CHEMICAL AND BIOLOGICAL FACTORS IN THE CONTROL OF
BRUCELLA AND BRUCELLOSIS.

Carlos Gamazo¹*, María Concepción Lecároz¹, Sandra Prior¹, Ana Isabel Vitas¹,
Miguel Angel Campanero², Juan Manuel Irache³, and María José Blanco-Prieto³

¹ Department of Microbiology, University of Navarra, 31080 Pamplona, Spain;
² Department of Clinical Pharmacology, Clinica Universitaria, 31080 Pamplona, Spain;
³ Department of Pharmacy and Pharmaceutical Technology, University of Navarra,
31080 Pamplona, Spain

*To whom correspondence should be addressed:
Tel:  ++34 948 425688
Fax:  ++34 948 425649
E-mail: cgamazo@unav.es
Address: Department of Microbiology, University of Navarra, Irunlarrea 1, 31080 Pamplona, Spain,
Running title: Factors in the Control of Brucellosis

Keywords: *Brucella*, brucellosis, chemotherapy, gentamicin, drug delivery systems

List of abbreviations used:

GEN: gentamicin

CDC: Centers for Disease Control

BSL: Biosafety Level

MIC: Minimum Inhibitory Concentration

THP-1: Human acute monocytic leukemia cell line

I/E: ratio of the intracellular concentration of antibiotic to the extracellular one

PLGA: poly (lactide-co-glycolide)

ABSTRACT

Brucellosis is a highly contagious bacterial zoonosis that affects millions of people worldwide. *Brucella* is highly infectious, especially when aerosolized. The infection induces severe protracted diseases, which are both debilitating and incapacitating, hence, *Brucella melitensis* has been considered a potential biological warfare agent. In the battle against *Brucella*, it is crucial to know its chemical-structure and biochemistry-metabolic characteristics. It is well known that *Brucella*, as well as many other intracellular bacterial pathogens, has evolved to survive and even proliferate within monocytes and macrophages cells. Depending on the route of entry (complement, Fc, lectin or fibronectin receptors), the fate of the bacteria will vary; it may even segregate from the endocytic route towards the endoplasmic reticulum. This intracellular “non
regular” behaviour of Brucella makes treatment difficult. Most antibiotics, although effective in vitro, do not actively pass through cellular membranes, or, once inside, may not reach the discrete intracellular niche where the bacteria is hidden. Therefore, complete eradication of the microorganisms is difficult to achieve, and the incidence of relapses is rather high. Taking these data into consideration, this review will evaluate the past, current and new trends in the control of brucellosis, paying special attention to the drug delivery systems as potential vectors for targeting these intracellular sites where the organisms are located.

INTRODUCTION

The parasitism of mononuclear phagocytes is an essential feature of diverse microorganisms [1]. For example, the recent recognition of Brucella spp. as facultative extracellular pathogens [2] emphasizes that these bacteria may have an extreme preference for the intracellular environment despite their ability to live outside host cells. This special feature may hamper the chemotherapeutic efficacy of many in vitro active antibiotics. In this review, we are going to consider Brucella and gentamicin (GEN) as the key actors of this special confrontation in order to learn lessons which would be applicable in other binomia pathogen-antibiotic.

Bacteria of the genus Brucella cause debilitating zoonotic infections in humans. Pathogenic brucellae are found worldwide. Traditionally, four major Brucella species are distinguished on the basis of their virulence for humans: B. melitensis, B. abortus, B. suis, and, rarely, B. canis. B. melitensis is the most infectious to man (infective dose, 1-10 (colony forming units) followed by B. suis (10,000), B. abortus (100,000), and, finally, B. canis (>1,000,000 in immuno-compromised people). Systemic infection can result in a chronic and debilitating febrile illness commonly known as Malta fever or
undulant fever [3]. People acquire this disease through direct contact with infected animals or their products. Humans may also be infected through exposure to the brucellae in the laboratory. What’s more, the CDC classifies *B. abortus*, *B. melitensis* and *B. suis* as "agents of mass destruction" BSL-3 organisms, due to their ability to infect humans through aerosol exposure.

