Uptake of *Helicobacter pylori* outer membrane vesicles
by gastric epithelial cells

Heather Parker¹, Kenny Chitcholtan¹, Mark B. Hampton², Jacqueline I. Keenan¹*

*Departments of¹Surgery & ²Pathology, University of Otago, Christchurch, New Zealand.

**Running title:** UPTAKE OF *H. PYLORI* OMV

*Corresponding Author: Dr Jacqueline Keenan, University of Otago, PO Box 4345, Christchurch, New Zealand. Phone: 64 3 3640 570. Fax: 64 3 3641 427. E-mail: jacqui.keenan@otago.ac.nz
Helicobacter pylori colonize the human stomach where they stimulate a persistent inflammatory response. *H. pylori* is considered non-invasive, however LPS-enriched outer membrane vesicles (OMV), continuously shed from the surface of this bacterium, are observed within gastric epithelial cells. The mechanism of vesicle uptake is poorly understood, and this study was undertaken to examine the roles of bacterial VacA cytotoxin and LPS in OMV binding, and cholesterol and clathrin-mediated endocytosis in vesicle uptake by gastric epithelial cells. OMV association was examined using a fluorescent membrane dye to label OMV and comparison was made between the association of vesicles from a VacA+ strain and OMV from a VacA− isogenic mutant strain. Within 20 minutes essentially all associated OMV were intracellular and vesicle binding appeared to be facilitated by the presence of VacA cytotoxin. Uptake of vesicles from the VacA+ strain was inhibited by *H. pylori* LPS (58% inhibition with 50 μg/ml LPS) while uptake of OMV from the VacA− mutant strain was less affected (25% inhibition with 50 μg/ml LPS). Vesicle uptake did not require cholesterol. However, uptake of OMV from the VacA− mutant strain was inhibited by a reduction in clathrin-mediated endocytosis (42% with 15 μg/ml chlorpromazine) while uptake of OMV from the VacA+ strain was less affected (~25% inhibition with 15 μg/ml chlorpromazine). We conclude that VacA toxin enhances the association of *H. pylori* OMV with cells and that the presence of the toxin may allow vesicles to exploit more than one pathway of internalization.
INTRODUCTION

Infection with the gastric pathogen *Helicobacter pylori* results in chronic gastritis (13) and is associated with increased risk for the development of peptic ulcer disease (35), gastric carcinoma (41, 57) and gastric lymphoma (5, 60). *H. pylori* persistence, in an environment where peristalsis and sloughing of cells are continually occurring, is mediated by a variety of adhesins present on the bacterial surface (14, 21, 36, 40). However, despite the ability to adhere to the gastric epithelium, the majority of organisms remain unattached to surface cells (32), leading to speculation that lipopolysaccharide (LPS)-enriched outer membrane vesicles (OMV) shed by these bacteria (15, 19, 26) contribute to *H. pylori* pathogenesis via the persistent delivery of bacterial virulence factors (including the vacuolating cytotoxin VacA) and antigens to the gastric mucosa (26, 27). Observations that *H. pylori* OMV modulate gastric epithelial cell proliferation (22), induce apoptosis (3), stimulate secretion of the pro-inflammatory cytokine interleukin-8 (22), increase micronuclei formation (8), and are observed at the luminal surface (15, 26) and within cells of the gastric epithelium (15) support this hypothesis.

OMV shedding by gram-negative bacteria is well described in the literature (reviewed by Kuehn and Kesty (33)), yet little is known of the mechanisms of vesicle adherence to and internalization within mammalian host cells. The adherence of enterotoxigenic *Escherichia coli* (ETEC) OMV to host cells is mediated via a heat-labile enterotoxin LT associated with these OMV (31), whereas leukotoxin, associated with OMV shed by *Actinobacillus actinomycetemcomitans*, is not involved in vesicle binding (12). The internalization of ETEC, *Porphyromonas gingivalis* and *Pseudomonas aeruginosa* OMV has been shown to involve cholesterol-rich lipid rafts (6, 16, 31) and recently, Kaparakis &
colleagues (25) reported that uptake of *H. pylori* OMV is also lipid raft-dependent. This is in contrast to the uptake of *Shigella flexneri* OMV which occurs via phagocytosis, with proposed subsequent fusion of OMV with the phagosomal membrane and release of vesicle contents into the cell cytoplasm (24).

