

MOLECULAR AND CELLULAR BASIS OF *BARTONELLA* PATHOGENESIS

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■ **Abstract** The genus *Bartonella* comprises several important human pathogens that cause a wide range of clinical manifestations: cat-scratch disease, trench fever, Carrión's disease, bacteremia with fever, bacillary angiomatosis and peliosis, endocarditis, and neuroretinitis. Common features of bartonellae include transmission by blood-sucking arthropods and the specific interaction with endothelial cells and erythrocytes of their mammalian hosts. For each *Bartonella* species, the invasion and persistent intracellular colonization of erythrocytes are limited to a specific human or animal reservoir host. In contrast, endothelial cells are target host cells in probably all mammals, including humans. Bartonellae subvert multiple cellular functions of human endothelial cells, resulting in cell invasion, proinflammatory activation, suppression of apoptosis, and stimulation of proliferation, which may cumulate in vasoproliferative tumor growth. This review summarizes our understanding of *Bartonella*–host cell interactions and the molecular mechanisms of bacterial virulence and persistence. In addition, current controversies and unanswered questions in this area are highlighted.

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INTRODUCTION

Bartonella bacilliformis has been known as a human pathogen for nearly one century (3), and it was the only species of the genus *Bartonella* until the beginning of the 1990s. Since then, the reclassification of previously described genera based on 16S rRNA sequences (i.e., *Grahamella* and *Rochalimaea*) (11, 13, 45, 95, 107, 121, 123) and the description of novel species isolated from various animal reservoirs (9, 10, 36, 44, 54, 55, 68–70, 79, 124) resulted in a major expansion of the genus *Bartonella* to currently 20 species, among which 8 have been associated with human disease (3, 31, 63, 68, 70, 91, 96, 121, 124) (Table 1). The human pathogenic species are extraordinarily adept at establishing long-term infections that can either manifest as acute or chronic disease, or be clinically asymptomatic with the potential to resurface later. Understanding the cellular interactions and factors that contribute to this long and complex relationship between pathogen and host is essential to our ability to modulate its clinical outcomes. In this review I discuss diseases caused by *Bartonella* species in the context of their host range, pathology, pathogen–host cell interaction, and pathogenesis. I highlight commonalities and differences that aid in the understanding of their pathogenic mechanisms, describe recent studies of *Bartonella* virulence factors that illuminate mechanisms of pathogenesis, and concentrate on recent advances and current outstanding issues.

NATURAL HISTORY AND EPIDEMIOLOGY

Bartonellae are small pleomorphic gram-negative bacilli that belong to the α -2 subdivision of *Proteobacteria*. These arthropod-borne pathogens cause a persistent, often-asymptomatic intraerythrocytic bacteremia in their mammalian reservoir hosts. As our knowledge progresses, we may learn that the chronic infection of erythrocytes and endothelial cells as major target cell types actually predisposes the host to mild, insidious manifestations or induces, in selected instances, severe diseases (27, 34, 39). A wide range of mammalian hosts, including domestic and wild animals, serves as reservoir hosts for the 20 described *Bartonella* species (Table 1). Depending on the ecology of the blood-sucking arthropod vector, intraerythrocytic bacteremia can be highly prevalent in the reservoir population, e.g., >80% of the individuals are infected in wild ruminant populations (9, 23, 36,

TABLE 1 *Bartonella* species, their natural reservoir and vector, and the resulting human diseases

Bartonella species	Reservoir	Vector	Human disease(s)
Human-specific species			
<i>B. bacilliformis</i>	Human	Sandfly	Carrión's disease: Oroya fever and verruga peruana
<i>B. quintana</i>	Human	Body louse	Trench fever, endocarditis, bacillary angiomatosis
Zoonotic species			
<i>B. clarridgeiae</i>	Cat	Cat flea	Cat-scratch disease
<i>B. elizabethae</i>	Rat	?	Endocarditis, neuroretinitis
<i>B. grahamii</i>	Mouse, vole	?	Neuroretinitis
<i>B. henselae</i>	Cat	Cat flea	Cat-scratch disease, endocarditis, bacillary angiomatosis, bacillary peliosis, neuroretinitis, bacteremia with fever
<i>B. vinsonii</i> subsp. <i>arupensis</i>	Mouse	Ticks	Bacteremia with fever
<i>B. washoensis</i>	Ground squirrels	?	Myocarditis
Animal-specific species			
<i>B. alsatica</i>	Rabbit	?	?
<i>B. birtlesii</i>	Mouse	?	?
<i>B. bovis</i> (= <i>B. weissii</i>)	Cattle/cat	?	?
<i>B. capreoli</i>	Roe deer	?	?
<i>B. chomelii</i>	Cattle	?	?
<i>B. doshiae</i>	Vole	?	?
<i>B. koehlerae</i>	Cat	?	?
<i>B. peromysci</i>	Deer, mouse	?	?
<i>B. schoenbuchensis</i>	Roe deer	Deer ked	?
<i>B. talpae</i>	Mole	?	?
<i>B. taylorii</i>	Mouse, vole	?	?
<i>B. tribocorum</i>	Rat	?	?
<i>B. vinsonii</i> subsp. <i>berkhoffii</i>	Dog	Ticks	?
<i>B. vinsonii</i> subsp. <i>vinsonii</i>	Vole	Vole ear mite	?

40). Humans serve as the reservoir host for *B. bacilliformis* and *B. quintana*, with clinical manifestations resulting from both intraerythrocytic bacteremia as well as endothelial cell association (27, 34, 39). Other *Bartonella* species, for which humans are not the natural reservoir, tend to cause persistent bacteremia only in immunodeficient individuals, and in this case without evidence for intraerythrocytic localization, as typically observed in the reservoir host. For these zoonotic infections, the severity of the clinical presentation correlates well with the status of the host's immune system (34, 39, 97) and often reflects a prominent endothelial cell association, i.e., by the formation of vasoproliferative lesions (35).

