Helicobacter pylori toxin VacA is transferred to host cells via a novel contact-dependent mechanism

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Summary

Helicobacter pylori **is the causative agent of peptic ulcer disease. A major virulence factor of** *H. pylori* **is VacA, a toxin that causes massive vacuolization of epithelial cell lines** *in vitro* **and gastric epithelial erosion** *in vivo*. **Although VacA is exported over the outer membrane and is released from the bacteria, a portion of the toxin remains associated with the bacterial surface. We have found surface-associated toxin to be biologically active and spatially organized into distinct toxin-rich domains on the bacterial surface. Upon bacterial contact with host cells, toxin clusters are transferred directly from the bacterial surface to the host cell surface at the bacteria–cell interface, followed by uptake and intoxication. This contact-dependent transfer of VacA represents a cost-efficient route for delivery of VacA and potentially other bacterial effector molecules to target cells.**

Introduction

The human-specific gastric pathogen *Helicobacter pylori* causes chronic gastritis and peptic ulcer disease (Blaser, 1998). It is also associated with development of gastric adenocarcinoma (Blaser and Parsonnet, 1994). The vacuolating cytotoxin, VacA, is a major virulence factor of *H. pylori* (reviewed by Reyrat *et al.*, 1999a), which, together with two other virulence factors, CagA (Covacci *et al.*, 1999) and the blood group antigen-binding adhesin BabA (Ilver *et al.*, 1998), is significantly associated with strains isolated from patients with more severe disease such as peptic ulcer or adenocarcinoma (Prinz *et al.*, 2001). VacA has been shown to cause gastric epithelial erosion when administered intragastrically to mice (Telford *et al.*, 1994).

In vitro, it causes massive vacuolar degeneration of subconfluent epithelial cell lines and a reduction in *trans*-epithelial resistance in polarized epithelial monolayers (reviewed by Reyrat *et al.*, 1999a).

VacA belongs to the autotransporter family of secreted proteins that are transported across the inner membrane of Gram-negative bacteria by a *sec*-dependent process and across the outer membrane by a transporter domain located at the carboxy-terminus of the precursor protein (Henderson *et al.*, 1998). The transporter domain is proteolytically removed from the protein during transport across the outer membrane. The VacA precursor polypeptide is ≈140 kDa and is processed during export to produce the mature toxin subunit of 87 kDa (Telford *et al.*, 1994; Nguyen *et al.*, 2001). The toxin is released from the bacteria and is found in the culture supernatant in the form of high-molecular-weight homo-oligomers containing 6–7 or 12–14 copies of the mature toxin polypeptide (Lupetti *et al.*, 1996; Cover *et al.*, 1997). The purified oligomeric form of the toxin is biologically inactive, but its vacuolating activity is revealed by dissociation of the oligomers by treatment at low or high pH (de Bernard *et al.*, 1995; Yahiro *et al.*, 1999). This is in contrast to VacA activity in concentrated culture supernatants, which is not increased by low pH activation. However, a considerable proportion of the mature 87 kDa toxin remains firmly associated with the bacteria (Telford *et al.*, 1994). This surface-associated toxin has been demonstrated to reduce TER in polarized epithelial cell monolayers, and it has therefore been proposed that it is in a monomeric form (Pelicic *et al.*, 1999). Each 87 kDa monomer has two functional domains, p37 and p58, connected by a hydrophilic loop (Telford *et al.*, 1994). The p58 domain is responsible for host cell binding (Reyrat *et al.*, 1999b) and host tropism (Pagliaccia *et al.*, 1998). The p37 domain, together with the N-terminal 150 amino acids of p58, is sufficient to cause vacuole formation when expressed in the cytoplasm of host cells (de Bernard *et al.*, 1998).

Most published research on the function of VacA has dealt with toxin isolated and purified from growth media. This paper focuses on the form of VacA that is located on the bacterial surface. Bacterially associated toxin was found to be fully active in the cell vacuolation assay and is transferred to host cells by a novel contact-dependent mechanism, thus presenting a possible functional connection between bacterial adhesion factors and VacA.

