a one-way ANOVA and a Tukey multiple comparison post hoc test (GraphPad software, InStat, Scottsdale, AZ, USA). A significant increase in cfu after 24 h in the non-treated infected control was used as a criterion for acceptance of the drug treatment sets. If a significant increase in cfu was not observed in the non-treated infected control, the results were judged non-valid and the experiment repeated until that criterion was satisfied. In all experiments reported here, there was a significant increase in cfu with regard to the time zero cfu and the cfu achieved in the untreated infected control at 24 h (P < 0.001–0.05).

Results and discussion

Brucellae are considered to be Select Agents by the CDC because of the debilitating nature of brucellosis, the lack of a safe and effective human vaccine, the ease with which the organism can be aerosolized and the low infectious dose (10–100 organisms).⁴,⁶,¹¹ These qualities increase the potential of the Brucella species to be used as agents of biological warfare and bioterrorism. Brucellae produce chronic and often lifelong infections in natural hosts due predominantly to their capacity to maintain intracellular residence in host macrophages. Antimicrobial therapy requires complete eradication of brucellae from this intracellular niche, necessitating prolonged treatment. Because of the intracellular nature of brucellae, it is equally
important to determine the efficacy of antimicrobials within the infected macrophages with relation to their MIC. The purpose of this study was to develop a method to evaluate intracellular drug efficacy of individual drugs for treatment of human brucellosis, using clinically relevant antibiotics. We chose to use MM6 because it is the only human cell line that constitutively expresses the phenotypic and functional characteristics of mature monocytes.\textsuperscript{13} MM6 may be more representative of macrophage infection in human brucellosis. This model will serve to aid in determining intracellular efficacy of novel compounds received through our NIH drug-screening contract.

**Drug toxicity testing of monocytic cell lines**

Prior to testing the ability of any of the clinically relevant antibiotics to kill intracellular brucelae, it was necessary to establish whether or not the antibiotics of choice had any adverse effects on the MM6 and J774 cell lines. These cells were treated exactly as infected cells and were then treated with drug at 8, 4, 1, 0.25 and 0.125 × the MIC of each of the antibiotics, with the exception of gentamicin and streptomycin that were used at 8 × their MIC. No adverse effects of the drugs were observed in uninfected cells after 24 h of drug treatment using the MTT assay (Table 1) as determined by viability in excess of 80% of untreated control cells. The MM6 cell line is generally less sensitive to drug toxicity than J774 cells, making it more amenable to high-throughput screening of novel compounds.

**MM6 and J774 cells generate an oxidative burst with repeated passaging**

The premise for using MM6 cells to test intracellular antibiotic efficacy as opposed to other human monocytic cell lines is that it constitutively expresses the phenotypic and functional characteristics of mature monocytes and therefore may be more representative of macrophage infection in human brucellosis. Mature macrophages exhibit the ability to mount an oxidative burst when stimulated by bacterial lipopolysaccharide or phorbol myristate.\textsuperscript{23} MM6 and J774 cells generate an oxidative burst, but macrophage cell lines can lose this ability with repeated passaging.\textsuperscript{24−26}

**Table 1.** Monocytic cell viability after 24 h in the presence of antibiotic or solvent control

<table>
<thead>
<tr>
<th>Average uninfected cell viability (%) of untreated</th>
<th>MM6 24 h</th>
<th>J774 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>88</td>
<td>85</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>89</td>
<td>82</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>99</td>
<td>90</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>99</td>
<td>90</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>98</td>
<td>100</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>97</td>
<td>100</td>
</tr>
</tbody>
</table>

Viability was determined by the MTT assay and drugs were at the highest concentration used in these studies (8 × their respective MIC). Treatment with DMSO or any of the antibiotics did not significantly impair monocyte viability as determined by >80% survival compared with untreated cells. The results are the averages of three experiments performed in triplicate.

We treated each passage of MM6 and J774 cells prior to infection with phorbol myristate according to the protocol described by Pick and found that these monocytes initially produce a robust oxidative burst upon stimulation after being stored at −120 °C (data not shown).\textsuperscript{25} Both MM6 and J774 were found to mount an oxidative burst past 10 passages (data not shown), however none of the monocytes used in this study were used beyond passage 6.

**Monocytic infection with B. abortus 2308**

No previous reports describing infection of MM6 cells with any species of *Brucella* have been reported. We found that MM6 infection with *B. abortus* 2308 proceeds without bacterial opsonization, forced physical contact by centrifugation or chemical treatment of the monocytes. This is a vast improvement over other human monocytic cell lines commonly used in *Brucella* research. It should be noted that 2308 has a smooth colony morphology due to its lipopolysaccharide and typical of wild-type virulent strains. In the absence of a need for bacterial opsonization in MM6 cells, it was necessary to use a previously described method that uses centrifugation to infect J774 cells with *B. abortus* so that the two methods would be comparable.\textsuperscript{27} An MOI was determined for MM6 and J774 monocytes such that nearly equivalent bacterial burden was achieved (Figure 1a and b).

