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Pathogenesis and pathobiology of zoonotic brucellosis in humans

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Summary
Although human brucellosis has protean clinical manifestations, affected tissues usually exhibit signs of inflammation. The cellular and molecular bases of some immunopathological phenomena probably involved in the pathogenesis of infection with brucellae have been elucidated recently. Human osteoblasts and fibroblast-like synoviocytes produce cytokines, chemokines and matrix metalloproteinases in response to infection with brucellae and/or to stimulation by brucellae-infected monocytes. In turn, released cytokines promote the secretion of the metalloproteinases and induce osteoclastogenesis. These phenomena may underlie the bone loss and cartilage degradation found in brucellar arthritis and osteomyelitis. *Brucella abortus* and its lipoproteins elicit an inflammatory response in the central nervous system of mice, leading to astrogliosis, a characteristic feature of neurobrucellosis. Brucellae can also replicate in human endothelial cells, inducing an inflammatory response with increased expression of chemokines, interleukin-6 and adhesion molecules. Persistent brucellar infection of the endothelium would support development of endocarditis and other vascular manifestations. Thus, although the inflammatory phenomena triggered by brucellae are relatively mild, they are long-lasting as a result of the prolonged intracellular persistence of the bacteria in infected tissues and eventually lead to tissue damage.

Keywords
Astrogliosis – Bacterial lipoprotein – Bone loss – Cartilage degradation – Intracellular persistence – Matrix metalloproteinase – Osteoclastogenesis – Pro-inflammatory cytokine – TLR2 signalling.

Clinical picture of human brucellosis

Species of *Brucella* infect both domestic and wild animals and are usually transmitted to humans through the consumption of contaminated unpasteurised dairy products, direct contact with infected animal tissues or inhalation of infected aerosols. Following the initial infection, brucellae disseminate throughout the body and establish a survival-and-replication niche in cells of the reticuloendothelial system, producing a systemic infection or preferentially affecting a given organ (localised disease) (42). The most common signs and symptoms of human brucellosis are fever, asthenia, myalgia, arthralgia, sweats, lymphadenopathy, hepatomegaly and splenomegaly. Osteoarticular manifestations (peripheral arthritis, sacroiliitis, spondylitis) are the most common forms of localised disease (29). The prevalence of neurobrucellosis ranges from 5% to 7% in areas of *B. melitensis* endemicity (10, 63); the reported frequency of liver involvement (granulomas, inflammatory infiltrations, parenchymal necroses) ranges from 5% to 52% (6). Less frequently, brucellosis may be accompanied by severe vascular complications such as infective endocarditis and mycotic aneurisms. A common characteristic of localised brucellosis is the inflammatory nature of the lesions.

Brucellae as inducers of inflammation

At variance with other pathogens, brucellae do not exhibit classic pathogenic factors that can directly harm eukaryotic cells, such as exotoxins, exoproteases, cytolysins or other exoenzymes (37). Tissue damage may therefore result from indirect mechanisms, probably through the activation of host immune responses after recognition of brucellar antigens by immunity receptors such as Toll-like receptors (TLR) or nucleotide oligomerisation domains. It has been shown that TLR2, TLR4 and TLR9 are involved in recognition of brucellae by professional phagocytes (41). The non-canonical structure of brucellar lipid A means that the biological activities induced by brucellar lipopolysaccharide differ from those of classic enterobacterial lipopolysaccharide, including a much lower potency (three orders of magnitude) as inducer of pro-inflammatory mediators (9, 21, 22, 36, 43).

The genomes of *Brucella* species contain several genes encoding outer membrane proteins (OMP) (56, 57), some of which (OMP10, OMP16, OMP19) are lipoproteins and are surface exposed (55). Lipoproteins, but not lipopolysaccharide, have been identified as the main brucellar antigens that induce pro-inflammatory cytokine release by human monocytes, a phenomenon that requires recognition through TLR2 (21). In spite of the protean nature of brucellosis, inflammation is a hallmark of the disease and the affected tissues usually exhibit inflammatory infiltrates (27, 49). Lesions in domestic animals also show infiltrates of different cell types, including neutrophils, monocytes/macrophages and lymphocytes, and different degrees of necrosis and vasculitis (3). Similar findings have been reported in the rhesus macaque model of airborne brucellosis (25, 62) and in the mouse model (52). Although the inflammation appears milder than in infections caused by other bacteria, due in part to molecular mechanisms exhibited by brucellae (46, 50), the long-term presence of viable bacteria in infected tissues could stimulate persistent low-level inflammation leading to tissue damage.

