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Free-soluble and outer membrane vesicle-associated VacA from *Helicobacter pylori*: Two forms of release, a different activity

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This paper is devoted to the memory of Dr. G. Menestrina, leading biophysicist and toxinologist, untimely died in a motorcycle accident in July 2004.

Abstract

Helicobacter pylori releases VacA both as free-soluble and as outer membrane vesicle (OMV)-associated toxin. In this study, we investigated the amount of VacA released in each of the two forms and the role of each form in VacA-induced cell vacuolation in vitro. We found that: (1) free-soluble toxin accounted for about 75% of released VacA, while the remaining 25% was OMV-associated; (2) although OMV-associated VacA caused a statistically significant vacuolation, virtually all the vacuolating activity of a *H. pylori* broth culture filtrate was due to free-soluble VacA. While it is widely accepted that OMVs may represent an important vehicle for delivering virulence factors to the gastric mucosa, our results suggest that OMV-associated VacA could play a pathobiological role different from that of free-soluble toxin. This conclusion fits with mounting evidence that VacA exerts a large pattern of pathobiological effects among which cell vacuolation might not be the main one.

Keywords: Cell vacuolation; Gastric epithelial cells; *Helicobacter pylori*; Outer membrane vesicles; VacA

Helicobacter pylori is a Gram-negative, microaerophilic, S-shaped bacterium that is free-living in the mucous layer of the human stomach. Only a small proportion of *H. pylori* attaches to gastric epithelial cells without invading them. Colonization of the stomach by *H. pylori* induces infiltration of the lamina propria and epithelium with immunocytes and inflammatory cells, a condition referred to as chronic gastritis or chronic active gastritis (reviewed in [1–3]). During the years or decades that follow the initial infection, chronic gastritis may remain asymptomatic or may evolve into more severe diseases, such as peptic ulcer or atrophic gastritis. In addition, *H. pylori* infection increases the risk for gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma [1,3,4].

Among several virulence factors produced by the bacterium, a pivotal role in *H. pylori* pathogenetic action is played by the vacuolating toxin VacA [1,2,5–7]. Encoded by the *vacA* gene, VacA is a protein toxin which induces cytoplasmic vacuoles in eukaryotic cells in culture [8,9]. When given to mice, VacA causes gastric epithelial damage closely resembling that found in *H. pylori*-colonized patients [10]. The structure of the *vacA* gene varies, especially in the region encoding the signal sequence (which may be type s1 or s2) and in the mid-region (which may be type m1 or m2) [6,7,11]. Infection with genotypes (especially the s1/m1) exhibiting a high vacuolating activity in vitro is correlated with the development of more severe diseases such as peptic ulcer and gastric cancer [5–7,12]. VacA toxin is composed by monomers of about 90 kDa synthesized as a 140 kDa precursor with a N-terminal signal sequence of 33 amino acids [6,7,10,11]. VacA belongs to

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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 C-terminus of the precursor protein and proteolytically removed during protein transport [10,12–14].

We and others have previously demonstrated that VacA is released by the bacterium both as soluble secreted protein and as VacA-containing vesicles derived from outer membrane blebs [15–18]. Both soluble secreted VacA and VacA-containing vesicles bind to, and are internalized by, gastric epithelial cells in culture and are detectable in the gastric mucosa from *H. pylori*-infected patients [15,16]. Therefore, the release of outer membrane vesicles (OMVs) by *H. pylori* may represent a mechanism, additional to the secretory pathway, for the delivery of bacterial virulence factors and antigens into the gastric mucosa [16,18]. However, both the amount of VacA released as free-soluble rather than OMV-associated toxin and their respective role in VacA-induced cell damage are still unknown. This study was designed to investigate the amount of VacA released in each of the two forms and the role of each form in VacA-induced vacuolation of human epithelial cells in culture.

