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Extracellular pH Modulates *Helicobacter pylori*-Induced Vacuolation and VacA Toxin Internalization in Human Gastric Epithelial Cells

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In this study we investigated whether an acidic extracellular pH may inhibit *H. pylori*-induced internalization of bacterial virulence factors by gastric epithelium, thus preventing ingestion of potentially dangerous luminal contents and resulting cellular damage. The interaction of *H. pylori* VacA toxin and ammonia (produced by *H. pylori* urease) with partly polarized gastric MKN 28 cells in culture was investigated at neutral and moderately acidic pH (6.2, compatible with cell viability) by means of neutral red dye uptake and ultrastructural immunocytochemistry. We found that acidic extracellular pH virtually abolished both VacA-dependent and ammonia-dependent cell vacuolation, as shown by the neutral red test, and caused a 50% decrease in VacA internalization into endosomal vesicles and vacuoles, as assessed by quantitation of immunogold particles. In addition, acidic pH blocked endosomal internalization of *H. pylori* outer membrane vesicles, a convenient indicator of endocytosis. Our data raise the possibility that suppression of gastric acid may increase *H. pylori*-induced gastric damage by enhancing epithelial internalization of *H. pylori* virulence factors through activation of endocytosis. Increased transmembrane diffusion of ammonia could also contribute to this process.

Key Words: **cell vacuolation; extracellular pH; *Helicobacter pylori;* ultrastructural immunocytochemistry; VacA internalization.**

Human gastric epithelium colonized by *Helicobacter pylori* is known to undergo luminal bulging, cytoplasmic swelling, and expansion and dilation of apical endosomes, whose reciprocal fusion may result in cytoplasmic vacuoles (1–3). A pivotal role in *H. pylori*-induced cell damage seems to be played by two *H. pylori* virulence factors: urease and VacA toxin. Urease catalyses the hydrolysis of urea to carbon dioxide and ammonia; ammonia is known to cause cell vacuolation (4–6). VacA is a protein toxin which causes cytoplasmic vacuolation in a variety of cultured cell lines (7–9). VacA-induced vacuole development is strictly dependent on the presence in the incubation medium of weak bases like ammonia (7, 10). When given to mice, VacA causes gastric epithelial damage resembling that found in *H. pylori*-colonized patients (11). Epithelial cell lines incubated with broth culture filtrate (BCF) from VacA-producing strains of *H. pylori* or with purified VacA show energy-dependent uptake of the toxin and its selective internalization into endosomes and related vacuoles (10, 12–14) likely by a process of receptor-mediated endocytosis (8, 9, 15). That endocytosis is involved in the process is confirmed by the fact that both soluble secreted VacA and VacA-containing outer membrane vesicles (OMVs) budding off from *H. pylori* cells adhere to the apical surface of human gastric cells *in vitro* and of human gastric epithelium *in vivo,* to be internalized into endosomal vesicles and vacuoles (13, 16).

Although gastric absorption of antigenic material by an active process compatible with endocytosis has been documented (17), little information is so far available on gastric luminal endocytosis—a potentially dangerous process given the luminal content of acid, pepsin and exogenous antigens—and on factors possibly controlling it. The recent demonstration in pancreatic acini that a moderately acidic luminal pH, not interfering with cell viability or secretory activity, blocks apical endocytosis (18) suggested us that a similar mechanism of acid-mediated inhibition of endocytosis

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might prevent ingestion of potentially dangerous luminal contents by gastric epithelium.

In vivo, the gastric surface epithelium is known to be covered by a protective layer of mucous-bicarbonate gel and phospholipids mostly resulting from its own secretory activity. As a result, a near-neutral pH has been reported at the epithelial surface when pH up to 2.0 is found in the gastric lumen (19, 20). With lower pH values, as regularly found during night in human stomach, especially of duodenal ulcer patients, the pH gradient is likely to be dissipated (20). In addition, the protective gel layer is known to be damaged by *H. pylori* proteases, phospholipase, urease-dependent ammonia production and other virulence factors (21). Thus, a direct contact between gastric acid content and surface epithelium seems likely to occur, at least in pathologic conditions.

