Antiangiogenic Effect of Erythromycin: An In Vitro Model of Bartonella quintana Infection

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Background. Bartonella quintana, the etiological agent of bacillary angiomatosis (BA), causes endothelial cell proliferation. Erythromycin has dramatic effects on BA, and the effects are largely unexplained by the compound’s bacteriostatic properties. Our aim here was to evaluate the possibility that erythromycin alters angiogenesis.

Methods. The effect of erythromycin on B. quintana–induced endothelial cell proliferation was studied using a wild-type strain and an erythromycin-resistant B. quintana mutant. The latter was generated by serial subcultures on blood agar plates.

Results. We show that erythromycin significantly inhibits the proliferation of dermal microvascular endothelial cells induced either by wild-type B. quintana or by our erythromycin-resistant mutant. Doxycycline and gentamycin failed to exert such an effect. Finally, we found that the resistant strain harbored a 27-bp insertion in the highly conserved region of the gene encoding the ribosomal protein L4; this insertion may explain the existence of the resistance to erythromycin.

Conclusion. The data presented here indicate that erythromycin profoundly down-modulates endothelial cell proliferation irrespective of its bacteriostatic effects and suggest that this may be a key component of the efficacy of the compound in the treatment of patients with BA.

Members of the genus Bartonella are facultative intracellular bacteria with a tropism for endothelial cells that leads to angioproliferative lesions. These include verruga peruana for B. bacilliformis and bacillary angiomatosis (BA) for B. quintana and B. henselae [1–3]. Angioproliferative lesions are unique and defining features of Bartonella infection [4]. Inoculation of B. quintana or B. henselae into endothelial cells increases the life span of the cultured cells, stimulates cell multiplication, and leads to morphological changes [5]. Endothelial cell proliferation has been previously linked to both mitotic induction and inhibition of apoptosis [6]. This angiogenic effect is related to an increased secretion of vascular endothelial growth factor (VEGF) by B. henselae–infected carcinoma [7] and monocytic [8] cell lines. Therefore, the bacteria may also promote angiogenesis indirectly by stimulating VEGF release from nonendothelial cells.

BA is a vasoproliferative disease that has been described in both immunocompetent and immunocompromised patients. When grown axenically, bacteria of the genus Bartonella are susceptible to many antibiotics, including penicillin and cephalosporin compounds, aminoglycosides, chloramphenicol, tetracyclines, and macrolide compounds, such as telithromycin, rifampin, and fluoroquinolones [9]. The first described patient with BA was treated empirically with erythromycin, and the lesions completely resolved [10]. Subsequently, erythromycin given for 3–4 months has become the drug of choice and has been used successfully to treat many patients with BA [11]. The response to treatment of BA can be dramatic, with the disappearance of subcutaneous lesions within hours [12]. Such an effect has not been reported for other forms of Bartonella infection [12]. Recent studies have demonstrated that 14-membered ring macrolides, of which erythromycin is the prototype, may have additional therapeutic activities [13]. Thus, the antibiotic effect of erythromycin on the pathogen itself may not explain the dramatic ben-
We therefore hypothesized that erythromycin modulates the B. quintana–induced pathological angiogenesis. To evaluate this hypothesis, and to distinguish the effects that are related to bacteriostatic properties from those that could alter host cells, we generated a B. quintana mutant that was resistant to erythromycin and assessed its effects on microvascular endothelial cells. We selected HMEC-1, a microvascular endothelial cell line of dermal origin, for our in vitro model of BA.

We show here that erythromycin significantly inhibits endothelial cell proliferation induced by either wild-type B. quintana or our erythromycin-resistant B. quintana mutant. The potential mechanism of resistance of B. quintana to macrolides and related antibiotics is not known, and so the molecular mechanism of resistance of the fully erythromycin-resistant mutant was also investigated via DNA sequencing and comparison of the nucleotide sequences of potential target gene candidates.

**MATERIALS AND METHODS**

**Bacterial strain.** The reference B. quintana strain Oklahoma (ATCC 51694), cultured in our lab for >100 passages, was used to select for an erythromycin-resistant mutant. B. quintana was grown at 37°C in a 5% CO2 atmosphere on Columbia 5% sheep blood agar plates (Biomerieux). Heat-killed bacteria were obtained by incubation for 1 h at 100°C.

