***Helicobacter pylori* VacA cytotoxin associated with the bacteria increases epithelial permeability independently of its vacuolating activity**

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Polarized epithelial monolayers of Madin-Darby canine kidney (MDCK) cells were used to study the pathogenicity of *Helicobacter pylori*, with an emphasis on the effect of VacA. The adherence of *H. pylori* to MDCK monolayers resulted in a decrease in trans-epithelial resistance (TER) across the cell monolayer. Isogenic vacA mutants did not lower the TER, demonstrating that the effect is strictly linked to the action of the toxin. A similar effect was observed with all VacA-producing strains, including those producing m2 toxins that are inactive in the vacuolating assay. In contrast to that seen with purified toxin, TER decrease was not enhanced by acid pH, which may indicate that the toxin associated to the bacterial surface is possibly in a monomeric state and therefore does not require a pH-induced conformation to be active. These data raise the possibility that one role of VacA in ulcerogenesis may consist of increasing the paracellular permeability of the gastric epithelium.

Keywords: VacA, *Helicobacter pylori*, epithelial permeability, gastritis, ulcerogenesis

INTRODUCTION

Helicobacter pylori has evolved sophisticated virulence determinants that have enabled it to colonize the gastric mucosa of more than 50% of the world's population, making it probably the most common chronic bacterial infection of humans (Blaser, 1993). This micro-organism establishes lifelong, mostly asymptomatic infections, though invariably accompanied by a mild inflammation of the gastric mucosa termed chronic superficial gastritis. Nevertheless, epidemiological studies have demonstrated that *H. pylori* infections can lead to severe gastric diseases including atrophic gastritis, peptic ulcers and gastric adenocarcinomas (Figura *et al.*, 1989; Parsonnet *et al.*, 1991).

Putative virulence factors that may contribute to different steps of *H. pylori* pathogenicity have been

identified (Labigne & de Reuse, 1996). Among these, genes from the *cag* pathogenicity island and the vacuolating cytotoxin VacA deserve a special mention because they are almost invariably expressed in the highly virulent strains isolated from patients with severe forms of gastric diseases (Xiang *et al.*, 1995). The 40 kb *cag* pathogenicity island (Akopyants *et al.*, 1998; Censini *et al.*, 1996), discovered during the genetic characterization of the immunodominant antigen CagA, encodes several genes which are involved in the induction of interleukin 8 secretion by target cells (Akopyants *et al.*, 1998; Segal *et al.*, 1997), a phenomenon thought to be essential for the onset of a strong and persistent inflammatory response. On the other hand, epidemiological (Figura *et al.*, 1989) and experimental evidence (Marchetti *et al.*, 1995; Telford *et al.*, 1994) has clearly demonstrated the association between VacA production and the occurrence of tissue damage. Indeed, using the mouse (Marchetti *et al.*, 1995; Telford *et al.*, 1994) or the beagle dog (Radin *et al.*, 1990) as animal models, it is possible to induce gastric erosion by orally administering either VacA-producing strains or purified VacA toxin.

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Abbreviations: CA, Columbia agar; DMEM, Dulbecco's modified Eagle medium; MDCK, Madin-Darby canine kidney; NRU, neutral-red uptake; TER, trans-epithelial resistance.