In this study, we sought to examine whether VacA cytotoxin associated with *H. pylori* OMV was involved in vesicle binding. We also examined the rate of OMV internalization and the involvement of LPS, cholesterol and clathrin-mediated endocytosis in vesicle uptake by AGS gastric epithelial cells. We report that within 20 min essentially all VacA+ OMV associated with AGS cells are intracellular and that uptake is enhanced by the presence of vesicle-associated cytotoxin. Excess *H. pylori* LPS reduced vesicle uptake having a more significant effect on VacA+ OMV than VacA- vesicle uptake. Uptake of both VacA+ and VacA- OMV did not require cholesterol. However, a reduction in clathrin-mediated endocytosis inhibited VacA- OMV uptake and to a lesser extent VacA+ OMV internalization.
MATERIALS AND METHODS

Bacterial strains. The well characterized \textit{H. pylori} clinical isolate 60190 (ATCC 49503) was used in this study. \textit{H. pylori} 60190 is a vacA s1m1 strain (2) producing a cytotoxin that induces extensive vacuolation in cultured epithelial cells (9). \textit{H. pylori} strain 60190:v1, in which the VacA gene has been disrupted by insertional mutagenesis resulting in both the absence of the 87-kDa protein and vacuolating cytotoxin production (11), was also used.

Harvesting and labeling of OMV. \textit{H. pylori} were grown in 2.8\% (wt/vol) \textit{Brucella} broth (Becton Dickinson and Company, Sparks, MD, USA) supplemented with 5\% fetal bovine serum (Invitrogen, Auckland, NZ) at 37°C under micro-aerobic conditions with constant rotation (120 rpm). At 72 h incubation, bacteria were removed by two centrifugations (12,000 × g, 15 min, 4°C) and the final supernatants ultracentrifuged (200,000 × g, 2 h, 4°C) to recover OMV. After three washes in phosphate-buffered saline (PBS) OMV were stored at −20°C until required. Vesicles to be labeled were washed once after recovery then, to standardize labeling, were suspended in 1 ml PBS/50 mg OMV pellet. OMV were labeled with 1\% (vol/vol) 3,3’-dioctadecyloxacarbocyanine perchlorate or 1,1’-dioctadecyl-3,3’,3’-tetramethylindodicarbocyanine perchlorate (Vybrant™ DiO & Vybrant™ DiD, respectively; Molecular Probes, Eugene, OR, USA) by incubation for 20 min at 37°C. Free dye was removed by two washes with PBS and labeled OMV were stored at 4°C for up to six weeks.

The protein concentration of OMV was determined (37) and preparations were examined by transmission electron microscopy (TEM) to confirm the absence of whole bacteria and flagella by overlaying aliquots of OMV suspension onto Formvar-coated 200 mesh copper grids and negatively staining with 1\% ammonium molybdate (pH 7.4).

Cell culture. The AGS human gastric adenocarcinoma cell line (ATCC CRL-1739) was
cultured in F-12 Nutrient Mixture (HAM) (+ L-glutamine) (Invitrogen, Auckland, NZ) and supplemented with 10% (vol/vol) fetal bovine serum and 1% (vol/vol) penicillin-streptomycin-glutamine supplement. Cells were cultured at 37°C with 5% CO₂.

**Cell proliferation.** Cell proliferation was determined using a colorimetric assay that measures the amount of 5-bromo-2'-deoxyuridine (BrdU) incorporated into cellular DNA (Roche Diagnostics, Manneheim, Germany). AGS cells (1 × 10⁴) were cultured overnight in 96-well plates then incubated for a further 24 h with labeled or unlabeled OMV from strain 60190 (0.05 – 20 µg). The BrdU assay was performed as per kit instructions. Briefly, individual wells were incubated with BrdU labeling solution (10 µM BrdU) for 2 h. After removal of the medium, a FixDenat solution was added at room temperature for 30 min then replaced with anti-BrdU-POD (diluted 1:100) for 2 h. After washing, substrate solution was applied and absorbance read at 370 nm every minute for 30 min with a SpectraMax plate reader (Molecular Devices, USA). The level of BrdU incorporation was calculated from the slope of the linear portion of the graph.