Results

VacA is associated with the bacterial surface in an active form

Figure 1 shows anti-VacA immunoblots of whole bacterial extracts and corresponding cell culture concentrates from liquid cultures of *H. pylori*. Whereas VacA is usually puri-fied from the culture supernatant, under conditions of mild agitation, more than 50% of the VacA remains associated with the bacteria (Fig. 1). From the apparent molecular weight of the toxin associated with the bacteria ($\approx$ 90 kDa), it is clear that the carboxy-terminal outer mem-brane exporter region has been proteolytically removed, thus indicating that the toxin has been secreted through the outer membrane to the surface of the bacteria. Anti-body-reactive material corresponding to the predicted pre-cursor (140 kDa) was not observed in this experiment and has never been described in the literature, suggesting that transport and processing of the precursor occurs rapidly after synthesis. To determine whether the toxin associated with the bacteria is biologically active, AGS cells were incubated with live bacteria for 30 min; then, the non-adhering bacteria were washed away, and incubation was continued for a further 7.5 h. Ammonium chloride (5 mM) was included in the culture medium in order to promote osmotic swelling of the vacuoles (Sommi *et al.*, 1998), which are otherwise barely detectable. Vacuolation, as assessed by the uptake of neutral red dye, was detected after incubation with the bacteria for 8 h in the absence of any activating treatment (Fig. 2A). Significant vacuolation was observed at a multiplicity of infection (MOI) as low as about 50 bacteria per eukaryotic cell. Although we did not assess the percentage of bacteria that remained adhering to the cells after the non-adhering bacteria were washed away, light microscope observation indicated that cells with as few as 10 bacteria adhering contained visible vacuoles (data not shown). In contrast, highly purified,
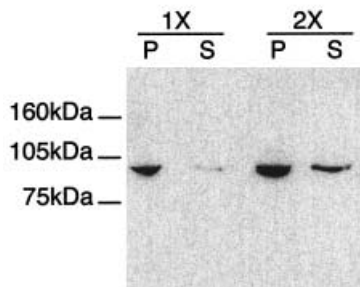

Fig. 1. VacA immunoblots of bacterial pellet and supernatant. Equal amounts of extracts of *H. pylori* (P) and the corresponding quantity of culture supernatant (S) were separated on SDS-PAGE and blotted with anti-VacA antiserum. The right two lanes (2X) were loaded with twice as much extract as the left two lanes (1X). Migration of molec-ular weight markers is shown.
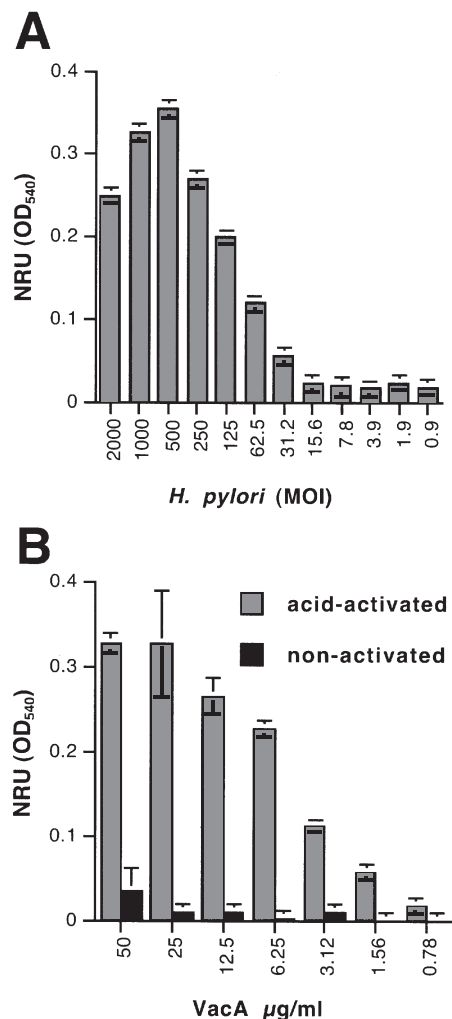

Fig. 2. Vacuolation of AGS cells by either purified VacA or infection with live *H. pylori*.
A. Neutral red uptake by AGS cells infected with live *H. pylori* at different multiplicity of infection (MOI). Cells were infected at the MOI indicated for 30 min. The non-adherent bacteria were then washed away, and the cells were incubated for a further 7.5 h before Neutral red uptake assay.
B. Neutral red dye uptake by AGS cells treated for 8 h with acid-activated or non-activated VacA as indicated.

oligomeric toxin had barely detectable activity even at a concentration of 50 µg ml^{-1} and only became active after treatment at pH 2.0 (Fig. 2B). These data indicate that VacA on the surface of the bacteria is presented to host cells in an active form.

VacA is organized into distinct domains on the bacterial surface

The spatial localization of VacA on the surface of *H. pylori* bacteria was investigated by confocal microscopy. A mon-oclonal antibody (mAb), C1G9, recognizing a native
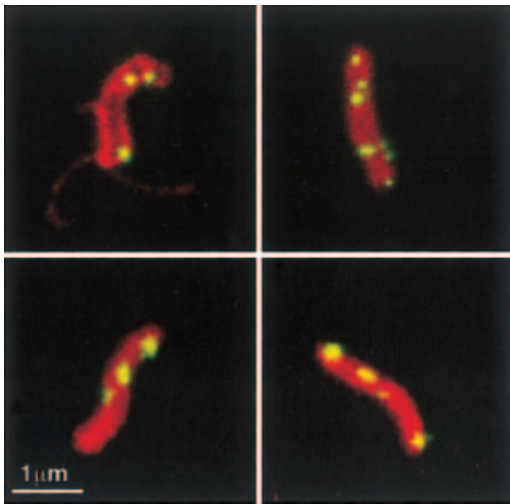
Fig. 3. Confocal micrographs of *H. pylori* stained with anti-VacA mAb. Four examples of single bacteria showing the typical punctate staining (green) seen when stained with either anti-VacA mAb C1G9 and fluorescent-labelled anti-mouse Ig secondary antibody (top) or C1G9 directly labelled with Alexa fluor 488 (bottom). The bacteria are counterstained with a rabbit antiserum raised against whole *H. pylori* and a labelled anti-rabbit Ig secondary antibody (red).

