Editor’s Choice

Invasion of endothelial cells and arthritogenic potential of endocarditis-associated Corynebacterium diphtheriae

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Although infection by Corynebacterium diphtheriae is a model of extracellular mucosal pathogenesis, different clones have been also associated with invasive infections such as sepsis, endocarditis, septic arthritis and osteomyelitis. The mechanisms that promote C. diphtheriae infection and haematogenic dissemination need further investigation. In this study we evaluated the association and invasion mechanisms with human umbilical vein endothelial cells (HUVECs) and experimental arthritis in mice of endocarditis-associated strains and control non-invasive strains. C. diphtheriae strains were able to adhere to and invade HUVECs at different levels. The endocarditis-associated strains displayed an aggregative adherence pattern and a higher number of internalized viable cells in HUVECs. Transmission electron microscopy (TEM) analysis revealed intracellular bacteria free in the cytoplasm and/or contained in a host-membrane-confined compartment as single micro-organisms. Data showed bacterial internalization dependent on microfilament and microtubule stability and involvement of protein phosphorylation in the HUVEC signalling pathway. A high number of affected joints and high arthritis index in addition to the histopathological features indicated a strain-dependent ability of C. diphtheriae to cause severe polyarthritis. A correlation between the arthritis index and increased systemic levels of IL-6 and TNF-α was observed for endocarditis-associated strains. In conclusion, higher incidence of potential mechanisms by which C. diphtheriae may access the bloodstream through the endothelial barrier and stimulate the production of pro-inflammatory cytokines such as IL-6 and TNF-α, in addition to the ability to affect the joints and induce arthritis through haematogenic spread are thought to be related to the pathogenesis of endocarditis-associated strains.

INTRODUCTION

Corynebacterium diphtheriae is the causative agent of diphtheria, a toxemic localized infection of the respiratory tract. Furthermore, C. diphtheriae is not only the aetiologic agent of diphtheria, but can cause other infections as well (Ott et al., 2010). Systemic infections caused by
C. diphtheriae (Patey et al., 1997; Mattos-Guarald & Formiga, 1998) suggest that diphtheria bacilli are able to penetrate respiratory epithelial cells and gain access to deeper tissues (Mattos-Guarald & Formiga, 1998; Hirata et al., 2004). Bacteraemia and endocarditis caused by both non-toxigenic and toxigenic C. diphtheriae strains have been reported with increased frequency (Hirata et al., 2004; Bertucetti et al., 2004). Endocarditis lethality due to non-toxigenic C. diphtheriae has been shown to be 64% and 19% for C. diphtheriae subsp. mitis and C. diphtheriae subsp. gravis, respectively (Bertucetti et al., 2004). Infective endocarditis due to C. diphtheriae may occur (37%) in patients with normal cardiac valves or without any known risk factors and on occasion may be an aggressive disease. Non-toxigenic strains seem to have a preference for left-side valves and tend to form large valvular vegetations, septic emboli and aneurysms (Belko et al., 1998). Septic arthritis caused by non-toxigenic C. diphtheriae has also been described (Guran et al., 1979; Appelbaum & Dossett, 1982; Tiley et al., 1993; Damade et al., 1993; Barakett et al., 1997; Patey et al., 1997; Puliti et al., 2006; Hirata et al., 2008). In a study conducted by Tiley et al. (1993), 50% of patients developed septic arthritis as a complication of endocarditis due to non-toxigenic C. diphtheriae subsp. gravis. Osteoarticular involvement due to non-toxigenic C. diphtheriae subsp. mitis infection was also observed in between 27.5–40.0% of patients (Tiley et al., 1993).

Internalization or translocation through endothelial cells results in invasion of the vascular system by human pathogens (Mishra et al., 2005). The role of endothelial cells in the pathogenesis of invasive and localized C. diphtheriae infections has not been documented until now. The main objective of the present study was to investigate the in vitro association and invasion mechanisms of C. diphtheriae with human umbilical vein endothelial cells (HUVECs) in addition to the arthritogenic potential observed using conventional Swiss Webster (SW) mice intravenously (i.v.) injected with C. diphtheriae subsp. mitis endocarditis-associated (HC01 and HC02) invasive strains and non-invasive (non-toxigenic type ATCC 27010 and homologous toxicigenic ATCC 27012) strains.