**Flow cytometry.** Cells (3 x 10⁵) were cultured overnight then washed once with PBS and the media replaced prior to addition of labeled OMV (200 µg OMV protein) for up to 6 h. After incubation, cells were washed to remove unbound OMV and lifted with trypsin/EDTA (Invitrogen). Fluorescence measurements were made using a FACS vantage flow cytometer (Beckman Coulter Cytomics FC 500 MPL, AUS) and CXP software (Beckman Coulter 2005). A total of ten thousand events were collected for each sample. Mean fluorescence intensity (MFI) values of cells incubated in the absence of OMV were subtracted from the values of OMV-treated cells.

To determine the proportion of internalized OMV, extracellular vesicle fluorescence was
quenched with trypan blue (0.025% final concentration). To confirm trypan blue quenched DiO fluorescence, cells were incubated with labeled OMV for 4 h then fixed and permeabilized using a Fix & Perm® Cell Permeabilization Kit (Caltag Laboratories, Burlingame, CA, USA) as per manufacturer’s instructions. Fluorescence was measured before and after the addition of trypan blue.

**Fluorescence microscopy.** AGS cells (3 × 10⁵), cultured overnight on glass coverslips, were incubated with 200 µg DiO-labeled OMV for 5 h. Following fixation (4% paraformaldehyde, 45 min), the cells were washed extensively in PIPES buffer, and coverslips mounted in SlowFade® without glycerol (Molecular Probes, Eugene, OR, USA). Where immunofluorescence microscopy was used to visualise intracellular OMV, the cells were blocked with 5% (wt/vol) BSA for 60 min at room temperature. After washing, the cells were permeabilised with 0.5% (vol/vol) Triton X-100 for 20 min and washed again before being incubated overnight at 4°C with a murine IgG1 subclass monoclonal antibody to *H. pylori* Lpp20 (27). Primary antibody binding was detected using a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody. Cells were examined and imaged using a Leitz Aristoplan microscope (Germany) fitted with a Photometrics KAF1400 CCD camera and QUIPS Smartcapture software, version 1.3 (Vysis Inc, IL, USA). Images were formatted in Adobe Photoshop (CS2).

**Cell treatments.** To investigate the role of LPS in OMV association, DiO-labeled OMV were added to cells at the same time as *H. pylori* LPS then cells were incubated for 4 h before fluorescence measurements. The effect of several pharmacological agents (cycloheximide, nystatin, chlorpromazine & MβCD; Sigma, St Louis, MO, USA) on OMV uptake was examined by pre-treating cells with each drug for the following times: nystatin and MβCD,
150 1 h; chlorpromazine, 30 min; cycloheximide, 15 min. Cells were then incubated with
151 labeled-OMV for 2 - 4 h. Control cells were incubated without inhibitor for the same period
152 of time. To assess drug cytotoxicity, AGS cells were incubated in media alone or with the
153 highest concentration of each inhibitor for 4 h then stained with 0.75% propidium iodide for
154 10 min and fluorescence measured by flow cytometry. Cytotoxicity was expressed as the
155 percentage of propidium-iodide positive cells. To assess inhibition of clathrin-mediated
156 endocytosis, cells were incubated with DiO-labeled LDL (15 µg) for 4 h.
157
158 **Preparation of H. pylori LPS.** Extraction of *H. pylori* LPS was performed using
159 conventional hot phenol-water treatment (23). Subsequent purification of this crude LPS was
160 performed by using enzymatic treatments with RNase A, DNase II and proteinase K (all from
161 Sigma) as described previously (38).
162
163 **Cholesterol quantification.** AGS cells (3 x 10^5) were cultured overnight, washed once
164 with PBS prior to incubation for 1 h at 37°C in F12 media containing 5 mg/ml of MβCD.
165 The cholesterol content of cells was measured using the cholesterol CHOD-PAP reagent
166 (Roche Diagnostics, Indianapolis, IN).
167
168 **Neutral red assay.** Cells (3 x 10^4) were cultured overnight in 96-well micro-titre plates
169 then incubated with OMV (10 – 50 µg OMV protein) for 6 h. Untreated AGS cells were
170 used as controls for background vacuolation. Vacuolation was assessed by staining with
171 neutral red as previously described (10).
172
173 **Statistical analysis.** Results are the means ± standard errors of the means (SE). Data
174 were analyzed by t-test, Pearson’s correlation, One Way Analysis of Variance (ANOVA) or
175 Two Way ANOVA. If the ANOVA *p* value was < 0.05, this was followed by Dunnett’s
176 post-hoc test.
RESULTS

