Providencia alcalifaciens strains translocate from the gastrointestinal tract and are resistant to lytic activity of serum complement

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The ability of Providencia alcalifaciens strains, isolated from patients with diarrhoeal disease, to translocate from the gastrointestinal tract and their resistance to serum complement lytic activity were investigated and compared with previously characterized differential invasive capabilities in HeLa cells. Translocation ability to several extraintestinal sites and resistance to lysis by human serum complement were observed in both highly invasive and non-invasive strains. These characteristics have not been previously described in P. alcalifaciens and their potential role in causing disseminated infections should therefore be considered.

INTRODUCTION

Providencia alcalifaciens is a member of the Enterobacteriaceae and has been implicated as an agent of diarrheal disease in children and adults (Haynes & Hawkey, 1989; Albert et al., 1992; Guth et al., 1996). Recently, a large outbreak of foodborne infection was attributed to P. alcalifaciens, providing definitive evidence of the role of this organism in causing gastroenteritis (Murata et al., 2001).

Several studies have shown that P. alcalifaciens is able to invade cultured epithelial cells (Albert et al., 1992; Guth et al., 1996; Janda et al., 1998); invasion was also observed in intestinal tissues by using an adult rabbit ileal loop model (Albert et al., 1992; Mathan et al., 1993).

Previous data from our laboratory showed that invasive P. alcalifaciens strains, although frequently found, did not predominate among isolates from patients with diarrhhea in São Paulo, Brazil, and that non-invasive P. alcalifaciens strains were also isolated from diarrheal stools in pure culture from which no other bacterial enteropathogens were identified (Guth et al., 1996). However, the presence of other virulence mechanisms and the role of non-invasive P. alcalifaciens strains as agents of disease have not been established.

Accumulating evidence has demonstrated that bacterial translocation, defined as the passage of viable indigenous bacteria from the gastrointestinal (GI) tract to the mesenteric lymph node (MLN) complex and other extraintestinal sites, is an important event in the pathogenesis of opportunistic infections that originate in the human GI tract. Several bacterial species, including members of the family Enterobacteriaceae, are likely to translocate from the GI tract in animal models (Berg, 1999; Sakamoto et al., 2000). Enterobacteriaceae are also the most common cause of infection in hospitalized patients (Sedman et al., 1994; Tani et al., 1997).

Although the mechanisms responsible for bacterial translocation have not been well-elucidated, several clinical conditions, as well as the presence of virulence-associated properties (including those that allow evasion of host defences), are all predisposing factors to bacterial translocation (Sedman et al., 1994; Tani et al., 1997).

The ability of P. alcalifaciens to translocate from the intestinal lumen to extraintestinal sites of the host, with possible aetiology for systemic infections, has not been studied. Thus, in the present study, we evaluate the capacity for translocation and resistance to lytic activity of serum complement of invasive and non-invasive P. alcalifaciens strains that were previously isolated from patients with diarrhea in our community.

METHODS

Bacterial strains. In total, 30 P. alcalifaciens strains were studied (Guth et al., 1996). Except for four strains that were isolated from asymptomatic patients, all other strains were isolated from diarrheal stools. Most strains were isolated in pure culture or from stool cultures in
which no other enteropathogenic bacteria were identified. Fifteen strains were able to invade HeLa cells; the other strains did not show this characteristic. Invasion assays in HeLa cells were performed as described previously (Guth et al., 1996), except that bacterial suspension (10⁹ c.f.u. ml⁻¹) was prepared and diluted 1/10 in Eagle’s Minimal Essential Medium (MEM; Sigma) supplemented with 10 % fetal bovine serum (FBS) and 2 % D-mannose. After 3 h incubation at 37°C, the bacterial suspension was removed, the cell monolayer was washed and reincubated for 1 h at 37°C in fresh MEM–FBS that contained 150 μg gentamicin ml⁻¹.

### Resistance to lytic activity of serum complement.

The turbidimetric assay described by Pelkonen & Finne (1987) was employed to test resistance of bacterial strains to serum bactericidal activity. Briefly, an overnight bacterial culture grown in Luria–Bertani (L)-broth was diluted 1/100 in fresh medium and grown until the exponential phase was reached. Bacterial cells were harvested and resuspended in L-broth to a concentration of ~10⁷ cells ml⁻¹ and 75 μl bacterial suspension was distributed between the microplate wells. A volume of a pool of sera, prepared from blood from healthy human volunteers, was added to each well to achieve final concentrations of 36 and 50 %. Each strain was tested in a separate experiment. The results are expressed as the percentage of surviving bacteria.

**Fig. 1.** Representative results on susceptibility to serum bactericidal activity of invasive and non-invasive *P. alcalifaciens* strains. (a) *P. alcalifaciens* 40448 (INV⁺); (b) *P. alcalifaciens* 2101–15 (INV⁻); (c) *P. alcalifaciens* 9938 (INV⁺); (d) *P. alcalifaciens* 0181–9 (INV⁻); (e) *Escherichia coli* K-12-711 (serum-sensitive); (f) *Enterobacter cloacae* 046 (serum-resistant). ▲, 50 % normal serum; ●, 36 % normal serum; ○, 50 % inactivated serum; ■, 36 % inactivated serum; △, bacterial suspension.
tested in triplicate with both normal and heat-inactivated (56 °C, 30 min) serum. The microplates were incubated at 37 °C and the growth ability of each strain was assessed by measurement of OD620 at 30 min intervals for 3 h. *Escherichia coli* strain K-12-711 and *Enterobacter cloacae* strain 046 (Keller et al., 1998) were used as negative and positive controls, respectively. *P. alcalifaciens* strains were considered to be resistant to the lytic activity of serum when they were able to grow equally in normal (complement-active) and heat-inactivated (complement-inactive) sera at both 36 and 50 °C.