Materials and methods

Bacterial strains, broth culture filtrate preparation, and OMV purification. Two well-characterized VacA-producing *H. pylori* strains (both with a s1a/m1 *vacA* genotype) were used: 60190 (ATCC 49503) and CCUG 17874 (from Culture Collection University of Göteborg, Göteborg, Sweden). VacA-containing broth culture filtrate ($VacA^+$ BCF) was produced as described by Ricci et al. [19,20]. Briefly, bacteria were grown in Brucella broth (Difco, Detroit, MI) supplemented with 1% Vitox (Oxoid, Basingstoke, UK) and 5% fetal calf serum (FCS; Gibco, Grand Island, NY) for 24–36 h at 37 °C under microaerobic conditions and continuous shaking. When bacterial suspensions reached 1.2 optical density units at 450 nm (corresponding to a bacterial concentration of 5×10^8 CFU/ml), bacteria were removed by centrifugation (12,000*g* for 10 min) and the supernatant sterilized by passage through a 0.22 μm cellulose acetate filter to obtain the broth culture filtrates (BCFs). To remove the ammonia content, BCFs were dialyzed against Hanks' balanced salt solution (HBSS) for 36 h in dialysis tubing with a 12 kDa molecular mass cut-off (Sigma, St. Louis, MO). Dialyzed $VacA^+$ BCF was ultracentrifuged (196,000*g* for 90 min) to separate OMV-associated VacA (i.e., the pellet) from free-soluble toxin (i.e., the supernatant). The pellet was then washed with HBSS and ultracentrifuged as above. To allow a correct comparison of vacuolating activity and toxin content of either OMV-associated or free-soluble VacA preparations with those of the total VacA preparation, both the pellet and the supernatant were brought to the same volume of the starting dialyzed $VacA^+$ BCF by using HBSS.

Human cell lines and cell vacuolation assay. We used the MKN 28 cell line, derived from a human gastric tubular adenocarcinoma, which is known to retain gastric type differentiation [21–23] and to provide a suitable model for the study of the response of gastric epithelial cells to *H. pylori* [23–25]. The MKN 28 cells were grown in DMEM/Ham's nutrient mixture F-12 (1:1) (Sigma) supplemented with 10% FCS (Gibco). We also used HeLa cells (from a human cervix carcinoma) which were cultured in DMEM supplemented with 10% FCS and 2 mM L-glutamine (all from Gibco). All the cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air and used at 30–40% confluency.

Subconfluent monolayers of MKN 28 or HeLa cells on six-well multiwell tissue culture dishes were washed twice with HBSS and then incubated at 37 °C, for either 6 or 18 h, with: (1) HBSS containing 4 mM NH_4Cl (control); (2) different dilutions of dialyzed $VacA^+$ BCF or its supernatant or its pellet in HBSS containing 4 mM NH_4Cl. This fixed amount of ammonia was added in order to obtain an identical ammonia level for each experimental condition, a crucial point since the presence of a weak base like ammonia is strictly required for the development of VacA-dependent cell vacuolation [25].

At the end of incubation, the degree of cell vacuolation was assayed by means of neutral red dye uptake (NRU) as previously described [20,25]. NRU is a widely used quantitative in vitro assay for *H. pylori*-induced cell vacuolation [22,25–30]. Results were expressed as nanograms of neutral red per microgram cell protein. Vacuolation of variously treated cell monolayers was also evaluated qualitatively using a phase-contrast inverted microscope (Diaphot 300, Nikon, Tokyo, Japan) equipped with a photocamera; representative microscopic fields were selected by an observer (V.N.) unaware of the treatment.

Electron microscopy. *Helicobacter pylori* bacterial pellets harvested from broth cultures and $VacA^+$ BCF-treated MKN 28 cell monolayers were washed twice with cacodylate buffer (pH 7.3), fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer for 40 min at 4 °C, postfixed in 1% osmium tetroxide for 1 h at room temperature, and then embedded in epon–araldite resin mixture [16,22].