Assessment of the rate of *H. pylori* OMV internalization by AGS cells. We and others have previously shown that *H. pylori* OMV are internalized by gastric epithelial cells (8, 15, 44, 45). In this study, we sought to examine the rate and mechanism of OMV internalization using the lipophilic fluorescent membrane dye DiO to monitor OMV localization. The addition of labeled OMV to AGS cells for 24 h induced a 73% decrease in proliferation (27 ± 4% of control) similar to that induced by unlabeled OMV (30 ± 2% of control) (means ± SE, n = 3) indicating that labeling did not interfere with the interaction of vesicles with AGS cells.

Internalization of OMV was assessed quantitatively by flow cytometry using the cell impermeant dye trypan blue to quench extracellular DiO-vesicle fluorescence. The effectiveness of trypan blue at quenching DiO-OMV fluorescence was confirmed by incubating cells with labeled OMV and comparing the fluorescence of permeabilized and non-permeabilized cells treated with this dye. Complete loss of DiO fluorescence was observed in permeabilized cells upon addition of trypan blue while non-permeabilized cells showed no change in fluorescence (Fig. 1A & B). To examine the rate of OMV internalization, AGS cells were incubated with DiO-labeled OMV for up to 4 h and fluorescence measured before (total associated OMV) and after (intracellular OMV) the addition of trypan blue. A linear increase in total associated OMV was observed over the 4 h, with Pearson’s coefficient correlation r value = 0.997 (p = 0.003; Fig. 1C). At each time point assessed the majority of the OMV associated with the cells were intracellular (Fig. 1C). Examination of earlier time points showed that internalization of OMV peaked at 20 min and then plateaued (Fig. 1D).
De novo protein synthesis is not required for vesicle binding. The steady increase in OMV association with cells over time could be explained by specific vesicle binding that is dependent on upregulation or recycling of receptors after internalization of OMV. To examine whether vesicle binding stimulates de novo synthesis of OMV receptors, AGS cells were pretreated with increasing concentrations of the protein synthesis inhibitor cycloheximide. Inhibition of protein synthesis had no effect on the association of OMV with AGS cells (ANOVA $p = 0.878$; Fig. 1E).

VacA enhances OMV association with AGS cells. We have previously shown that VacA is localized on the surface of $H. pylori$ OMV (26) and that OMV-associated cytotoxin is biologically active (22). These findings, plus evidence that heat-labile enterotoxin has a role in the internalization of OMV shed from enterotoxigenic $E. coli$ (31), led us to consider that vesicle-associated VacA may also play a role in the uptake of $H. pylori$ OMV. Uptake of OMV from the vacuolating strain 60190 was compared to its isogenic mutant 60190:v1 that does not produce a toxin (11). The vacA gene has been disrupted in this mutant by insertion of a kanamycin cassette preventing formation of the cytotoxin (11). Although the proteome of $H. pylori$ OMVs has not been defined, OMV from this mutant strain are assumed to have the same phenotype as wild-type OMV, except for the absence of the VacA protein. Examination by fluorescence microscopy after 5 h confirmed a previous observation (8) that vesicles shed from both wild type and VacA- mutant strains are internalized by AGS cells (Fig. 2A & B). To confirm that the observed fluorescence was OMV and not free dye, we labeled vesicles with DiO then, after incubation with AGS cells, used a polyclonal antibody directed against $H. pylori$ OMV to detect vesicles. Immuno-labeled OMV colocalized with DiO-labeled OMV (Fig. 2C).
While the data obtained by fluorescence microscopy demonstrated that uptake of VacA\(^{-}\) mutant OMV can occur, there was no indication of the rate of association of these OMV. To investigate this, AGS cells were incubated with labeled OMV from either strain for up to 6 h and examined at regular time points by flow cytometry. As shown in Figure 3A, wild type OMV associated with AGS cells at a faster rate than OMV from the VacA\(^{-}\) strain (\(p < 0.01\) at 6 h).