epitope in the p58 region of VacA (Reyrat *et al.*, 1998) was used in combination with a secondary fluorescence-labelled antibody to detect the toxin. To visualize the bacteria, antiserum raised against whole *H. pylori* was used. Confocal microscopy revealed a distinct pattern of staining in which the toxin appears to be clustered in discrete packets on the surface of the bacteria (Fig. 3). To exclude the possibility of an artifactual antibody-mediated capping of VacA being responsible for the observed spotted pattern of VacA, bacteria were fixed with paraformaldehyde before staining with the antibodies. In addition, the same pattern of staining was observed when a polyclonal antiserum raised against purified VacA (Manetti *et al.*, 1995) or directly labelled C1G9 antibody was used, and no staining was observed on *H. pylori* in which the *vacA* gene was inactivated by replacement with an antibiotic resistance cassette (data not shown). The nature of the toxin packets is not clear; however, the data suggest that the VacA is not randomly distributed on the surface.

Direct transfer of VacA from bacteria to host cells upon contact

The apparently organized nature of the toxin in its active state motivated us to study the mode of transfer of VacA from the bacterial surface to host cells. In particular, we were interested to ascertain whether the toxin was delivered directly to the cells from the surface of the bacteria or whether it was first released into the culture medium then subsequently taken up by the cells. To address this, we incubated AGS cells with *H. pylori* at an MOI of 100

bacteria per cell for 4 h and followed uptake of VacA by confocal microscopy. In these experiments, ammonium chloride was excluded from the culture medium in order to avoid the loss of cell integrity resulting from massive swelling of the vacuoles. Figure 4 shows *H. pylori* adhering to intact AGS cells and stained with C1G9 anti-VacA mAb (green) and antiserum raised against intact *H. pylori* (red). Although there were sufficient bacteria per cell to cause vacuolation (see Fig. 2), very little toxin was observed on the surface of the cells. In contrast, bright staining was observed when the cells incubated with bacteria were permeabilized by treatment with 0.2% Triton X-100 before staining (Fig. 4). No staining was observed when AGS cells were infected with the *H. pylori* strain deleted for the *vacA* gene and treated in an identical manner (data not shown). Cells treated with highly purified, soluble VacA, even at a concentration of VacA below that required for vacuolation (1 μg ml^{-1}; see Fig. 2), showed bright staining in both permeabilized and non-permeabilized cells (Fig. 4). Hence, these data suggest that VacA can be transferred to the target cells directly from the bacteria without first being released into the culture medium.

To confirm this, we have carried out similar experiments using very few bacteria per cell. Figure 5 shows VacA and whole bacterial staining of permeabilized AGS cells incubated for 30 min with *H. pylori* at an MOI of 20 bacteria per cell, then washed and incubated for a further 3.5 h. VacA can clearly be observed in cells with only a few adherent bacteria, indicating efficient delivery of the toxin. Strikingly, nearby cells to which there are no bacteria adhering show no VacA staining. These data demonstrate unequivocally that VacA is delivered to the cells directly from the bacteria without release into the culture supernatant and suggest that delivery is contact dependent.