In this study we tested the human gastric cell line MKN 28, which shows partly polarized differentiation of a luminal surface closely mimicking that of gastric surface epithelium, while lacking a substantial mucous secretion and mucous gel layer formation (16, 22). Under these experimental conditions, the direct interaction of *H. pylori* VacA toxin and ammonia with gastric epithelium was investigated at neutral as well as moderately acidic pH (6.2) compatible with cell viability. The partly polarized cell line used at subconfluency also allowed to evaluate VacA-dependent cell vacuolation, which is not observed in confluent monolayers of fully polarized epithelial cells (23).

MATERIALS AND METHODS

Gastric Epithelial Cells

We used the MKN 28 cell line, derived from a human gastric tubular adenocarcinoma, which is known to retain gastric type differentiation (22, 24) and to provide a suitable model for the study of the response of gastric epithelial cells to *H. pylori* (10, 25). The MKN 28 cells were grown as monolayers in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 supplemented with 10% foetal calf serum (FCS; Gibco, Grand Island, NY, USA) in 35 mm plastic Petri dishes (Corning Glass Works, Corning, NY, USA) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Effect of pH on Cell Viability

After 16 h incubation with Hanks' balanced salt solution (HBSS) at different pH values, cell viability was evaluated by means of a live/dead fluorescent assay (26). Each monolayer was washed once with HBSS and then incubated at room temperature with 1 ml of ethidium bromide (250 μg/ml)/acridine orange (75 μg/ml) solution in HBSS. After 1 min, cells were observed under a fluorescence inverted microscope (Diaphot 300, Nikon Co., Tokyo, Japan) at 200×. Ethidium bromide is excluded by intact cell membranes, but stains the nuclei of dead cells orange fluorescent; acridine orange enters living cells giving the nuclei a green fluorescence. For each monolayer (in triplicate for each experimental condition) three microscopical fields were photographed by using a 400 ASA colour film. Cells were counted and viability was calculated as percentage of nuclei fluorescing green as a proportion of the total (orange plus green) nuclei.

Bacterial Strains, Filtrate Production, and Cell Incubation

Two *H. pylori* strains were used: (1) the VacA-producing CCUG 17874 strain (from Culture Collection University of Göteborg, Göteborg, Sweden), and (2) the VacA-negative G21 strain (a kind gift from N. Figura, Siena, Italy) (25). Bacteria were grown in Brucella broth (Difco, Detroit, MI) supplemented with 1% Vitox (Oxoid, Basingstoke, UK) and 5% FCS for 24–36 h at 37°C in a thermostatic shaker under microaerophilic conditions. Bacteria were then removed by centrifugation and the supernatants were sterilized by passage through a 0.22 μm cellulose acetate filter (Nalge Co., Rochester, NY, USA) to obtain the broth culture filtrates (BCFs) (13, 25). Uninoculated broth filtrate served as a control. To remove the ammonia content, control and BCFs were dialyzed against HBSS for 36 h in dialysis tubing with a 12 kDa molecular mass cut-off (Sigma, St. Louis, MO, USA). The presence and the absence of VacA in the respective BCFs from CCUG 17874 and G21 *H. pylori* strains (respectively referred to as $VacA^+$ BCF and $VacA^-$ BCF) was assessed by means of SDS–PAGE, followed by immunoblotting with the αpKH C3 anti-VacA serum (10). The αpKH C3 polyclonal rabbit antiserum (kindly given by J. L. Telford, Siena, Italy) was raised against the carboxy-terminal portion of the vacuolating toxin, expressed as a recombinant fragment in *Escherichia coli* (11).