Bartonella bacilliformis

B. bacilliformis was described by the Peruvian physician Alberto Barton in 1909. He observed the presence of intraerythrocytic bacilli in blood smears of patients suffering from Carrión's disease (3). This biphasic disease consists of Oroya fever and verruga peruana and is transmitted by the sandfly *Lutzomyia verrucarum*. Carrión's disease occurs endemically in the valleys of the South American Andes of Peru, Columbia, and Ecuador (4). Oroya fever is a highly fatal febrile anemia resulting from the massive invasion of erythrocytes. Patients surviving this acute intraerythrocytic phase may develop the verruga peruana as the chronic secondary tissue phase: Bacteria colonize the vascular endothelium and cause vasoproliferative eruptions of the skin, which develop within 1 to 2 months and persist for months to years. Since pre-Incan times, *B. bacilliformis* has caused debilitating illness, e.g., from 1869 to 1873, when 8,000 of 10,000 railroad workers operating in an endemic area died from Oroya fever (4). However, aside from causing disease, infection may also result in a chronic, asymptomatic bacteremia (22, 27). Bacterial or host factors that determine whether infection results in clinical disease or the asymptomatic carrier state are unknown.

Bartonella quintana

B. quintana was a leading cause of infectious morbidity among Allied troops during World War I (4). Trench fever caused by *B. quintana* is characterized by an intraerythrocytic bacteremia (100) with recurrent, five-day cycling fever, headache, and leg pain (4). The disease is transmitted among humans by the human body louse, *Pediculus humanus* (4). Although almost forgotten by medical science since the end of World War II, *B. quintana* re-emerged toward the end of the twentieth century as an agent of disease among the homeless, drug addicts, and other disfavored social groups who are at least occasionally infested by body lice. However, many of the infected individuals do not suffer from disease but develop a chronic asymptomatic, bacteremic carrier state (94). The contemporary disease manifestations include "urban" trench fever and several new disease manifestations such as endocarditis and, specifically in HIV patients, a vasoproliferative disorder called bacillary angiomatosis (BA). Typical manifestations of BA are vasoproliferative skin lesions resembling pyogenic granulomas, hemangiomas, or Kaposi's sarcoma (4), with bacteria found in close association with proliferating endothelial cells (80). The vasoproliferative infection is usually progressive and fatal unless treated by antibiotic therapy (4).

Bartonella henselae

B. henselae is a zoonotic pathogen distributed worldwide. It causes intraerythrocytic bacteremia in cats, which are the natural reservoir hosts (67, 101). The rate of bacteremia (7–43%) and the seroprevalence to the *B. henselae* antigen (4–81%) is higher in cats living in warm, humid geographic regions (51). Cat-to-cat

transmission occurs mainly by the cat flea, *Ctenocephalides felis* (26). In correlation with the infestation with cat fleas, feral cats are more likely to be infected than domestic cats from the same region (25). Although some of these animals may be bacteremic over a period of more than one year, cats are typically asymptomatic (65). On the basis of serological results, however, investigators have determined that naturally infected cats are more likely to suffer from lymphadenitis, gingivitis, and stomatitis, and are predisposed to urological diseases (27).

B. henselae transmission from cat to humans occurs indirectly by the cat flea or directly by cat scratch or bite (24). This pathogen can cause a variety of clinical manifestations in humans. Cat-scratch disease (CSD) was first described in 1950 by the French physician Debré in patients suffering from suppurative lymphadenitis following cat scratches (33). CSD occurs worldwide in all age groups. In the United States, *B. henselae* causes 22,000 diagnosed cases of CSD per year, requiring hospitalization of the infected patient in about 10% of cases. Most cases occur in late fall and winter, when a closer and prolonged contact to indoor cats occurs. Regional lymphadenopathy is the most common clinical manifestation of CSD, but atypical manifestations with involvement of various organs such as the eyes (i.e., neuroretinitis), liver, spleen, central nervous system, skin, and bones may occur. In these immunocompetent hosts, the pathologic response is granulomatous, suppurative, extracellular and intracellular, and generally self-limited (5).

Similar to *B. quintana*, *B. henselae* is a major cause of endocarditis. Homeless and chronically alcoholic individuals are particularly susceptible to the disease. It is estimated that *Bartonella* species account for 3 to 4% of all endocarditis cases (27, 74).

BA is the most common clinical manifestation in immunocompromised, especially HIV-positive, patients of infection with *B. henselae*. The clinical and the histopathological picture of this cutaneous vasoproliferative disease is indistinguishable from BA caused by *B. quintana* (80). However, *B. henselae* can also cause a related vasoproliferative disease in inner organs. This bacillary peliosis (BP) is characterized by vascular proliferation in liver and spleen, which results in the formation of blood-filled cysts (113, 115). Similar to BA, BP is found in association with proliferating endothelial cells (80). Other disease manifestations in the immunocompromised are persistent or relapsing bacteremia with fever (5).

Other Human-Pathogenic Bartonellae

B. bacilliformis, *B. quintana*, and *B. henselae* are responsible for most human infections with bartonellae. However, in recent years at least five additional zoonotic species have been associated with human disease. *B. elizabethae*, which naturally infects rats (45), was isolated from a patient with endocarditis (31). Similar to *B. henselae*, *B. clarridgeiae* colonizes cats as the reservoir host and may also cause CSD in humans (68, 102). *B. grahamii* naturally infects rats and other rodents (45, 66) and may cause neuroretinitis in humans (63). *B. vinsonii* spp. *arupensis* infecting mice as the reservoir host was isolated from a bacteremic patient

with fever (124). Finally, *B. washoensis*, which typically infects ground squirrels, causes human myocarditis (70).

Our increasing appreciation of the widespread distribution of *Bartonella* species in mammalian hosts will probably lead to the description of novel *Bartonella* species. Likewise, the improvement of diagnostic methods for novel and known species should aid in associating *Bartonella* infection with human disease and may thereby help to recognize the pathogenic potential of these enigmatic pathogens.

BARTONELLA–HOST CELL INTERACTIONS

Erythrocyte Interaction

The common theme in *Bartonella* ecology is a prolonged period of intraerythrocytic bacteremia in the specific mammalian reservoir host, associated with transmission by blood-sucking arthropod vectors (34). Selected aspects of the initial interaction of *B. bacilliformis* and human erythrocytes have been studied in vitro (i.e., bacterial binding and invasion). However, investigating the complex dynamics of erythrocyte parasitism in the long-term course of a hemotropic *Bartonella* infection requires the establishment of appropriate animal models.