Time course for toxin transfer

The process of VacA transfer from bacteria to host cells was studied over time by stopping the toxin transfer at different times after infection. AGS cells were infected with *H. pylori* and, at different time points, samples were fixed and processed for immunodetection of VacA and analysed by confocal microscopy. In order to have sufficient VacA to detect at the very short time point, an MOI of 200 bacteria per cell was used, and the non-adherent *H. pylori* were not washed away until the end of the incubation period. For the longer time points, a lower MOI (100 bacteria per cell) was used, and the non-adherent bacteria were washed away after 30 min. As seen in Fig. 6, shortly after attachment of wild-type *H. pylori* to host cells, VacA could be detected in small domains on the surface of the bacteria. However, after 1 h, the VacA-rich domains were no longer visible on the bacterial surface (Fig. 6). Instead,
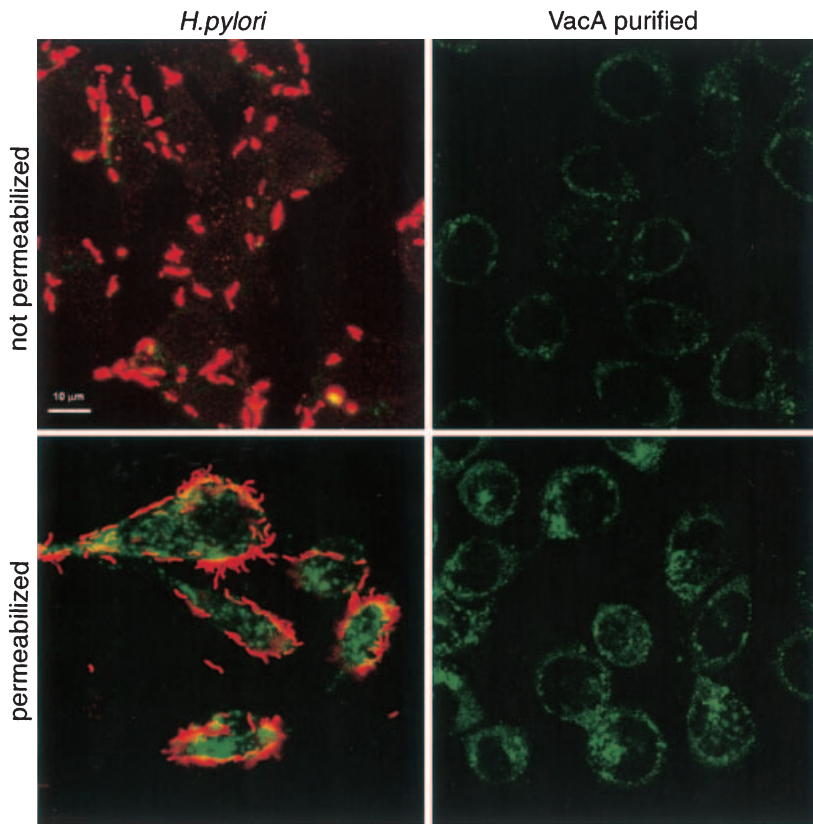
Fig. 4. VacA staining in AGS cells infected with live *H. pylori* or treated with purified VacA. AGS cells were infected with live *H. pylori* at an MOI of 100 (left) or acid-activated, purified VacA at 1 μg ml^{-1} (right). Cells were then fixed directly (top) or first permeabilized with 0.2% Triton X-100 before fixing (bottom). VacA was stained with mAb C1G9 (green), and bacteria were counterstained with anti-*H. pylori* antiserum (red).

the toxin was relocated to the interface between bacteria and cells. Interestingly, the VacA staining remained in spots similar to its organization on the bacterial surface. The clustered location of VacA in the cells underneath the bacteria supports a direct transfer of toxin from bacteria to the same cell that it is attached to. After 8 h incubation, the majority of the toxin had been transferred into the host cell, and practically no toxin could be detected on the bacteria or at the cell surface (Fig. 6).

We have described previously a truncated VacA protein, p58, which lacks most of the 37 kDa amino-terminal domain (Reyrat *et al.*, 1999b). This mutant, which completely lacks vacuolating activity, still binds efficiently to target cells but fails to be internalized. When AGS cells were infected with bacteria expressing p58, at early times, C1G9 staining had a similar punctate appearance on the bacterial surface to that observed with full-length VacA (Fig. 7). After 2 h, the staining began to accumulate at the cell membrane underneath the bacteria adhering to the cell (data not shown). After 8 h of incubation, most of the toxin had been depleted from the bacteria and had accumulated at the cell–bacteria interface but, in line with
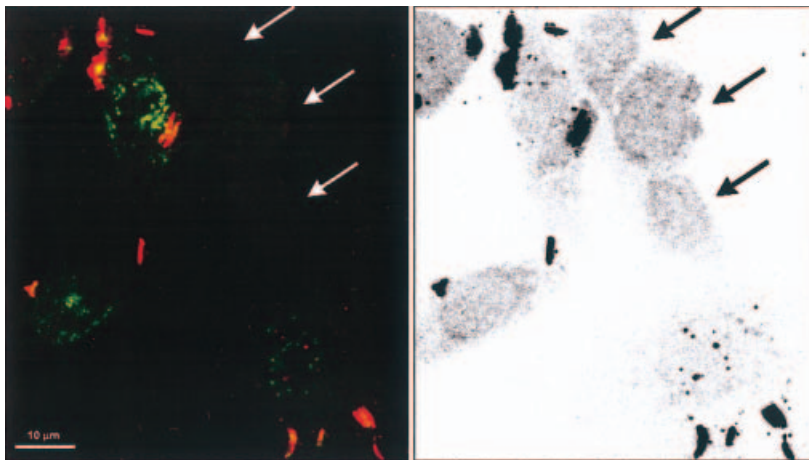

