Persistence of Helicobacter pylori VacA toxin and vacuolating potential in cultured gastric epithelial cells

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Sommi, Patrizia, Vittorio Ricci, Roberto Fiocca, Vittorio Necchi, Marco Romano, John L. Telford, Enrico Solcia, and Ulderico Ventura. Persistence of Helicobacter pylori VacA toxin and vacuolating potential in cultured gastric epithelial cells. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G681–G688, 1998.—The vacuolating toxin A (VacA) is one of the most important virulence factors in Helicobacter pylori-induced damage to human gastric epithelium. Using human gastric epithelial cells in culture and broth culture filtrate from a VacA-producing H. pylori strain, we studied 1) the delivery of VacA to cells, 2) the localization and fate of internalized toxin, and 3) the persistence of toxin inside the cell. The investigative techniques used were neutral red dye uptake, ultrastructural immunocytochemistry, quantitative immunofluorescence, and immunoblotting. We found that VacA 1) is delivered to cells in both free and membrane-bound form (i.e., as vesicles formed by the bacterial outer membrane), 2) localizes inside the endosomal-lysosomal compartment, in both free and membrane-bound form, 3) persists within the cell for at least 72 h, without loss of vacuolating power, which, however, becomes evident only when NH4Cl is added, and 4) generally does not degrade into fragments smaller than ~90 kDa. Our findings suggest that, while accumulating inside the endosomal-lysosomal compartment, a large amount of VacA avoids the main lysosomal degradative processes and retains its apparent molecular integrity.

outer membrane vesicles; VacA internalization; VacA metabolism; VacA immunocytochemistry

HELCOBACTER PYLORI is a gram-negative curved or spiral bacterium that plays a major role in the development of chronic gastritis, peptic ulcer, and gastric cancer (22, 25, 34, 38, 39). H. pylori is specifically suited to the colonization of the human stomach, in which it causes an inflammatory reaction and epithelial damage with cellular swelling and cytoplasmic vacuolation, both in vivo and in vitro (4, 11, 13, 16, 24, 29, 30, 37). The two main bacterial factors involved in this cellular damage are urease (14, 20) and vacuolating toxin A (VacA) (2), as expressed by 100% and 50–60%, respectively, of H. pylori clinical isolates. Urease acts by producing ammonia via urea hydrolysis. The mechanism through which ammonia (and other weak bases) exerts its cytopathic effect is well known (9, 23, 26). Ammonia crosses cell membranes in an uncharged state, is trapped by protonation within acidic intracellular compartments, and thereafter induces osmotic swelling of these compartments, which in turn causes cell vacuolation.

VacA seems to play a key role in epithelial damage induced by H. pylori infection (2, 13, 37), but the mechanism through which vacuolation occurs remains poorly understood. Monomeric VacA, with a molecular mass of ~90 kDa (3), is synthetized by H. pylori as a 139-kDa protoxin (2, 37), which is rapidly processed to form the native toxin released in the extracellular environment. In bacterial culture, and probably in vivo too, monomers of ~90 kDa gather together to form high molecular mass (1,000 kDa) dimers (2, 3, 10). Moreover, it has been suggested that the monomeric toxin of ~90 kDa is further processed to produce a 37-kDa NH₂-terminal fragment and a 58-kDa COOH-terminal fragment and that these fragments remain associated after deavage (36, 37). VacA is believed to exert its cytotoxic activity by acting inside the cell (12), but the molecular species involved in vacuolation and the fate of the internalized toxin remain unclear. Furthermore, it is unknown how long the toxin and/or its fragments persist inside the cell.

In the present study, through using human gastric epithelial cells in culture, we attempt to clarify the uptake, localization, and fate of the internalized toxin and the time of persistence of toxin inside the cell.