ANIMAL MODELS OF ERYTHROCYTE PARASITISM Hemotropic infection by bartonellae has been experimentally established in several animal models (1, 12, 50, 66, 71, 72, 110); however, the course of erythrocyte parasitism was most precisely described for *B. tribocorum* in a rat model (110). Upon experimental infection in the tail vein, the bacterial inoculum was rapidly cleared from circulating blood, which then stayed sterile for at least three days. On day four or five postinfection, bacteria reappeared in the bloodstream in a synchronous infection wave. The primary niche that allows bacterial replication in the first three days of infection (when the circulating blood is sterile) and then seeds the hemotropic infection is yet unknown. However, circumstantial evidence suggests that this primary niche includes endothelial cells as the major target cells of bartonellae (34, 104, 111, 112) (see below). Bacteria seeded from the primary niche to the bloodstream adhere to mature erythrocytes and subsequently invade them. Following invasion, intracellular multiplication occurs in a membrane-bound compartment over a period of several days. Thereafter, the bacterial cell density remains static for the remaining life span of the infected erythrocytes, which is not shortened by bacterial colonization (110). Because of this nonhemolytic course of erythrocyte parasitism, *B. tribocorum* can persist for several weeks in a protected intracellular compartment of circulating blood. Moreover, recurrent waves of erythrocyte infection seeded from the primary niche at intervals of ~five days (110) further extend the duration of the intraerythrocytic bacteremia. This periodicity was interpreted as a result of a five-day-lasting infection cycle in the primary niche, liberating bacteria capable of reinfecting the primary niche as well as infecting mature erythrocytes (34). The resulting long-lasting intraerythrocytic bacteremia is considered to represent

a unique adaptation to the mode of transmission by blood-sucking arthropod vectors.

Intraerythrocytic bacteremia in the *B. tribocorum*–rat model subsides spontaneously after 8 to 10 weeks. Consistently, a prolonged intraerythrocytic course of bacteremia was observed in a murine model of *B. grahamii*, terminating 8 to 11 weeks postinoculation (66). Infection in these immunocompetent mice triggered a strong IgG antibody response (66). In contrast to the limited hemotropic period in immunocompetent animals, the infection of B cell–deficient mice led to persistent bacteremia (66). Immune serum transfer to these immunocompromised animals resulted in a transient course of bacteremia indistinguishable from that in immunocompetent mice (66). These observations indicate an important role for immunoglobulins in controlling blood-stage infection, although the effector mechanism remains unclear. Erythrocyte colonization by *B. tribocorum* showed a nonhemolytic course and persisted within infected cells for the average life span of mature erythrocytes. Thus, antibodies probably do not operate against bacterial antigens eventually exposed on the surface of infected erythrocytes, as such mechanisms should result in a more rapid clearance of parasitized erythrocytes. Rather, antibodies may interfere with the infection of mature erythrocytes by capturing extracellular bacteria seeded from the elusive primary niche.

The animal models for both *B. tribocorum* and *B. grahamii* have been useful for dissecting the course of hemotropic infection in animal reservoirs of bartonellae (66, 110). They might prove particularly relevant for designing appropriate strategies to prevent infection of animals serving as reservoirs for zoonotic bartonellae (e.g., for controlling infection of the major human pathogen *B. henselae* in cats as their natural reservoir). In addition, these models gain value as surrogate systems for better understanding human disease caused by intracellular erythrocyte parasitism of *B. quintana* (trench fever) and *B. bacilliformis* (Oroya fever). Remarkably, the feverish relapses of trench fever (also known as five-day fever) coincide with the ~five-day periodicity of erythrocyte infection waves in the *B. tribocorum*–rat model (112). The frequency of parasitized erythrocytes is lower in trench fever patients (between 0.001% and 0.005%) (100) than in the *B. tribocorum*–rat model (~0.1%) (110). However, this difference may result from experimental infection of rats, as *B. tribocorum* appears to cause a weaker bacteremia in naturally infected wild rats (55). In sharp contrast to *B. quintana* and *B. tribocorum*, *B. bacilliformis* invades up to 80% of erythrocytes in the course of Oroya fever. The massive hemolysis of parasitized erythrocytes resulting in severe hemolytic anemia—the major symptom of Oroya fever—is also clearly distinguished from the nonhemolytic intraerythrocytic infection course of *B. tribocorum* (110) and probably also of *B. quintana* and other *Bartonella* species.

IN VITRO STUDIES OF ERYTHROCYTE PARASITISM Most *Bartonella* species do not appear to interact strongly with erythrocytes in vitro (60, 84; R. Schulein & C. Dehio, unpublished data), suggesting that the colonization of the elusive primary niche is required to gain competence for erythrocyte interaction (34). The exception

is *B. bacilliformis*, which infects most of the erythrocyte population during natural infection in human and which also binds to and invades human erythrocytes in vitro (8, 15, 85, 103, 126). This in vitro model has been used mainly to characterize factors of *B. bacilliformis* involved in mediating erythrocyte invasion and hemolysis, and is discussed below (see Bacterial Factors Involved in Pathogenesis).

Endothelial Cell Interaction

B. bacilliformis, *B. quintana*, and *B. henselae* are unique among all known bacterial pathogens in their ability to cause vasoproliferative lesions, a process of pathological angiogenesis resulting in the formation of new capillaries from pre-existing ones. The fascinating angioproliferative process of bartonellae has attracted considerable interest as both a basic and clinically important biological system, representing a paradigm for pathogen-triggered tumor formation (35, 61, 75). The typical manifestations of *Bartonella*-triggered vasoproliferation are either Kaposi's sarcoma-like lesions of the skin, known as verruga peruana (*B. bacilliformis*) or BA (*B. quintana* and *B. henselae*), or a cystic form in liver and spleen referred to as BP (*B. henselae*). These lesions comprise proliferating endothelial cells, bacteria, and mixed infiltrates of macrophages/monocytes and polymorphonuclear neutrophils (PMN) (73, 80). Bartonellae are found as aggregates both surrounding and within endothelial cells, indicating that the vascular endothelium represents a target tissue for intra- and extracellular colonization in vivo (80). Clearance of infection by antibiotic treatment results in complete regression of vascular lesions (80). Together, these findings suggest that bartonellae specifically invade and colonize the vascular endothelium and produce a mitogenic factor that acts locally and temporarily. Primary human umbilical vein endothelial cells (HUVEC) have been used as an in vitro system to study these interactions with the human vascular endothelium, i.e., (a) cell invasion and the associated cytoskeletal rearrangements and (b) endothelial cell proliferation (35). Other endothelial cell interactions studied in this in vitro system include (c) the inhibition of endothelial cell apoptosis and (d) NF- κ B activation and proinflammatory response, which are considered to contribute indirectly to *Bartonella*-triggered vasoproliferation (35).