The mechanisms underlying the way in which Brucella is able to enter and survive within the host cells, such as the macrophages, are not yet clear. Within macrophages, *Brucella* appears to be so well adapted from a physiological standpoint that it is able to resist the harsh environmental conditions encountered. In fact, the primary basis for the chronicity of *Brucella* infections lies in the capacity of the brucellae to persist for prolonged periods in the phagosomal compartment of these host phagocytes. As a consequence, the choice of antibiotic for brucellosis treatment is based on the drug’s facility to enter into these cells. Some data will be introduced in this review, regarding the understanding of the antibiotic chemotherapy struggle against intracellular bacteria.

Several antimicrobials, such as tetracycline, doxycycline, aminoglycosides, rifampicin, trimethoprim-sulfamethoxazole, quinolones, cephalosporins, chloramphenicol and macrolides, have been tested on laboratory animals and humans against brucellosis [4-12]. Monotherapies have produced unsatisfactory results. A prolonged administration of a tetracycline-aminoglycoside combination (i.e. doxycycline 100 mg twice/day for 45 days and streptomycin 1 g/day for 14 days or gentamicin 5-6 mg/Kg/d for 7 days) has proven to be the best treatment for the disease [10, 13]. However, with relapses of 3-5% [10], plus serious side effects due to the tetracyclines, especially in children and pregnant women [4-14], there is the need to press on in search of new alternatives.
Aminoglycosides are extremely active antimicrobial agents, particularly against bacteraemia caused by aerobic gram-negative bacilli [15]. Gentamicin, in particular, is an aminoglycoside with a wide spectrum of antibacterial activity and is more active \textit{in vitro} against clinical isolates of \textit{Brucella} than the more toxic streptomycin [3, 16, 17]. These properties make gentamicin an attractive antimicrobial candidate for the treatment of brucellosis. However, as a highly water-soluble drug, it penetrates cells poorly. This constitutes a particularly important drawback for the therapy of intracellular bacterial infections [18-20]. These major hurdles may be solved by the use of Drug Delivery Systems. These vectors have been proposed for delivering antimicrobial agents and targeting intracellular sites of infection, thereby helping to increase the therapeutic index of antimicrobials in intracellular niches [21-24]. At the end of this manuscript, we review the work on the development of new approaches to an effective pharmaceutical dosage form of gentamicin for the treatment of brucellosis. Liposomes and biodegradable microspheres loaded with antibiotics appear useful for targeting monocytes and reducing intracellular \textit{Brucella} infection; these delivery systems should represent a promising alternative approach for the treatment of intracellular bacterial infections.

**ENTRY AND FATE OF \textit{BRUCELLA} IN MACROPHAGES**

Bacteria and antibiotics must come into contact with each other, and this obvious presumption is critical in the treatment of intracellular bacteria. A better understanding of the basis for the successful survival and replication of \textit{Brucella} in the monocytic-macrophagic cells should provide us with critical information for improving chemotherapeutic treatments. Thanks to the genomic sequence data [25], the expected ability of \textit{Brucella} cells to express special strategies to adapt to the environmental
conditions encountered within these host cells has been confirmed. Thus, not only brucellae inside the macrophage are protected from the extracellular environment (antibodies, complement, antibiotics, etc.) but also the new physicochemical intracellular conditions induce strategical, structural and metabolic changes, making them resistant to, for instance, in vitro active antibiotics. In the same context of the confrontation, it should be stressed that the intracellular activity of antibiotics may also change. As a consequence, we should bear in mind that the final encounter between Brucella and the antibiotic was not expected after in vitro studies.

Human monocytes readily internalize Brucella using different phagocytosis-promoting receptors, forming so-called brucellomes (Brucella phagosomes) (Figs. 1 and 2). However, in contrast to other intracellular pathogens, Brucella cells do not avoid the typical steps of acidification and phagosome-lysosome fusion. On the contrary, the survival of internalized Brucella depends on an acidic intraphagosomal pH. After that, in this acidic compartment, poor in nutrients and microaerobic, it becomes partially dormant. The new expressed metabolism associated with this “stationary phase” induces a generalized resistance to a variety of intramacrophagic stresses, including exposure to acidic pH and reactive oxygen intermediates [26, 27]. The pleotropic consequence is that it may reduce their sensitivity to other chemical agents, such as antibiotics. Finally, the brucellae course themselves to a new intracellular compartment that is favourable for survival and replication: the endoplasmic reticulum [28-33].