For the ultrastructural immunolocalization of *H. pylori* VacA toxin, we used the colloidal gold technique as previously described [25]. Briefly, ultrathin sections collected on 300 mesh nichel grids were pretreated with satured water solution of sodium metaperiodate for 10 min, washed with buffer A (0.45 M NaCl, 1% Triton X-100, and 0.05 M Tris–HCl, pH 7.4), and incubated in non-immune goat serum at room temperature for 1 h, to prevent non-specific binding of immunoglobulins. The sections were then incubated at 4 °C overnight with an anti-VacA polyclonal rabbit serum (serum 123; kindly given by T.L. Cover, Nashville, TN), diluted 1:600 in buffer B (0.45 M NaCl, 1% bovine serum albumin, 0.5% sodium azide, and 0.05 M Tris–HCl, pH 7.4). After a further wash with buffer B, binding of primary immunoglobulins was revealed by gold-labelled goat anti-rabbit IgG (EM GAR 20, British Bio Cell, Cardiff, UK) diluted 1:20 in buffer B. The sections were stained with uranyl and lead before electron microscopy investigation using a Zeiss EM 902 electron microscope.

Western blotting and densitometric analysis. VacA content in either dialyzed $VacA^+$ BCF (i.e., the overall toxin content) or its pellet (i.e., the OMV-associated VacA) or its supernatant (i.e., the free-soluble VacA) was evaluated by SDS–PAGE (under reducing conditions) followed by Western blotting as previously described [15]. The anti-VacA polyclonal rabbit serum 958 (kindly given by T.L. Cover, Nashville, TN) at a dilution of 1:20,000 and the enhanced chemiluminescence revelation system (ECL Advance; from Amersham, UK) were used. Taking that the response of the antibody may be not linear with the protein on the nitrocellulose, different volumes per lane (i.e., 5, 10, 20, and 40 μl) for either dialyzed $VacA^+$ BCF or its pellet or its supernatant were analyzed. VacA immunoreactive bands were quantitated by densitometric analysis (area-density method) of ECL-exposed films using the 1Dscan EX software (Scanalytics, Fairfax, VA).

Statistics. Results were expressed as means ± SEM of three independent experiments. The statistical significance of the differences was evaluated by analysis of variance (ANOVA) followed by Newman–Keuls' *Q* test.

Results and discussion

Mounting evidence suggests an important role for OMVs in the pathogenesis of bacterial diseases. OMVs are released by several Gram-negative bacteria, including *Neisseria meningitidis*, *Haemophilus influenzae*, *Borrelia burgdorferi*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, and enterotoxigenic *Escherichia coli* [16,31–33]. OMVs, consisting of outer membrane lipids and a subset of outer membrane proteins and soluble periplasmic components, have been proposed to be vehicles for virulence factor