CELL INVASION AND ASSOCIATED CYTOSKELETAL REARRANGEMENTS Within hours, all three vasoproliferative *Bartonella* species adhere to and enter HUVEC by an actin-dependent process, resulting in small clusters of bacteria residing in membrane-bound compartments that typically localize to the perinuclear region (14, 38, 118). For *B. bacilliformis*, this uptake route depends on the small GTPases Rho, Rac, and CDC42, which are key regulators of actin reorganization (118–120). These characteristics, presumably shared by all three vasoproliferative bartonellae, are reminiscent of bacterium-directed phagocytosis as previously described for other intracellular pathogens (43). In addition to this classical uptake process, *B. henselae* can enter HUVEC via the “invasome”-mediated mechanism (38). This slow invasion process (lasting 24 h) is initially characterized by the

formation of a bacterial aggregate on the cell surface that subsequently is engulfed and internalized by an actin-dependent mechanism. While the *in vivo* relevance of invasome-mediated uptake remains to be demonstrated, the bacterial aggregates formed *in vitro* could correspond to the clumps of bacteria observed in close association with the atypically proliferating endothelial cells in BA lesions (73, 80).

ENDOTHELIAL CELL PROLIFERATION Angiogenesis is a complex process involving several carefully orchestrated steps: Proliferation and migration of endothelial cells are followed by their reorganization into new capillaries. The capacity to stimulate HUVEC proliferation is the simplest criterion for *in vitro* angiogenesis. Live *B. bacilliformis*, *B. quintana*, and *B. henselae*, as well as cell-free extracts of these bacteria, are capable of stimulating the proliferation of HUVEC, indicating that soluble and possibly secreted factors are responsible for these bacterial activities (47, 48). *B. henselae* and *B. quintana* further stimulate HUVEC migration through a porous membrane, an additional criterion for *in vitro* angiogenesis (29). The molecular characterization of the angiogenic activity of the vasoproliferative bartonellae is described below (see Bacterial Factors Involved in Pathogenesis).

INHIBITION OF ENDOTHELIAL CELL APOPTOSIS Cell death by apoptosis is a common response of mammalian cells to bacterial infection. However, some pathogens alleviate or even inhibit apoptosis (52, 75). Likewise, *B. henselae* specifically inhibits apoptosis of HUVEC *in vitro* by suppressing both early and late events in apoptosis, namely caspase activation and DNA fragmentation, respectively (64). An antiapoptotic activity is also produced by *B. quintana*, but not by the two species *Bartonella vinsonii* and *Bartonella elizabethae*, which have not been associated with angioproliferative lesions (64). Although an antiapoptotic activity alone cannot account for the increase in cell number observed for *Bartonella*-triggered proliferation of endothelial cells *in vitro*, the enhanced survival of endothelial cells may contribute, at least in part, to the vascular proliferation observed *in vivo*.

NF- κ B ACTIVATION AND PROINFLAMMATORY RESPONSE The mixed infiltrate of macrophages/monocytes and PMN found in *Bartonella*-triggered vasoproliferative lesions is indicative of chronic inflammation. An acute inflammatory reaction triggered by the *Bartonella*-infected endothelium may be crucial for initiating chronic inflammation. In general, an acute inflammatory response is thought to induce a mediator cascade that activates the endothelium, resulting in the release of proinflammatory chemoattractants and the sequential establishment of receptor-ligand interactions between the activated endothelium and circulating PMN. Selectins (e.g., E-selectin) mediate the initial attachment and the rolling of PMN. Subsequently, firm adhesion of PMN to the vessel wall occurs via interaction of activated CD11/CD18 (β 2-integrin) receptors with endothelial ligands such as intercellular adhesion molecule-1 (ICAM-1). Finally, transendothelial migration of PMN into tissue occurs in response to chemoattractants [i.e., interleukin (IL)-8]

and the engagement of platelet-endothelial adhesion molecule-1 (PECAM-1) (18). The transcription nuclear factor NF- κ B is considered the primary regulator of this proinflammatory cascade (32).

Infection of HUVEC by *B. henselae* triggers the activation and nuclear translocation of NF- κ B. The resulting upregulation of ICAM-1 and E-selectin leads to PMN rolling on and adhering to the infected endothelial cell monolayers (46, 78). IL-8 secretion, as shown to occur by the human microvascular endothelial cell line-1 (HMEC-1) in response to *B. henselae* infection (98), should stimulate transendothelial migration of PMN in vivo. The transformation from acute to chronic inflammation can be triggered by the monocyte chemoattractant protein-1 (MCP-1). Thrombin-activated endothelial cells produce MCP-1 in response to a complex of soluble IL-receptor-6 alpha (sIL-6R α), released from PMN membranes, and IL-6, produced in an autocrine manner (81, 98). The macrophages/monocytes recruited by MCP-1 to vasoproliferative lesions would likely propagate chronic inflammation by releasing proinflammatory cytokines. *B. henselae*-infected macrophages (either the mouse cell line J774 or the human cell line THP-1) released high levels of tumor necrosis factor alpha (TNF- α), IL-1 β , and IL-6 (92, 98). Taken together, the acute inflammatory response of *Bartonella*-infected endothelium mediated by NF- κ B appears to be the first step in initiating chronic inflammation.