Mature VacA can be processed into two associated subunits of 37 and 58 kDa (Telford *et al.*, 1994) and oligomerizes into rosette-shaped structures with six or seven petals (Cover *et al.*, 1997; Lupetti *et al.*, 1996). The toxin is bound and internalized by target cells following a process that is specific and saturable, suggesting that it is mediated by still-unidentified high-affinity cell-surface receptors (Garner & Cover, 1996; Massari *et al.*, 1998). Moreover, transfection experiments where *vacA* has been expressed intracellularly have indicated that the toxin induces vacuole formation from the cytoplasm (de Bernard *et al.*, 1997). These data suggest that VacA may belong to the family of AB di-chain toxins, with a catalytic A domain (37 kDa subunit) acting on an as-yet unknown intracellular target after membrane binding and translocation into the cytosol assisted by the B moiety (58 kDa subunit) (Montecucco *et al.*, 1994). The best known VacA effect is its ability to induce cytoplasmic vacuoles in various eukaryotic cell lines (Leunk *et al.*, 1988) by altering intracellular membrane traffic at the late endosomal/prelysosomal level (Molinari *et al.*, 1997; Papini *et al.*, 1994, 1997). The toxin is also responsible for functional defects preceding vacuolar degeneration such as (i) mistargeting of acidic hydrolases (Satin *et al.*, 1997), (ii) impairment of proteolytic degradation inside lysosomes (Satin *et al.*, 1997) and (iii) inhibition of li-dependent antigen processing (Molinari *et al.*, 1998a). In addition to these alterations seen in non-polarized target cells, a new effect has been recently identified *in vitro* using polarized epithelial cell monolayers. Purified VacA was found to increase the permeability of such monolayers to low-molecular-mass molecules by an uncharacterized molecular mechanism (Papini *et al.*, 1998). Nevertheless, it has been shown that the *trans*-epithelial resistance (TER) decrease does not result from (i) disruption of the intercellular junctions, (ii) acute cytotoxicity or (iii) intracellular vacuolation. Whether any of the above toxic effects is actually relevant to pathogenesis remains to be determined under more physiological conditions, using whole bacteria. In the absence of gastric cell lines able to differentiate into sealed monolayers *in vitro*, epithelial cells that can differentiate into tightly sealed monolayers represent a valuable alternative to complex animal models.

In this study, we have tested whether polarized monolayers of Madin-Darby canine kidney (MDCK) cells could be a suitable *in vitro* model of infection by *H. pylori* and examined any possible role of other bacterial factors, in addition to VacA, on the integrity of such monolayers. We found that MDCK monolayers are indeed suitable for studying *H. pylori* pathogenicity because the bacterium readily binds to their apical surface. Following bacterial adhesion, the TER of the monolayers decreases in a dose-dependent fashion. This effect is due exclusively to VacA expression, as clearly demonstrated by a mutational analysis, and is readily apparent at very low bacterial doses. Moreover, we provide evidence that this effect is due exclusively to the toxin exposed on the bacterial surface. Interestingly, a

similar effect was observed with all VacA-producing strains, including those producing m2 toxins that are commonly considered as inactive because they are inactive in the vacuolating assay. The possible relevance of these phenomena in ulcerogenesis is discussed.

METHODS

Bacterial strains and culture conditions. *H. pylori* strains used in this study and their relevant characteristics are listed in Table 1. *vacA* isogenic *H. pylori* mutants were constructed by allelic exchange. The vector used for mutagenesis (pJMR1) was constructed by inserting the kanamycin-resistance cassette from pBK53K (Trieu-Cuot *et al.*, 1985), present on a *Sma*I fragment, into the blunt-ended *Eco*NI site present in the *vacA* gene cloned into pTOX140 (Manetti *et al.*, 1995). Bacteria were naturally transformed with 1 µg pJMR1. Several Km^r clones, selected on plates containing 20 µg kanamycin ml⁻¹, were analysed by Southern and Western blotting and were shown to be allelic exchange mutants. Bacteria were grown on Columbia agar (CA) plates supplemented in the following order with 5 mg vancomycin l⁻¹, 10 mg trimethoprim l⁻¹, 6 mg cefsulodin l⁻¹, 9 mg amphotericin B l⁻¹, 0.2% cyclodextrin, 5% defibrinated horse blood and 100 mg cycloheximide l⁻¹ (Marchini *et al.*, 1994). The plates were incubated for 2 d at 37 °C under moist atmosphere in an Oxoid anaerobic jar containing a gas-generating kit (Unipath). To avoid any secondary variation, all the infection assays were performed with jar-grown cultures of single frozen bacterial stocks conserved at -80 °C in BHI medium supplemented with 20% glycerol, 0.2% cyclodextrin and 10% foetal calf serum.