To further examine the role of VacA in OMV binding, vesicles from strain 60190 were labeled with DiO (green) and vesicles from strain 60190:v1 with DiD (red). Cells were incubated with OMV from either strain or a 50:50 mix of both. Co-incubation of wild type OMV with VacA\(^{-}\) OMV had no effect on the association of wild type vesicles with cells (Fig. 3B). In contrast, the association of OMV from the VacA mutant strain was significantly inhibited (\(p = 0.002\), Fig. 3B). This observation, that incubation with wild type OMV inhibited the association of VacA\(^{-}\) OMV, suggests the presence of VacA enhances vesicle binding.

**OMV uptake is inhibited by LPS.** LPS has been implicated as a potential *H. pylori* adhesin (14). AGS cells were co-incubated with wild type or VacA\(^{-}\) OMV and increasing concentrations of purified *H. pylori* LPS. OMV uptake was inhibited in a dose-dependent manner by *H. pylori* LPS, with the effect most evident with VacA\(^{+}\) OMV (58% ± 2.5 inhibition of wild type OMV uptake compared with 25% inhibition ± 2.4 of VacA\(^{-}\) OMV uptake with 50 µg/ml LPS) (Fig. 4) (Dunnett’s post-hoc test \(p < 0.05\)). In combination with Fig. 3, this suggests that VacA is important in facilitating the uptake of OMV by an LPS-inhibitable mechanism, while in the absence of VacA LPS plays a less significant role.
Cholesterol is not required for OMV uptake. Depletion of plasma membrane cholesterol, which disrupts lipid rafts (34), has been reported to inhibit entry of purified VacA into host cells (42, 53). To examine whether depletion of plasma membrane cholesterol could likewise reduce the uptake of VacA+ OMV, AGS cells were pretreated with 5 mg/ml methyl-β-cyclodextrin (MβCD), a cholesterol sequestering agent (47). MβCD-treatment resulted in no difference in cholesterol levels per mg of total cell protein (37.74 ± 1.87 for untreated cells, 33.16 ± 2.21 for MβCD-treated cells) (means ± SE, n=3; p = 0.133). This was unexpected as a reduction in cholesterol of up to ~50% has been reported with similar concentrations of MβCD (42, 53). However, pretreatment of AGS cells with as little as 1 mg/ml MβCD was sufficient to significantly inhibit vacuole formation in response to 60190 OMV (Fig. 5A), while vesicle uptake was increased by MβCD at all concentrations tested (Fig. 5B) (Dunnett’s post-hoc test p < 0.001 and p < 0.05, respectively). Higher concentrations of MβCD were not used as, in addition to disrupting lipid rafts, these have been reported to inhibit clathrin-mediated endocytosis (47, 55) and to induce marked changes in cellular morphology in HeLa cells (53). Instead, cells were treated with nystatin, a cholesterol-binding agent (50), that disrupts lipid rafts but does not affect clathrin-mediated endocytosis (49). Pretreatment with nystatin (25 μg/ml) inhibited wild-type OMV-mediated cell vacuolation (Fig. 5C) (Dunnett’s post-hoc test p < 0.05) but had no effect on wild-type or VacA- mutant OMV uptake (Fig. 5D) (ANOVA p = 0.114 and p = 0.811, respectively).

Inhibition of clathrin-mediated endocytosis reduces OMV uptake. Clathrin-mediated endocytosis was explored as a potential pathway for vesicle internalization. This pathway can mediate the constitutive uptake of ligands such as transferrin or low-density lipoprotein as well as ligand-triggered receptor uptake of proteins (17). Chlorpromazine, a known
inhibitor of clathrin-mediated endocytosis (59), significantly inhibited vacuole formation in AGS cells treated with 60190 OMV when added at a concentration of 15 µg/ml (Dunnett’s post-hoc test \( p < 0.001 \); Fig. 6A) and had a dose-response effect on wild-type OMV uptake (ANOVA \( p = 0.011 \); Fig. 6B). Chlorpromazine (15 µg/ml) reduced the uptake of VacA-OMV to a greater extent (42% ± 8.9) (Dunnett’s post-hoc test \( p < 0.05 \); Fig. 6C). A reduction in clathrin-mediated endocytosis was confirmed by measurement of LDL uptake (Dunnett’s post-hoc test \( p < 0.05 \); Fig. 6D). We were not able to completely inhibit clathrin-mediated endocytosis with chlorpromazine as concentrations of this drug greater than 15 µg/ml were cytotoxic, as assessed by propidium iodide staining (data not shown).
DISCUSSION

The *H. pylori* vacuolating cytotoxin is secreted in a soluble form and is also associated with OMV (45). Vesicle-associated VacA has been observed at the surface (26) and within the gastric mucosa (15), however, little is known of the interactions of OMV-associated cytotoxin with host cells. The data presented in this study indicates that VacA is not essential for *H. pylori* OMV uptake but the presence of the cytotoxin increases the rate of vesicle association with cells.