Inflammation-driven mechanisms of tissue damage

Osteoarticular brucellosis

As mentioned above, osteoarticular brucellosis is the most common localisation of active brucellosis. A series of studies have begun to elucidate the cellular and molecular pathogenic mechanisms of joint and bone damage caused by brucellae. In osteomyelitis and septic arthritis caused by other bacteria, inflammatory cells are known to play a significant role in bone and synovial damage (24, 51). The finding of a non-specific inflammatory infiltrate in the synovial membranes and bones of patients with brucellar arthritis and osteomyelitis (29) suggests that inflammation may also be involved in the osteoarticular damage caused by brucellae.

Recruitment of inflammatory cells to osteoarticular tissues is of utmost importance in development of damage to bone and cartilage, and osteoblasts may play a central role in this process, as they respond to bacterial infection and bacterial antigens by secreting pro-inflammatory cytokines and chemokines (30). Notably, the invasion and replication of different species of *Brucella* has been demonstrated in human osteoblast cell lines (17, 48). The osteoblasts respond to the infection with a limited pro-inflammatory response (interleukin [IL]-8, monocyte chemotactic protein [MCP]-1) but exhibit a significant increase of MCP-1, IL-8 and IL-6 secretion following stimulation with culture supernatants from *Brucella*-infected human monocytes (THP-1 cell line). Conversely, granulocyte-macrophage colony-stimulating factor (GM-CSF) released by *Brucella*-infected osteoblasts stimulates THP-1 cells to produce IL-8, IL-1β, IL-6 and tumour necrosis factor (TNF)-α. These findings suggest that osteoblasts and macrophages present at the site of osteoarticular brucellar infection can mutually amplify their inflammatory responses to the pathogen through secreted soluble factors.

The tissue damage in septic arthritis and osteomyelitis involves the action of matrix metalloproteinases (MMP). Following infection, increased production of pro-inflammatory cytokines may lead to increased levels of MMP activity in osteoarticular tissue, causing damage; MMP-2 and MMP-9 are particularly important in osteoarticular diseases since they can degrade a variety of collagens. Notably, increased levels of MMP-9 have been found in synovial fluid in a human case of prepatellar bursitis caused by *Brucella* spp. (59).

Osteoblasts can secrete several MMP, including MMP-2, which degrades type I collagen in bone and type II collagen in cartilage (11). Significant release of MMP-2 is triggered in *B. abortus* infection of human osteoblasts, mediated in part by the autocrine action of GM-CSF (48). Matrix metalloproteinases can also be produced by professional phagocytes. In THP-1 cells, infection with *B. abortus* or stimulation with heat-killed *B. abortus* (HKBA) or an outer membrane lipoprotein of the bacterium (lipidated OMP19 [L-OMP19]) induces a high level of MMP-9 secretion. These effects are mediated by TLR2 recognition and by the autocrine action of TNF-α produced by these cells. The latter cytokine also induces MMP-2 secretion in uninfected osteoblasts. In addition, GM-CSF produced by infected osteoblasts induces the production of TNF-α by monocytes, which,

as mentioned above, induces these cells to secrete MMP-9. Thus, both osteoblasts and monocytes produce MMP in response to infection with brucellae and such responses can be amplified by a network of reciprocal stimulations.

