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Helicobacter pylori Vacuolating Toxin Exhibits Polar Activity of Cl^- Secretion and Secretory Response to Carbachol in T84 Cells

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To investigate whether VacA (vacuolating toxin) produced by *Helicobacter pylori Korean stain 99* induces intestinal secretion, purified VacA was added to T84 cell monolayers mounted in Ussing chambers, and electrical parameters were monitored. Mucosal addition of low pH-pretreated VacA increased short circuit current (Isc). The effect was time- and dose-dependent and saturable. The time-to-peak Isc was concentration-dependent. Chloride channel inhibitors, niflumic acid or 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), inhibited VacA-stimulated Isc. Carbachol (CCh)-induced increase of Isc was prolonged by the addition of VacA to the mucosal side only. The effect was unaltered by the addition of niflumic acid. VacA did not show cytopathic effects. These studies indicate that VacA is a nonlethal toxin that acts in a polar manner on T84 monolayers to potentiate Cl^- secretion and the response to CCh secretion without decrease in monolayer resistance. VacA may contribute to diarrhea diseases in human intestinal epithelial cells.

Key Words: *Helicobacter pylori*, VacA, Short circuit current, Secretion, T84 cells

INTRODUCTION

The protein toxin VacA produced by pathogenic *Helicobacter pylori* strains plays a major role in the pathogenesis of gastroduodenal diseases associated with infection by this bacterium (Blaser, 1993; Cover et al, 1993; Marchetti et al, 1995; Xiang et al, 1995). VacA exhibits variations in signal sequence and midgene coding sequence that are related to the intensity of inflammation and epithelial injury (Atherton et al, 1997). Consistently, gastric epithelial erosion observed in these pathologies is mimicked in animal models by oral administration of VacA (Telford et al, 1994). *Helicobacter pylori* is also associated with conditions such as short stature, suggesting that its effects are not confined to the stomach (Patel et al, 1994). The bacterium has been isolated from stool, indicating that infective organisms may be present in the intestine (Thomas et al, 1992), and may contribute to diarrhea disease associated with Helicobacter pylori infection in human intestine by altering epithelial barrier function and stimulating ion secretion. Previous observations on the identity of VacA with the toxic factor, isolated from stool samples with mild diarrhea in which no other pathogens were detected (Luzzi et al, 1993), are consistent with Guarino's results (Guarino et al, 1998). However, the effect of VacA on Isc and secretagogues induced secretion in the Cl^--secreting T84 epithelial cell monolayers is unknown.

In the present study, we investigated the effects of VacA produced by *Helicobacter pylori Korean strain 99* in T84 cell monolayers and tested the hypothesis that VacA induces ion secretion.

METHODS

Preparation of VacA

VacA was purified from culture supernatants of *Helicobacter pylori Korean strain 99* by affinity chromatography and gel filtration, as described (Manetti et al, 1995). The toxin was activated by pre-treatement at pH 2.0 and room temperature (22~25°C) for 30 min. The vacuolating activity of each preparation was assayed in HeLa cells by measuring the uptake of neutral red dye, as described (Papini et al, 1995).

Cell culture

T84 cells were grown in DMEM medium supplemented with 10% fetal calf serum and 1% antibiotics at 37°C in a 5% CO_2 atmosphere. For Ussing experiments, T84 cells were seeded on Transwell porous filters (0.4-μm pore size, 12.0-mm diameter), at a density of 1.0×10^6 cells/cm^2 to form monolayers, as described previously (Matthews et al, 1995), and stable levels (>1,000 $\Omega \cdot$ cm^2) of transepithelial

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ABBREVIATIONS: Isc, short circuit current; VacA, vacuolating toxin produced by *Helicobactor pylori*; CCh, carbachol; G, conductance; TEER, transepithelial electrical resistance; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoate

resistances were attained after 7~15 days.