Formation of cell monolayers and integrity assays. MDCK cells can form polarized monolayers on Transwell polycarbonate membrane filters (Corning Costar). The barrier function of such monolayers, depending on the formation of tight junctions, can be assessed by monitoring the TER with a Millicell-ERS apparatus (Millipore). Monolayers with high TER values, between 11000 and 14000 Ω per cm², were obtained within 10–14 days as described (Papini *et al.*, 1998).

Infection of monolayers. Before the infection, the medium was removed from both compartments of a Transwell unit and replaced with fresh DMEM (Dulbecco's modified Eagle medium) containing no gentamicin. In order for the TER to stabilize, the filters were incubated for approximately 1 h at 37 °C under 5% CO₂. In the meantime, bacteria were harvested from CA plates in DMEM, resuspended by 2 passages through a gauge needle and diluted in the same medium to a final OD₆₀₀ of 1 (approx. 2.6 × 10⁸ c.f.u. ml⁻¹). The medium from the Transwell unit top compartment was removed, 500 µl bacterial inocula of defined concentration were added, and the filters were further incubated at 37 °C under 5% CO₂. The integrity of the monolayers was assessed by measuring the TER every hour.

When indicated, the CCUG isogenic *vacA* mutant was functionally complemented with purified VacA toxin as follows. Bacterial suspension (1 ml) at OD₆₀₀ of 1 was mixed with 100 nM purified toxin (Manetti *et al.*, 1995) and incubated for 5 min at 37 °C. Whether the toxin was pre-treated by a short exposure at pH 2 or not, the final pH of the solution was around neutrality. Bacteria were then pelleted by centrifuging at 1000 g for 5 min, gently resuspended in 1 ml fresh DMEM and used to infect the monolayers.

Table 1. *H. pylori* strains used in this study and their relevant characteristics

Strain	Relevant characteristics	VacA production*	VacA activity†
CCUG 17874	Strain producing s1m1 VacA	+	+
CCUG <i>vacA</i>	CCUG 17874 <i>vacA</i> ::Km isogenic mutant	—	—
SPM 326	Strain producing s1m1 VacA	+	+
SPM 326 <i>vacA</i>	SPM 326 <i>vacA</i> ::Km isogenic mutant	—	—
95-54	Strain producing s1m2 VacA	+	—
95-54 <i>vacA</i>	95-54 <i>vacA</i> ::Km isogenic mutant	—	—

* Assessed by immunoblotting.

† Ability of bacterial water extracts to induce vacuolation in HeLa or MDCK cells.

Quantification of adherent bacteria. Viable bacteria adhering to the monolayers were quantified as described by Corthésy-Theulaz *et al.* (1996). In brief, the adhesion was allowed to proceed at 37 °C under 5% CO₂ for 1, 2, 4 or 21 h. The monolayers were then washed eight times with sterile PBS to remove all non-adherent bacteria. The cells with adherent bacteria were recovered by adding a freshly thawed solution of trypsin/EDTA to both compartments of the Transwell unit and incubating for 10 min at 37 °C under 5% CO₂. Adherent bacteria were dispersed by vigorous pipetting and enumerated by plating serial dilutions on CA plates that were incubated for 4–5 d at 37 °C under a 5% CO₂ atmosphere.

Quantification of cell vacuolation. Bacteria were harvested from CA plates in distilled water and diluted to a final OD₆₀₀ of 1. Surface proteins were separated from the bacteria by vortexing at room temperature for 2 min. Supernatants that were recovered after a 5 min centrifugation at 14000 g were subsequently filter-sterilized using 0.2 µm pore-size filters. The extent of vacuolation induced in MDCK cells by serial dilutions of the water extracts was quantified spectrophotometrically by measuring the neutral-red uptake (NRU) as previously described for HeLa cells (Papini *et al.*, 1993), except that cells were seeded on 96 well plates at a density of 8000 cells per well.