Macrophage Interaction: The Paracrine Loop of Vasoproliferation

As part of the mixed inflammatory infiltrates in BA lesions (73, 80), cells of the mononuclear phagocyte lineage (i.e., macrophages) may also represent target cells for the vasoproliferative bartonellae. *B. henselae* was reported to be effectively phagocytosed by J774 cells and to survive within this mouse macrophage cell line for at least several hours (92). However, as macrophages are capable of producing potent angiogenic factors upon activation (116), their primary role during interaction with vasoproliferative bartonellae may be as effector cells that release angiogenic substances and thereby contribute to the process of vasoproliferation in a paracrine fashion. Bartonellae may thereby provoke vasoproliferation by two independent mechanisms: directly, by triggering proliferation and inhibiting apoptosis of endothelial cells, or indirectly, by stimulating a paracrine loop of vasoproliferative host factors. Two independent reports support such a paracrine loop model for *Bartonella*-triggered vasoproliferation (62, 98). Both vascular endothelial growth factor (VEGF), one of the most potent inducers of angiogenesis, and IL-1 β , a potentiator of VEGF, are released from the human macrophage cell line THP-1 in response to infection with *B. henselae*. Importantly, such a conditioned culture medium was able to trigger proliferation of HMEC-1 (98). Likewise, *B. henselae* infection of Ea.hy 926 cells (a fusion clone of HUVEC and the lung carcinoma cell line A549) triggers secretion of VEGF at levels supporting HUVEC proliferation, while neutralizing anti-VEGF antibody blocked most of

the angiogenic activity contained in the conditioned culture medium (62). These data are in favor of a paracrine angiogenic loop with VEGF representing the major vasoproliferative substance released by macrophages in response to *B. henselae* infection (35, 97). PMNs, which represent another major constituent of the mixed inflammatory infiltrates of *Bartonella*-triggered vasoproliferative lesions, are also capable of secreting VEGF in response to bacterial infection, as recently demonstrated for *Streptococcus pneumoniae* (117). However, it is unknown to what extent bartonellae may stimulate VEGF secretion by PMN. In contrast to activated inflammatory cells, endothelial cells are generally considered poor VEGF producers (62, 77, 98), and no evidence is available indicating that *B. henselae* can stimulate an autocrine loop of VEGF-mediated endothelial proliferation.

The proposed role of VEGF as a paracrine mediator of *Bartonella*-triggered vasoproliferation is supported by a recent study on mice implanted with VEGF-secreting melanoma cells. In this model the mice developed a vasoproliferative syndrome in liver, strongly resembling BP as caused by *B. henselae* in human liver (125). These lesions were interpreted as a paraneoplastic syndrome caused by the production of VEGF at the tumor site and the induction of endothelial proliferation in the liver. The authors speculate that there could be a common pathway in this mouse melanoma model and *B. henselae*-triggered BP. The immunohistochemical demonstration of increased VEGF levels in BP and BA lesions supports this notion (62).

BACTERIAL FACTORS INVOLVED IN PATHOGENESIS

In recent years, studies of *Bartonella* pathogenesis have taken a big step forward. The combination of bacterial genetics (7, 37, 41, 49, 76, 99, 108) and appropriate cell culture (8, 38, 46, 47, 64) or animal models (110) of *Bartonella* infection provided the first glimpses of the molecular mechanisms that govern the host cell interactions of these elusive pathogens. Table 2 summarizes the bacterial factors considered to contribute to pathogenesis.

Type IV Secretion Systems

A growing number of bacterial pathogens are known to encode type IV secretion systems (T4SS). These versatile transporters have evolved from bacterial conjugation systems and mediate translocation of bacterial effector molecules during interaction with host cells (21). The prototype of these "adapted conjugation machines" is the VirB-D4 system of *Agrobacterium tumefaciens*. Eleven proteins encoded by the *virB* operon and one encoded by *virD4* build up a pilus and pore complex, which spans both gram-negative bacterial membranes and possibly the host cell membrane, allowing translocation of a nucleoprotein complex from the bacterial cytoplasm directly into the host cell cytoplasm. Human pathogens have adapted the type IV machinery for transport of toxins to the extracellular milieu (i.e., pertussis toxin secreted by *Bordetella pertussis*) or directly into the

TABLE 2 A synopsis of pathogenicity factors of *Bartonella* spp. in alphabetical order

Factor(s)	Function	Reference(s)
Angiogenic factor	Stimulates endothelial cell proliferation	(29, 47, 48, 77, 88)
Deformin	Deformation of erythrocyte membranes	(8, 42, 58, 126)
Flagella	Motility, binding to and invasion of erythrocytes	(8, 102, 103)
Hemolysin	Contact-dependent hemolysis	(57)
Hbp/Pap 31	Omp family, hemin-binding proteins	(19, 87, 129)
IalA-B	Putative invasins of erythrocytes	(20, 30, 89, 90)
Iba	Autotransporter, putative adhesins	(34, 112)
LPS	Lipopolysaccharide, detoxified	(128)
Omp43	Putative adhesin for endothelial cells	(16, 17)
pili	Type IV-like pili, twitching motility, cell adhesion	(6, 62)
Trw	T4SS, establishment of intraerythrocytic infection	(111)
VirB-D4-Bep	T4SS, subversion of endothelial cell function	(104, 108; R. Schulein, P. Guye, T.A. Rhomberg, M.C. Schmid & C. Dehio, unpublished results)

host cell cytosol (e.g., delivery of CagA protein of *Helicobacter pylori* into gastric epithelial cells) (21). *Bartonella* species encode two distinct T4SS, VirB-D4 and Trw, that are both essential for infection in an animal model (108, 111). These T4SS represent to date the only bona fide pathogenesis factors of *Bartonella*.

VirB-D4 T4SS: SUBVERSION OF ENDOTHELIAL CELL FUNCTION A T4SS displaying homology to the prototypic VirB-D4 system of *A. tumefaciens* in *B. henselae* (93, 105; R. Schulein, P. Guye, T.A. Rhomberg, M.C. Schmid & C. Dehio, unpublished results), *B. tribocorum* (108), and *B. quintana* has recently been identified. The *virB-D4* locus of these bartonellae is encoded by an operon of 10 genes (*virB2-10*) and a downstream-located *virD4* gene. The *virB5* and *virB7* genes are atypical, as they are poorly conserved when compared with their orthologs in the T4SS loci of other bacteria. Interestingly, *virB5* encodes a 17-kDa protein that was identified as an immunodominant protein in patients diagnosed for cat-scratch disease (2) and is well conserved across the genus *Bartonella* (114). The function of the 17-kDa antigen is unknown; however, in regard to its immunogenicity and in analogy to the VirB5 homologs from other T4SS, it is reasonable to assume that it localizes as a minor component of the T4SS pilus, where it could serve as an adhesin for mediating interaction with mammalian target cells (127).