Ussing experiments

Transepithelial transport studies were carried out across T84 confluent monolayers in a simplified apparatus for measuring electrophysiological parameters (surface area 1.0 cm^2). The electrodes were connected to an automatic voltage clamp (DVC 1000; World Precision Instruments, New Haven, CT). The transepithelial potential difference (PD) was recorded every 10 min under open-circuit conditions, and the voltage was then clamped and the Isc was recorded. Resistance of the monolayer was calculated from the Isc and open-circuit PD according to Ohm's law. Ringer's solution contained (in mM): NaCl 116, $NaHCO_3$ 24, $CaCl_2$ 1.5, $MgCl_2$1, KCl 4.5, glucose 5, bubbled with CO_2 (pH 7.4), which was consistently gassed with 95% O_2 and 5% CO_2, and kept at 37°C with a thermostat-regulated circulating pump. Isc was expressed as $\mu A/cm^2$, G as mS/cm^2, and PD as mV.

To investigate the sensitivity of VacA to heating, the toxin preparation was exposed to 100°C for 20 min before being added to cell monolayers.

A resistance monitoring apparatus (Millicel-ERS; Millipore, USA) was used to measure the transepithelial electrical resistance (TEER), as previously described (Dharmsathaphorn et al, 1986). This experimental model made it possible to investigate the cytopathic effects of biomolecules in both short and long terms, since TEER is a sensitive marker of epithelial damage (Dharmsathaphorn et al, 1986).

Chemicals

All chemicals were of analytical grade and purchased from Sigma (St. Louis, M.O., USA). Culture media were from GIBCO (Grand island, N.Y., USA), and transwell filters were from Costar (Corning, N.Y., USA).

Statistics

Data were expressed as means±SE. n stands for number of observations. Statistical analysis was done by Student's paired and unpaired t test; a value of $P<0.05$ was considered to be statistically significant.

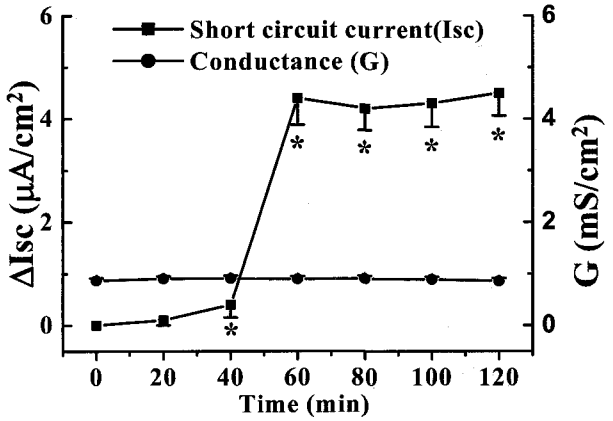

Fig. 1. Time-course effect of mucosal addition of VacA (0.1 μg/ml) on short circuit current (Isc) and Conductance (G) of T84 cell monolayers mounted in Ussing chambers. VacA increased Isc dose- and time-dependently without G changes of T84 cell monolayers. Data are mean±SE of 6 separate observations. *, $P<0.05$ vs. control cells.