Western blotting. Bacteria were resuspended to a final OD₆₀₀ of 1 in DMEM without foetal calf serum. After a 5 min centrifugation at 10000 g, pellets were resuspended in 200 µl loading buffer. The supernatants, first precipitated with trichloroacetic acid, were resuspended in 200 µl loading buffer as well. Western blotting was then performed using an anti-VacA serum as previously described (de Bernard *et al.*, 1997) and the ECL analysis system (Amersham) for detection.

Measurement of [¹⁴C]mannitol paracellular flux. Changes in paracellular permeability of the monolayers in response to bacterial infection were assayed by measuring the penetration of [¹⁴C]mannitol, a marker molecule of small size (M_r 182.2). In brief, after overnight bacterial infection, 4 × 10⁶ c.p.m. [¹⁴C]mannitol was added to the Transwell lower compartment and the monolayers were incubated at 37 °C. At defined time periods, the medium in the apical compartment was removed, replaced with fresh medium and counted by liquid scintillation counting.

Statistics. Each experiment was done at least in triplicate. Results are reported as means ± standard deviation. Bars, representing the standard deviations, are generally too small to be visible on the graphs.

RESULTS

H. pylori adheres to MDCK monolayers

H. pylori binds *in vitro* to numerous cell lines of various origins (Labigne & de Reuse, 1996), including epithelial cells that can form polarized monolayers on porous surfaces (Corthésy-Theulaz *et al.*, 1996). To study the pathogenicity of *H. pylori*, with an emphasis on potential VacA effects, we opted for MDCK cells because they form the most tightly sealed polarized monolayers (with the highest TER). When MDCK monolayers were infected with 1.3 × 10⁸ c.f.u. CCUG 17874 (hereafter referred to as CCUG), a strain producing an s1m1 toxin (Telford *et al.*, 1994), bacteria adhered to the monolayer extremely rapidly, reaching within the first hour of infection a plateau of ~1.5 × 10⁶ bound bacteria (1% of the initial inoculum) that remained constant over the time of the experiment (Fig. 1). A comparable efficiency of binding was observed

Fig. 1. Adherence of *H. pylori* CCUG and its isogenic *vacA* mutant to MDCK monolayers at various times after infection. The number of adherent bacteria is expressed as a percentage of the initial bacterial inoculum. Note that 1% represents ~1.5 × 10⁶ bacteria. White bars show CCUG cells, black bars, CCUG *vacA*.

with a *vacA* isogenic mutant of CCUG. To estimate the m.o.i., defined as the number of bound bacteria per cell in the monolayer, cells forming a monolayer typically used for infection (10–14 days of growth) were recovered by trypsin treatment and counted. Three million cells per well were present, indicating that a fivefold multiplication took place after seeding. From the number of bound bacteria, 1.5×10^6 , the m.o.i. can be estimated at 0.5 bound bacteria per MDCK cell. These data indicate that monolayers of MDCK cells are a suitable model for studying *H. pylori* pathogenicity *in vitro*.

Low doses of adherent *H. pylori* strongly decrease the TER of the monolayers

To assess whether the infection by *H. pylori* had an effect on the integrity of the monolayers, we followed the variation of the TER, which inversely correlates with the paracellular permeability. Under the above infection conditions, with an inoculum of 1.3×10^8 c.f.u. CCUG, we observed a rapid and very marked decrease of the TER (Fig. 2a). After 4 h infection, the effect on the TER was already pronounced, with a decrease of 60 % of the initial TER values. After 21 h infection, the effect was maximal with an 85 % decrease. This effect is almost exclusively due to adherent bacteria, because washing away the unbound bacteria does not change the final TER decrease (data not shown). When the experiment was performed with smaller bacterial loads, an effect on the TER was still observed with similar kinetics but was less pronounced (Fig. 2a). The dose-response curves indicate that an effect can be seen with an m.o.i. as low as 0.05.