**METHODS**

**Bacterial strains and growth conditions.** Clinical and microbiological characteristics of partially studied endocarditis-associated (HC01 and HC02) C. diphtheriae subsp. mitis blood isolates evaluated in this study are displayed in Table 1: non-toxigenic ATCC 27010 (C7s (−) tox; NCTC 11397) type strain and the homologous toxicigenic ATCC 27012 strain from the American Type Culture Collection were also included in the study. The non-adherent and non-virulent DH5-α Escherichia coli strain was used as negative control in a cytoskeleton mobilization test. Stock cultures in 10% skimmed milk with 25% glycerol added were maintained at −70 °C and recovered as required by cultivation in trypticase soy broth (TSB; Difco; Hirata et al., 2002; Santos et al., 2005).

**Human umbilical vein endothelial cell culture.** Primary human umbilical vein endothelial cells (HUVECs) were obtained by treatment of umbilical veins with 0.1% collagenase IV solution (Sigma) as previously described (Menzies & Kourteva, 2000; Cossart & Sansonetti, 2004). HUVECs were maintained in 199 medium (M199)/HEPES (Sigma), supplemented with 20% FCS, 2 mM glutamine, 50 µg gentamicin ml⁻¹ and 250 µg amphotericin ml⁻¹ at 37 °C in a humidified 5% CO₂ atmosphere until monolayers reached confluence. Cells were cultured in 24-well tissue culture plates and only cells collected after the first or second passage were assayed. Confluent cultures were treated with 0.025% trypsin/0.2% EDTA solution prepared in Dulbecco’s mineral salt solution (PBSS), rinsed in serum-depleted culture medium, and used for the experiments.

**Bacterial adherence and intracellular viability assays.** Aliquots (500 µl) of bacterial suspensions in a concentration of 10⁷ c.f.u. ml⁻¹ were used to infect HUVEC monolayers grown to about 95% confluence on 24-well tissue culture plates (approximate m.o.i. of 100 : 1). After incubation periods of 0, 30, 60, 120, 360 min and 24 h, infected HUVECs were washed six times with PBSS, lysed with 0.1% Triton X-100 (Sigma) in PBSS (PBSS-T), diluted and cultured on trypticase soy agar (TSA; Difco) at 37 °C, 24 or 48 h for bacterial viable counting (c.f.u. ml⁻¹). Bacterial inoculum corresponded to the number of viable bacterial cells in supernatant plus the number of viable bacteria associated with HUVEC monolayers (intracellular plus extracellular; Hirata et al., 2002). The total number of cell-associated bacteria (intracellular plus extracellular) was expressed as the percentage of the inoculum recovered from monolayers after 3 h incubation.

For bright-field microscopic analysis, bacterial infection was performed in semi-confluent HUVEC monolayers grown on circular coverslips (13 mm diameter). Following a 180 min incubation, infected cells were washed three times with PBSS, fixed with methanol and stained by Giemsa (Hirata et al., 2004).

The ability of C. diphtheriae to enter HUVECs was assessed by the gentamicin protection assay as previously described (Hirata et al., 2002). Briefly, cells were gently washed five times with PBSS and subsequently incubated with 500 µl M199 medium containing gentamicin (150 µg ml⁻¹) and incubated for 1 h at 37 °C. The wells were then washed three times with PBSS. Cell lysis was carried out as described above and the lysates (100 µl) from each well were diluted and plated onto TSA-agar for determination of viable intracellular bacteria. The number of intracellular bacteria was determined by viable counts after lysis of gentamicin-pretreated HUVEC monolayers with 0.5 ml 0.1% Triton X-100 in PBS. The percentage of intracellular bacteria was deduced from HUVEC-associated bacteria. All assays were performed in triplicate and repeated at least three times.

**FITC-labelled phalloidin assay (FAS test).** Infected semiconfluent HUVECs monolayers (3 h) were rinsed six times with PBS, fixed with 3% formaldehyde for 15 min, and then treated with PBSS-T for 10 min. Monolayers were washed three times with PBS and treated with 5 µM fluorescein conjugated phalloidin ml⁻¹ (Sigma) for 30 min. FAS test results were considered positive when foci of intense fluorescence corresponded to areas of bacterial adherence observed under phase-contrast microscopy (Hirata et al., 2002). The control strains E. coli E2348/69 (FAS-positive) and E. coli DH5-α (FAS-negative) were included in this study. The experiments were performed in triplicate.