Subconfluent monolayers of MKN 28 cells were washed twice with HBSS and then incubated, at either 6.2 or 7.4 final pH, at 37°C for 16 h with 1) uninoculated broth filtrate diluted 1:3 in HBSS, in the absence (control) or in the presence of 4 mM NH_4Cl; 2) $VacA^+$ BCF or $VacA^-$ BCF diluted 1:3 in HBSS in the presence of 4 mM NH_4Cl. This fixed amount of ammonia was added to dialyzed BCFs in order to obtain an identical ammonia level for each experimental condition, a crucial point considering that the presence of a weak base like ammonia is strictly required for the development of VacA-dependent cell vacuolation (10).

Neutral Red Dye Uptake Assay

At the end of incubation, the degree of cell vacuolation was assayed by means of neutral red dye uptake as previously described (6). Results were expressed as ng of neutral red per μg cell protein. Neutral red uptake is a widely used *in vitro* assay for *H. pylori*-induced cell vacuolation (5–7, 10, 14, 27, 28).

Electron Microscopy

After cell incubation, the medium was discarded, and cell monolayers were washed twice with cacodylate buffer (pH 7.3) and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde 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, postfixed in 1% osmium tetroxide, and then embedded in Epon-Araldite mixture (6). Uranyl-lead stained ultrathin sections were viewed with a Zeiss EM 902 electron microscope.

For the ultrastructural immunolocalization of *H. pylori* VacA toxin, we used the colloidal gold technique as previously described (10). 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, 0.05 M Tris–HCl, pH 7.4), and incubated in non-immune goat serum at room temperature for 1 h, to prevent nonspecific binding of immunoglobulins. The sections were then incubated at 4°C overnight with either αpKH C3 or 123 anti-VacA serum, diluted 1:600 in buffer B (0.45 M NaCl, 1% bovine serum albumine, 0.5% sodium azide, 0.05 M Tris–HCl, pH 7.4). The anti-VacA polyclonal rabbit serum 123 (kindly given by T. L. Cover, Nashville, TN) was raised against

purified native VacA (7). 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. Quantitative evaluation of gold labelling and areas was performed by means of an IBAS 2 image analyzer (Zeiss), and expressed as mean ± SEM of immunogold particles per μm^2 of endosomal vesicles-vacuoles, nuclei or extracellular area.

Statistics

All data were expressed as the mean ± SEM of 4 independent experiments. The statistical significance of the differences was evaluated by the Student's *t*-test, and by analysis of variance followed by Newman–Keuls' *Q*-test.

RESULTS

Effect of pH on Cell Viability

We found that after 16 h incubation at pH 6.2 MKN 28 cells exhibited a viability degree virtually identical to that exhibited after incubation at pH 7.4 (94 ± 3% and 97 ± 2%, respectively; $P = 0.44$). On the contrary, after 16 h incubation at pH 5.6, 5.0, or 4.4, MKN 28 cells exhibited a viability degree (63 ± 3, 28 ± 4, and 12 ± 2%, respectively) significantly lower ($P < 0.05$) than that at pH 7.4. Thus, we chose pH 6.2 as an acidic pH value suitable for this study since it was the lowest pH value not affecting cell viability in our experimental conditions.

Neutral Red Dye Uptake

Figure 1 shows the results of neutral red uptake experiments. When MKN 28 cells were incubated at pH 7.4, $VacA^+$ BCF caused a neutral red uptake significantly higher (about 2.5-fold; $P < 0.001$) than that induced by $VacA^-$ BCF in the presence of the same NH_4Cl concentration (4 mM). At this pH, $VacA^-$ BCF induced a neutral red uptake significantly higher (about 2-fold; $P < 0.001$) than that caused by dialyzed uninoculated broth filtrate (control), but virtually identical to that induced by control supplemented with 4 mM NH_4Cl. This finding confirms our previous observations (6) that cell vacuolation induced by $VacA^-$ BCF is essentially accounted for by its ammonia content.