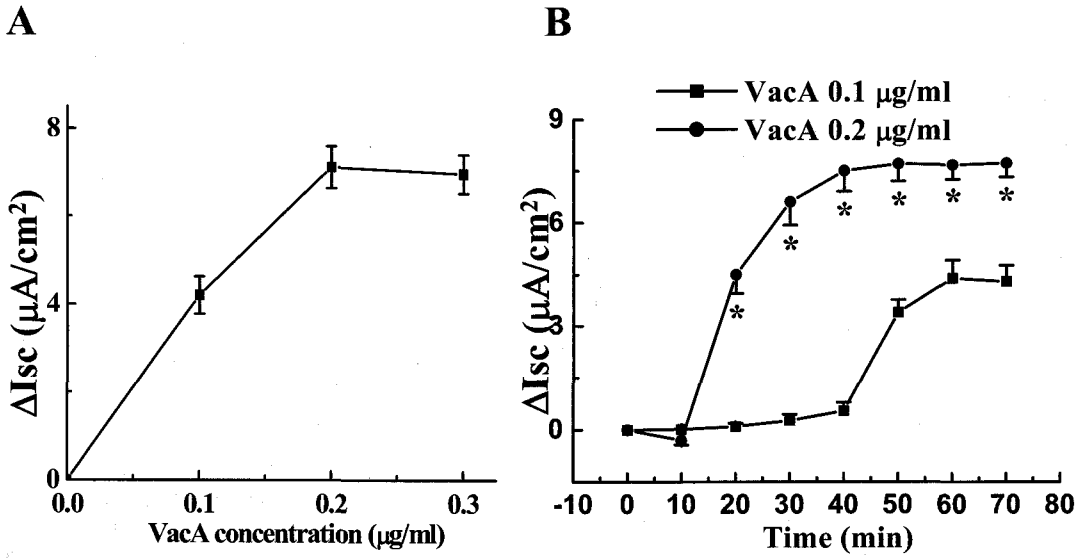

Fig. 2. Dose (A) and time course (B) of Isc response to VacA. (A) concentration response with VacA. Peak change (Δ) in Isc stimulated by VacA toxin added to mucosal side of T84 cell monolayers was recorded. Data are mean±SE of 4 separate observations/concentration. (B) time course of ΔIsc response to 0.1 μg/ml (n=6) or 0.2 μg/ml (n=4) concentration of VacA, illustrating the earlier Isc peak response with increasing VacA concentration. *, $P<0.05$ vs. ΔIsc values stimulated by 0.1 μg/ml VacA.

RESULTS

Effect of VacA on intestinal transport in T84 cell monolayers

When VacA was pre-exposed to low pH and then added to the apical (Fig. 1) bathing solution of T84 cell monolayers, an increase in Isc was observed. The electrical response was slow in the first 30~40 min (Fig. 1), and then Isc rapidly increased with a peak about 60 min after toxin addition (0.1 μg/ml). This increase was sustained for $\geq$2 h after the toxin addition due to an effect on PD, since no modifications of G values were recorded (Fig. 1). The rise in Isc was dose-dependent with a maximal concentration of 0.2 μg/ml VacA (Fig. 2A). Addition of higher concentrations of VacA did not induce further increase in Isc, indicating a saturation of the effect. The time-to-peak Isc was also concentration-dependent, because increasing the concentration of VacA shifted the Isc peak to an earlier time (Fig. 2B): The maximal response was reached by about 40 min after the addition of VacA, and the effect persisted for at least 2 hours.

The electrical effect of VacA in T84 cells was observed with the addition of the toxin only to the mucosal, but not to the serosal side (Fig. 3), indicating that VacA exhibits a polar activity. The addition of not low pH-pretreated VacA to a concentration of 0.2 μg/ml, either to the mucosal or serosal side of T84 cell monolayers, did not induce modifications of electrical parameters in Ussing chamber experiments (Fig. 3). Subsequently, studies of VacA were performed with apical addition alone. When the toxin was exposed to 100°C for 20 min before being added to T84 cells, the electrical parameters were unaltered, indicating that the effect was heat-labile (Fig. 3).

To further investigate the Cl^- dependence of enterotoxic effect, Cl^- channel inhibitors, NPPB (Fig. 4A) and niflumic acid (Fig. 4B), were added to the mucosal side. Both NPPB (n=3) and niflumic acid (n=3) inhibited the increase in Isc induced by VacA (0.2 μg/ml), suggesting that VacA induces secretion through the opening of apical Cl^- channels.

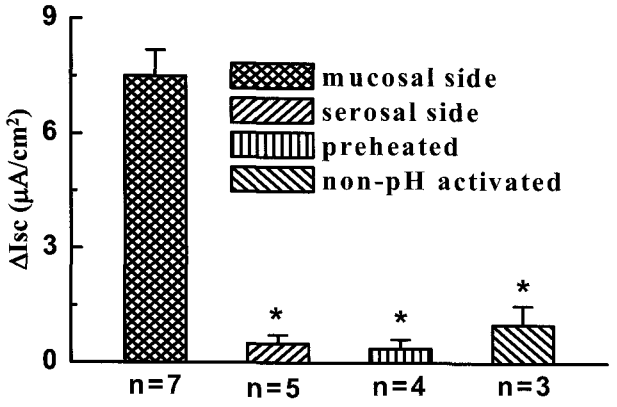

Fig. 3. Effect of VacA (0.2 μg/ml) on ΔIsc, when VacA was added to mucosal side, serosal side, after exposure to heat, or non-pH activated. The increase in Isc was induced by the addition of pH-preactivated VacA to the apical side, suggesting that VacA exhibits polar activity and is heat-sensitive. *, $P<0.05$ vs. low pH-pretreated VacA-stimulated values.