**Treatment of HUVECs with cytochalasin E, colchicine and genistein.** Final concentrations of inhibitors and solvents were used as follows: 5 µM cytochalasin E in DMSO; 2 µg colchicine in 70% ethanol; 100 µM genistein in DMSO. Monolayers were pre-incubated with each inhibitor at 37 °C for 30 min prior to bacterial infection as described above. All chemicals were purchased from Sigma (von Hulosten et al., 2003). None of the inhibitors tested affected either bacterial viability or growth (as assessed by standard counts of viable
Table 1. Clinical and microbiological aspects of endocarditis-associated *Corynebacterium diphtheriae* subsp. *mitis* strains evaluated in this study.

Both strains exhibited an aggregative adherence pattern in HUVEC, similar to those observed in human endothelial (Hep-2) cells (Hirata *et al.*, 2008). ND, not determined; F, female; HA, agglutination of human B erythrocytes; A%, percentage of viable adherent bacteria; I%, percentage of intracellular viability.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gender/ age (y)</th>
<th>Arthritis/other complications</th>
<th>Prior heart defect</th>
<th>Outcome</th>
<th>Tox gene*</th>
<th>Biofilm formation on abiotic surface†</th>
<th>HA Hep-2 cells (3 h incubation)</th>
<th>HUVEC cells (3 h incubation)</th>
<th>SW mice i.v. infection (3 days after infection)</th>
<th>Arthritis index</th>
<th>Infected mice with arthritis clinical signs (%)</th>
<th>IL-6/ TNF-α plasma levels (pg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC01</td>
<td>F/14</td>
<td>ND/Peripheral emboli of legs and arms; microaneurism with brain haemorrhage</td>
<td>No</td>
<td>Death</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>ND/ND</td>
<td>3.5</td>
<td>ND/ND</td>
<td>86.66 / 115.6 (P=0.001)</td>
</tr>
<tr>
<td>HC02</td>
<td>F/9</td>
<td>Arthritis/Myositis; valvar abscess; acute renal failure</td>
<td>No</td>
<td>Valve replacement</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>35.40</td>
<td>0.06</td>
<td>1.5</td>
<td>50.00</td>
<td>75.97 / 79.75 (P&lt;0.001)</td>
</tr>
</tbody>
</table>

*Detected by Multiplex PCR (Pimenta *et al.*, 2008).
†Polystyrene (negatively charged).
bacteria), or affected HUVEC viability (as assessed by Trypan blue staining) or morphology.

**Transmission electron microscopy (TEM).** Infected monolayers were rinsed and fixed with 0.1 M cacodylate buffer pH 7.2 containing 2 % glutaraldehyde, 4 % paraformaldehyde, 5 mM CaCl₂ for 1 h at 4 °C. Monolayers were post-fixed with 1 % OsO₄, 5 mM CaCl₂ and 0.8 % K₄[Fe(CN)₆] in cacodylate buffer for 1 h, 22 °C, and dehydrated through a graded ethanol series. They were then embedded in Epon, thin sectioned, and examined with an EM 906 Zeiss transmission electron microscope (Zeiss; Hirata et al., 2002).

**Experimental model of bacterial infectious arthritis in mice.** Conventional healthy SW mice, which were sex-matched and weighed 18–22 g, from CAECAL-FIOCRUZ were anaesthetized with intraperitoneal injection of ketamin (50 mg kg⁻¹). The study was performed in compliance with guidelines outlined in the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for the International Organizations of Medical Sciences and with the Brazilian government’s ethical guidelines for research involving animals (Fiocruz Ethic Committee for Animal Experiments – CEUA/FIOCRUZ – L-034/07). For clinical evaluation of arthritis and histological assessment, induced- septic arthritis experiments were carried out in triplicate based on methods previously described (Puliti et al., 2000). Mice injected i.v. with 4 × 10⁶ ml⁻¹ of tox+ or tox- C. diphtheriae strains were evaluated daily for up to 1 month for signs of arthritis, in particular for the presence of joint inflammation, and scored for arthritis severity (macroscopic score). Arthritis was defined as visible erythema and/or swelling of at least one joint. To evaluate the intensity of arthritis, the following clinical scoring (arthritic index) was used for each limb: 1 point, mild swelling and erythema; 2 points, moderate swelling and erythema; 3 points, marked swelling, erythema, and/or ankylosis. Thus, a mouse could have a maximum score of 12. The arthritis index (mean ± SD) was constructed by dividing the total score (cumulative value of all paws) by the number of animals in each experimental group.