Fibroblast-like synoviocytes (FLS) have been increasingly recognised as a key source of MMP in inflammatory arthritides. In particular, TNF-α and IL-1β are key inducers of MMP production by FLS. In addition, FLS produce some cytokines and chemokines in response to inflammatory or infectious stimuli (33). *Brucella abortus* has been shown to infect and replicate in human FLS *in vitro* (SW982 cell line), triggering the production of MMP-2 and pro-inflammatory mediators (IL-6, IL-8, MCP-1, GM-CSF) (47). Moreover, culture supernatants from *Brucella*-infected FLS induce the *in vitro* migration of monocytes and neutrophils. In addition, GM-CSF and IL-6 produced by infected FLS induce the secretion of MMP-9 by monocytes and neutrophils respectively. Conversely, TNF-α released by *Brucella*-infected monocytes and neutrophils induces FLS to produce MMP-2. The secretion of pro-inflammatory mediators and MMP-2 by FLS is also induced by HKBA and L-OMP19 via TLR2 recognition. Moreover, the injection of HKBA or L-OMP19 into the knee joint of mice results in local induction of pro-inflammatory mediators MMP-2 and MMP-9 and in generation of a mixed inflammatory infiltrate. Overall, these findings suggest that joint damage in brucellar arthritis can result, at least in part, from the action of MMP and pro-inflammatory mediators released by *Brucella*-infected FLS and recruited phagocytes.

One of the most serious complications of brucellar osteomyelitis is the presence of localised osteolysis leading to bone loss (7, 14, 29). Under normal physiological conditions, bone resorption is mediated by osteoclasts, which can be identified by their multinucleate phenotype and expression of tartrate-resistant alkaline phosphatase and other markers. The generation of osteoclasts from precursors of the monocyte/macrophage lineage present in bone marrow can be induced by pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (54), which contribute to bone loss in chronic inflammatory bone diseases (24, 26, 32, 40, 61). Macrophages in inflamed tissues can contribute to bone resorption through their differentiation to osteoclasts (2) and through the production of pro-inflammatory cytokines (13).

The TLR2- and myeloid differentiation (MyD) 88-mediated production of TNF-α by murine macrophages in response to *B. abortus* antigens stimulates bone marrow-derived monocytes to undergo osteoclastogenesis (16). Furthermore, the response can be induced by L-OMP19, used as a model lipoprotein. This observation has been corroborated *in vivo*, since injection of HKBA or L-OMP19 into the tibiae of mice induced the formation of tartrate-resistant alkaline phosphatase-positive multinucleated osteoclasts, whereas this did not occur in TLR2-deficient mice (16). The finding was confirmed using human monocytes, indicating that this mechanism could also play a role in human brucellosis. Overall, these findings, which are summarised in Figure 1, shed light on how the interactions of *B. abortus* with cells involved in bone metabolism, and their interaction with cells of the innate immune system, play a role in the pathogenesis of osteoarticular brucellosis.

Neurobrucellosis

Invasion of the central nervous system by brucellae results in the inflammatory disorder neurobrucellosis, which is perhaps the most morbid form of the disease (23, 42). Neurobrucellosis may manifest as meningoencephalitis (the most common form), meningovascular disease, brain abscesses, demyelinating syndromes or myelitis and can present with or without systemic symptoms. Neurobrucellosis can associate with other focal forms of brucellosis (10, 58). Encephalitis and myelitis are directly related to the presence of the bacterium in cerebral tissue and spinal cord (28). Other pathological manifestations of neurobrucellosis, such as polyradiculoneuritis, anterior poliomyelitis or Guillain-Barré syndrome, have also been described (15, 53). The relatively few microscopic descriptions of central nervous system pathology in brucellosis patients consistently report a diffuse involvement of the white matter, together with astrogliosis and reactive microgliosis (27, 49). Direct action of the bacterium together with the effect of pro-inflammatory cytokines and a demyelinating immunopathological process have been proposed as mechanisms involved in damage to the central nervous system during neurobrucellosis (20). However, as no secreted proteases, toxins or lytic enzymes have yet been described in *Brucella* spp., a direct deleterious effect seems unlikely.