The Effect of VacA on carbachol (10^{-4} M)-induced Isc

Next, we tested whether VacA might potentiate responses also to other known chloride secretagogues such as the muscarinic agonist. For these experiments, carbachol (CCh) was added at a concentration of 10^{-4} M, which induced a maximal Isc response, and VacA at a maximal concentration of 0.2 μg/ml. Similar to the results previously reported (Dharmsathaphorn et al, 1986), the Cl^- secretory response to CCh (added to the basolateral membrane) was rapid and transient with a peak Isc of 20.10 ± 1.63 μA/cm^2 at 2 min, and returned nearly to baseline by 10 min (Fig. 5). In contrast, VacA (0.2 μg/ml) alone showed no effect on Isc within 10 min, however, persistently increased Isc with a peak of 7.5 ± 0.6 μA/cm^2 at 40 min (Fig. 2B). Serial addition of CCh and VacA at 2 min (Fig. 5) resulted in additive response of CCh plus VacA that was apparent at 50 min with a peak Isc of 29.20 ± 1.56 μA/cm^2, which persisted for at least 60 min (predicted additive effect, 29.92 ± 2.1 μA/cm^2; n=4, $P>0.05$). When CCh was removed from basolateral solution in the presence of VacA in the apical

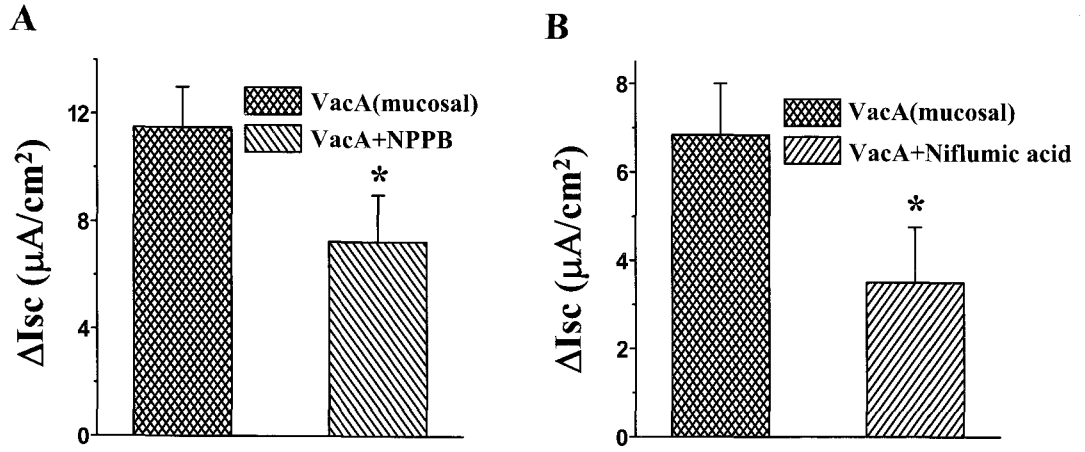

Fig. 4. Effect of NPPB (A) or niflumic acid (B) on VacA-induced Isc in T84 cell monolayers. NPPB (10^{-4} M) or niflumic acid (10^{-4} M) added to apical bath inhibited peak VacA-stimulated Isc. *, $P<0.05$ vs. VacA-stimulated values in standard condition (n=3).