Histopathological procedures to evaluate the features of the disease were based on methods previously described (Dias et al., 2011). Briefly, mice inoculated i.v. with non-toxigenic C. diphtheriae were sacrificed after 9 days. Control mice groups were infected with saline. Arthritic paws (one per mouse) were removed aseptically, fixed with 10 %, v/v, formalin for 24 h and then decalcified in 10 % EDTA in phosphate buffer 100 mM for 7 days. Subsequently, the specimens were dehydrated, embedded in paraffin, sectioned at 5–7 μm and stained with haematoxylin and eosin. Joints were examined for synovitis (defined as synovial membrane thickness of more than two cell layers), extent of infiltrate (presence of inflammatory cells in the subcutaneous and/or periarticular tissues), cartilage damage, bone erosion and loss of joint architecture. Arthritis severity was classified as mild (mild synovial hypertrophy and minimal infiltrate), moderate (moderate synovial hypertrophy, presence of infiltrate, minimal exudate, integrity of joint architecture) and severe (marked synovial hypertrophy, presence of massive infiltrate/exudate, cartilage and bone erosion and disrupted joint architecture) (Dias et al., 2011).

**Serum sample preparation and evaluation of cytokine concentration.** Blood samples from C. diphtheriae-infected mice were obtained by retro-orbital sinus bleeding at different time points (day 0, 1, 3, 15 and 20). They were left for 30 min at room temperature to clot and then were centrifuged at 2000 g for 10 min. Sera were collected and stored at −80 °C until analysed. The levels of IL-6 and TNF-3 in the serum samples were determined by using specific ELISA kits according to the manufacturer’s instructions (R&D Systems). The results were expressed as the concentration of cytokine as pg (ml serum)⁻¹, as extrapolated from a standard curve constructed using recombinant cytokine (Dias et al., 2011).

**Statistical analysis.** All assays were performed in triplicate and repeated at least three times. Results are presented as the mean ± SD. One way ANOVA analysis with Tukey test was used to compare means of columns, with P-value <0.05 considered significant (dos Santos et al., 2010).

**RESULTS**

**Adherence and entry of C. diphtheriae strains into HUVECs**

Percentages of both viable cell-associated bacteria (extracellular and intracellular) and intracellular bacteria at different periods of incubation (non-toxigenic ATCC 27010 type strain and endocarditis-associated HC01 strain) are displayed in Table 2. Data indicated that C. diphtheriae isolates were able to adhere to and survive within HUVECs in varied periods of incubation and at different levels. Viable associated and internalized bacteria were detected immediately post-infection of monolayers with the HC01 strain and ATCC 27010 strain, respectively. The non-toxigenic ATCC 27010 strain was the only one capable of survival (0.45 %) within the intracellular compartment 24 h post-infection.

All C. diphtheriae strains showed a maximum level of ability to enter and survive within HUVECs at 3 h post-infection. A higher ability to enter and survive within endothelial cells was observed for the endocarditis-associated HC01 strain when compared to the ATCC 27010 type strain (P<0.05). Interaction with HUVECs was higher for the non-toxigenic HC01 (51.80 %) and HC02 (35.40 %) blood/endocarditis isolates.

After reaching the highest level of interaction, adherence seemed independent of toxin production, as evidenced by comparison of the adherence rates of the non-toxigenic ATCC 27010 type strain (30.80 %) and the toxigenic homologous strain (22.60 %; P>0.05). The invasion rates of the non-toxigenic ATCC 27010 type strain (0.92 %) and the toxigenic homologous ATCC 27012 strain (1.33 %; P=0.0012) 3 h post-infection suggested an influence of tox gene in the internalization process by HUVECs. However, the non-toxigenic HC01 endocarditis-associated isolate demonstrated the highest number of viable internalized bacteria followed by the toxigenic ATCC 27012 isolate (1.79 % and 1.33 %, respectively; P=0.0009). Intracellular viability was lower (0.06 %) for the HC02 blood/endocarditis isolate.

The non-toxigenic HC01 blood/endocarditis and ATCC 27010 strains demonstrated the highest mean level of adherent (31.16 % and 16.80 %, respectively) and internalized (0.58 % and 0.40 %, respectively; P=0.0003) viable bacteria (Table 2).