**Bacterial translocation.** Four *P. alcalifaciens* strains that presented different invasive abilities and different resistance profiles to the lytic activity of serum were selected for the test. Translocation assays were performed as described by Koh & Silva (1996); each *P. alcalifaciens* strain was tested six times on different days, with a total of 24 assays. Briefly, Wistar-EPM rats were fasted for 2 days and given water *ad libitum*. All surgical procedures were performed under aseptic conditions and the animals received ether-inhalation anaesthesia. Following orocholedochal cannulation, 10 ml bacterial suspension in PBS, which contained $10^{10}$ c.f.u. ml$^{-1}$, was inoculated into the intestinal lumen between the duodenum and ileum. MLNs, spleen, liver and blood were collected 2 h after inoculation. Aliquots of minced organs were cultured in MacConkey’s agar at 37 °C for 24 h. Bacterial counts are expressed as log$_{10}$ c.f.u. g$^{-1}$.

Colonies were confirmed as inoculated bacteria by morphological characteristics and biochemical tests (Ewing, 1986). Enteric pathogens were classified as invasive or non-invasive strains (Cornaglia et al., 1995; Pinto et al., 1999), although *P. alcalifaciens* strains have also been isolated from patients with diarrhoea (Guth et al., 1996). However, the potential pathogenic role of these strains has not been described.

**RESULTS AND DISCUSSION**

The role of *P. alcalifaciens* as an enteric pathogen has been observed by several authors (Haynes & Hawkey, 1989; Guth et al., 1996; Murata et al., 2001) and as it has been reported that these bacteria can invade eukaryotic cell lines (Albert et al., 1992; Guth et al., 1996), such invasion has been proposed as a virulence mechanism. Although invasive ability seems to be strain-dependent (Guth et al., 1996; Janda et al., 1998; Sobreira et al., 2001) and, in our previous study, it was suggested to be clonally determined (Guth et al., 1999), several non-invasive *P. alcalifaciens* strains have also been isolated from patients with diarrhoea (Guth et al., 1996). However, the potential pathogenic role of these strains has not been described.

Resistance to human serum and bacterial translocation from the GI tract have been considered to be important virulence characteristics for several species, including members of the family *Enterobacteriaceae* (Taylor, 1983; Sedman et al., 1994; Sakamoto et al., 2000). In the present study, we report for the first time that invasive and non-invasive *P. alcalifaciens* strains demonstrated resistance to human serum bactericidal activity and the ability to translocate from the GI tract.

Resistance to lytic activity of serum complement was observed in nine of 30 (30 %) *P. alcalifaciens* strains studied, these included five invasive and four non-invasive strains. Complement-resistant invasive and non-invasive *P. alcalifaciens* strains were able to survive and multiply in 36 and 50 % human serum, showing a growth rate 8–20 % higher than the initial inoculum (Fig. 1). All other *P. alcalifaciens* strains were susceptible to serum bactericidal activity.

By using a small-animal model to study bacterial translocation, we observed that *P. alcalifaciens* strains demonstrated the ability to translocate from the intestinal lumen to extraintestinal sites; they were detected in MLNs, liver and spleen (Table 1). Moreover, the results showed that invasion of eukaryotic cells and resistance to human serum bactericidal activity do not appear to be determining factors in the occurrence of *P. alcalifaciens* translocation.

Although a strong correlation between serum resistance and the ability of a variety of Gram-negative bacteria to invade and survive in the bloodstream has been demonstrated in several studies (Roantree & Rantz, 1960; Taylor, 1983; Keller et al., 1998), the survival of apparently serum-susceptible bacteria in the blood of bacteraemic patients has also been reported; this may be related to serum ineffectiveness at killing the infectious agent or bacterial circumvention of the serum bactericidal system by adoption of an intracellular location in the blood or reticuloendothelial system (Roantree & Rantz, 1960; Taylor, 1983).

*Providencia* spp., have been implicated as agents of bacteremia and other extraintestinal infections (Cornaglia et al., 1995; Pinto et al., 1999), although *P. alcalifaciens* strains have

<table>
<thead>
<tr>
<th>Strain</th>
<th>Invasion of HeLa cells</th>
<th>Susceptibility to serum bacterial activity</th>
<th>Bacterial count (c.f.u. g$^{-1}$)</th>
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<tr>
<td></td>
<td></td>
<td>MLN (n = 6)</td>
<td>Spleen (n = 2)</td>
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<tr>
<td>40448</td>
<td>+</td>
<td>R</td>
<td>$8 \times 10^3$</td>
</tr>
<tr>
<td>9938</td>
<td>+</td>
<td>R</td>
<td>$2.8 \times 10^4$</td>
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<tr>
<td>0181-9</td>
<td>–</td>
<td>S</td>
<td>$4.8 \times 10^4$</td>
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<tr>
<td>2011-15</td>
<td>–</td>
<td>R</td>
<td>$2.2 \times 10^4$</td>
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*, Invasive; –, non-invasive.
†R, Resistant; S, susceptible.
been related mainly to diarrhoeal diseases (Guth et al., 1996; Janda et al., 1998; Murata et al., 2001). Despite this know-
ledge, P. alcalifaciens is not recognized as a potential pathogen in most clinical laboratories and can therefore pass easily through routine screening, although a selective medium for P. alcalifaciens has been reported (Senior, 1997). The results obtained in the present study reinforce the importance of P. alcalifaciens as a pathogen by showing that some strains were able to translocate from the GI tract and survive the killing action of complement, giving support to the suggestion that these bacteria may have the potential to cause more disseminated infection.

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