EVENTS IN THE ENCOUNTER BETWEEN GENTAMICIN AND BRUCELLA

Considering the antibiotic in face of Brucella cells, the first target for GEN is the outer membrane. The large size of GEN (above 1 nm) precludes its penetration through the
outer membrane by porin channels. On the contrary, its cationic nature facilitates its passage by a self-promoted pathway involving the disruption of Mg$^{2+}$ bridges between adjacent lipopolysaccharide molecules [34-36] (Figs. 3 and 4). Subsequent transport across the inner membrane is an energy-dependent process, being inhibited or even blocked by divalent cations, hyperosmolarity, low pH, and anaerobiosis. Once in the cytoplasm, GEN binds irreversibly to the ribosomal 30S subunit, again through a potentially inhibitable energy-dependent process. This binding prevents the elongation of the polypeptide nascent chain by blocking the transfer of the peptidyl tRNA from the A-site to the P-site (Fig. 4) [37].

Aminoglycosides are bactericidal antibiotics that show a high activity against *Brucella in vitro* [38, 39]). However, the above-mentioned environmental factors related to the physical location of *Brucella*, may affect the bactericidal activity of the antibiotic. Lets recall that the pathogen transits through the endocytic pathway, evading its fusion with lysosomes, reaching, finally, its replicative niche, the endoplasmic reticulum [28, 31, 32]. This last compartment, where *Brucella* mostly resides, shows a neutral pH of 7.2; endosomes (the first niche for the pathogen after being uptaken) have pH 6; and in lysosomes where aminoglycosides accumulate, a pH of 5 is found [40].

On the other hand, studies in the intracellular traffic of GM, show that it transits principally to lysosomes, but 10% of the antibiotic transits through Golgi [41], then to endoplasmic reticulum and, finally, to cytoplasm, where it associates with mitochondrial membranes and the nucleus [42] (Fig. 2). Therefore, it is exposed to a range of pH between neutral (7.2 for cytoplasm and endoplasmic reticulum) and acid values (around 6 for endosomes and Golgi, 5.0 for lysosomes) [40]. Aminoglycosides are known to be lysosomotropic, because of their protonation state at acidic pH, but different aminoacid transporters have been described in lysosomal membranes [43, 44].
which could help antibiotic translocation to the cytoplasm and subsequently enter the pathogen’s compartment. These findings agree with Sandoval et al. [41, 45, 46] who proposed that the antibiotic would follow a retrograde traffic from Golgi to endoplasmic reticulum, and, after its release in cytoplasm, would bind to mitochondria and nucleus.

At low pH, these antibiotics increase their MIC due to changes in their degree of ionization. Thus, an acidification from pH 7.5 to 6.5 would increase GEN’s MIC 16-fold and at pH 5, 64-fold [47]. Under our experimental conditions, at neutral pH, bactericidal concentration of the GEN (0.5 μg/mL) eliminated all infective brucellae within 8 h. In contrast, at pH 5 and 6 it was unable to reduce bacterial counts. Lack of GEN activity is related to the protonation of the molecule at acidic pH [47] and since antibiotic enters the bacteria by active transport, factors affecting this mechanism (divalent cations, hiperosmolarity, anaerobiosis and acid pH) would reduce GEN antibacterial activity [37].

Similarly, azithromycin is not active against Brucella, neither on animal models nor in vitro conditions when mimicking the intracellular ones [8, 11, 48]. It is a well known fact that in alkali media, the macrolides are very active, but its activity is reduced by between 50 and 200 times when the test is performed under acidic conditions [8]. A series of determinations of azithromycin were made at different pH values and it could be seen that at pH ≤ 6.5 (close to the intraphagosomal pH) the MIC was >128 μg/ml. That is to say, Brucella in intracellular conditions would be resistant to azithromycin (>8 μg/ml) [48].