**Intracellular antibiotic efficacy against B. abortus 2308**

Previously determined MIC values by our group were used for evaluations of treatment parameters for rifampicin, ciprofloxacin, tetracycline, doxycycline, gentamicin and streptomycin.\textsuperscript{12} Infected MM6 and J774 cells were treated with 8, 4, 1, 0.25 and 0.125 × their MIC. Gentamicin and streptomycin were used at 8 × their MIC because they are believed to enter monocytes poorly if at all. These MIC values (rifampicin = 1.0 mg/L, ciprofloxacin = 1.0 mg/L, tetracycline = 0.5 mg/L, doxycycline = 0.25 mg/L, gentamicin = 0.5 mg/L and streptomycin = 1.0 mg/L) fall within those established by the CLSI and other published reports.\textsuperscript{28−30}

Intracellular and extracellular bacteria were enumerated after 24 h. Extracellular bacterial numbers were insignificant as compared with intracellular burden (data not shown). Significant decreases in bacterial loads (P < 0.05 or greater) were consistently observed at or above the MIC for rifampicin, ciprofloxacin, tetracycline and doxycycline evaluated in both cell lines (Figures 1–4), whereas none was observed with the DMSO solvent control (Figure 1), gentamicin (data not shown) or streptomycin (data not shown). The results from treatment with gentamicin and streptomycin were not unexpected given that they have limited ability to permeate cell membranes. The bacterial burden was reduced by 2 logs on average with rifampicin, ciprofloxacin, tetracycline and doxycycline in MM6 and 1.5–2 logs in J774 cells. Interestingly, bacterial burden was not significantly reduced in J774 cells at the MIC for ciprofloxacin and tetracycline, while this effect was observed in infected MM6. However, rifampicin and doxycycline treatment significantly reduced the number of viable intracellular brucellae at their respective MIC in J774 macrophages. Furthermore, rifampicin and tetracycline significantly decreased the number of bacteria in MM6 at 0.25 × their respective MIC. This phenomenon was not observed in the J774 cells. Perhaps the differences observed could be due to drug penetration in these different cell lines.
It is notable that none of the antibiotics tested completely eradicated intracellular *B. abortus* 2308. This is not unexpected given that clinical therapy for brucellosis is recommended for 6 weeks and that the assay was performed for 24 h. Additionally, incomplete killing of *Listeria monocytogenes* and *Staphylococcus aureus* in J774 cells treated with fluoroquinolones for 24 h has been reported.

Figure 2. Intracellular efficacy of ciprofloxacin in MM6 and J774 monocytes. The bars represent the number of viable brucellae at time zero (initial) and after 24 h of treatment with ciprofloxacin at 8, 4, 1, 0.25 and 0.125× its MIC (8, 4, 1, 0.25 and 0.125 mg/L) in (a) MM6 cells and (b) J774 murine cells. Macrophages infected for 24 h but not exposed to drug are labelled ‘untreated’. MM6 cells were infected at an MOI of 50:1, whereas J774 cells were infected at an MOI of 25:1. The graphs represent the average intracellular bacteria from three experiments performed in triplicate. The error bars represent the standard deviation between those three experiments. Asterisks indicate the statistical significance values (*P* < 0.05 or **P** < 0.01) obtained between the experimental group and its corresponding untreated group. A significant difference in cfu was observed with regard to the initial infection and the untreated infected control at 24 h (*P* < 0.001 for MM6 and <0.05 for J774; data not shown in graph).

Figure 3. Intracellular efficacy of tetracycline in MM6 and J774 monocytes. The bars represent the number of viable brucellae at time zero (initial) and after 24 h of treatment with tetracycline at 8, 4, 1, 0.25 and 0.125× its MIC (4, 2, 0.5, 0.125 and 0.063 mg/L) in (a) MM6 cells and (b) J774 murine cells. Macrophages infected for 24 h but not exposed to drug are labelled ‘untreated’. MM6 cells were infected at an MOI of 50:1, whereas J774 cells were infected at an MOI of 25:1. The graphs represent the average intracellular bacteria from three experiments performed in triplicate. The error bars represent the standard deviation between those three experiments. Asterisks indicate the statistical significance values (*P* < 0.05 or **P** < 0.01) obtained between the experimental group and its corresponding untreated group. A significant difference in cfu was observed with regard to the initial infection and the untreated infected control at 24 h (*P* < 0.001 for MM6 and <0.05 for J774; data not shown in graph).
Intracellular efficacy model for *Brucella*

![Graph](image)

**Figure 4.** Intracellular efficacy of doxycycline in MM6 and J774 monocytes. The bars represent the number of viable brucellae at time zero (initial) and after 24 h of treatment with doxycycline at 0.125, 0.25, 1, 4 and 8 times its MIC (0.063 mg/L) in (a) MM6 cells and (b) J774 murine cells. Macrophages infected for 24 h but not exposed to drug are labelled ‘untreated’. MM6 cells were infected at an MOI of 50:1, whereas J774 cells were infected at an MOI of 25:1. The graphs represent the average intracellular bacteria from three experiments performed in triplicate. The error bars represent the standard deviation between those three experiments. Asterisks indicate the statistical significance values (\(* P < 0.05\) or \(**P < 0.01\)) obtained between the experimental group and its corresponding untreated group. A significant difference in cfu was observed with regard to the initial infection and the untreated infected control at 24 h (\(P < 0.01\) for MM6 and \(<0.001\) for J774; data not shown in graph).

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**Transparency declarations**

None to declare.

**References**