Brucella abortus and its lipoproteins activate the innate immunity of the central nervous system, both *in vivo* and *in vitro*, eliciting an inflammatory response that leads to astrogliosis, a characteristic feature of neurobrucellosis (19). Injection of HKBA or L-OMP19 into mice striatum induces astrogliosis accompanied by a neutrophilic infiltrate. *In vitro* infection of astrocytes and microglia with *B. abortus,* or stimulation of these cells with HKBA or L-OMP19, induces secretion of IL-6, IL-1b, TNF-α, MCP-1 and a keratinocyte-derived chemokine (KC, chemoattractant for neutrophils). Infection with *B. abortus* also induces apoptosis in astrocytes but not in microglia. Heat-killed *B. abortus* and L-OMP19 elicit astrocyte apoptosis and proliferation, two features observed during astrogliosis. Astrocyte apoptosis depends on TNF-α signalling, since it is completely suppressed in cells of mice lacking the p55 subunit of the TNF-α receptor. Taken together, these findings suggest that lipoproteins of brucellae may contribute to the pathogenesis of neurobrucellosis by eliciting an inflammatory response

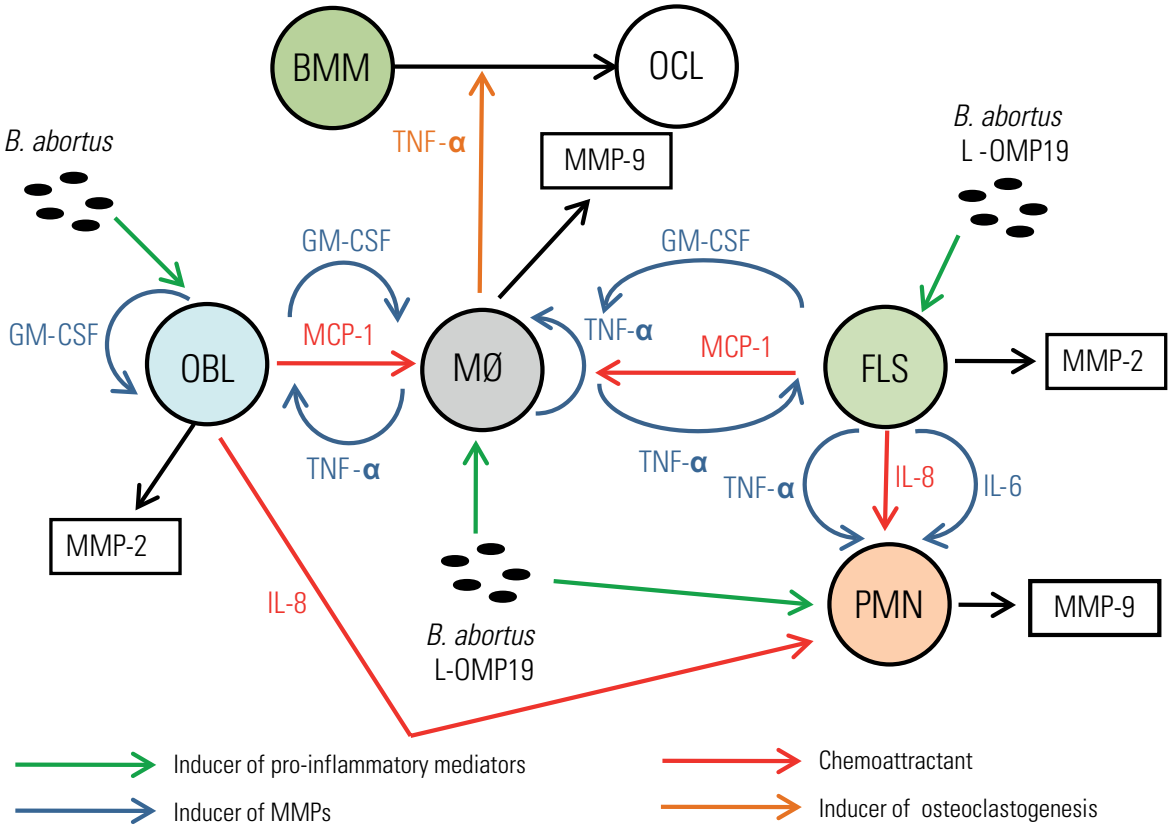

Fig. 1

Potential mechanisms of *Brucella*-induced osteoarticular damage

Following infection with *B. abortus* or stimulation with brucellar lipoproteins (L-OMP19), osteoblasts (OBL) and fibroblast-like synoviocytes (FLS) produce matrix metalloproteinase-2 (MMP-2), pro-inflammatory cytokines and chemokines, as well as granulocyte-macrophage colony-stimulating factor (GM-CSF). Chemokines attract macrophages and/or neutrophils, which respond to the presence of brucellae or to factors produced by infected OBL or FLS with the secretion of MMP-9 and TNF-α. The latter stimulates secretion of MMP-2 by OBL or FLS and promotes generation of osteoclasts (OCL) from their precursors (bone marrow-derived monocytes, BMM)

that leads to astrogliosis. In addition, TNF-α produced by astrocytes and/or microglia in response to infection with brucellae may induce glial and neuronal apoptosis, probably explaining the neurological deficits observed in neurobrucellosis (20, 31, 44).

As mentioned above, some patients with neurobrucellosis may present Guillain-Barré syndrome. It has been postulated that the association between bacterial infections and this syndrome results from molecular mimicry between the outer core structures of bacterial lipo-oligosaccharides and human gangliosides (34, 64). A lipo-oligosaccharide with a GM1 ganglioside-like structure is localised on the surface of *B. melitensis* (not *B. abortus*) (60), and hyperimmunisation with formalin-killed *B. melitensis* causes Guillain-Barré syndrome-like symptoms in BALB/c mice, suggesting a causal association.

Vascular manifestations