GENTAMICIN BIODISPONIBILITY AND DRUG DELIVERY SYSTEMS

The goal is to reach a sufficiently high concentration in the target organs to cope with the loss of activity caused by low pH. The intracellular concentration depends on
penetration, accumulation and disposition [20, 49]. However, as discussed above, GEN, being polar, does not pass efficiently across membranes, and therefore is taken up by endocytosis, which results in an exclusively lysosomal localization helped by its lysosomotropism. Lysosomotropic substances are weak organic bases that can pass through biological membranes and concentrate in the cell compartments of low pH values (i.e., lysosomes).

Aminoglycosides have shown such limited intracellular activity compared to their strong bactericidal potential in extracellular medium [50] that they have been used in vitro to eliminate extracellular bacteria without compromising intracellular survival [51, 52]. Nevertheless, some authors have observed that aminoglycosides are readily incorporated into phagocytes when these cells are in contact for prolonged periods [50]. Supporting these observations, our own experimental experience using THP-1 human monocytes, revealed that only a small amount of GEN is able to enter these cells and that ratio of the intracellular concentration of antibiotic to the extracellular one (I/E) was 0.001 at all concentrations studied (Lecároz et al, unpublished results). The linear increase in intracellular accumulation of the antibiotic with extracellular concentration suggested that the pinnocytosis proposed for GEN entrance [53] was not a saturated process, even at the high concentrations studied. Previous works performed in macrophages using a lower concentration of antibiotic (18 µg/mL), obtained higher I/E ratios, between 0.3-1.0 [50] compared to our ratio (0.001). However, different cell lines, incubation times and antibiotic concentrations were used. Besides, the washing of cell monolayers to remove extracellular antibiotic, potentially underestimates antibiotic cell uptake since most antibiotics reemerge from the cell very rapidly during the washing procedure [50]. Other studies performed in fibroblasts had shown that after the incubation of high antibiotic concentrations (500 and 100 µg/mL) for several days, an
accumulation of the drug occurs with I/E ratios of 6. Therefore, very long incubation periods required for GEN accumulation would mean the maintenance of high circulating GEN concentrations. Since free GEN is rapidly eliminated showing a plasma half-life of 2-3 h [54], sustained drug concentrations could not be achieved. Therefore, in order to get in vivo sustained bactericidal concentrations, the use of “drug delivery systems” has been suggested. Besides, these vectors may help to drive the antibiotic to Brucella’s niche in the target liver and spleen organs.

Liposome-encapsulated aminoglycosides offer possibilities for increasing the therapeutic index of this class of antibiotics. Liposomes with a membrane-like structure favour good cell interaction and their versatility in terms of structure and composition grant them their main advantages. Moreover, in our work, important therapeutic activity in experimental models of brucellosis [55, 56] have been exhibited. However, stability issues, both during storage and after injection, and reproducibility in terms of the production of a well-defined and consistent formulation, still need attention. Conversely, microspheres represent a more stable system and offer the advantage of providing controlled release of the encapsulated GEN which could minimize the need for multiple injections. The intracellular antibiotic efficiency of GEN-loaded microspheres in the context of Brucella-infected monocytes was first examined in vitro. Our results indicated that GM containing poly (lactide-co-glycolide) (PLGA) 50:50H microspheres, significantly reduced the number of intracellular bacteria [57]. However, the experimental conditions used were not the most appropriate to extrapolate the results to the human brucellosis condition, since murine monocytes and B. abortus were used for the infection studies. Besides, the method of preparation of the microspheres was based on the spray-drying technique that rendered a high degree of aggregation among particles [58]. Therefore, further experiments were performed with the more
virulent *B. melitensis* strain to *in vitro* infect THP-1 human monocytes. Furthermore, we developed a solvent-evaporation method to successfully encapsulate GEN in PLGA microparticles, avoiding hydrophobic aggregations [59]. Different co-polymers of PLGA were used, 502H and 75:25H being the most appropriate. The results demonstrated that PLGA microparticles were efficiently captured by the macrophages (Figs. 5 and 6), and that the GEN released from these particles was active, being able to exert its bactericidal effect inside the macrophagic cells.