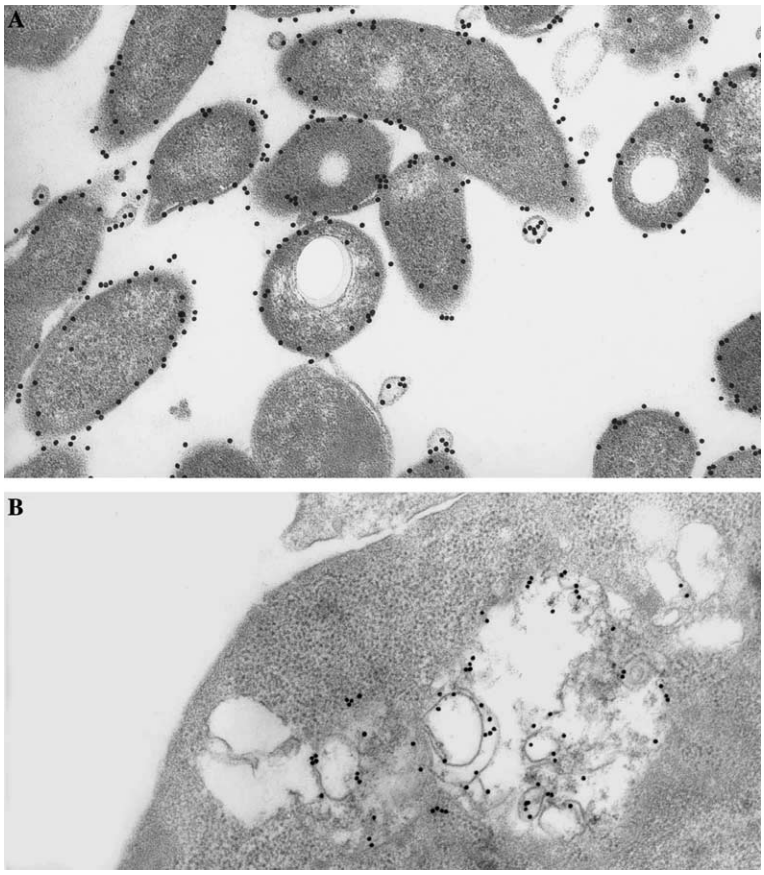

Fig. 1. Ultrastructural immunocytochemistry of (A) bacterial cultures of 60190 *H. pylori* strain and (B) MKN 28 cells incubated with dialyzed VacA$^+$ BCF (from 60190 *H. pylori* strain) diluted 1:3 in HBSS containing 4 mM NH_4Cl. Note immunogold deposition over bacterial outer membranes and related vesicles in (A) as well as over endocytosed fragments of bacterial membranes in (B). Immunoreactivity not bound to bacterial membranes is also evident in both the panels. Original magnification: (A) 44,000× (B) 33,000×.

delivery to host cells [16,31–33]. For instance, greater than 95% of the catalytically active heat-labile enterotoxin secreted by enterotoxigenic *E. coli* is represented by OMV-associated toxin [34]. Very recently, Kesty et al. [33] demonstrated a role in virulence for OMVs from enterotoxigenic *E. coli* showing that such OMVs serve as specifically targeted transport vehicles that mediate entry of active enterotoxin and other virulence factors into host cells.

It has been previously reported that *H. pylori* releases OMVs during growth in vitro and in vivo, and that these vesicles contain VacA toxin [15–18]. *H. pylori* OMVs bind to, and are internalized by, human gastric epithelial cells in vitro and in vivo, and may represent an important vehicle whereby *H. pylori* virulence factors are delivered to the gastric mucosa [15,16]. In this respect, Ismail et al. [18] recently demonstrated that OMVs from *H. pylori* alter cell proliferation and cause vacuolation, cell death, and production of the proinflammatory cytokine interleukin 8 in AGS gastric epithelial cells. However, it is still unknown both how much VacA is released by *H. pylori* as OMV-associated toxin and the role of this vehicle-delivered toxin in the overall VacA-induced cytotoxicity.

In the present study, ultrastructural immunocytochemistry confirms that *H. pylori* releases VacA both as free-soluble and as OMV-associated toxin (Fig. 1A); both forms of VacA are taken up by MKN 28 cells and localize inside cytoplasmic tubulovesicles and vacuoles (Fig. 1B). By SDS–PAGE followed by Western blotting, we then evaluated (Fig. 2) how much of the total toxin (i.e., in the dialyzed VacA$^+$ BCF) (lane A) is released either as OMV-associated (i.e., in the pellet) (lane B) or free-soluble toxin (i.e., in the supernatant) (lane C). Densitometric analysis of VacA immunoreactive bands from different gels carried out using different volumes per lane (i.e., 5, 10, 20, and 40 μl) for either dialyzed VacA$^+$ BCF or its pellet or its supernatant revealed that OMV-associated VacA accounted for about 25% of total VacA, while the remaining 75% was represented by free-soluble VacA. Both the *H. pylori* strains used gave virtually the same results (not shown). It is worth noting that OMV-associated VacA was repre-