Unlike wild-type *B. tribocorum*, mutants deleted for *virB4* or *virD4* are unable to cause intraerythrocytic bacteremia in the experimental rat model (108). The VirB-D4 T4SS is required at an early infection stage before the onset of intraerythrocytic bacteremia occurs (108). On the basis of the observation that the *virB* operon of *B. henselae* is induced during infection of endothelial cells in vitro (106), it was suggested that the primary niche may correspond to the vascular endothelium and that the VirB-D4 system may thus be involved in mediating endothelial cell interaction in both the mammalian reservoir and the incidental human host (35). Indeed, mutations in either *virB4* or *virD4* of *B. henselae* are incapable of mediating most of the known physiological changes associated with *B. henselae* infection of HUVEC. These include (a) massive rearrangements of the actin cytoskeleton, resulting in the formation of bacterial aggregates and their internalization by the invasome structure; (b) NF- κ B-dependent proinflammatory activation, leading to cell adhesion molecule expression and chemokine secretion; and (c) inhibition of apoptotic cell death, resulting in enhanced endothelial cell survival. Moreover, the VirB-D4 system mediates cytostatic and cytotoxic effects at high bacterial titers, which interfere with the potent VirB-independent mitogenic activity of *B. henselae* (104, 108). The VirB T4SS is thus a major virulence determinant for *B. henselae* required for targeting multiple endothelial cell functions exploited by this vasculotropic pathogen.

The downstream region of the *virB* locus in *B. henselae* harbors several genes encoding effector proteins translocated by the VirB-D4 T4SS into endothelial cells (R. Schulein, P. Guye, T.A. Rhomberg, M.C. Schmid & C. Dehio, unpublished results). These Bep (*Bartonella*-translocated effector proteins) proteins share a conserved C-terminal translocation domain, while their heterologous N-terminus is considered to serve effector functions within endothelial cells. Deletion of the locus encoding the Bep proteins results in a mutant phenotype indistinguishable from the *virB4* and *virD4* mutants, demonstrating that the Bep proteins mediate all VirB-D4-dependent host cell phenotypes (R. Schulein, P. Guye, T.A. Rhomberg, M.C. Schmid & C. Dehio, unpublished results). It will be interesting to test whether the conserved VirB-D4 T4SS of other bartonellae translocate a similar set of Bep proteins and whether this results in subversion of the same endothelial cell functions as demonstrated for *B. henselae*.

THE Trw T4SS: ESTABLISHING INTRAERYTHROCYTIC BACTEREMIA The second T4SS of *Bartonella*, Trw, was initially identified in a screen for *B. henselae* promoters, which are specifically activated during in vitro infection of endothelial cells (111). Likewise, expression of the *B. tribocorum* *trw* locus is upregulated during endothelial cell infection (111). On the basis of the availability of the rat infection model and the complete sequencing of the locus, most of the functional analysis has been done in *B. tribocorum*. The Trw system of *B. tribocorum* shares an extremely high level of sequence identity with the Trw conjugation machinery of the broad-host-range antibiotic-resistance plasmid R388 of enterobacterial origin (up to 80% amino acid identity for individual T4SS components). The highly

conserved T4SS loci are colinear except for the presence of numerous tandem gene duplications in *B. tribocorum*, which mostly encode variant forms of presumable surface-exposed pilus subunits. Conservation is not only structural, but also functional: R388 mutated in either *trwD* or *trwH* encoding essential T4SS components can be *trans*-complemented for conjugation by the homologs of the *B. tribocorum* system. Conservation even includes the transcription regulatory circuit: Both T4SS loci encode a highly homologous and interchangeable KorA/KorB repressor system that negatively regulates expression of all T4SS components. These data suggest that the Trw T4SS of *Bartonella* and R388 have a short history of adaptive evolution from a common ancestor locus. Recent acquisition of the *trw* locus of *B. tribocorum* by horizontal gene transfer is suggested by the presence of a cryptic phage-like integrase at a distal position of the locus, as well as by the fact that flanking chromosomal regions are contiguous in the genomes of related α -proteobacteria (111). By these criteria, the *trw* locus is a pathogenicity island (53).

An essential role of the Trw T4SS in the infection process was demonstrated by genetic means: Unlike wild-type *B. tribocorum*, a mutant deleted for *trwE* was unable to cause intraerythrocytic bacteremia in the experimental rat model (111). Preliminary data indicate that the *trwE* mutant may still be capable of colonizing the primary niche, although after seeding to the blood it is quickly cleared without establishing erythrocyte infection (A. Seubert & C. Dehio, unpublished results). Moreover, the *trw* locus appears to be upregulated in the primary niche (similarly as shown *in vitro* for HUVEC), as it is already expressed when bacteria are seeded into the bloodstream (A. Seubert & C. Dehio, unpublished results). These data suggest that the Trw T4SS is not required in the primary niche, which may in fact represent the vascular endothelium, as it might already be expressed in this niche to be functional in erythrocyte interaction immediately after release into the blood.

The pilus subunit variants encoded in several copies by the Trw T4SS have been proposed to serve in mediating host cell tropism, i.e., by binding to various erythrocytic blood group antigens present in the reservoir population (111). To date there are no substrates known to be translocated by the Trw system. Moreover, although the Trw system encodes all components required to build up a pore complex across the two bacterial membranes and a T4SS pilus on the surface, this system lacks a VirD4 homolog (111), which is required for substrate transport by most other T4SS (21), i.e., the closely related Trw system of R388 (111). It thus appears reasonable to assume that the primary role of the Trw T4SS is to establish contact to erythrocytes by surface-expressed T4SS pili rather than to translocate effector proteins into host cells as demonstrated for the VirB-D4 T4SS of *B. henselae* (R. Schulein, P. Guye, T.A. Rhomberg, M.C. Schmid & C. Dehio, unpublished results). Clarification of the precise molecular function of the Trw T4SS in establishing erythrocyte infection awaits further experimental evidence.