When cell incubation was carried out at pH 6.2, both $VacA^+$ BCF and $VacA^-$ BCF failed to induce any significant increase in neutral red uptake compared to control. At the same pH, supplementation of control with 4 mM NH_4Cl also failed to increase neutral red uptake. Compared to pH 7.4, cell incubation at pH 6.2 significantly ($P < 0.001$) decreased neutral red uptake induced by $VacA^+$ BCF (of about 80%) as well as that induced by $VacA^-$ BCF or NH_4Cl-supplemented control (of about 45% for both). On the contrary, no difference was found in neutral red uptake by MKN 28 cells incubated with control broth filtrate at pH 6.2 or pH 7.4.

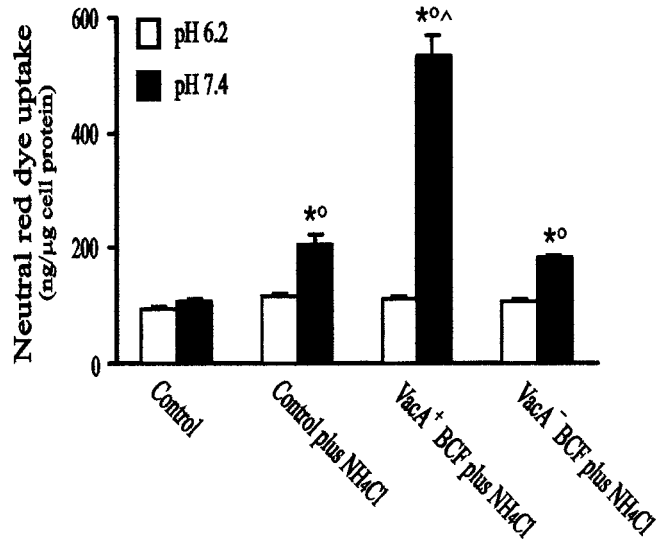

FIG. 1. Neutral red dye uptake induced in MKN 28 cells incubated for 16 h at pH 6.2 or 7.4 by dialyzed uninoculated broth filtrate in the absence (control) or in the presence of 4 mM NH_4Cl, and by $VacA^+$ BCF (from the CCUG 17874 *H. pylori* strain) or $VacA^-$ BCF (from the G21 *H. pylori* strain) in the presence of 4 mM NH_4Cl. All values shown are means ± SEM of four independent experiments. *, $P < 0.001$ vs control at the same pH; °, $P < 0.001$ vs the same condition at pH 6.2; ^, $P < 0.001$ vs control plus NH_4Cl at the same pH.

Electron Microscopy

Ultrastructural analysis of subconfluent cultures of MKN 28 cells in control medium showed polar differentiation of plasma membranes into luminal type, with relatively short, about 0.1 μm thick, gastric type microvilli, and lateral type with long, thin, undulating and interdigitating phylopodes. Cellular membranes adhering to the plastic dishes were mostly of smooth, undifferentiated type, whereas those forming the free part of the cells were usually of luminal type. MKN 28 cells incubated for 16 h at pH 7.4 with $VacA^+$ BCF from the CCUG 17874 strain showed expansion, dilation and fusion of endosomal tubulovesicles with large vacuoles formation (Fig. 2A) as reported in details elsewhere (6, 10). In addition, small (100–200 nm) vesicles formed by *H. pylori* OMVs were observed, either adhering to luminal type membrane or inside endosomal vesicles and vacuoles (Fig. 2B). Ultrastructural immunocytochemistry confirmed the presence of VacA within endosomal tubulovesicles and related cytoplasmic vacuoles, with concentrations significantly higher than in the extracellular space or the nucleus, taken as control (Fig. 2 and Table 1).