**Selection of an erythromycin-resistant mutant.** An erythromycin-resistant mutant was selected by serial passages of B. quintana strain Oklahoma on blood agar plates containing a disk of erythromycin (15 μg; Bio-Rad) on the corner. Bacteria were subcultured every 2 weeks, and the diameter of the zone of inhibition was recorded. MICs for erythromycin were determined using the Etest (AB Biodisk) [14] or a disk-diffusion assay before and after obtaining the erythromycin-resistant mutant.

**Polymerase chain reaction (PCR) amplifications and DNA sequencing.** The erythromycin-susceptible strain and the erythromycin-resistant mutant were screened for the erythromycin-resistance candidate genes by PCR amplification [15]. Oligonucleotide primers specific for the 23S rRNA gene and the genes encoding the B. quintana ribosomal proteins L4 and L22 were designed on the basis of the complete genome sequence of strain Toulouse (GenBank accession number NC_005955) and are shown in table 1.

Genomic DNA was extracted from the B. quintana cultures by use of the QIAamp Tissue Kit (QIAGEN), in accordance with the manufacturer’s instructions. PCRs were done in a PTC-200 automated thermalcycler (MJ Research), with a 1 μmol/L concentration of each primer, Taq DNA polymerase (Eurobio), and 2 μL of the DNA preparation. The following conditions were used for amplification: an initial 3 min of denaturation at 94°C was followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at various temperatures (indicated in table 1), and extension for 1 min at 72°C. Amplification was completed by holding the reaction mixture for 3 min at 72°C, to allow complete extension of the PCR products. PCR products were purified using a QIAquick Spin PCR Purification Kit (QIAGEN), in accordance with the manufacturer’s instructions. Sequencing reactions were done using the d-Rhodamine Terminator cycle sequencing ready reaction kit with AmpliTaq Polymerase FS (PerkinElmer), in accordance with the manufacturer’s instructions. Sequencing products were resolved using an ABI 3100 automated sequencer (PerkinElmer). Sequence analysis was performed using the software package ABI Prism DNA Sequencing Analysis Software (version 3.0; PerkinElmer). For all PCR products, sequences from both DNA strands were determined twice. Sterile water was used as a negative control in each assay.

**Quantification of B. quintana DNA.** Quantification of B. quintana DNA copies was done using a real-time quantitative PCR assay with primers for the 16S–23S interspaced region, as described elsewhere [16].

**Sequence analysis.** The nucleotide sequences of the 23S rRNA gene and the genes encoding the ribosomal proteins L4 and L22 of the erythromycin-susceptible strain and the erythromycin-resistant strain as well as the B. quintana Toulouse strain were aligned and compared, to look for possible mutations known to be associated with macrolide resistance [15], using the CLUSTALW program (available at: http://www.infobiogen.fr) supported by the Infobiogen Web site. Sequence alignments were done in comparison with known original sequences of either Escherichia coli (for 23S rRNA gene comparison) or Streptococcus pneumoniae (for L4 and L22 gene comparison; GenBank accession number NC_003098) retrieved from the Kyoto Encyclopedia of Genes and Genomes Web site. Because the S. pneumoniae numbering system was the most...

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**Table 1. Sequences of the primers used in the present study.**

<table>
<thead>
<tr>
<th>Gene, primer</th>
<th>Sequence (5′–3′)</th>
<th>Annealing temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>23S rRNA gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BQ23SF1</td>
<td>ATCAAGTGTCTAAAGGGC</td>
<td>52</td>
</tr>
<tr>
<td>BQ23SR1</td>
<td>TCCACGACAACTAAGGCA</td>
<td>54</td>
</tr>
<tr>
<td>BQ23SF2</td>
<td>GTCCGAACCCAGATTGTTT</td>
<td>60</td>
</tr>
<tr>
<td>BQ23SR2</td>
<td>TGTCAGCATCTCAGCTC</td>
<td>54</td>
</tr>
<tr>
<td>BQ23SF3</td>
<td>CGAAAATGAACGAGGCT</td>
<td>54</td>
</tr>
<tr>
<td>BQ23SR3</td>
<td>GGAAAGCTCGTACCGATT</td>
<td>54</td>
</tr>
<tr>
<td>BQ23SF4</td>
<td>TGGAGGTTAGAGGAGA</td>
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</tr>
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<td>54</td>
</tr>
<tr>
<td>BQ23SR5</td>
<td>AATGGGAAGCATAAGGTC</td>
<td>52</td>
</tr>
<tr>
<td>L4 gene</td>
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<td></td>
</tr>
<tr>
<td>BQL4F</td>
<td>ATGGATCTTGTAATAGAAC</td>
<td>52</td>
</tr>
<tr>
<td>BQL4R</td>
<td>TCATTAAACGCTCCTCAA</td>
<td>54</td>
</tr>
<tr>
<td>L22 gene</td>
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<td></td>
</tr>
<tr>
<td>BQL22F</td>
<td>ATGGGAAAGCTAAGGTTCC</td>
<td>56</td>
</tr>
<tr>
<td>BQL22R</td>
<td>TTATGCGGCCCTCACATT</td>
<td>54</td>
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</tbody>
</table>
commonly used for comparison of ribosomal proteins, we have used these sequences for alignments with sequences obtained for Bartonella species.