Angiogenic Factor: Trigger of Vasoproliferation

All three vasoproliferative *Bartonella* species, *B. bacilliformis*, *B. quintana*, and *B. henselae*, display vasoproliferative activities during infection of HUVEC (29,

35, 47, 48, 77). Early studies suggested that the angiogenic factor of *B. bacilliformis* is a protein because it is heat sensitive, larger than 14 kDa, and can be precipitated with 45% ammonium sulfate (47). A recent study used biochemical and immunological analyses and identified GroEL as a candidate for the observed mitogenic activity (88). A *groEL* deletion mutant could not be generated, possibly because GroEL is essential in *B. bacilliformis* as reported for other bacterial species. However, a strain containing the intact *groES-groEL* operon on a multicopy plasmid was generated and used to demonstrate a correlation between HUVEC mitogenicity and GroEL levels in the lysate. Antiserum to GroEL significantly inhibited mitogenicity of the lysate. Data also indicate that GroEL is located in the soluble and insoluble fractions (including inner and outer membranes) of *B. bacilliformis* and may also be actively secreted (88). Interestingly, a recent study on the zoonotic pathogen *Brucella abortus* suggested that GroEL is actively secreted by its VirB system (122), a T4SS related to the *Bartonella* VirB-D4 system (108). It might be interesting to test whether secretion of GroEL depends on a functional VirB-D4 T4SS in *B. bacilliformis*. Another open question is whether GroEL is directly involved in mediating endothelial proliferation, or whether it may act as the chaperone for the actual angiogenic factor. Additional experimental evidence is required to unequivocally demonstrate the molecular role of GroEL in vascular proliferation.

Compared with *B. bacilliformis*, much less is known regarding the angiogenic activity of *B. henselae* and *B. quintana*. Susceptibility to trypsin digestion suggests that the angiogenic activity of at least *B. henselae* is also of a proteinaceous nature (29). To what extent the vasoproliferative factor(s) of *B. bacilliformis*, *B. henselae*, and *B. quintana* is related in structure and function, and whether they are benign endothelial mitogens or trigger proliferation indirectly via an autocrine loop, await further analysis.

Deformin: Deformation of Erythrocyte Membranes

One striking feature of *B. bacilliformis* interaction with human erythrocytes is the marked production of deeply invaginated pits and trenches in the erythrocyte membrane (8), which are considered entry portals for invading bacteria. This phenomenon appears to be triggered by a secreted bacterial factor termed deformin, which has been identified in the culture supernatants of *B. bacilliformis* and *B. henselae* (60). Deformin-induced invaginations can be reversed by treatment with the ATPase inhibitor vanadate, the phospholipid dilauroylphosphatidylcholine, or by increasing intracellular Ca^{2+} levels with ionophores (85, 126). Originally reported to be a protein (126), deformin is a small hydrophobic molecule with a high affinity for albumin, as indicated by more recent work by the same group (42). Deformin can be extracted from albumin as a heat- and protease-resistant, water-soluble molecule with a molecular mass of about 1.4 kDa. A very recent study indicates that a group of proteins of about 36 kDa present in the supernatant of *B. bacilliformis* are also required for high levels of deforming activity. When these proteins are missing, as in the supernatant of a variant strain, or are removed

by filtration or ammonium sulfate precipitation, the levels of deforming activity are extremely low or abolished (58). The 36-kDa proteins are considered either directly responsible for deformation of human erythrocytes—they may be part of a deforming protein complex—or may be necessary for secretion of the deforming activity (58). Further work is required to reveal the precise molecular mechanisms of erythrocyte deformation by *B. bacilliformis*.

Flagella: Strengthening Erythrocyte Interaction

B. bacilliformis is highly motile due to the expression of multiple unipolar flagella (103). In vitro studies with human erythrocytes showed that bacterial motility correlates with bacterial adherence. Early work suggested that nonmotile variants bind poorly and do not invade erythrocytes (8, 85). Consistently, antibodies raised against the flagellin subunit partially inhibited erythrocyte binding and almost completely abolished invasion (103). Recently, a nonflagellated mutant obtained by site-directed mutagenesis of the flagellin gene displayed a 75% reduced binding capacity for human erythrocytes, and this phenotype could be partially rescued by complementation with the wild-type locus (7, 86). It remains to be demonstrated whether flagella directly bind to erythrocytes or whether the energy-dependent process of motility simply enhances bacteria-erythrocyte collisions. Early studies favor the latter possibility by showing that reagents inactivating proton motive force (N-ethylmaleimide) or respiration (KCN) significantly reduce bacterial binding to human erythrocytes (8, 103). The flagella of *B. bacilliformis* appear critical for the particularly high erythrocyte invasion rate observed in Oroya fever (up to 80% infected erythrocytes) (59). However, considering that several *Bartonella* spp. (e.g., *B. henselae*, *B. quintana*, and *B. tribocorum*) are nonflagellated but capable of invading erythrocytes (6, 55, 67, 77, 100, 101, 110), flagella cannot be essential for the process of erythrocyte invasion in these species.

Lipopolysaccharide: Unique Structure with Low Endotoxicity

The remarkable interaction of *B. henselae* and *B. quintana* with endotoxin-sensitive endothelial cells, which eventually leads to vasoproliferation, and the apparent lack of septic shock as a result of bacteremia suggest that the lipopolysaccharide (LPS) of these pathogens may display a reduced endotoxic activity (82). The endotoxic activity of highly purified LPS from *B. henselae* is 1,000 to 10,000 lower than that of enterobacterial LPS as measured by IL-8 secretion in response to TLR4 signaling (128). The complete structural analysis of this *B. henselae* LPS revealed a short-chain structure with several unusual features, including (a) a very short carbohydrate portion (a glucose residue attached to a 3-deoxy-D-manno-oct-2-ulosonic acid disaccharide), (b) an unusual core structure of lipid A (2,3-diamino-2,3-dideoxy-glucose disaccharide bisphosphate), and (c) penta-acetylation of lipid A including a long-chain fatty acid (25-hydroxyhexacosanoic or 27-hydroxyoctacosanoic acid) (128). Some of the unusual structural features of *B. henselae* LPS, such as the long-chain fatty acid of lipid A, are shared by the LPS of other intracellular bacteria that cause chronic infections (i.e., *Legionella*

spp. and *Chlamydia* spp.). These features may provide the structural basis for the low endotoxic potency of these LPS structures (128).

Pili: Binding to Nucleated Cells

Pili appear to play an important role in adhesion to nucleated cells, which is thought to precede bacterium-directed phagocytosis and possibly also invasome-mediated invasion (6, 38). The presence of *B. henselae* pili was also associated with host cell production of VEGF, as a Pil-mutant was unable to induce VEGF production by infected Ea.hy 926 cells (62). Pili structures in *B. henselae*, *B. quintana*, *B. tribocorum*, and *B. alsatica* have been demonstrated by electron microscopy (6, 54, 55, 77). These organisms may possess type IV pili owing to the presence of a number of properties typical for this type of pili, e.g., twitching motility and self-aggregation (6).