When cell incubation was performed at pH 6.2, OMVs were seen in contact with luminal membranes but not intracellularly; only scanty vacuoles were observed and dilated endosomal structures showed scarce evidence of reciprocal fusion (Fig. 3). Ultrastructural immunocytochemistry showed a significant re-

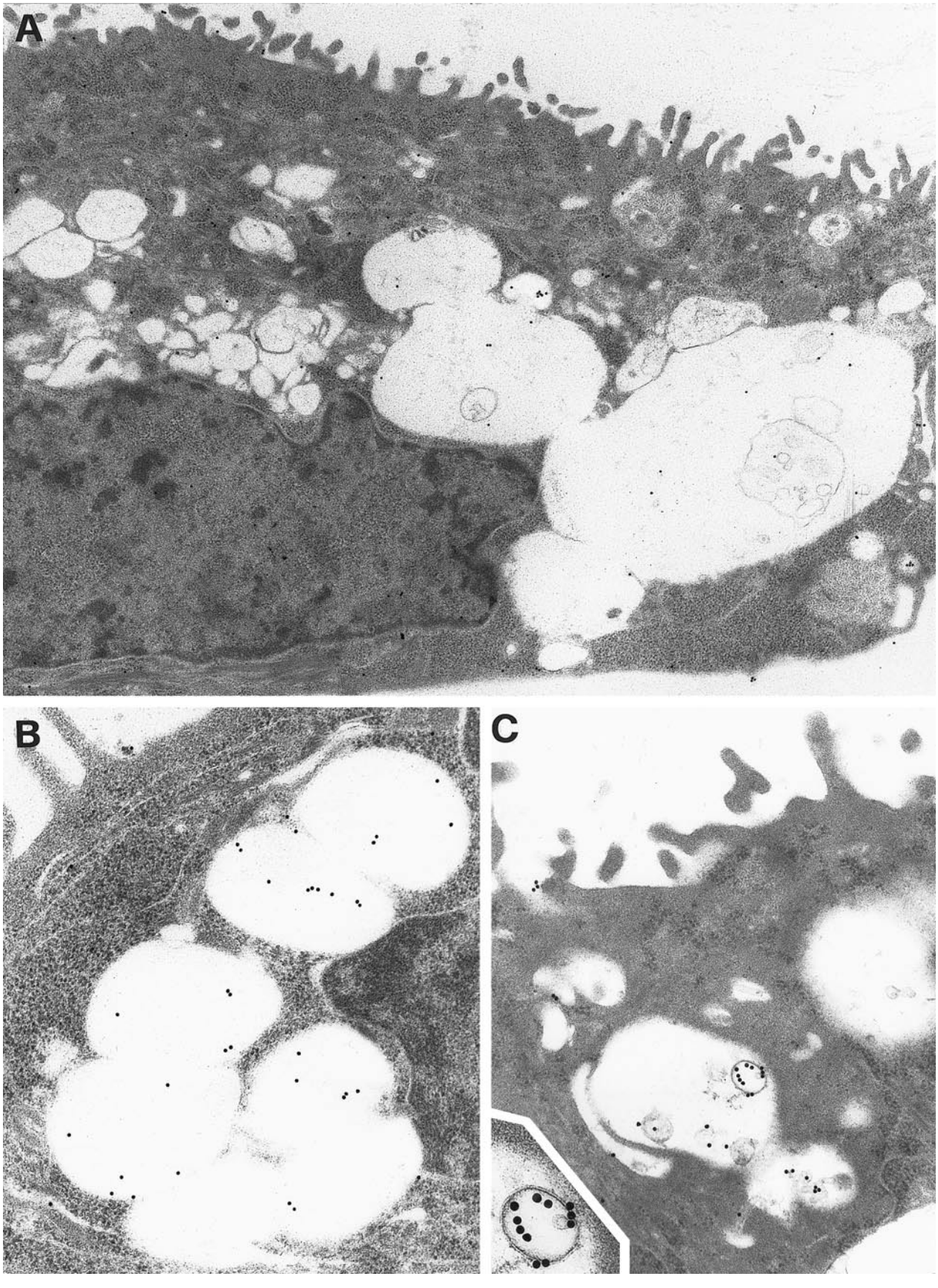
A
B
C

TABLE 1

Mean Number (±SEM) of Immunogold Particles per μm^2 of Endosomal Vesicles-Vacuoles, Nuclei or Extracellular Area of MKN 28 Cells Incubated at pH 7.4 or 6.2 with *H. pylori* $VacA^+$ BCF in the Presence of 4 mM NH_4Cl

	pH 7.4	pH 6.2	*P*
Endosomes	2.37 ± 0.21	1.30 ± 0.16	0.000
Nuclei	0.77 ± 0.08	0.35 ± 0.05	0.003
Extracellular area	1.14 ± 0.13	1.14 ± 0.18	NS