MATERIALS AND METHODS

Bacterial strains and filtrate production. The VacA-producing H. pylori strain used was CCUG 17587 (from Culture Collection University of Göteborg, Göteborg, Sweden). Bacteria were grown in Brucella broth supplemented with 5% FCS (GIBCO, Grand Island, NY), for 24–36 h at 37°C in a thermostatic shaker under microaerophilic conditions. As previously described (35), to obtain the broth culture filtrate (BCF), we then removed bacteria by centrifugation and sterilized the supernatants by passage through a 0.22-µm cellulose acetate filter (Nalgé, Rochester, NY). Uninoculated broth filtrate served as a control. To remove ammonia, we dialyzed control and BCF against Hanks’ balanced salt solution (HBSS) for 36 h in dialysis tubing with a 12-kDa molecular mass cutoff (Sigma Chemical, St. Louis, MO). The presence of VacA in the BCF was tested by means of SDS-PAGE, followed by immunoblotting with anti-VacA serum (27).

Gastric epithelial cells and cell incubation. For this study, we used the MKN 28 cell line. This cell line, derived from a human gastric tubular adenocarcinoma, shows moderate gastric-type differentiation (15, 31). MKN 28 cells were grown as monolayers in DMEM/Ham’s nutrient mixture F-12 (Sigma
Chemical) supplemented with 10% FCS (GIBCO) in 35-mm petri dishes (Corning Glass Works, Corning, NY) at 37°C in a humidified atmosphere of 5% CO2 in air.

Subconfluent cell monolayers were washed twice with HBSS before incubation for 16 h (loading period; step 1) with either un inoculated broth filtrate (diluted 1:3 in HBSS), BCF (diluted 1:3 in HBSS, both with and without 4 mM NH4Cl), or 4 mM NH4Cl (dissolved in HBSS). After the initial loading, cell monolayers were incubated for 5 h (step 2) with either HBSS or NH4Cl and were finally treated for 16 h (step 3) in Hanks' balanced salt solution (HBSS) or NH4Cl. At the end of each step, cell vacuolation was quantitated by neutral red dye uptake assay. Each SE was <10% of the respective mean. *P < 0.05, **P < 0.001 vs. control at the same step.

For electron microscopy study, MKN 28 cells were loaded for 16 h with either BCF or uninoculated broth filtrate (diluted as above) were extensively washed and then maintained in HBSS alone for 5, 21, 48, or 72 h. In some experiments, to study the intracellular persistence of VacA activity, cells previously incubated with BCF or uninoculated broth filtrate (diluted as above) were extensively washed and maintained in HBSS alone for 5, 21, 48, or 72 h and then in HBSS or 4 mM NH4Cl for an additional 16 h.

Neutral red dye uptake. At the end of each step, the degree of cell vacuolation was assayed by means of neutral red dye uptake, in accordance with the method of Cover et al. (5), and was expressed as micrograms of neutral red dye per microgram of cell protein (29). The protein content of cell monolayers was measured in accordance with the method of Lowry et al. (18). Neutral red dye is an acidotropic, membrane-permeant amine that accumulates in the vacuolar lumen (5, 23). Neutral red dye uptake is a widely accepted in vitro assay for H. pylori-induced cell vacuolation (5, 24, 29, 30).

SDS-PAGE and immunoblotting. Cells loaded for 16 h with BCF and then incubated in HBSS for 21 or 72 h were washed extensively with HBSS and finally lysed with lysis buffer (1.5 M Tris·HCl, pH 6.8, 8% SDS, and 40% glycerol) supplemented with 20% 2-mercaptoethanol. Controls consisted of 1) cells incubated for 16 h without BCF, maintained in HBSS for...
21 h, and lysed as above, and 2) BCF from H. pylori strain CCUG 17874. Each sample (40 µl) was subjected to SDS-PAGE in 7% polyacrylamide gel with a 3% stacking gel. Proteins were then blotted onto nitrocellulose (Bio-Rad Laboratories, Richmond, CA); the subsequent immunologic analysis used polyclonal antisera. The following rabbit antisera raised against native VacA or its fragments (as obtained by recombinant DNA techniques) were used: αII, directed against native VacA; αB, against region B (amino acid residues 262–428 of the toxin), αBK, against region BK (amino acids 34–751); and αC, against region C (amino acids 751–1,000) (37). Serum αII has been shown to block the vacuolating activity of purified VacA in in vitro tests (19).