By transmission electron microscopy and immunocitochemistry (gold-labelled antibodies against GEN) the lack of fusion between, both, microspheres and the pathogen’s niche (Fig. 6) was noted. However, the antibiotic released from the particles was observed in the cytoplasm and nucleus and also entered the vacuoles harbouring *Brucella*. Once inside the lysosomes, upon micro particle degradation [60], the antibiotic would be released to the cytoplasm and other intracellular compartments (see above) [61].

Regarding particle distribution *in vivo*, microparticles prepared using PLGA 502H or PLGA 75:25H were successfully delivered to the liver and spleen. Furthermore, microparticles of 502H and 75:25H PLGA released their content in a sustained manner. Pharmacokinetics parameters illustrated the markedly altered distribution of PLGA - loaded GEN compared to the free drug, observing higher concentrations of GEN in the spleen and liver when it was administered loaded in microspheres. At the same time, undetectable concentrations were obtained in the serum samples, precluding drug accumulation in the kidneys. Distribution studies showed that after two weeks, only 75:25H intact microspheres were observed in the spleen, and, in discrete quantities, in the liver. However, GEN was detected up to four weeks later in the liver and spleen after a single dose of the microsphere formulations. This long persistence is probably
due to the nature of the aminoglycoside. These drugs are highly stable and are not metabolized in the liver. Because of their polar nature, they penetrate cells very poorly, but, once inside, their intracellular retention in very high [24].

Concentrations of GEN in the liver and spleen were in the range of the in vitro MBC for B. melitensis (Lecároz et al., unpublished results). When BALB/c mice were chronically infected with the virulent strain B. melitensis and treated with the selected GEN containing formulations, both significantly reduced the splenic infection. Results also indicated that the treatment with free GEN was ineffective, in agreement with the undetectable levels of GEN found in the liver and spleen.

The data reviewed here support the use of drug delivery systems as an alternative therapeutic approach for the treatment of Brucella infections.

CONCLUDING REMARKS

Treatment of intracellular bacterial infection remains both a medical and economic challenge. Brucellae, which thrive in macrophages, are more protected against many antibiotics. This explains why Brucella cells are not only harmful for the host but may also constitute a reservoir for recurrence and reinfection.

Gaining a better understanding of the basis for the successful survival and replication of the brucellae in the host cell should provide us with critical information that can be used for designing and improving better chemotherapeutic strategies against brucellosis.

Because of their strong antibacterial properties, aminoglycosides remain useful for the treatment of serious infections, but drug monitoring has to be strict to preserve antibacterial activity while avoiding toxicity as far as possible. A drug delivery system that helps to increase the therapeutic index of the aminoglycosides by increasing the concentration of the drug at the site of infection and/or reducing the nephro- and
ototoxicity would attract considerable interest. Liposomal and microparticle encapsulation of aminoglycosides provide relatively high GM entrapment efficiencies and efficient interaction with monocyte-macrophages; key achievements compared with current therapies against *Brucella*.

Finally, in the battle against bacteria, it is vital to know your enemy. As a consequence, a key point that should be stressed from its intracellular habitat, is that *Brucella* is highly variable when comparing it with its structure in extracellular (*in vitro*) environments. Therefore, when testing new drugs, it is always necessary to study its in vitro behaviour mimicking *in vivo* conditions. All these data, together with pharmacokinetics and pharmacodynamics (in animal and in cell) of the administered form of GM are of great significance for effective treatment of intracellular *Brucella* infections.

**ACKNOWLEDGMENTS**

We would like to express special gratitude to Blanca Goñi and Marian Burrell for technical assistance in the electron microscopy studies. The work was supported by grants from PIUNA (Fundación Universidad de Navarra) and the Program “Redes Temáticas de Investigación Cooperativa del FIS – Brucellosis (Ref. No. G03/201)”.

**REFERENCES**