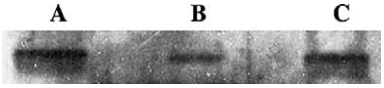

Fig. 2. Western blot showing VacA immunoreactivity of (A) dialyzed VacA$^+$ BCF (from 60190 *H. pylori* strain) (i.e., total toxin) or (B) its pellet (i.e., OMV-associated VacA) or (C) its supernatant (i.e., free-soluble VacA). Sample per lane: 20 μl.

sented by an immunoreactive band showing the same molecular mass (about 90 kDa) of the free-soluble VacA. This finding confirms the previous observation by Ilver et al. [12] and suggests that the C-terminal outer membrane exporter region of the precursor protein is rapidly proteolytically removed during protein transport so that virtually all OMV-associated VacA is represented by mature monomers.

By treating, for 6 or 18 h, human gastric epithelial cells (MKN 28 cell line) with different dilutions of either VacA$^+$ BCF (from 60190 *H. pylori* strain) or its supernatant or its pellet, we quantitatively evaluated (using the NRU assay) the efficiency of free-soluble and OMV-associated VacA in causing cell vacuolation. We found that virtually all the vacuolating activity of VacA$^+$ BCF was accounted for by free-soluble VacA, while OMV-associated VacA caused a statistically significant ($P < 0.05$) NRU only when the highest concentrations were used and the longest time of cell treatment was performed (Fig. 3). VacA$^+$ BCF from CCUG 17874 *H. pylori* strain gave virtually the same results (not shown).

To rule out the possibility that the difference in the vacuolating activity of free-soluble and OMV-associated VacA was depending on the specific cell line used, we carried out another set of experiments using HeLa cells, the most widely used cell model in studying VacA interaction with human epithelial cells [26,28–30]. As shown in Fig. 4, the results obtained with HeLa cells were almost identical to those showed for MKN 28 cells. Also morphological observation of variously treated HeLa cells by phase-contrast microscopy (Fig. 5) showed that, after 18 h of cell incubation, even though the vacuolating activity of VacA$^+$ BCF seemed to be completely accounted for by its content in free-soluble VacA, OMV-associated VacA was able to induce some cell vacuolation.

To explore the possibility that the poor vacuolating activity of OMV-associated VacA might be due to its presence in OMVs as oligomers which require

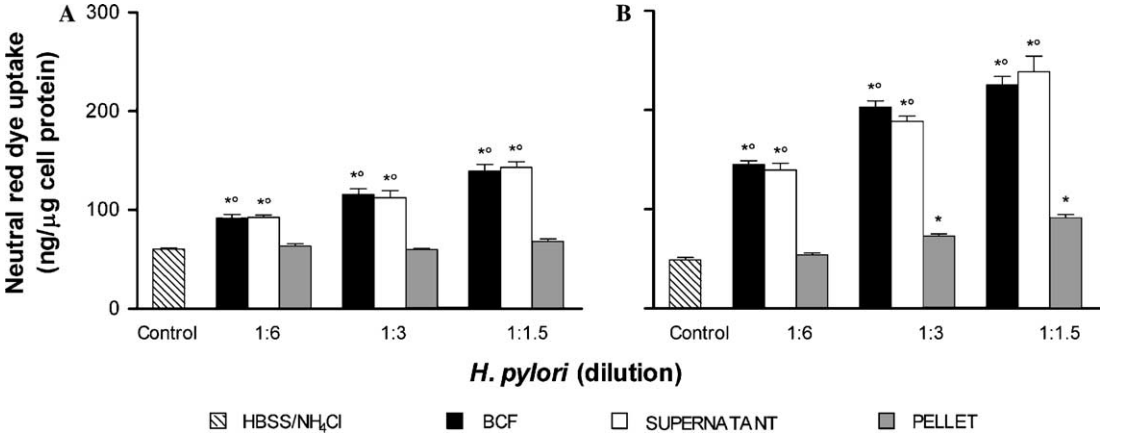

Fig. 3. Neutral red dye uptake induced in MKN 28 cells by 6 h (A) or 18 h (B) incubation with: (1) HBSS containing 4 mM NH_4Cl (control); (2) different dilutions of dialyzed VacA$^+$ BCF (from 60190 *H. pylori* strain) or its supernatant or its pellet in HBSS containing 4 mM NH_4Cl. Means ± SEM ($n = 3$). *$P < 0.05$ vs control; °$P < 0.05$ vs pellet.