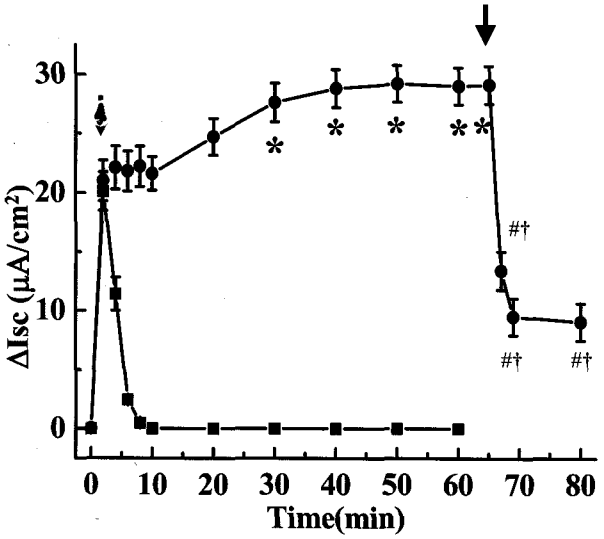

Fig. 5. Effect of CCh alone or serial addition of CCh and VacA on Isc (n=4). ■ CCh alone (10^{-4} M) added to basolateral bath solutions at 0 min. ● CCh (10^{-4} M) added to basolateral bath solutions at time 0, followed by VacA (0.2 μg/ml) added to mucosal side at 2 min (indicated as arrow). CCh alone induced increase in Isc was rapid and transient with a return nearly to baseline by 10 min. In contrast, serial addition of CCh and VacA at 2 min persistently increased Isc and resulted in a CCh plus VacA response, which was apparent at 50 min without CCh-induced Isc returning to baseline, showing additive effects of VacA and CCh on the increase of Isc. When CCh was removed (indicated as arrow on the right), the component of CCh induced increase in Isc was eliminated immediately and that of VacA induced Isc increase was retained. *#, $P<0.05$ vs. CCh-stimulated values at 2 min (n=4). †, $P<0.05$ vs. CCh plus VacA-stimulated values (n=4).

side, the fraction of CCh-induced increase of Isc was eliminated after 3.75±0.33 min, and the residual increase of Isc had no significant difference from the VacA fraction observed in the presence of CCh (n=4, $P>0.05$). Apical addition of niflumic acid (0.1 mM) inhibited the VacA component, whereas the component of CCh induced Isc increase was unaltered (data not shown). Furthermore, addition of VacA to the basolateral side had no effect on the response to CCh (data not shown). These results suggest that the effects of VacA and CCh on increase of Isc are additive, and that the action of VacA and CCh in the presence of VacA is mediated through different pathways. The exact mechanism still remains unknown.

Effect of VacA on epithelial integrity

Previous studies report that agents such as rotavirus, which induce intestinal epithelial cell damage attenuate TEER (Guarino et al, 1996). In the present study, when TEER of T84 cell monolayers reached the steady state, their exposure to 0.2 μg/ml VacA, which was added every 24 hours, did not modify TEER values by incubating up to 72 hours (data not shown).

DISCUSSION

Our results show that the addition of low pH-pretreated VacA to the mucosal side of T84 cell monolayers results in an increase of Isc in a dose- and time-dependent manner. And, the time-to-peak Isc is also concentration-dependent. These maximal effects were saturable without modifications of G values, although they were delayed in comparison with that observed with other toxins (Grasset et al, 1985; Guarino et al, 1995). These responses were not observed, when the VacA toxin was preheated or non-pH-activated or added to basolateral side, suggesting that only low pH-pretreated VacA toxin may act through a limited number of receptors located on the mucosal surface of polarized intestinal cells. VacA was found to result in typical gastrointestinal toxic effect, implicated by a rise of Isc in T84 monolayers, suggesting that the toxin may induce water and electrolyte intestinal secretion *in vivo*.