Purified VacA is reported to bind to multiple cell surface components, including sphingomyelin (18), glycosphinglipids (46), glycosylphosphatidylinositol (GPI)-anchored protein(s) (43) and receptor-like protein tyrosine phosphatases β (39), with subsequent clustering of the receptor-bound toxin in cholesterol-rich lipid rafts (39, 42, 53). Horstman *et al.* speculated that such binding of vesicle-bound bacterial toxins to multiple binding sites acts to bring vesicles into closer proximity with host cells, thus allowing secondary adhesions to increase the “intimacy” between the two (20). In support of this, VacA⁺ OMV associated with cells quicker than VacA⁻ OMV and inhibited the association of VacA⁻ vesicles suggesting the toxin increases the avidity of vesicle binding. In addition, *H. pylori* LPS inhibited OMV uptake to a greater extent with VacA⁺ OMV. We speculate that binding of vesicle-associated VacA to cells increases the interaction of OMV LPS with cells, increasing the avidity of the overall vesicle binding reaction. In studies of other bacteria, the aminopeptidase PaAP, one of the major protein constituents of OMV from *P. aeruginosa* cystic fibrous isolates, was recently shown to increase the rate of association of *P. aeruginosa* OMV with lung epithelial cells (4). In addition, a heat-labile enterotoxin (LT) associated with ETEC OMV increases the association of *E. coli* OMV with cells (31). In the
absence of LT, *E. coli* OMV binding is significantly reduced (31).

Uptake of vesicles from several bacterial genera has been shown to be cholesterol-dependent (6, 25, 31). In addition, Kaparakis and colleagues (25) recently reported that *H. pylori* OMV uptake is cholesterol-rich lipid raft-dependent. However, in our study binding of cholesterol with MβCD or nystatin, disruptors of lipid raft formation (25, 49), did not significantly inhibit *H. pylori* vesicle uptake. The disparity between the Kaparakis study and ours may be a reflection of the uptake of vesicles from different *H. pylori* strains and/or the use of serum-free cell culture medium (25).

MβCD, although having no significant effect on AGS cell cholesterol levels, significantly decreased vacuolation in cells treated with VacA+ OMV. Low concentrations of MβCD have also been shown to inhibit vacuolation in HeLa cells treated with purified VacA (53), adding weight to evidence that membrane cholesterol is essential for VacA toxigenesis (39, 42). A reduction in vacuolation in the absence of an effect on OMV uptake may be explained by a greater sensitivity of toxin-mediated vacuole biogenesis to membrane cholesterol levels than vesicle uptake.

VacA translocation to lipid rafts following binding to receptor-like protein tyrosine phosphatases β receptors in non-lipid raft domains on the cell surface is reportedly inhibited by treatment with the same concentration of MβCD (5 mg/ml) used in our study (39). Thus, if lipid rafts are required for VacA+ OMV uptake we expected to see reduced vesicle internalization. Instead we observed that OMV uptake in MβCD-treated cells was increased. The reason for this is not immediately apparent and requires further investigation.

Disruption of clathrin-mediated endocytosis with chlorpromazine reduced the uptake of OMV. To our knowledge this is the first report demonstrating OMV internalization via
clathrin-mediated endocytosis. Internalization of ETEC and *P. aeruginosa* OMV is unaffected by chlorpromazine and these OMV rarely colocalize with clathrin (4, 31). Indeed, ETEC OMV frequently colocalize with caveolae (31). In addition, *P. gingivalis* OMV internalize via a Rac1-regulated lipid raft-dependent pathway that is independent of caveolin and clathrin (16).

The observation that VacA+ OMV uptake was less inhibited by chlorpromazine than uptake of VacA− OMV suggests that toxin-containing *H. pylori* OMV may be taken up into gastric epithelial cells via more than one pathway. That nystatin had no significant effect on VacA+ OMV uptake also supports this. However, further investigation is required to confirm this hypothesis. Of note, several bacterial toxins (including anthrax, cholera and Shiga toxins) are reportedly endocytosed by both clathrin-dependent and clathrin-independent mechanisms (1, 52, 56).