Other Putative Pathogenesis Factors of *Bartonella*

The following paragraphs describe bacterial factors presumably involved in pathogenesis.

HEMIN-BINDING PROTEIN Hbp/Pap 31 The growth of *B. henselae* and *B. quintana* strongly depends on hemin (19, 129). At least five membrane-associated proteins of *B. henselae* and eight of *B. quintana* bind hemin, with the homologous proteins Pap31 and HbpA representing the dominant hemin-binding proteins of *B. henselae* and *B. quintana*, respectively (19, 129). The gene encoding Pap31 was heterologously expressed in the outer membrane of *Escherichia coli*. Complementation of a *hemA*-deficient *E. coli* mutant with Pap31 demonstrated that this protein is involved in heme acquisition and may thus be an important pathogenicity factor of *B. henselae* (129). The hemin-binding protein HbpA of *B. quintana* displayed typical features of outer membrane proteins (e.g., heat modification) (19). In addition to their close relation to Pap31 in *B. henselae*, HbpA homologs from a variety of gram-negative pathogens, including *Brucella* spp. outer membrane protein (Omp)31, *Agrobacterium tumefaciens* Omp25, and neisserial opacity proteins (Opa), were also identified (87). Moreover, HbpA is encoded as part of a five-member gene family (*hbpA-E*) (87). Mutagenesis of *hbpA* by allelic exchange produced a strain that exhibited an enhanced hemin-binding phenotype relative to the parental strain. Analysis of the mutant by quantitative RT-PCR showed elevated transcript levels for the other *hbp* family members, suggesting that compensatory expression occurs (87).

HEMOLYSIN Following erythrocyte invasion by bartonellae, the subsequent intracellular colonization has typically a nonhemolytic course (67, 110). A striking exception is the massive hemolysis observed in Oroya fever caused by *B. bacilliformis* (59). A recent report has demonstrated contact-dependent hemolysis by *B. bacilliformis*, but not by *B. henselae* (57). The hemolytic activity is protease sensitive, suggesting that it corresponds to a surface-exposed protein. Whether this

hemolysin is the sole factor distinguishing the hemolytic course of *B. bacilliformis* bacteremia from the nonhemolytic blood-stage infection by other bartonellae remains to be investigated.

INDUCIBLE *Bartonella* AUTOTRANSPORTER The genes encoding the novel *iba* (inducible *Bartonella* autotransporter) family of related autotransporter proteins (56) were identified in a genetic screen for *B. henselae* genes, which were specifically upregulated during HUVEC infection (112). Strikingly, in the *B. tribocorum*–rat model the *ibaB*-promoter is fully activated at the onset of blood-stage infection but is quickly downregulated following erythrocyte invasion. These data suggest that the promoter is activated either in the primary niche or when bacteria are released from this niche into the bloodstream. The in vivo regulation pattern of the *ibaB* gene is indicative for a role of the encoded autotransporter in establishing hemotropic infection (112).

INVASION-ASSOCIATED LOCUS The invasion-associated locus (*ial*) of *B. bacilliformis*, composed of *ialA* and *ialB*, was identified by a genetic approach in *E. coli* as a putative virulence determinant implicated in erythrocyte invasion. Introduction of the *ialA* and *ialB* genes into *E. coli* conferred a minimally invasive phenotype for human red blood cells in vitro (89). The *ial* locus is highly conserved across the genus *Bartonella* (90) (C. Gille & C. Dehio, unpublished results). The *ialA* gene encodes a nucleoside polyphosphate hydrolase of the MutT motif family (20, 30). These enzymes are believed to eliminate toxic nucleotide derivatives from the cell and to regulate the levels of important signaling nucleotides and their metabolites (83). How this enzymatic activity of IalA relates to the process of erythrocyte invasion is unknown. The *ialB* gene encodes an 18-kDa protein that has been localized to the bacterial inner membrane (28). Site-directed mutagenesis of *ialB* in *B. bacilliformis* results in a 50% reduced capacity to interact with human erythrocytes in vitro, whereas the mutant phenotype was rescued by complementation with the wild-type locus (28). The mechanism by which the inner membrane protein IalB contributes to erythrocyte invasion remains elusive.

OUTER MEMBRANE PROTEIN 43 Multiple outer membrane proteins (Omps) of *B. henselae* were reported to bind to endothelial cells (16), particularly a 43-kDa protein (Omp43) displaying amino acid sequence similarity to the Omp2b porin of *Brucella* spp. (17). Recombinant Omp43 binds to HUVEC (17). However, whether native Omp43 in the bacterial outer membrane can mediate the binding of *B. henselae* to endothelial cells remains to be demonstrated.

CONCLUDING REMARKS

The molecular and genetic tools now available for studying *Bartonella*, coupled with the recent establishment of cell culture and animal infection models, have improved our understanding of *Bartonella*–host cell interactions and have identified the first pathogenicity factors of this pathogen, e.g., the VirB/D4 T4SS and their

translocated protein substrates, which play a crucial role in subverting host cell function. Despite these significant advances, our understanding of *Bartonella* pathogenesis is still incomplete. Important questions regarding *Bartonella* infection in the reservoir host include the nature of the primary niche, its relation to endothelial cells, and the molecular mechanisms determining host-specific intraerythrocytic parasitism. In respect to *B. bacilliformis* it is unclear how this deadly pathogen causes hemolysis. A major open question in regard to human pathology is to what extent vasoproliferative tumor formation depends on the two described mechanisms of vasoproliferation: either the direct stimulation of endothelial cell proliferation and antiapoptosis or the indirect stimulation of endothelial cell proliferation by a paracrine angiogenic loop established by the release of VEGF from infected macrophages. Finally, we are likely unaware of several bacterial factors that play important roles in these pathogenic processes. At least some of these open issues may be addressed by an ongoing systematic search for novel *Bartonella* virulence determinants by signature-tagged mutagenesis (H. Saenz, M. Stockli & C. Dehio, unpublished data). Moreover, at least three *Bartonella* genome-sequencing projects are at or near completion (<http://www.genomesonline.org/>; S. Schuster & C. Dehio, unpublished data). The advent of comparative genomics will likely reveal important clues regarding the evolution and pathogenic mechanisms of bartonellae and should promote experiments to improve our understanding of these enigmatic pathogens.

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