Fig. 5. VacA staining in AGS cells infected with very few bacteria. AGS cells were infected with live *H. pylori* at an MOI of 20 bacteria per cell for 30 min. The non-adherent bacteria were then washed away, and the incubation continued for 8 h. After permeabilization with 0.2% Triton X-100, VacA was stained with anti-VacA mAb C1G9 (green), and bacteria were stained with anti-*H. pylori* antiserum (red). As very few bacteria were used, not all cells had adherent bacteria. Left, a PHOTOSHOP-enhanced image to visualize the position of unstained cells with no bacteria adhering (arrows).
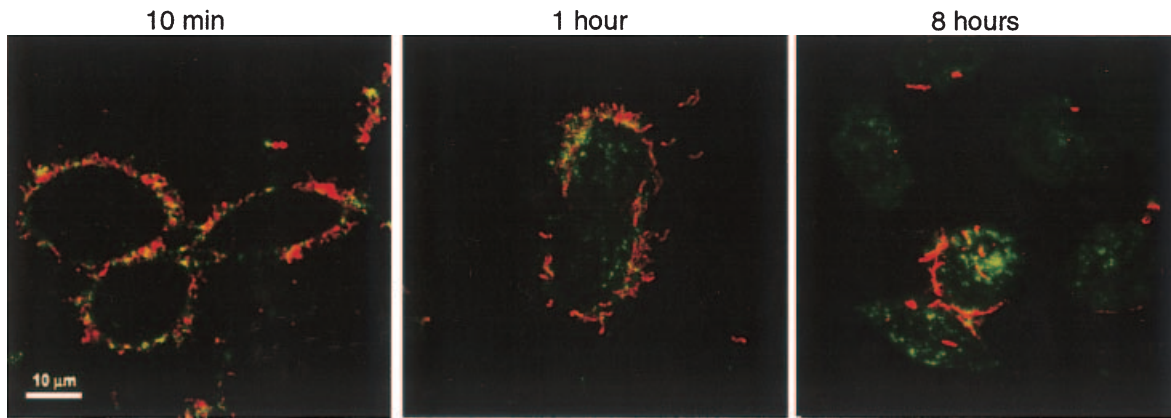
Fig. 6. Time course of VacA transfer from live *H. pylori* to AGS cells. AGS cells were infected with live *H. pylori* for the times shown then permeabilized with 0.2% Triton X-100, fixed and stained with anti-VacA mAb C1G9 (green) and anti-*H. pylori* antiserum (red). For the 10 min time point, an MOI of 200 was used. For the 1 h and 8 h time points, an MOI of 100 was used, and the non-adherent bacteria were washed away after 30 min incubation.

previous studies on p58, no intracellular staining was observed (Fig. 7). Thus, even though p58 is inactive and fails to be internalized, it is still transferred from the bacteria to the cell in a manner similar to the wild-type toxin.

Discussion

We have demonstrated that VacA is delivered directly to infected target cells in an active form. This indicates that the inactive high-molecular-weight soluble oligomeric form that is found in the culture supernatant is not assembled on the bacteria. Ricci *et al.* (1997) have demonstrated VacA activity in concentrated culture supernatants that is not increased by low pH activation, but this activity may result from VacA associated with released outer membrane vesicles (Fiocca *et al.*, 1999a). It appears therefore that there are two independent ways of delivering VacA, and this raises the question of the relevance of the oligomeric form. Although the pH of the region below the gastric mucous layer is close to neutral and the released oligomer could

be formed, it is unclear what function it may have. In order to become active, it must first traverse the mucous layer to the lumen of the stomach where the pH is sufficiently low to cause dissociation of the oligomer and activation of the toxin. It is possible that this mechanism permits the toxin to be dispersed from the site of colonization to attack cells some distance away, but it is difficult to see why this should be an advantage to the bacteria. The possibility cannot be excluded that the oligomeric form is, in fact, an artifact formed from the interaction of hydrophobic regions of the monomer released from the bacteria by vigorous stirring in the artificial conditions of laboratory growth.

Delivery of the active, presumably monomeric, form of the toxin directly at the site of adhesion of the bacteria to the gastric epithelium appears to make more sense in terms of advantage to the bacteria. The toxin forms channels in target cell membranes that allow the passage, not only of certain essential cations such as bicarbonate and pyruvate, but also of urea (Szabo *et al.*, 1999; Tombola *et al.*, 2001). Urea is an important source of nitrogen for
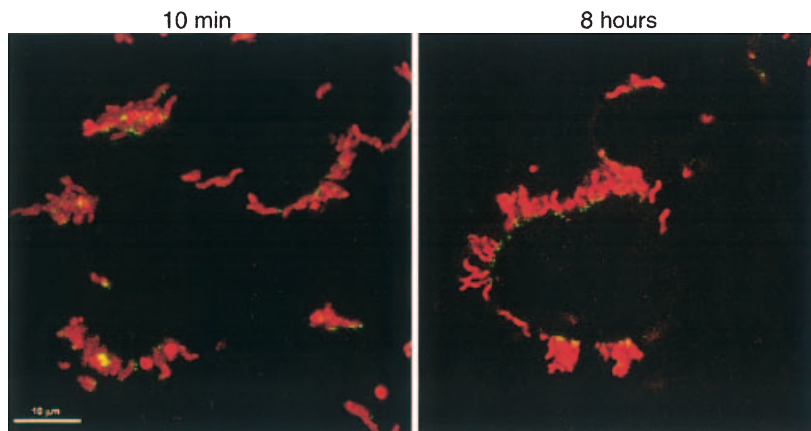

Fig. 7. Direct transfer of a non-internalized VacA deletion mutant from *H. pylori* to AGS cells. AGS cells were infected with the mutant *H. pylori* strain SPM326-P58 for the times shown then permeabilized, fixed and stained with anti-VacA mAb C1G9 (green) and anti-*H. pylori* antiserum (red).