**Endothelial cells.** HMEC-1 cells were provided by E. W. Ades (Centers for Disease Control and Prevention) [17]. These cells were grown in endothelial cell growth medium (EGM-2; Clonetics) supplemented with 10% fetal calf serum, 1% hydrocortisone, 1 mmol/L l-glutamin, and 1% endothelial cell growth factor (Clonetics). Cells were seeded on tissue-culture flasks coated with 2% gelatine. Cells were detached at 37°C with 2 mL of trypsin/EDTA in PBS. Cells were collected in prewarmed culture medium. Cultures were grown to confluency at 37°C in a 5% CO₂ atmosphere, and medium was replaced every other day.

**Antibiotic treatment and infection of endothelial cells.** Endothelial cells were seeded in 24-well culture plates at 1 × 10⁴ cells/well and were infected with B. quintana (1 × 10⁴ bacteria/cell) by centrifugation at 700 g for 15 min [18, 19]. Infected cells were incubated at 37°C for 1 h. Cells were then washed 3 times to remove free bacteria; this time was designated as hour 0. Infected cells were again cultured for 24, 48, 72, and 96 h in the presence or absence of erythromycin (10 µg/mL; Pfizer), doxycycline (4 µg/mL; Abbott), or gentamycin (10 µg/mL; Dakota Pharm). Doxycycline and gentamycin were used as controls in the endothelial cell proliferation assay.

**Endothelial cell proliferation assay.** Proliferation of endothelial cells was determined by direct counting (using a hemacytometer) of trypsinized cells and by trypan blue dye exclusion.

**Statistical analysis.** Results were expressed as means ± SDs. Quantitative data were compared by the Mann-Whitney U test. Differences were considered to be significant when P<.05.

**RESULTS**

**Inhibition of B. quintana–induced endothelial cell proliferation by erythromycin.** Monolayers of HMEC-1 cells were incubated with the erythromycin-sensitive B. quintana strain, and endothelial cell proliferation was assessed by microscopic enumeration. In the absence of B. quintana, the number of HMEC-1 cells increased after 48 h and, at 96 h, reached a level that was 3 times higher than the initial level. In the presence of B. quintana, the number of HMEC-1 cells was already significantly higher by 24 h and steadily increased during the 96-h culture. At 96 h, the number of HMEC-1 cells had increased >5-fold relative to the initial number and was nearly twice that observed in the absence of bacteria (figure 1). From 24 to 96 h, the number of HMEC-1 cells was significantly higher (2-fold) in the presence of B. quintana than in their absence (P<.01 to P<.001). Heat-killed B. quintana had a similar effect on the proliferation of HMEC-1 cells (figure 1). The addition of erythromycin to the monolayer completely abolished the B. quintana–induced proliferation. Hence, the numbers of HMEC-1 cells at 48, 72, and 96 h were not significantly different from those for HMEC-1 cells cultured alone (figure 1). Erythromycin also significantly reduced spontaneous HMEC-1 cell proliferation, suggesting a dissociation between the antibiotic effects and the antiendothelial growth effects of the drug.

**Generation of an erythromycin-resistant B. quintana mutant.** We wondered whether the erythromycin-induced down-modulation of endothelial cell proliferation occurred irrespective of the drug’s bacteriostatic effect. For that purpose, we generated an erythromycin-resistant B. quintana mutant. After 16 subcultures (8 months) of the parental strain, Oklahoma, on blood agar plates containing a disk of erythromycin (15 µg) (figure 2A), a mutant resistant to erythromycin and related antibiotics was obtained. After each subculture, the diameter of the zone of inhibition around the disk of erythromycin slowly decreased, from 86 to 17 mm (the lower critical concentration as determined by the Clinical and Laboratory Standards Institute [formerly known as the NCCLS]) over the 8 months. After the 16th passage, the strain suddenly became completely resistant to erythromycin, with growth directly in contact with the disk. The erythromycin-selected mutant was found to be completely resistant to erythromycin both by disk diffusion assay (diameter, <6 mm) and by Etest (MIC, 64 mg/L).