Electron microscopy. At the end of incubation, cell monolayers were washed twice with cacodylate buffer [0.2 M (CH3)2AsO2Na·3H2O, pH 7.3 with HCl] and fixed with a freshly prepared mixture of one part 2.5% glutaraldehyde and two parts 1% osmium tetroxide in cacodylate buffer for 40 min at 4°C. Fixed monolayers were scraped and collected in cacodylate buffer, centrifuged at 10,000 g for 10 min, and then embedded in Epon-Araldite mixture. Uranyl lead-stained ultrathin sections were viewed with a Zeiss EM 902 electron microscope (Oberkochen, Germany).

For the ultrastructural immunolocalization of VacA, we used the colloidal gold-labeling technique. Briefly, ultrathin sections were collected on 300-mesh nickel grids, washed with buffer A (0.45 M NaCl, 1% Triton X-100, and 0.05 M Tris·HCl, pH 7.4), and incubated in nonimmune goat serum at room temperature for 1 h to prevent nonspecific binding of immunoglobulins. The sections were then incubated at 4°C overnight with αII polyclonal rabbit antisera directed against native oligomeric VacA, diluted 1:600 in buffer B (0.45 M NaCl, 1% BSA, 0.5% sodium azide, and 0.05 M Tris·HCl, pH 7.4). After further washing in buffer B, primary immunoglobulin binding was revealed by gold-labeled goat anti-rabbit IgG (EM GAR 20, British BioCell, Cardiff, United Kingdom) diluted 1:20 in buffer B. The sections were stained with uranyl and lead before electron microscopy investigation (30).

Quantitative immunofluorescence analysis. In accordance with Chavrier et al. (1), after incubation cell monolayers were washed once with PBS and permeabilized by treatment for 15 min with 0.5% saponin in 80 mM PIPES (pH 6.8), 5 mM EGTA, and 1 mM MgCl2. The cells were fixed for 15 min with 3% formaldehyde in PBS (pH 7.4). After fixation, the cells were washed for 5 min with 0.5% saponin in PBS (saponin-PBS), and free aldehyde groups were quenched for 10 min with 50 mM NH4Cl in PBS. Cell monolayers were washed with saponin-PBS for 5 min and then incubated with αII serum in saponin-PBS for 20 min. After triple rinsing of the cells and 20 min incubation with goat anti-rabbit IgG conjugated with tetramethylrhodamine isothiocyanate (TRITC) (Sigma Chemical) (1:400 in saponin-PBS), primary antibody binding was visualized. After being washed in saponin-PBS, the petri dishes were mounted on an uprising microscope (AxioLab, Zeiss) equipped with a 100-W mercury lamp, a water-immersion objective (Achromat, Zeiss), and a standard filter set for TRITC (filter set 15, Zeiss). Image acquisition was by means of a high-sensitivity camera (Extended-ISIS camera, Photonic Science, Millam, United Kingdom) interfaced by a frame grabber (CX100, ImageNation, Beaverton, Oregon).
OR) to a high-end personal computer. Locally developed software was used for the recording and the simultaneous analysis, in triplicate, of the fluorescence obtained from 50 cells for each condition.

Statistics. All data were expressed as means ± SE of four independent experiments. The statistical significance of the differences was evaluated by Student's t-test and by ANOVA followed by Newman-Keuls Q-test (33). Data expressed as a percentage of control were analyzed before being normalized vs. control.