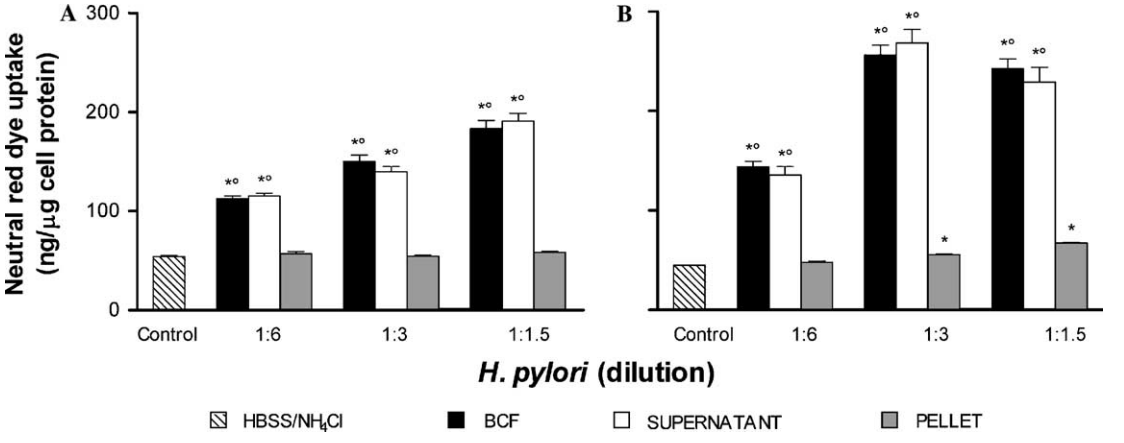

Fig. 4. Neutral red dye uptake induced in HeLa cells by 6 h (A) or 18 h (B) incubation with: (1) HBSS containing 4 mM NH_4Cl (control); (2) different dilutions of dialyzed VacA$^+$ BCF (from 60190 *H. pylori* strain) or its supernatant or its pellet in HBSS containing 4 mM NH_4Cl. Means ± SEM ($n = 3$). *$P < 0.05$ vs control; °$P < 0.05$ vs pellet.