H. pylori, and bicarbonate and pyruvate are essential molecules in a number of metabolic pathways in many bacteria. Hence, the primary function of VacA may be to assist the bacteria in the acquisition of important nutrients. In this context, the reduction in integrity of the gastric epithelial monolayer suggested by the VacA-induced reduction in TER observed in epithelial monolayers *in vitro* may play a similar role.

The nature of the VacA on the surface of the bacteria is still unclear. The punctate staining suggests that the protein accumulates in some kind of outer membrane subdomain. These may be budding outer membrane vesicles that are reported to contain VacA (Fiocca *et al.*, 1999b). The fact that the toxin is active, however, indicates that it is not organized in the high-molecular-weight regular oligomers found in culture supernatant. Neither is the mechanism of transfer of the protein to the eukaryotic cell known. What is clear is that, on adhesion to the target cell, the surface of the bacteria becomes depleted of toxin staining. This process may not necessarily require an active transport mechanism. A simple explanation may be that the toxin packets are free to diffuse over the surface of the bacteria until they come to the interface between it and the eukaryotic cell, at which point they may become trapped by interaction with specific receptors or by direct interaction with cell membrane components.

On the other hand, in biopsies from infected patients, it has been observed that a large proportion of resident *H. pylori* are located in the mucin layer rather than attached to the host surface mucus cells. The demonstration that toxin is quickly transferred to the host cell from the bacterial surface upon adherence may suggest that the surface-located VacA is also a storage form of toxin ready to be transferred at bacterial–host cell contact.

In conclusion, we have described a novel delivery of the VacA cytotoxin directly from the adhering bacteria to its target cells. This means of delivery appears to be very efficient and ensures that the toxin reaches the relevant cells where its activity is required. Furthermore, it highlights the importance of adhesion of the bacteria to the cells of the gastric epithelium in the disease process. It is of note that adhesion is also necessary for the transfer of CagA through the *cag* type IV secretion system to the cytoplasm of the cells where it plays a major role in disease (Covacci *et al.*, 1999). It is thus less surprising that there is a strong statistical association of strains expressing all three virulence factors, VacA, CagA and the BabA adhesin, with severe *H. pylori*-induced disease (Prinz *et al.*, 2001).

Experimental procedures

Bacteria and culture conditions

Helicobacter pylori strains SPM326 (Marchetti *et al.*, 1995) and

SPM326p58 (Reyrat *et al.*, 1999b) were maintained in 5% CO_2 at 37°C on Brucella agar plates supplemented with 5% horse blood. For infection experiments, bacteria were adapted to grow in cell culture media (50% DMEM, 50% Ham's F12 mixture) with 5% FCS for at least 3 days, changing the media and diluting the culture daily by 20-fold to keep bacteria in the logarithmic growth phase. Only spiral-shaped swimming bacteria were used for infection experiments.

Cell culture

AGS cells were grown in a 50% DMEM and 50% Ham's F12 mixture supplemented with 10% FCS, 2-mM L-glutamine, 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin at 37°C in 5% CO_2. Cells were seeded at a density of 1.5×10^5 ml^{-1}, 20 h before the infection experiments. Cells were seeded on Laboratory Teck II chamber slides (Nunc).

Cell vacuolation assay

AGS cells were seeded at 1.5×10^5 ml^{-1} in 96-well microtitre plates in medium without antibiotics 20 h before the assay. The extent of vacuolation was determined quantitatively by measuring the uptake of Neutral red dye (Cover *et al.*, 1992). VacA purified from *H. pylori* culture supernatant (Manetti *et al.*, 1995) was acid activated by adjusting to pH 2.0 for 5 min at room temperature, then neutralized before incubation with the cells as described previously (de Bernard *et al.*, 1995). Live *H. pylori* from liquid culture were centrifuged at 5000 r.p.m. in a microfuge and washed once before adding to the cells. After 30 min, incubation chambers were washed with culture media to remove unbound bacteria, then incubated at 37°C for a further 7.5 h.