RESULTS

Neutral red dye uptake. To investigate VacA activity in the presence or absence of ammonia, we studied MKN 28 cells loaded in the following four different
conditions (step 1): 1) uninoculated broth filtrate (control), 2) BCF plus NH₄Cl, 3) BCF alone, or 4) NH₄Cl alone. At the end of each treatment, cell monolayers were extensively washed and further incubated for 5 h (step 2) and then for 16 h (step 3) in HBSS or NH₄Cl as depicted in Fig. 1. We found that 1) NH₄Cl alone induced a slight but significant neutral red dye uptake, whereas BCF alone did not; 2) simultaneous treatment with BCF and NH₄Cl greatly enhanced neutral red uptake compared with NH₄Cl alone; 3) treatment with BCF alone in step 1 significantly enhanced neutral red dye uptake induced by subsequent treatment with NH₄Cl, whereas neutral red dye uptake induced by treatment with NH₄Cl in step 1 was not increased by
BCF plus NH₄Cl caused neutral red dye uptake to a potentiation. We incubated MKN 28 cells with NH₄Cl weak base was important for VacA activity and/or (data not shown).

Finally, when cells were loaded with BCF plus NH₄Cl subsequent treatments with NH₄Cl. The enhancing effect of BCF on neutral red dye uptake was suppressed by previous incubation with αI anti-native VacA serum (data not shown).

To investigate whether preloading of cells with a weak base was important for VacA activity and/or potentiation, we incubated MKN 28 cells with NH₄Cl before BCF (alone or with addition of NH₄Cl) treatment. Figure 2 shows that 1) incubation with BCF (step 3) was ineffective if cells were loaded with NH₄Cl (step 1) and then incubated with HBSS for 5 h (step 2) and 2) BCF plus NH₄Cl caused neutral red dye uptake to a similar extent, irrespective of pretreatment with NH₄Cl. Finally, when cells were loaded with BCF plus NH₄Cl and washed in HBSS, subsequent incubation with BCF alone was ineffective. Altogether, these results suggest that the vacuolating activity of VacA was evident only when the weak base and VacA were added simultaneously to the cells or when NH₄Cl was added to cells previously loaded with VacA.

In addition, we studied the persistence of the vacuolating activity of VacA in cells that had been incubated for 16 h with BCF, maintained in HBSS for differing time spans, and finally incubated in HBSS or NH₄Cl for an additional 16 h. Figure 3 shows that incubation with NH₄Cl invariably increased neutral red dye uptake in MKN 28 cells pretreated with either unincubated broth filtrate or BCF and subsequently maintained in HBSS for differing time spans. However, the increase in neutral red dye uptake, as evaluated for each time span considered, was 100% for cells previously loaded with control (Fig. 3A) and 400% for cells previously incubated with BCF (Fig. 3B), representing a statistically significant difference (P < 0.001).

Electron microscopy. The ultrastructure of the MKN 28 cell line and its vacuolar changes after incubation for 16 h with BCF from VacA⁺ H. pylori strains have been reported in detail previously (29, 30). In the presence of ammonia, H. pylori toxin gave rise to large vacuoles by expansion and fusion of endosomes. Ultrastructural immunocytochemistry confirmed the presence of internalized VacA within endosomal tubulovesicles and related cytoplasmic vacuoles (Fig. 4). In addition, VacA-immunoreactive bacterial outer membrane vesicles (OMV), 50–300 nm in size, were detected in MKN 28 cell cultures incubated with H. pylori BCF or with unfiltered supernatant of H. pylori broth culture but not in cell cultures incubated with unincubated broth filtrate. The OMV were found to interact closely with the luminal-type surface of MKN 28 cells (Fig. 5), to enter invaginations of cell membrane and small endocytic vesicles immediately beneath the cell surface (Fig. 6), and to accumulate into dilated endosomes and related vacuoles, together with VacA not bound to OMV (Fig. 4). At the end of 21 h and, especially, 72 h of HBSS treatment, both free and membrane-bound VacA immunoreactivity levels were substantially reduced in endosomes and vacuoles, while they were concentrated in discrete vacuolar structures storing membranous or amorphous material and resembling lysosomes. VacA-immunoreactive OMV and fragments were prominent in such structures (Fig. 7).