Sequences of the 23S rRNA gene of the parental strain, Oklahoma; the erythromycin-resistant strain; and the strain Toulouse did not display any nucleotide differences at positions 754, 2057, 2058, 2059, and 2611 (E. coli numbering known to confer resistance to erythromycin [15]). The nucleotide sequences of the gene encoding L22 were exactly the same for the 3 strains. Conversely, the erythromycin-resistant strain, ob-
Figure 2. Generation of the erythromycin-resistant mutant of Bartonella quintana. A, Kinetics of the decrease in the diameter of the zone of inhibition around the disk of erythromycin during the selection of the mutant strain of B. quintana by culture on blood agar plates. B, Comparison of deduced amino acid sequences of the conserved portion of the L4 gene from the parental (susceptible) and resistant strain of B. quintana. A portion of the L4 sequence, from aa 63 to aa 109 of Streptococcus pneumoniae, was aligned for comparison. Insertion of the 9 aa between aa R71 and aa A72 is indicated in boldfaced italics. The highly conserved region in S. pneumoniae is indicated in bold and is underscored.

To confirm this dissociation, we then assessed the effect of erythromycin on B. quintana replication. We quantified, by real-time PCR, the number of bacterial DNA copies at 72 h in HMEC-1 cells proliferating under stimulation with the erythromycin-sensitive B. quintana strain or the erythromycin-resistant mutant. The replication of the erythromycin-resistant strain was slower than that of the sensitive strain. Indeed, whereas the sensitive-strain DNA copy number reached 15,000 copies, only 6000 DNA copies of the resistant strain were detected (figure 3B). Although the number of DNA copies from the resistant strain was similar in the presence or absence of erythromycin, the number of DNA copies from the sensitive strain was significantly decreased in the presence of erythromycin (figure 3B).

Effect of other antibiotics on endothelial cell proliferation in the absence or presence of B. quintana. We wondered whether the erythromycin-mediated inhibition of endothelial cell proliferation was specific to the drug. HMEC-1 cells were, therefore, cultured with B. quintana in the presence or absence of erythromycin, gentamycin, and doxycycline. Only erythro-
Figure 3. Endothelial cell proliferation in response to the erythromycin-resistant *Bartonella quintana* mutant and to erythromycin. HMEC-1 cells were enumerated after 72 h of culture with either medium alone (−), erythromycin-sensitive wild-type *B. quintana* (ES Bq WT), the erythromycin-resistant *B. quintana* mutant (ER Bq mut), erythromycin (Ery), or combinations. A, Endothelial cell proliferation. B, Quantification of bacterial DNA copies per well. Results are the means ± SDs of 5 experiments.

Erythromycin was able to reduce both spontaneous and *B. quintana*-induced HMEC-1 cell proliferation (figure 4A and 4B). In the presence of gentamycin or doxycycline, HMEC-1 cell proliferation remained similar to that found when the cells were cultured alone, indicating that, among these antibiotics, the inhibition of endothelial cell proliferation is specific to erythromycin. In the supernatants of resting or *B. quintana*-stimulated HMEC-1 cell cultures, VEGF concentrations remained under the limit of detection, 5 pg/mL (data not shown).

**DISCUSSION**

In the present study, we have shown that erythromycin is able to abolish the proliferative effect that *B. quintana* has on microvascular endothelial cells of dermal origin. This was seen both with wild-type *B. quintana* and with a novel erythromycin-resistant mutant. The precise enumeration of bacterial DNA copies indicated that erythromycin impeded endothelial cell proliferation irrespective of its bacteriostatic effect. Interestingly, the proliferation of endothelial cells induced by the erythromycin-resistant mutant was lower than that induced by the wild-type strain. The fitness of the erythromycin-resistant mutant for the endothelial cell may be changed over time, given that this mutant was obtained after 16 passages. Indeed, these multiple passages may initiate such mutations as loss of pilus expression (which has been observed for *B. henselae* on epithelial cells after being passaged in vitro [20]) and loss of non-fimbrial adhesin expression (such as *Bartonella* adhesin A, which is known to be involved in the adhesion and proliferation of endothelial cells [21]). Nevertheless, our *B. quintana* parent strain had already been passaged >100 times in our laboratory before antibiotic selection, and it is known that *B. quintana* does not significantly adhere to Hep-2 cells and does not express pili [20]. Conversely, we believe that the slower rate of growth of our erythromycin-resistant mutant (70% reduction) was connected to the major L4 modification and may account for this lesser proliferation of endothelial cells. Mutations in genes for ribosomal proteins have been previously shown to alter the rate of growth of bacteria [22]. This hypothesis is supported by the description of 2 erythromycin-resistant *S. pneumoniae* isolates, one containing a 6-bp insertion and the other containing an 18-bp insertion in the highly conserved region of the L4 gene, that respectively displayed a 60% increase and an 80% increase in generation time, compared with that of their isogenic parent [23, 24].