TER decrease is due to the production of VacA

Because it was recently demonstrated that VacA purified from CCUG decreases the TER of polarized monolayers (Papini *et al.*, 1998), we performed a mutational analysis to confirm that the effect we observed with entire bacteria indeed resulted from the action of the toxin. We constructed an isogenic *vacA* mutant of CCUG by allelic exchange, where the *vacA* gene was disrupted by the insertion of a kanamycin-resistance cassette. The mutant does not produce VacA, as assessed by Western blotting, and is consequently inactive in the NRU assay on MDCK cells. When MDCK monolayers were infected with the *vacA* mutant under standard conditions (m.o.i. 0.5), no significant decrease of the TER was observed: the initial 10 % decrease was only due to the change of the medium (Fig. 2b). No TER decrease was observed even with five times more concentrated inocula (data not shown). These results clearly indicate that the decrease in the TER of MDCK monolayers observed in response to the infection by CCUG depends only on the production of VacA toxin. The effect we observed was not specific to the toxin of CCUG: infections with *H. pylori* SPM 326, another strain producing an s1m1 toxin, also led to a decrease in TER. After 21 h infection, SPM 326 induced a 65 % decrease

Fig. 2. Effect of (a) various doses of *H. pylori* CCUG and (b) its *vacA* isogenic mutant on the TER of MDCK monolayers. TER values are expressed as a percentage of the initial TER, prior to infection. The media-alone control (■) shows that the TER of uninfected monolayers is stable during the time of the experiment. Symbols: (a) ▽, 1.3×10^7 c.f.u.; ◇, 4.3×10^7 c.f.u.; □, 6.5×10^7 c.f.u.; ○, 1.3×10^8 c.f.u.; (b) ○, CCUG; ●, CCUG *vacA*.

of the initial TER values, compared with the 85 % decrease for CCUG (Fig. 3). As above, no effect was seen when an isogenic *vacA* mutant of SPM 326 was used to infect the monolayers. From these data, it can be concluded that all m1 VacA-producing strains induce a decrease of the TER of MDCK monolayers.

m2 toxins decrease the TER of the monolayers

Some *H. pylori* strains produce toxins presenting a particular mid-region termed m2, which are inactive in the classical vacuolation assay on HeLa cells (Atherton *et al.*, 1995). Nevertheless, their *vacA* gene is conserved, suggesting that the physiological properties of VacA may be different from the well-known vacuolation effect. *H. pylori* 95-54, a strain presenting an s1m2 *vacA*

Fig. 3. TER of MDCK monolayers 21 h after infection with *H. pylori* strains. White bars indicate wild-type, black bars show the *vacA* mutants.

Fig. 4. (a) Western-blot detection of VacA produced and released by *H. pylori* strains CCUG, SPM 326 and 95-54. P, pellet; S, supernatant. (b) NRU spectrophotometric quantification of vacuolation induced in MDCK cells by successive dilutions of the water extracts of the various *H. pylori* strains used in this study. NRU values are the difference of absorbance at 534 nm and 405 nm after subtraction of the background optical density of CCUG *vacA*. White bars show values for CCUG, hatched bars, SPM 326 and black bars, 95-54.

genotype, was isolated from a patient with peptic ulcer. It produces a toxin (Fig. 4a) that, in contrast to CCUG and SPM 326, does not induce vacuoles in sparse MDCK

Fig. 5. Kinetics of [^{14}C]mannitol penetration through MDCK monolayers 21 h after infection with CCUG and its isogenic *vacA* mutant. Values are defined as the percentage of radioactivity in the apical compartment relative to the total radioactivity added to the basolateral compartment. $\times$, control; $\circ$, CCUG; $\bullet$, CCUG *vacA*.

cells (Fig. 4b). This VacA isoform was, however, perfectly active since infection of the MDCK monolayers with 95-54 induced a notable decrease of the TER: after 21 h infection, the TER was only 50% of the initial values (Fig. 3). As before, no effect on the TER was obtained with a 95-54 *vacA* isogenic mutant constructed by allelic exchange. It can be concluded from these data that the decrease in TER of MDCK monolayers is a more reliable assay for VacA toxicity than the vacuolation activity and that the m2 toxins are perfectly active.