Since the maximum interaction occurred at about 3 h post-infection for the C. diphtheriae strains tested, this incubation period was chosen for further investigation into the mechanisms of bacterial adherence and internalization within endothelial cells (Table 1; Figs 1–4).
Table 2. Percentage of viable bacterial cells associated to (A%) and internalized by (%) HUVECs at different periods post-infection of non-toxigenic Corynebacterium diphtheriae strains. Results are presented as the mean ± SD.

<table>
<thead>
<tr>
<th>Infection period (h)</th>
<th>Control</th>
<th>C. diphtheriae strains</th>
<th>Endocarditis-associated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATCC 27010</td>
<td>HC01</td>
<td>HC02</td>
</tr>
<tr>
<td>A%</td>
<td>1%</td>
<td>A%</td>
<td>1%</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.11 ± 0.00</td>
<td>0.60 ± 0.59</td>
</tr>
<tr>
<td>0.5</td>
<td>7.70 ± 0.50</td>
<td>0.15 ± 0.03</td>
<td>23.30 ± 0.82</td>
</tr>
<tr>
<td>1</td>
<td>17.77 ± 0.50</td>
<td>0.34 ± 0.08</td>
<td>34.30 ± 9.02</td>
</tr>
<tr>
<td>2</td>
<td>28.70 ± 1.00</td>
<td>0.41 ± 0.04</td>
<td>44.40 ± 6.85</td>
</tr>
<tr>
<td>3</td>
<td>30.80 ± 13.74*</td>
<td>0.92 ± 0.01</td>
<td>51.80 ± 9.55</td>
</tr>
<tr>
<td>24</td>
<td>15.90 ± 0.60</td>
<td>0.45 ± 0.09</td>
<td>32.60 ± 1.26</td>
</tr>
<tr>
<td>Mean value</td>
<td>16.80</td>
<td>0.40</td>
<td>31.16</td>
</tr>
</tbody>
</table>

*Comparative analysis between data presenting statistically significant differences with P<0.05.

Microscopic examination of infected endothelial cells
Light micrographs demonstrating the aggregative adherence pattern in HUVECs, characterized by clumps of bacteria with a ‘stacked-brick’ appearance of endocarditis-associated C. diphtheriae subsp. mitis HC01 and HC02 blood isolates (3 h incubation) are displayed in Fig. 1a and 1b, respectively. TEM assays illustrated the close association between bacteria and endothelial cell surfaces for both non-toxigenic ATCC 27010 and endocarditis-associated HC01 C. diphtheriae strains after 3 h of incubation (Fig. 1c, d). Micro-organisms were found attached by focal linkages to HUVEC membranes (Fig. 1d). Membrane invagination of the host cell at the site of bacterial attachment is shown in Figs. 1e–h. Fibrillar-like structures were visible on bacterial surfaces during the internalization process (Fig. 1f–h). Internalized bacteria were also found in a host-membrane-confined compartment as a single micro-organism (Fig. 1g, h) and/or free in the cytoplasm (Fig. 1i, j).

FAS test
Fluorescence and phase-contrast microscopy showed positive FAS tests of invasive C. diphtheriae subsp. mitis (HC01 and HC02) strains isolated from the blood of patients with endocarditis, the toxicogenic C. diphtheriae strain ATCC 27012 and the ATCC 27010 non-toxicogenic type strain. Cytoskeletal changes were observed directly beneath the adherent bacteria with accumulation of polymerized actin. Foci of intense fluorescence corresponded to areas of bacterial adherence observed under phase-contrast microscopy (Fig. 2).

The effects of eukaryotic inhibitors on the C. diphtheriae internalization process
Analysis of the effect of cytochalasin E (a known inhibitor of eukaryotic microfilament formation) showed a complete inhibition of bacterial internalization for all four C. diphtheriae (HC01, HC02, ATCC 27010 and ATCC 27012) strains tested. To investigate whether the invasion process involved actin polymerization via a phosphotyrosine-signalling cascade, bacterial interaction with HUVECs was evaluated in the presence of genistein, a tyrosine kinase inhibitor. This showed a reduction of bacterial internalization to ≤0.10% (P<0.05) for all strains tested. Pretreatment of HUVECs with the microtubule-disrupting drug colchicine also caused a significant reduction of internalization to ≤0.15% (P<0.05) of all C. diphtheriae strains tested.