*Bartonella* infections are characterized by a high frequency of relapses after brief courses of antibiotic therapy. It is to be noted that, although *Bartonella* species are highly susceptible to antibiotics, only the aminoglycosides have proved to be bactericidal in vitro [25]. However, erythromycin is the treatment of choice for BA, even though this antibiotic is only bacteriostatic [12]. This led us to evaluate the possibility that erythromycin could also act on the *B. quintana*-induced proliferation of endothelial cells. Some antibiotics are known to have the potential to down-modulate angiogenesis. The first observation involved an *in vitro* model using corneal endothelium [26]. The concept of pathological angiogenesis originated from the ophthalmologist Michaelson and dates to the 1940s. Such antibiotics as cefuroxime, dicloxacillin, and erythromycin inhibit DNA and protein synthesis in bovine endothelial cells [27]. Interestingly, in our model, erythromycin clearly displayed an antiproliferative effect, whereas other antibiotics did not (figure 4). Macrolides have a particular tropism for endothelial
cells, as has been shown in the context of tumor angiogenesis with clarithromycin [28] and roxythromycin [29, 30]. Some of the macrolides have been proposed to be antiangiogenic compounds, particularly 14-membered ring macrolides, including erythromycin, roxithromycin, and clarithromycin, which have immunomodulating or anti-inflammatory effects in a variety of settings [13]. Indeed, not only can erythromycin reduce the proliferation of endothelial cells, it can also reduce its adhesive properties for neutrophils [31]. Besides, roxithromycin can down-regulate the cytokine-induced expression of endothelial leukocyte adhesion molecule (E-selectin) and intracellular adhesion molecule 1 on endothelial cells of the dermal microvasculature [32]. The macrolide azithromycin can more widely affect endothelial cell functions in patients with chlamydial infection [33, 34]. Aside from antibacterial agents, antimalarial compounds (such as chloroquine and artesunate) have also been shown to have antiproliferative effects on endothelial cells [35]. The results we present here suggest that the antiangiogenic effect of erythromycin, in combination with its anti-inflammatory effect, could explain why this antibiotic can induce such a dramatic response when therapy is initiated in patients with BA [12]. Along similar lines, it has been demonstrated that clarithromycin significantly reduces the incidence of HIV-related neoplastic diseases [36].

In the erythromycin-resistant mutant we generated, the difference in susceptibility to erythromycin was not due to either a substitution or a deletion in the 23S rRNA gene or the L22 gene. Compared with the parental strain of B. quintana, among the previously identified gene targets for erythromycin, we found that the resistant strain harbored a 27-bp insertion in the highly conserved region of the L4 gene, resulting in a 9-aa repeat. We believe that this insertion in the highly conserved region of the L4 gene may explain the crude appearance of the resistance to erythromycin. Furthermore, this mutation was stable—it was maintained after 10 culture passages without erythromycin. To the best of our knowledge, such a molecular mechanism of resistance has never been reported in clinical isolates or laboratory mutants of Bartonella species. The repeated insertion found in our resistant mutant may have resulted from a slippage of the DNA polymerase, because the preceding nucleotides are identical to those of the insertion previously reported for Mycoplasma pneumoniae [37]. Similarly, it has been demonstrated that an erythromycin-resistant clinical strain of S. pneumoniae isolated in Canada contained an 18-bp insertion, resulting in the addition of 6 aa in this highly conserved region [23]. Therefore, we compared this sequence to that of our resistant mutant. The repeated insertion of 6 aa in this conserved region also conferred resistance to telithromycin [23], as in our resistant B. quintana mutant (J.-M.R. and D.R., unpublished data).

The present study suggests the possibility that 14-membered ring macrolides might be considered for future clinical development as antiangiogenic drugs. Further studies are needed to better understand the mechanism by which these drugs inhibit angiogenesis. In the future, our laboratory-derived B. quintana mutant may play a useful role in angiogenesis models and help.
to clarify the pathophysiology and treatment of *B. quintana* infection. In particular, our mutant may be useful in the establishment of an animal model of BA, such as the one recently reported [38].

**Acknowledgment**

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**References**