TER decrease, concomitant with an increased paracellular permeability, is due to the toxin associated with the bacteria

To test whether the decrease in TER led to an increase of paracellular permeability, as reported for the purified toxin (Papini *et al.*, 1998), we measured the flux of [^{14}C]mannitol, a marker molecule that uses mostly a paracellular route to permeate through a polarized epithelium. MDCK monolayers infected with CCUG for 21 h and showing an 85% decrease of TER presented a fivefold increase of mannitol flux over 6 h when compared to monolayers that were uninfected or infected with an isogenic *vacA* mutant (Fig. 5).

Acidic pH strongly potentiates the vacuolating activity (de Bernard *et al.*, 1995) and the TER-decreasing activity (Papini *et al.*, 1998) of the purified toxin, and hence this was tested with whole bacteria. When MDCK monolayers were infected with CCUG preincubated at pH 5.4, essentially the same effect on the TER as in the experiments performed at pH 7.4 was detected. Thus,

Fig. 6. Comparison of the TER effects induced by (a) the secreted and bacteria-bound VacA toxin and (b) an isogenic CCUG *vacA* mutant 'functionally complemented' with acid-activated purified toxin. TER values are expressed as the percentage of the initial TER. Symbols: (a) □, CCUG supernatant; ◇, CCUG pellet; ○, CCUG; (b) ●, CCUG *vacA*; ◇, CCUG *vacA* + VacA; ○, CCUG.

under the present experimental conditions, the toxin did not require the low-pH activation which is known to be associated with a dissociation of the secreted oligomeric toxin into monomers (Cover *et al.*, 1997). This suggested the possibility that the TER decrease we observed was due to the toxin that remained associated with the bacteria, that may be present in its active monomeric form. Bacteria were centrifuged prior to infection to remove any toxin released in the medium, and the bacterial pellet was used to infect the monolayers. The decrease in TER was only slightly smaller than that observed with non-centrifuged bacterial cultures (Fig. 6a). Accordingly, no decrease in TER was observed when the supernatant, containing roughly half of the toxin produced by the bacteria, was added to the

monolayers (Fig. 6a). Moreover, the effect was not due to toxin released by the bacterium upon contact with the cells, as the medium from the upper compartment taken at 1 and 5 h after infection was unable to induce any decrease in TER when added to new monolayers (data not shown). Moreover, when an isogenic *vacA* mutant was incubated with purified toxin prior to infecting the monolayers (a process that can be viewed as functional complementation), the TER-decreasing activity was partly restored (Fig. 6b). Interestingly, this was only observed when the toxin was preincubated at acid pH, a treatment known to induce the dissociation of the oligomeric VacA into monomers (Cover *et al.*, 1997). Taken together, these data suggest that the effect of VacA on the TER, corresponding to an increase of paracellular permeability, is due exclusively to constitutively active toxin molecules that remain associated with the bacteria.

DISCUSSION

In vitro models of infection are likely to help in solving crucial but still obscure aspects of infections with various bacteria, including *H. pylori*. Cellular models based on monolayers of polarized epithelial cells, which mimic the situation encountered by *H. pylori* *in vivo*, provide an additional insight over the non-polarized cells in culture that are most frequently used. Recently, Corthésy-Theulaz *et al.* (1996) used T84 cells and suggested that MDCK cells could also be used. Indeed, we have shown that *H. pylori* adheres very rapidly to MDCK monolayers, reaching a saturation level within 1 h of infection. This is comparable to what was reported for T84 monolayers and it confirms that appropriate receptors for *H. pylori* (Labigne & de Reuse, 1996) are present on the surface of these cells. In addition, the cell biology of MDCK is better studied than that of T84 cells, and MDCK cells form more tightly sealed monolayers that permit the testing of bacterial effects on the permeability properties of the monolayer.