Recently, VacA is shown to form anion-selective channels in the plasma membrane of HeLa cells and to increase the anion permeability. VacA-dependent increase of current conduction was effectively inhibited by the chloride channel blocker NPPB (Szabo et al, 1999). In the present study, niflumic acid or NPPB, the inhibitors of Cl^- channels, significantly attenuated the increase of Isc, suggesting that VacA may form a chloride channel in the apical side of T84 cell monolayers and induce a chloride current. This current was not caused by the activation of known endogenous Cl-channels, such as swelling-activated or Ca^{2+}-activated chloride channels (Szabo et al, 1999), consistent with our unpublished data obtained in AGS (adenocarcinoma cells of stomach, ATCC CRL 1739) cells. Therefore, this is not a typical bacterial toxin, such as cholera toxin (Guarino et al, 1998). However, we cannot role out the possibility at present that a hither-to-unknown endogenous channel, having biophysical and pharmacological properties very similar to VacA, is activated by the toxin, or that other pathways such as hormones or second messengers may be involved, because the significant increase in Isc was accompanied with a long latency. It is of interest to note here that VacA toxin indeed influences intestinal secretion induced by secretagogues. VacA-induced potentiation of secretory responses to CCh has not previously been described. Ca^{2+}-dependent neurohormonal agonists (e.g., carbachol, histamine, and serotonin) act at the basolateral membrane of intestinal epithelial cells and induce only very brief increase of Isc. In the present study, exposure of T84 monolayers to VacA produced a prolonged Isc response to CCh (Fig. 5), and acted additively with the Ca^{2+}-dependent agonist CCh. The increase of Isc induced by CCh plus VacA occurred only when VacA was added to mucosal side. Apical addition of niflumic acid only inhibited the component of VacA-induced increase in Isc without influencing that of CCh-induced effect in the presence of VacA, indicating that VacA toxin exhibits polar activity of the secretory response to CCh in T84 cells. The pathway activated by CCh was not sensitive to niflumic acid, while that activated by VacA was. Furthermore, the effects of VacA and CCh on the increase of Isc were additive, indicating that the mechanism of the effect of VacA is different from that of CCh.

The enterotoxic effect in the present study suggests that *Helicobacter pylori Korean strain 99* may be associated with diarrhea. *Helicobacter pylori* specific seropositivity was detected in 41 of 77 (53%) infants with chronic diarrhea and malnutrition (mean age 19 months, range 5~36), compared with 18 of 70 (26%) of age matched healthy controls and nearly a quarter (12/49, 24%) of age matched undernourished (marasmic) subjects without diarrhea in

Gambia, indicating that this infection is associated with chronic diarrhea and malnutrition in infancy (Sullivan et al, 1990). Furthermore, a cytotoxin inducing vacuolation was detected in 19 (3.1%) of 618 stool specimens from children with diarrhea, but in none of 135 from control children. Common enteric pathogens were found in only two (10.5%) of the 19 cytotoxin-positive stool specimens. The vacuoles induced by stool filtrates resembled those induced by VacA toxin, suggesting that microorganisms of the gastrointestinal tract produce a Helicobacter-like vacuolating toxin and may be responsible for childhood diarrhea (Luzzi et al, 1996). Finally, *Helicobacter pylori* infection was found in an HIV-seropositive patient with diarrhea. It was the only pathogen detected, and symptoms were resolved promptly with specific antimicrobial treatment (Dorrell et al, 1993).

It should be noted that the VacA toxin produced by *Helicobacter pylori Korea stain 99* did not induce T84 cell damage without the modifications of G and TEER under our experimental conditions, whereas AGS cell viability was significantly attenuated by the addition of this VacA to the mucosal side (our unpublished data). This suggests that enterocytes are insensitive to this toxin compared with gastric epithelial cells.

In summary, VacA produced by *Helicobacter pylori Korea stain 99* is a nonlethal toxin in the present study, which acts in a polar manner on T84 monolayers to stimulate Cl-secretion and to potentiate the response to CCh secretion without epithelial damage. The preliminary clinical evidence together with our *in vitro* data in human intestinal epithelial cells support the hypothesis that VacA may be associated with diarrhea.

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REFERENCES

Atherton JC, Peek RMJ, Tham KT, Cover TL, Blaser MJ. Clinical and pathological importance of heterogeneity in VacA, the vacuolating cytotoxin gene of Helicobacter pylori. *Gastroenterology* 112: 92-99, 1997

Blaser MJ. *Helicobacter pylori*: microbiology of a "slow" bacterial infection. *Trends Microbiol* 1: 255-260, 1993

Cover TL, Cao P, Lind CD, Tham KT, Blaser MJ. Correlation between vacuolating cytotoxin production by Helicobacter pylori isolates in vitro and in vivo. *Infect Immun* 61: 5008-5012, 1993

Dharmsathaphorn K, Pandol SJ. Mechanisms of chloride secretion induced by carbachol in a colonic epithelial cell line. *J Clin Invest* 77: 348-354, 1986

Dorrell L, Wardropper AG, Ong ELC. Helicobacter pylori infection in an HIV-seropositive patient with diarrhoea. *AIDS* 7: 900-901, 1993