Immunofluorescence. The presence and persistence of internalized VacA inside the cell were also assessed by quantitative immunofluorescence analysis. MKN 28 cells incubated for 16 h with BCF and then maintained in HBSS for 5, 21, 48 or 72 h exhibited a specific fluorescence (ranging from 270% to 320% of paired control; P < 0.05 vs. paired control) that was stable (no statistically significant differences between differing time points) throughout the entire time course considered (not shown).

Immunoblotting. Cell uptake of VacA was further confirmed by immunoblotting analysis of MKN 28 cell lysates at the end of 16 h of incubation with BCF plus an additional 21 or 72 h of HBSS treatment (Fig. 8). Cells not loaded with BCF were negative controls. As shown in Fig. 8, all anti-VacA sera tested recognized an immunoreactive ~90-kDa protein in cell lysates, with...
the exception of cells not loaded with BCF. In each Western blot, BCF-treated cells (Fig. 8, lanes 1 and 2) clearly differed from control cells (Fig. 8, lane 3, MKN 28 cells not loaded with BCF), because BCF-treated cells possessed this ~90-kDa band (Fig. 8). The persistence of VacA as a ~90-kDa peptide indicates that at least a large amount of internalized VacA was not degraded into fragments of smaller molecular mass.

**DISCUSSION**

Using human gastric epithelial cells in culture and BCF from a well-characterized VacA1 H. pylori strain, we attempted to clarify the mechanisms of VacA internalization, action, and fate in the present study. Our main findings were that 1) only in the presence of NH₄Cl does VacA induce significant neutral red dye uptake, 2) both free and membrane-bound (attached to bacterial OMV) VacA is present in BCF and both forms interact with the cell membrane and are internalized by the cell, 3) VacA accumulates inside cells, and does so specifically in endosomal vesicles and related endosomal vacuoles, 4) a large part of internalized VacA retains apparent molecular integrity, and 5) a vaculating potential persists in VacA-storing cells.

The neutral red dye uptake study showed that VacA does not induce large vacuoles in the absence of ammonia, while enhancing NH₄Cl vaculating power. In agreement with previous findings (30), the specific role of VacA in enhancing NH₄Cl vaculating power is supported by our tests using BCF preincubated with neutralizing anti-VacA serum. We also confirmed previous observations (28, 30) that VacA enters cells independently of ammonia but that its vaculating action is fully expressed only when NH₄Cl is added.

Internalized VacA persists inside the cell (for up to 72 h) and seems to preserve a latent vaculating power that can be activated by the addition of a weak base. An alternative hypothesis is that VacA induces an unknown permanent cell change that allows the weak base to cause cellular vacuolation. It should be outlined that other weak bases not investigated here, such as nicotine or trimethylamine, have been shown to give the same VacA potentiating effect as NH₄Cl (7).

Immunoblotting analysis showed that the bulk of internalized VacA, as localized into the endosomal compartments, does not undergo cleavage. The endosomal acidic environment possibly induces some modifications in the toxin itself. An acid-induced increase in the stability of VacA has recently been reported by de Bernard et al. (8). It is possible that protonation of VacA (predicted isoelectric point of 9.1 and 12% arginine content (6)) takes place inside the acidic endosomal compartments. This could prevent toxin cleavage (17).

In addition, we should consider the possibility that internalized toxin resists the low concentration of hydrolitic enzymes present in the endosomal compartment and never reaches the hydrolase-rich lysosome. This is supported by the observation that H. pylori toxin interferes with processes controlling the late stages of the endocytic pathway (24, 36). Our findings fit with recent observations (21, 32) that VacA induces the accumulation of a postendosomal hybrid compartment, resembling both late endosomes and lysosomes, but with a reduced proteolytic activity compared with normal late endosomes and lysosomes.

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**REFERENCES**


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