Immunoblots

Liquid cultures of *H. pylori,* grown to OD$_{600}$ of 1.2 were harvested and centrifuged at 13 000 r.p.m. in a microfuge. The pellet was resuspended in lysis buffer (8 M urea, 100 mM NaH$_2$PO$_4$, 10 mM Tris, pH 7.4) and incubated for 1 h at 4°C. The corresponding culture supernatants were concentrated by precipitating with a 50% saturated solution of ammonium sulphate. Samples of pellet and supernatants were loaded on 7.5% SDS-polyacrylamide gels in amounts corresponding to identical initial volumes of the culture. After transfer of the proteins to polyvinylidene difluoride (PVDF) membrane (Bio-Rad), the membranes were blocked with 5% BSA in PBS containing 0.05% Tween for 1 h at room temperature. VacA was detected using a rabbit polyclonal antibody raised against the toxin diluted in the same buffer. An anti-rabbit peroxidase-conjugated antibody was used as secondary antibody. Blots were developed using 4-chloro-1-naphthol and H_2O_2.

Cell infection experiments

Spiral *H. pylori* SPM326 or SPM326p58 in logarithmic growth phase were harvested, washed and resuspended in culture medium (50% DMEM, 50% Ham's F12 mixture + 5% FCS) and diluted to the appropriate MOI. Unbound bacteria were removed after 30 min incubation by washing wells twice with culture media. Incubations varied from 10 min to 8 h at 37°C and 5% CO_2. In

the case of the 10 min incubations, bacteria were not removed. For immunofluorescence experiments with purified toxin, VacA was acid activated and added to AGS cells at 1 μg ml^{-1} final concentration.

Immunofluorescence and confocal microscopy

For indirect immunofluorescence analysis, AGS cells were grown on chamber slides (Nunc). Incubation with either purified toxin or *H. pylori* was stopped by fixing with 3.7% paraformaldehyde in PBS plus 5 mM Ca^{2+} and Mg^{2+} for 15 min at room temperature. Aldehyde groups were quenched with 0.2 M glycine for 5 min at room temperature. Slides were than washed three times with PBS for 10 min each.

For cytosolic (internal) staining, cells were permeabilized with 0.2% Triton X-100 for 10 min at room temperature. PBS 5% FCS was used as blocking solution. Bacteria were stained with a rabbit serum raised against whole bacteria. For VacA staining, a mouse monoclonal antibody against the native toxin was used at 2 μg ml^{-1} (Reyrat *et al.*, 1998). Alexa fluor 568 goat anti-rabbit and Alexa 488 goat anti-mouse (Molecular Probes) fluorescent-labelled secondary antibodies were used. Where indicated, C1G9 was directly labelled with Alexa fluor 568 using a monoclonal antibody labelling kit (Molecular Probes) according to the manufacturer's instructions. Slides for confocal analysis were mounted with *SlowFade light* antifade kit (Molecular Probes).

Confocal images were obtained using a Leica TCS4D confocal microscope equipped with a krypton/argon laser (Leica Microsystems). Images were collected using low laser emission to attenuate photobleaching, and eight frame-averaged scans were made per image to improve the signal:noise ratio. Images collected at several focal planes were superimposed and merged into a single file and imported into Adobe PHOTOSHOP to adjust the size.

References

de Bernard, M., Papini, E., de Filippis, V., Gottardi, E., Telford, J., Manetti, R., *et al.* (1995) Low pH activates the vacuolating toxin of Helicobacter pylori, which becomes acid and pepsin resistant. *J Biol Chem* **270:** 23937–23940.

de Bernard, M., Burroni, D., Papini, E., Rappuoli, R., Telford, J., and Montecucco, C. (1998) Identification of the *Helicobacter pylori* VacA toxin domain active in the cell cytosol. *Infect Immun* **66:** 6014–6016.

Blaser, M.J. (1998) *Helicobacter pylori* and gastric diseases. *BMJ* **316:** 1507–1510.

Blaser, M.J., and Parsonnet, J. (1994) Parasitism by the 'slow' bacterium *Helicobacter pylori* leads to altered gastric homeostasis and neoplasia. *J Clin Invest* **94:** 4–8.

Covacci, A., Telford, J.L., Del Giudice, G., Parsonnet, J., and Rappuoli, R. (1999) *Helicobacter pylori* virulence and genetic geography. *Science* **284:** 1328–1333.

Cover, T.L., Vaughn, S.G., Cao, P., and Blaser, M.J. (1992) Potentiation of *Helicobacter pylori* vacuolating toxin activity by nicotine and other weak bases. *J Infect Dis* **166:** 1073–1078.

Cover, T.L., Hanson, P.I., and Heuser, J.E. (1997) Acid-induced dissociation of VacA, the *Helicobacter pylori* vacuolating cytotoxin, reveals its pattern of assembly. *J Cell Biol* **138:** 759–769.

Fiocca, R., Necchi, V., Sommi, P., Ricci, V., Telford, J., Cover, T.L., and Solcia, E. (1999a) Release of *Helicobacter pylori* vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium. *J Pathol* **188:** 220–226.

Fiocca, R., Necchi, V., Sommi, P., Ricci, V., Telford, J., Cover, T.L., and Solcia, E. (1999b) Release of *Helicobacter pylori* vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium. *J Pathol* **188:** 220–226.