Brucellosis patients present perivascular leukocytic infiltrates in all the affected organs (5), and some present vasculitis in different localisations (1, 39) that also exhibit infiltrates of neutrophils, monocytes and lymphocytes. Endothelial cells may have a central role in these phenomena, as it is well established that they express adhesion molecules and chemokines that mediate extravasation of circulating leukocytes to perivascular tissues. Endothelial cells may also be involved, both as target cells and as inflammation players, in severe vascular complications of brucellosis such as infective endocarditis and mycotic aneurysms. Brucellae have been isolated from the aortic and/or mitral valves of patients with endocarditis (45) and from resected brucellar mycotic aneurysms (4).

Endothelial cells cover the surface of cardiac valves and are the target of direct invasion by bacteria during infectious endocarditis; these cells also contribute to disease through secretion of chemokines and up-regulation of adhesion molecules (35, 38). It has been shown that *B. abortus* and *B. suis* can infect and replicate in primary human umbilical-vein endothelial cells and in the human microvascular endothelial cell line HMEC-1 (18). Both cell types respond to infection with brucellae by increased production of

chemokines (IL-8 and MCP-1) and IL-6 and increased expression of the adhesion molecule CD54 (ICAM-1). *Brucella*-infected umbilical vein endothelial cells also exhibit up-regulation of the adhesion molecules CD106 (VCAM-1) and E-selectin (CD62E). These pro-inflammatory effects on human endothelial cells can also be induced by L-OMP19 but not by the unlipidated version of the protein. The up-regulation of adhesion molecules and chemokines in response to brucellae results in increased migration of leukocytes. It has been shown that transmigration of neutrophils through *Brucella*-infected monolayers of HMEC-1 cells increased by 5-fold to 8-fold in comparison with migration through uninfected endothelial cells. Similarly, stimulation of endothelial cells with live *B. abortus* from the basolateral side increased the transmigration of neutrophils by up to 20-fold (18).

Overall, these studies suggest that interaction of brucellae with endothelium may enhance transmigration of phagocytes to infected tissues, possibly explaining the presence of perivascular leukocytic infiltrates in the affected organs of patients with brucellosis. However, since brucellae can survive intracellularly, persistent infection may also result in a long-lasting pro-inflammatory response. In infection with other pathogens, the pro-inflammatory response of infected endothelium has a central role in the development of endocarditis lesions (8, 12). Thus, the ability of brucellae to survive in endothelial cells and activate an endothelial pro-inflammatory response might be a factor in the pathogenesis of the vascular manifestations of brucellosis.