The increase in OMV association over time, in conjunction with the observation that the majority of associated OMV were intracellular 20 min and thereafter suggests to us, that OMV are taken up and that binding is the rate limiting step, possibly due to a limited numbers of receptors. The steady increase in fluorescence over time also suggests that, once internalized, OMV persist within AGS cells for some time. This is supported by the observation of intact OMV within cells after >72 h co-incubation (54). The association of ETEC and *P. aeruginosa* OMV with cells has also been shown to increase with time (4, 31). In agreement with Bauman & Kuehn (4) we consider this is consistent with (i) vesicle binding initiating upregulation of OMV receptors; or (ii) that receptors are recycled back to the plasma membrane after internalization of their cargo (7). We found OMV association was unaffected by cycloheximide indicating that if specific receptors are involved they are
already translated. We have observed that perturbation of the actin cytoskeleton inhibits vesicle association (H. Parker and J. Keenan, unpublished data) suggesting disruption of receptor recycling.

The small size of OMV (50 – 300 nm) makes their enumeration difficult. In this study OMV were quantified based on protein concentration. While this may vary between strains it is one of the more commonly used methods for OMV enumeration (16, 24, 25, 48, 58). As an alternative, OMV may be quantified based on the concentration of lipopolysaccharide (51) or toxin (6) in vesicle preparations. However, the amount of LPS and VacA cytotoxin also varies between H. pylori strains (26, 29), as well as within a strain grown under different conditions (28, 30). In addition, use of a VacA\(^-\) strain in this study precluded OMV enumeration based on cytotoxin concentration. Immunolabelling of VacA\(^+\) OMV shows relatively few (< 10) toxin molecules per OMV (15, 26, 54). Therefore, we considered that its absence from OMV may not significantly change the overall vesicle protein concentration thus allowing for reasonable comparison between OMV from wild type and VacA\(^-\) OMV.

In summary, we have shown that VacA toxin enhances the association of H. pylori OMV with cells and that VacA\(^-\) OMV are internalized via clathrin-mediated endocytosis while VacA\(^+\) OMV may be able exploit more than one pathway of internalization. We propose that constitutive release (26) and uptake (15) of OMV from the surface of these predominantly non-invasive bacteria contributes to H. pylori pathogenesis via the persistent delivery of virulence factors and antigens to gastric epithelial cells.
ACKNOWLEDGEMENTS

We thank Tim Cover (Division of Infectious Diseases, Vanderbilt University School of Medicine, USA) for the kind gift of the VacA mutant.

This work was supported by a Bright Futures Doctoral Scholarship to HAP and funding from the Cancer Society of New Zealand (to JIK and MBH).
REFERENCES