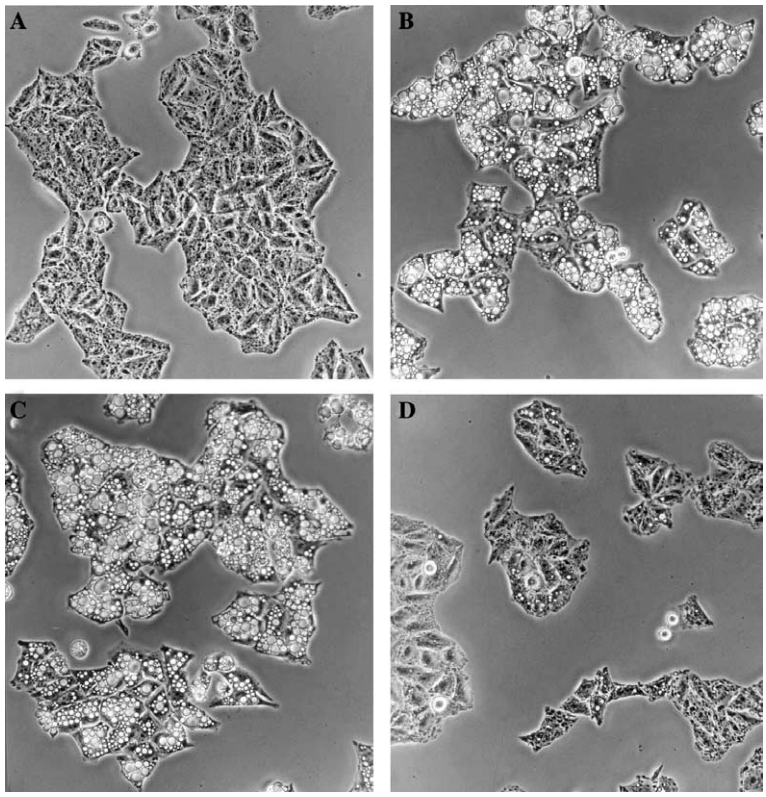

Fig. 5. Phase-contrast microphotographs showing the cell vacuolation induced in HeLa cells by 18 h incubation with: (A) HBSS containing 4 mM NH_4Cl (control); (B) dialyzed $VacA^+$ BCF (from 60190 *H. pylori* strain) or (C) its supernatant or (D) its pellet, each diluted 1:3 in HBSS containing 4 mM NH_4Cl. Original magnification: 100×.

disassembling into monomers by a strongly acidic or alkaline pH pulse to become active [7–9,30], HeLa cells were incubated with different dilutions of OMV-associated VacA either pretreated or not with an acidic pH pulse [19,30]. By means of NRU assay after 6 or 18 h of incubation, we found that acid pretreatment did not induce any increase in the vacuolating efficiency of OMV-associated VacA (not shown). Although we cannot rule out the possibility that OMV-association makes VacA oligomers not sensitive to the acidic pH pulse, our observation suggesting that OMV-associated VacA might be monomeric fits very well with previous findings showing that VacA molecules bound to *H. pylori* cell surface are in the monomeric form [7–9,12].

Taken together, the above findings suggest that, although representing about 25% of total toxin released by *H. pylori* in broth cultures, OMV-associated VacA is poorly vacuolating, virtually all the cell vacuolation caused by $VacA^+$ BCF being accounted for by free-soluble toxin. While it is widely accepted that OMVs may be important for delivering virulence factors to the gastric mucosa, the possibility arises that OMV-associated VacA could play a pathobiological role different from that of free-soluble toxin. This may fit with the increasing body of evidence that VacA exerts a large pattern of pathobiological effects, among which cell vacuolation might not be the main one. In recent years, there has indeed been increased recognition that VacA, like other bacterial toxins, can have pleiotropic effects on human cells [35]. Cellular effects produced by VacA include stimulation of different cellular signalling pathways, alteration of late endocytic compartments, and impairment of mitochondrial functionality [6–9,35]. Moreover, VacA can exert a pathobiologically relevant action not only on epithelial cells but also on a variety of different cell types, especially cells of the immune system such as antigen-presenting cells, T lymphocytes, phagocytic cells, and mast cells [7,35]. In this respect, it is noteworthy that VacA and other *H. pylori* antigens have been detected in expanded endosomal vesicles of *H. pylori*-colonized gastric epithelium as well as in mesenchymal cells of immediately underlying lamina propria ([16,36], Solcia et al., unpublished data). This finding is suggestive of active antigen transcytosis through the epithelium, which may be crucial for the modulation of mucosal immune-inflammatory response.

Providing a protective environment for the transmission of virulence factors from bacteria into the host target cells both locally and away from the site of colonization [33], OMVs may represent an important vehicle in delivering VacA and other *H. pylori* antigens to either epithelial or non-epithelial gastric cells where they can play their pathogenic actions. Further studies directed toward characterizing both the transepithelial trafficking and the vacuolation-independent effects of OMV-associated VacA are however required for a better understanding of the role that VacA toxin plays in *H. pylori* infection.

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