Grasset E, Bernabeu J, Pinto M. Epithelial properties of human colonic carcinoma cell line Caco-2: effect of secretagogues. *Am J Physiol* 248: C410-418, 1985

Guarino A, Bisceglia M, Canani RB, Boccia MC, Mallardo G, Bruzzese E, Massari P, Rappuoli R, Telford J. Enterotoxic effect of the vacuolating toxin produced by Helicobacter pylori in Caco-2 cells. *J Infect Dis* 178: 1373-1378, 1998

Guarino A, Canani RB, Casola A, Pozio E, Russo R, Bruzzese E, Fontana M, Rubino A. Human intestinal cryptosporidiosis: secretory diarrhea and enterotoxic activity in Caco-2 cells. *J Infect Dis* 171: 976-983, 1995

Guarino A, Casola A, Bruzzese E, Saini M, Nitsch L, Rubino A. Human serum immunoglobulin counteracts rotaviral infection in Caco-2 cells. *Pediatr Res* 40: 881-887, 1996

Luzzi I, Covacci A, Censini S, Pezzella C, Crotti D, Facchini M, Giammanco A, Guglielmetti P, Piersimoni C, Bonamico M, Mariani P, Rappuoli R, Caprioli A. Detection of a vacuolating cytotoxin in stools from children with diarrhea. *Clin Infect Dis* 23: 101-106, 1996

Luzzi I, Pezzella C, Caprioli A, Covacci A, Bugnoli M, Censini S. Detection of vacuolating toxin of Helicobacter pylori in human faeces [letter]. *Lancet* 341: 1348, 1993

Manetti R, Massari P, Burroni D, de Bernard M, Marchini A, Olivieri R, Papini E, Montecucco C, Rappuoli R, Telford JL. Helicobacter pylori cytotoxin: importance of native conformation for the induction of neutralizing antibodies. *Infect Immun* 63: 4476-4480, 1995

Marchetti M, Arico B, Burroni D, Figura N, Rappuoli R, Ghiara P. Development of a mouse model of Helicobacter pylori infection that mimics human disease. *Science* 265: 1656-1658, 1995

Matthews JB, Tally KJ, Smith JA, Zeind AJ, Hrnjez BJ. Activation of Cl secretion during chemical hypoxia by endogenous release of adenosine in intestinal epithelial monolayers. *J Clin Invest* 96: 117-125, 1995

Papini E, Bugnoli M, De Bernard M, Figura N, Rappuoli R, Montecucco C. Bafilomycin A1 inhibits Helicobacter pylori induced vacuolization of HeLa cells. *Mol Microbiol* 7: 323-327, 1995

Patel P, Mendall MA, Khulusi S, Northfield TC, Strachan DP. Helicobacter pylori infection in childhood: risk factors and effect on growth. *Br Med J* 309: 1119-1123, 1994

Sullivan PB, Thomas JE, Wight DGD, Neale G, Eastham EJ, Corrah T, Lloyd-Evans N, Greenwood BM. Helicobacter pylori in Gambian children with chronic diarrhoea and malnutrition. *Arch Dis Child* 65: 189-1891, 1990

Szabo I, Brutsche S, Tombola F, Moschioni M, Satin B, Telford JL, Rappuoli R, Montecucco C, Papini E, Zoratti M. Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of Helicobacter pylori is required for its biological activity. *EMBO* 18: 5517-5527,1999

Telford JL, Ghiara P, Dell'Orco M, Comanducci M, Burroni D, Bugnoli M, Tecce MF, Censini S, Covacci A, Xiang Z, Papini E, Montecucco C, Parente L, Rappuoli R. Gene structure of the Helicobacter pylori cytotoxin and evidence of its key role in gastric disease. *J Exp Med* 179: 1653-1658, 1994

Thomas J, Gibson G, Darboe M, Dale A, Weaver L. Isolation of Helicobacter pylori from human faeces. *Lancet* 340: 1194-11195, 1992

Xiang Z, Censini S, Bayeli PF, Telford JL, Figura N, Rappuoli R, Covacci A. Analysis of expression of CagA and VacA virulence factors in 43 strains of Helicobacter pylori reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. *Infect Immun* 63: 94-98, 1995