Arthritogenicity and histopathological findings
SW mice were infected with invasive (HC01, HC02) or non-invasive (ATCC 27010 and ATCC 27012) C. diphtheriae strains. Viable bacteria were recovered from joints, blood, kidneys, liver, and spleen but not from the heart and lungs of mice. Data displayed in Fig. 3a, b showed that non-toxigenic C. diphtheriae strains were capable of inducing arthritis through haematogenic spread at different levels. The invasive (HC01, HC02) strains were arthritogenic at a dose of 4 x 10^8 c.f.u. per mouse. The highest percentages (85.42%) of clinical signs of arthritis in affected mice (arthritis index) were observed for the endocarditis-associated HC01 (85.42%) and HC02 (50%) strains (Fig. 3a, b; Table 1). Arthritis index, indicating the clinical severity of septic arthritis caused by C. diphtheriae, was graded on a scale of 0–3 for each paw, according to changes in erythema and swelling (0, no change; 1, mild swelling and/or erythema; 2, moderate swelling and erythema; 3, marked swelling, erythema and/or ankylosis; Fig. 3b). Clinical signs of arthritis were evident, starting 3 days after i.v. injection. The most frequently affected joints were ankle and wrist. To confirm clinical signs of arthritis, histopathological studies of joints of invasive non-toxigenic...
C. diphtheriae-infected mice were performed. Three affected paws were examined in each mouse group after 9 days of infection. The most severe histopathological features of arthritis in mice were caused by the HC01 strain, with subcutaneous oedema, inflammatory infiltrate and damage to bone tissue and synoviocyte hypertrophy (data not shown).

**Cytokine production during C. diphtheriae infection**

Cytokines appear to play a central role in the development of articular lesions in experimental models of septic arthritis. To assess the involvement of cytokines in C. diphtheriae-induced arthritis, systemic levels of production of IL-6 and TNF-α were investigated at different time points after infection using different strains. Time-course experiments showed that there was a positive correlation between IL-6 and TNF-α release and time after infection (Fig. 4). A significant (\(P < 0.001\)) increase of TNF-α (up to 116 pg ml\(^{-1}\)) and IL-6 (up to 134 pg ml\(^{-1}\)) production was evident on day 3 after infection. A progressive decrease was observed from these peaks until day 20. High systemic levels of IL-6 were found in the mice groups infected with the HC01 and HC02 invasive strains in comparison to the non-toxigenic ATCC 27010 and toxigenic ATCC 27012 strains after infection. The toxigenic ATCC27012 strain showed the lowest capacity to induce IL-6 and TNF-α after infection. A correlation between the arthritis index (Fig. 3b) and IL-6 and TNF-α production was also observed for all invasive C. diphtheriae strains (Fig. 4), where the HC01 strain had the highest arthritogenic potential and the ability to induce cytokine release after mice infection.
DISCUSSION

In addition to diphtheria, *C. diphtheriae* has been also associated with invasive infections such as sepsis and endocarditis (Mattos-Guaraldi et al., 2000). Bacteraemia followed by attachment of bacteria to an endocardial surface precedes the establishment of endocarditis (Plotkowski et al., 1994; Mishra et al., 2005). The fact that *C. diphtheriae* endocarditis involves normal heart valves in many instances underscores the importance of using viable undamaged endothelial cell surfaces as the substrate in experimental models (Ogawa et al., 1985). For this reason, confluent monolayers of HUVECs were used in tissue culture as an analogue of the host surface encountered in vivo. This is the first report on the adherence and intracellular survival within endothelial cells by *C. diphtheriae* strains. Presently, viable bacteria were found on the surface of endothelial cells for all strains tested. *C. diphtheriae* strains displayed various degrees of invasive properties.

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**Fig. 3.** Experimental model of bacterial infectious arthritis in mice. (a) Percentage of *C. diphtheriae*-infected SW mice exhibiting clinical signs of arthritis. Mice were challenged i.v. with $4 \times 10^8$ c.f.u. of endocarditis-associated HC01, HC02 strains and of ATCC 27010 and ATCC 27012 control strains. Results are means ± SD of three separate experiments; n, 15 mice per group. (b) Severity of septic arthritis in SW mice infected with $4 \times 10^8$ c.f.u. of different *C. diphtheriae* strains per mouse: the toxigenic (○) ATCC 27012 strain, the non-toxigenic homologous (●) ATCC 27010 strain and the endocarditis-associated (★) HC01 and (■) HC02 strains. Clinical severity of arthritis was graded on a scale of 0–3 for each paw, according to changes in erythema and swelling (0, no change; 1, mild swelling and/or erythema; 2, moderate swelling and erythema; 3, marked swelling, erythema and/or ankylosis. The arthritis index was constructed by dividing the total score (cumulative value of all paws) by the number of animals in each experimental group. Results are means ± SD of three separate experiments. P<0.05 Tukey test (one-way ANOVA) with Graphpad Prism; n, 15 mice per group.