Henderson, I.R., Navarro-Garcia, F., and Nataro, J.P. (1998) The great escape: structure and function of the autotransporter proteins. *Trends Microbiol* **6:** 370–378.

Ilver, D., Arnqvist, A., Ogren, J., Frick, I.M., Kersulyte, D., Incecik, E.T., *et al.* (1998) *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science* **279:** 373–377.

Lupetti, P., Heuser, J.E., Manetti, R., Massari, P., Lanzavecchia, S., Bellon, P.L., *et al.* (1996) Oligomeric and subunit structure of the *Helicobacter pylori* vacuolating cytotoxin. *J Cell Biol* **133:** 801–807.

Manetti, R., Massari, P., Burroni, D., de Bernard, M., Marchini, A., Olivieri, R., *et al.* (1995) *Helicobacter pylori* cytotoxin: importance of native conformation for induction of neutralizing antibodies. *Infect Immun* **63:** 4476–4480.

Marchetti, M., Arico, B., Burroni, D., Figura, N., Rappuoli, R., and Ghiara, P. (1995) Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science* **267:** 1655–1658.

Nguyen, V.Q., Caprioli, R.M., and Cover, T.L. (2001) Carboxy-terminal proteolytic processing of *Helicobacter pylori* vacuolating toxin. *Infect Immun* **69:** 543–546.

Pagliaccia, C., de Bernard, M., Lupetti, P., Ji, X., Burroni, D., Cover, T.L., *et al.* (1998) The m2 form of the *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. *Proc Natl Acad Sci USA* **95:** 10212–10217.

Pelicic, V., Reyrat, J.M., Sartori, L., Pagliaccia, C., Rappuoli, R., Telford, J.L., *et al.* (1999) *Helicobacter pylori* VacA cytotoxin associated with the bacteria increases epithelial permeability independently of its vacuolating activity. *Microbiology* **145:** 2043–2050.

Prinz, C., Schoniger, M., Rad, R., Becker, I., Keiditsch, E., Wagenpfeil, S., *et al.* (2001) Key importance of the *Helicobacter pylori* adherence factor blood group antigen binding adhesin during chronic gastric inflammation. *Cancer Res* **61:** 1903–1909.

Reyrat, J.M., Charrel, M., Pagliaccia, C., Burroni, D., Lupetti, P., de Bernard, M., *et al.* (1998) Characterisation of a monoclonal antibody and its use to purify the cytotoxin of *Helicobacter pylori*. *FEMS Microbiol Lett* **165:** 79–84.

Reyrat, J.M., Pelicic, V., Papini, E., Montecucco, C., Rappuoli, R., and Telford, J.L. (1999a) Towards deciphering the *Helicobacter pylori* cytotoxin. *Mol Microbiol* **34:** 197–204.

Reyrat, J.M., Lanzavecchia, S., Lupetti, P., de Bernard, M., Pagliaccia, C., Pelicic, V. *et al.* (1999b) 3D imaging of the

58 kDa cell binding subunit of the *Helicobacter pylori* cytotoxin. *J Mol Biol* **290:** 459–470.

Ricci, V., Sommi, P., Fiocca, R., Romano, M., Solcia, E., and Ventura, U. (1997) *Helicobacter pylori* vacuolating toxin accumulates within the endosomal-vacuolar compartment of cultured gastric cells and potentiates the vacuolating activity of ammonia. *J Pathol* **183:** 453–459.

Sommi, P., Ricci, V., Fiocca, R., Necchi, V., Romano, M., Telford, J.L., *et al.* (1998) Persistence of *Helicobacter pylori* VacA toxin and vacuolating potential in cultured gastric epithelial cells. *Am J Physiol* **275:** G681–G688.

Szabo, I., Brutsche, S., Tombola, F., Moschioni, M., Satin, B., Telford, J.L., *et al.* (1999) Formation of anion-selective channels in the cell plasma membrane by the toxin VacA of *Helicobacter pylori* is required for its biological activity. *EMBO J* **18:** 5517–5527.

Telford, J.L., Ghiara, P., Dell'Orco, M., Comanducci, M., Burroni, D., Bugnoli, M., *et al.* (1994) Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *J Exp Med* **179:** 1653–1658.

Tombola, F., Morbiato, L., Del Giudice, G., Rappuoli, R., Zoratti, M., and Papini, E. (2001) The *Helicobacter pylori* VacA toxin is a urea permease that promotes urea diffusion across epithelia. *J Clin Invest* **108:** 929–937.

Yahiro, K., Niidome, T., Kimura, M., Hatakeyama, T., Aoyagi, H., Kurazono, H., *et al.* (1999) Activation of *Helicobacter pylori* VacA toxin by alkaline or acid conditions increases its binding to a 250-kDa receptor protein-tyrosine phosphatase beta. *J Biol Chem* **274:** 36693–36699.