OMV association (% of control) (% of total Dio fluorescence) (mean fluorescence intensity)
FIG. 1. Flow cytometry assessment of the association of DiO-labeled OMV (strain 60190) with AGS cells. (A & B) To confirm that trypan blue quenches DiO-OMV fluorescence, cells were incubated with labeled OMV for 4 h then (A) fixed and permeabilized or (B) not permeabilized. Fluorescence was measured before and after the addition of trypan blue. (C) The proportion of intracellular OMV was monitored over time. Cells were incubated with DiO-labeled 60190 OMV for 1 – 4 h then fluorescence measured before (total associated) and after (intracellular) the addition of trypan blue. (D) The proportion of intracellular OMV during the first hour incubation. (E) Inhibition of de novo protein synthesis does not inhibit the association of OMV with AGS cells. Cells were pretreated with cycloheximide for 15 min prior to incubation with labeled 60190 OMV for 2 h then fluorescence measured by flow cytometry. Results for (C - E) are ± SE for three independent experiments.
FIG. 2. OMV internalization occurs in the presence and absence of VacA. OMV from (A) strain 60190 and (B) its VacA- isogenic mutant 60190:v1 within AGS cells. Green – OMV; red – actin cytoskeleton. Images are a side view of composite Z-stack series. (C) To confirm DiO fluorescence was OMV-associated, AGS cells were incubated with DiO-labeled OMV for 5 h, fixed and immunolabeled with an antibody to *H. pylori* OMV detected with a PE-conjugated secondary antibody. No fluorescence was observed when cells were incubated without OMV (Control). Image shows two adjacent cells whose cytoplasm contains labeled OMV (n – nucleus; c – cytoplasm). (A – C) images are a representative of three independent experiments.
FIG. 3. The presence of VacA increases vesicle association with AGS cells. (A) Cells were incubated for up to 6 h with DiO-labeled OMV from strain 60190 or 60190:v1 and the increase in cell-associated vesicle fluorescence was measured by flow cytometry. Data are presented as fold increase of the fluorescence measured at 2 h for each experiment. (B) AGS cells incubated for 4 h with DiO-labeled wild type OMV (black bar) or DiD-labeled VacA- mutant OMV (grey bar) or a 50:50 mix of labeled OMV (hatched bar) from both strains. Measurement of DiO fluorescence is shown over the bar designated 60190 while measurement of DiD fluorescence is shown over the bar designated 60190:v1. Results are ± SE for three (A) or four (B) independent experiments. (A) *, results are statistically
different at the 6 h time point ($p < 0.01$). (B) **, significantly fewer VacA OMV are
internalized when mixed 50:50 with wild-type OMV. ($p = 0.002$).
FIG. 4. *H. pylori* LPS competitively inhibits OMV uptake by AGS cells. LPS (3.125 – 50 µg/ml) was added to cells at the same time as labeled OMV from either wild type or VacA⁻ mutant strains. Cells were then incubated for 4 h and fluorescence measured after the addition of trypan blue. Results are ±SE for three independent experiments performed in triplicate. Overall, purified LPS had a significant effect on wild type and VacA⁻ mutant OMV uptake (p < 0.0001 and p = 0.0002, respectively). *, results are statistically significant from controls not treated with purified LPS (p < 0.05).
FIG. 5. Effect of MβCD and nystatin on vacuole formation and vesicle uptake in AGS cells.

(A) Cells were pre-treated with MβCD (1 – 5 mg/ml) for 1 h then incubated with 60190 OMV for 6 h. Vacuolation was measured by neutral red assay. (B) Treatment with MβCD increased the uptake of 60190 OMV with AGS cells. Cells were pre-treated with MβCD for 1 h then incubated with DiO-labeled OMV for 4 h. Fluorescence was measured after the addition of trypan blue. (C) Cells were pre-treated with nystatin (5 – 50 mg/ml) for 1 h then incubated with OMV for 6 h. Vacuolation was measured by neutral red assay. (D) Cells were pre-treated with nystatin for 1 h then incubated with DiO-labeled 60190 OMV or labeled 60190:v1 OMV for 4 h. Fluorescence was measured after the addition of trypan blue. Results for (A & C) are ±SE for three separate experiments performed in triplicate; (B & D) are ±SE for three separate experiments. Overall, MβCD and nystatin had a significant
effect on vacuole formation ($p < 0.0001$ and $p = 0.027$, respectively). *, **, *** results are statistically significant from controls not treated with MβCD or nystatin ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).
FIG. 6. Effect of chlorpromazine on vacuole formation and vesicle uptake in AGS cells. (A) Cells were pre-treated with chlorpromazine (5–15 μg/ml) for 30 min then incubated with 60190 OMV for 6 h. Vacuolation was measured by neutral red assay. (B & C) Chlorpromazine reduces OMV uptake. Cells were pretreated with chlorpromazine for 30 min prior to 4 h incubation with labeled-OMV from either strain 60190 (B) or 60190:v1 (C) or with fluorescently labeled LDL (D). Fluorescence was measured after the addition of trypan blue. Results for (A) are ±SE for three independent experiments performed in triplicate. Overall, vacuolation decreased in 60190 OMV treated cells in response to a dose-
dependent increase in chlorpromazine ($p < 0.0001$). Results for (B) and (C) are ±SE for six independent experiments for OMV and three separate experiments for LDL (D). Overall, there was a significant decrease in 60190 ($p = 0.011$), 60190v:1 ($p = 0.003$) and LDL ($p = 0.002$) uptake in response to increasing concentration of chlorpromazine. *, ***, results are statistically significant from controls not treated with chlorpromazine ($p < 0.05$ and $< 0.001$, respectively).