**Fig. 4.** Induction of IL-6 and TNFα in SW mice exhibiting clinical signs of arthritis due to *C. diphtheriae* endocarditis-associated (■) HC01, (●) HC02 strains and control (●) 27010 and (○) 27012 strains. Cytokine levels were measured by ELISA and calculated as the means ± SD of three independent experiments; values are in pg (ml serum)$^{-1}$. P<0.05 Tukey test (one-way ANOVA) with Graphpad Prism; n, 5 mice per group per time point. So values, which were usually <10% have been omitted.
attachment and internalization to human endothelial cells indicating differences in the ability to cause invasive blood-borne infection. In fact, the HC01 and HC02 blood culture isolates which, by definition, have caused invasive blood-borne infection and therefore have had the potential to infect endothelial surfaces, consistently adhered most efficiently to HUVECs, with similar adherence patterns observed to HEp-2 cells (Hirata et al., 2008). Data suggest that potential adherence to normal endothelium may help to elucidate the ability of C. diphtheriae to cause endocarditis, as for other Gram-positive pathogens, including Staphylococcus aureus, Streptococcus agalactiae, Streptococcus sanguis and Listeria monocytogenes (Vercellotti et al., 1984; Gibson et al., 1993; Drevets, 1998; Matussek et al., 2005).

Invasion is a common strategy of pathogens to establish a privileged niche in the host. Earlier investigations showed the ability of both toxigenic and non-toxigenic clinical C. diphtheriae isolates to enter and survive within cultured respiratory epithelial cells (Plotkowski et al., 1994; Bertuccini et al., 2004). The ability of C. diphtheriae to survive intracellularly would benefit the bacterium, as it would be protected against host immune control mechanisms and many antimicrobial agents. It is likely that internalization and intracellular survival of C. diphtheriae is involved in throat colonization, thus contributing to the failure of bacterial eradication and asymptomatic carriage.

Several bacterial species are internalized by endothelial cells, even though these cells are not normally considered phagocytic (Finlay & Falkow, 1997). In the present study, it was demonstrated that C. diphtheriae also has the ability to enter and survive within endothelial cells at a variety of levels. Although some C. diphtheriae strains seem to have a preference for attaching to vascular endothelium, microorganisms (HC01 and HC02 strains) that have caused invasive blood-born infection also differed in their potential to infect HUVECs: the HC01 strain was found to be more adherent and invasive to HUVECs than the HC02 strain. This fact may help to explain the aggressive nature of the disease leading to death or valve replacement in patients with endocarditis despite antimicrobial therapy in many instances, including in cases related with HC01 and HC02 strains, respectively (Mattos-Guaraldi & Formiga, 1998; Belko et al., 2000).

This current study showed that non-toxigenic strains were highly adherent to and invasive into HUVECs. Furthermore, live intracellular bacterial cells detectable 24 h post-infection were observed only for the non-toxigenic ATCC 27010 type strain. These data together strengthen clinical findings showing invasive infections caused by non-toxigenic C. diphtheriae strains (Patey et al., 1997; von Hunolstein et al., 2003).

Positive FAS test results indicated the involvement of host cell cytoskeletal rearrangements in C. diphtheriae uptake by endothelial cells. The internalization of C. diphtheriae by HUVECs was found to be an active process that can be blocked by the phagocytic inhibitor cytochalasin E. Actin polymerization along with phospho-tyrosine signalling events seems to be a mechanism required during the C. diphtheriae internalization process into HUVECs, as previously observed with human respiratory epithelial cells (Plotkowski et al., 1994; Bertuccini et al., 2004). Inhibition assays with colchicines suggested that there are either two pathways (microtubule dependent and microfilament dependent) or one complex pathway involving polymerization of microtubules and/or microfilaments in C. diphtheriae uptake by endothelial cells.