By using monolayers of epithelial cells, including MDCK cells, purified VacA was recently found to selectively increase paracellular permeability to ions and low-molecular-mass molecules, presumably by loosening tight junctions between cells (Papini *et al.*, 1998). Here we extend these findings under more physiological conditions, using viable bacteria. We show that extremely small numbers of bacteria (m.o.i. as low as 0.5) are sufficient to obtain a decrease in TER similar to that seen with very large amounts of purified toxin (Papini *et al.*, 1998), suggesting that the process is much more efficient with viable bacteria. One possible explanation is that an intimate contact between the bacteria and the cells may favour the delivery of the toxin to the cells. A mutational analysis clearly demonstrates that VacA is necessary and sufficient to induce an increase in the permeability of the monolayer. Indeed, isogenic *vacA* mutants did not induce any noticeable decrease of the TER, unless they had been complemented with purified

toxin. Moreover, this study presents two novel findings that may be physiologically relevant to *H. pylori* pathogenicity. Firstly, a TER decrease was observed with all VacA-producing strains tested, independently of their vacuolating activity. For example, 95-54, which produces an s1m2 toxin that induces no vacuoles in MDCK sparse cells (Fig. 4b), was able to induce a TER decrease on MDCK monolayers. Thus, in contrast to the vacuolation activity, which is dependent on the cell line used (Pagliaccia *et al.*, 1998), the decrease in TER in MDCK monolayers may be a more reliable assay for VacA toxicity. This effect may be particularly relevant to the role of VacA *in vivo* and would also account for the fact that *vacA* m2 alleles have been conserved throughout the evolution. Secondly, the decrease in TER, presumably caused by toxin that remains associated with the bacterial surface, is not, in contrast to other described activities of VacA (de Bernard *et al.*, 1995; Papini *et al.*, 1998), potentiated by acid pre-treatment. Since acid treatment results in the dissociation of the oligomeric VacA into monomers (Cover *et al.*, 1997; Molinari *et al.*, 1998b), suggesting that the monomers are the active form of the toxin, it is tempting to speculate that the toxin present on the bacterial surface is already in its active, possibly monomeric, form. This idea is strengthened by the fact that acid-activated toxin was the only form able to functionally complement a *vacA* mutant in the TER decrease assay (Fig. 6a). However, using monolayers of MDCK cells (or T84), Corthésy-Theulaz *et al.* (1996) did not observe any TER decrease after bacterial infection. This absence of effect may be due to a lack of toxin production, since the bacterial strains they used were not genetically characterized, or to the fact that TER values of the monolayers they used were more than 5 times lower than those routinely reached in the present study. This last discrepancy was possibly due to differences in culture methods or to the use of slightly different cell lines. Another report showed that sonicates of *H. pylori* were able to induce a TER decrease in T84 monolayers (Terrés *et al.*, 1998), but this effect was different from the present one because it was rapidly reversible and non-dependent on *vacA* expression.

Taken together, previous (Papini *et al.*, 1998) and present results allow one to envisage a likely scenario directly linking a defined VacA effect *in vitro* to toxin-induced tissue damage seen *in vivo*. Briefly, intimate contact between *H. pylori* and the polarized monolayers of gastric epithelial cells could favour the delivery of a monomeric form of the toxin, associated with the bacterial surface, into the epithelial cell cytosol. As a consequence of a still-uncharacterized intracellular action, the toxin would lead to an increase of the paracellular permeability, rendering the gastric barrier more fragile and susceptible to later attack. It should be emphasized that unlike other bacterial toxins, such as fragilysin from *Bacteroides fragilis* (Obiso *et al.*, 1997), VacA does not completely disrupt the tight junctions between cells, as the TER values after infection are indicative of a sealed monolayer. Therefore, the

loosening of the intercellular junctions induced by VacA is probably only an initial step in the ulcerogenesis. As described for other pathogens (McCormick *et al.*, 1997, 1998), a subsequent *trans*-epithelial migration of polymorphonuclear leukocytes could accelerate the disorganization of the gastric epithelium and ultimately lead to its disruption, as observed in patients with peptic ulcers.

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