Pathogénie et pathobiologie de la brucellose zoonotique chez l'homme

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Résumé

La brucellose chez l'homme peut se manifester par une grande diversité de signes cliniques, l'inflammation des tissus atteints étant l'un des plus fréquents. Les fondements cellulaires et moléculaires de certains phénomènes immunopathologiques jouant un rôle probable dans la pathogénie de l'infection brucellique ont été élucidés il y a peu. Les ostéoblastes et les synoviocytes humains de type fibroblastique produisent des cytokines, des chimiokines et des métalloprotéinases matricielles en réponse à une infection brucellique et /ou à une stimulation par les monocytes infectés par *Brucella*. À leur tour, les cytokines libérées favorisent la sécrétion de métalloprotéinases et induisent le développement d'ostéoclastes. Ces phénomènes peuvent expliquer la perte osseuse et la dégradation du cartilage associées à l'arthrite et l'ostéomyélite brucelliennes. *Brucella abortus* et ses lipoprotéines suscitent une réponse inflammatoire au sein du système nerveux central de la souris, évoluant en une astrogliose, trait caractéristique de la neurobrucellose. Les *Brucella* peuvent également se multiplier dans les cellules endothéliales humaines, induisant une réaction inflammatoire avec un accroissement de l'expression des chimiokines, de l'interleukine-6 et des molécules d'adhésion. Une infection brucellique persistante de l'endothélium favoriserait l'apparition d'endocardite et d'autres manifestations vasculaires. Ainsi, bien que les phénomènes inflammatoires associés à l'infection à *Brucella* soient peu sévères, ils sont durables en raison de la persistance de la bactérie dans les cellules des tissus infectés et ils risquent d'entraîner à terme une dégradation des tissus.

Mots-clés

Astrogliose – Cytokine pro-inflammatoire – Dégradation du cartilage – Développement d'ostéoclastes – Lipoprotéine bactérienne – Métalloprotéinase matricielle – Persistance intracellulaire – Perte osseuse – Signaux des récepteurs TLR2.

Patogénesis y patobiología de la brucelosis zoonótica en el ser humano

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Resumen

Aunque la brucelosis humana tiene manifestaciones clínicas variables, los tejidos afectados suelen mostrar signos de inflamación. En fechas recientes se han dilucidado las bases celulares y moleculares de ciertos fenómenos inmunopatológicos que probablemente intervienen en la patogénesis de la infección por brucelas. Los osteoblastos y sinoviocitos tipo fibroblasto del ser humano responden a la infección por brucelas y/o a la estimulación por monocitos infectados por brucelas sintetizando citoquinas, quimioquinas y metaloproteinasas de la matriz. Las citoquinas liberadas, a su vez, promueven la secreción de las metaloproteinasas e inducen la osteoclastogénesis. Es posible que estos fenómenos subyazcan a la pérdida de hueso y la degradación del cartílago observadas en las artritis y osteomielitis causadas por brucelas. *Brucella abortus* y sus lipoproteínas provocan una respuesta inflamatoria en el sistema nervioso central del ratón que conduce a la astrogliosis, rasgo característico de la neurobrucelosis. Las brucelas también pueden replicarse en las células endoteliales humanas, induciendo una respuesta inflamatoria que conlleva una mayor expresión de quimioquinas, interleuquina-6 y moléculas de adhesión. La infección persistente del endotelio por brucelas favorecería la aparición de endocarditis y otras manifestaciones vasculares. Por ello, aunque relativamente leves, los fenómenos inflamatorios inducidos por brucelas son duraderos, porque las bacterias persisten dentro de las células de los tejidos infectados hasta acabar dañándolos.

Palabras clave

Astrogliosis – Citoquina proinflamatoria – Degradación de cartílago – Lipoproteína bacteriana – Metaloproteinasa de la matriz – Osteoclastogénesis – Pérdida de hueso – Persistencia intracelular – Señales TLR2.

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