The mechanisms underlying bacterial entry, phagosome maturation, and dissemination reveal common strategies as well as unique tactics evolved by individual species to establish infection. Invasive bacteria actively induce their own uptake by phagocytosis in normally non-phagocytic cells and then either establish a protected niche within which they survive and replicate, or disseminate from cell to cell by means of an actin-based motility process (Cossart & Sansonetti, 2004). The C. diphtheriae internalization process in HUVECs appeared to proceed in a sequential fashion as it is observed with professional phagocytes. Bacterial adherence to the endothelial cell was followed by the appearance of endothelial appendages which elongated and enclosed the bacteria into apparent phagosomes. Previous studies showed a similar sequence of ultrastructural events for endothelial phagocytosis of Staphylococcus aureus (Menzies & Kourteva, 2000). Like Staphylococcus aureus (Menzies & Kourteva, 2000; Matussek et al., 2005), ultrastructural analysis of C. diphtheriae-infected HUVECs demonstrated intracellular bacteria free in the cytoplasm or within vacuoles. Cytoplasmic vacuolization and degradation suggest death of infected endothelial cells that would probably provide a route for the dissemination of C. diphtheriae to distant sites where metastatic infections could be established. A previous report showed the ability of these bacteria to cause apoptosis in human U-937 macrophages (dos Santos et al., 2010). Studies are in progress to elucidate the molecular mechanisms of C. diphtheriae-induced cell death in endothelial cells. Independently of toxin production, C. diphtheriae strains may have diverged regarding mechanisms of interaction with the endothelial barrier, possibly developing different pathways of entry. Differences in the ability of C. diphtheriae strains to adhere to HUVECs are suggestive of specific mechanisms for the binding of the pathogen to endothelial surfaces.

Active uptake of bacteria by endothelial cells may not only assist in C. diphtheriae virulence in endovascular infections, independently of toxin production, but can be important to establish metastatic infection, like polyarthritis.

In an attempt to better understand endocarditis-associated C. diphtheriae subsp. mitis pathogenesis, a bacterial arthritis infection experimental model which showed similarities with human disease (Goldenberg & Reed, 1985) was prepared using conventional SW mice injected i.v. with strains isolated from animals and humans. The current study using this experimental mouse model showed the ability of
invasive non-toxigenic *C. diphtheriae* strains to affect the joints and to induce arthritis through haematogenic spread. The joint histopathological analysis of the *C. diphtheriae*-infected mice (subcutaneous oedema, inflammatory infiltrate, damage to bone tissue and synovioyte hypertrophy) strongly demonstrated the arthritogenic potential of the invasive non-toxigenic *C. diphtheriae*. Nevertheless, no signs of arthritis were observed for the toxigenic *C. diphtheriae* ATCC27012, showing that the diphtheria toxin might not be crucial in arthritogenic pathogenesis. These data are in accordance with recently published studies also showing the arthritogenic potential of non-toxigenic *C. diphtheriae* strains.

The non-toxigenic *C. diphtheriae* strains were different in the incidence and the severity of arthritis. The appearance of arthritis lesions is undoubtedly a multifactorial process involving bacterial virulence factors and the host immune system (Puliti et al., 2006). To the best of our knowledge, this is the first report showing not only the ability of non-toxigenic *C. diphtheriae* strains isolated from patients with endocarditis and arthritis to invade and survive into endothelial cells, but to cause arthritis in a mouse model. Interestingly, osteoarthritis has been described as an important clinical feature in 27–50% of patients with *C. diphtheriae* systemic infection (Patey et al., 1997).

In conclusion, *C. diphtheriae* isolates behave differently when they meet host surfaces, with regard to the cells they efficiently infect and the kind of inflammatory response they trigger. Adherence to and internalization by endothelial cells may be critical steps during blood-barrier disruption and systemic dissemination of both non-toxigenic and toxigenic *C. diphtheriae* strains. In a similar way to *Staphylococcus aureus* (Vercellotti et al., 1984), the ability of *C. diphtheriae* to invade and survive within endothelial cells may also contribute to its propensity to cause persistent endovascular infection with endothelial destruction and metastatic infections. Furthermore, a model of *C. diphtheriae*-induced septic arthritis in SW conventional mice could be a useful tool in studies on the pathogenicity and characterization of virulence factors other than diphtheria toxin.

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