Note. Endosomes vs nuclei: $P < 0.001$ at both pH 7.4 and 6.2. Endosomes vs extracellular area: $P < 0.001$ at pH 7.4, NS at pH 6.2. Nuclei vs extracellular area: NS at pH 7.4, $P < 0.002$ at pH 6.2.

duction ($P < 0.001$) of internalized VacA compared to pH 7.4 incubation, so that at pH 6.2 no significant difference was found between VacA concentrations of endosomal and extracellular compartments (Fig. 3 and Table 1). In a first evaluation using αpKH C3 anti-VacA serum, 2.01 ± 0.19 immunogold particles were counted per μm^2 of endosomal area from 24 ultrastructural fields of different cells incubated at pH 7.4, compared with 0.81 ± 0.10 particles per μm^2 from 23 ultrastructural fields of cells incubated at pH 6.2 ($P <$ 0.001 versus pH 7.4 incubation). Results of a second experiment using the anti-VacA serum 123 are detailed in Table 1.

DISCUSSION

This study shows that incubation of a gastric cell line with *H. pylori* $VacA^+$ BCF at a moderately acidic pH preserving cell viability markedly inhibits both VacA internalization into endosomal vesicles and vacuole formation, when compared with incubation at neutral pH values. Incubation with *H. pylori* $VacA^-$ BCF, whose vacuolating activity is essentially accounted for by its ammonia content (6, this study), at pH 6.2 also caused a cell vacuolation significantly lower than that caused at pH 7.4, possibly due to the increased protonation and reduced transmembrane diffusion of ammonia known to occur at acidic pH. However, the much higher neutral red uptake and ultrastructural vacuolation caused at pH 7.4 by $VacA^+$ BCF compared to $VacA^-$ BCF ensures that at near-neutral pH most epithelial vacuolation is VacA related.

It should be outlined that the MKN 28 cell line we used showed a luminal type membrane differentiation mimicking that of normal gastric epithelium in both ultrastructural morphology and capacity to selectively bind and internalize *H. pylori* VacA toxin (16, this study) and that several *in vitro* studies (6, 9, 10, 12, 14, 27) suggested endocytosis as a likely mechanism of cellular VacA internalization. Our detection of intact OMVs inside endosomes and related vacuoles of MKN 28 cells after incubation at pH 7.4 with *H. pylori* BCF strongly indicates that epithelial endocytosis is operative *in vitro* at this pH, while their lack at pH 6.2 suggests that environmental acidity blocks endocytic process. Parallel investigation of *H. pylori*-colonized gastric mucosa biopsies by ultrastructural immunocytochemistry showed adhesion of VacA and OMVs to the luminal surface of the epithelium and their accumulation into endosomes and related cytoplasmic vacuoles, thus suggesting that endocytosis may also be involved in toxin internalization by gastric epithelium *in vivo* (16). It has also been proposed that VacA may act as a channel-forming toxin (28, 29) and that VacA channels may play a direct role in cell vacuolation. Indeed, endocytosed VacA channels could stimulate the turnover of endosomal V-ATPase by increasing the permeability of the endosomal membrane to anions (28). This would lead to the accumulation of osmotically active species causing an osmotic imbalance of late endosomes with subsequent vacuole formation.

Whatever the precise mechanism of VacA/endosomes interaction, the possibility arises that the inhibition of VacA and OMVs internalization as well as the reduced VacA-dependent cell vacuolation we observed at moderately acidic pH result from acid-mediated inhibition of endocytosis. In this respect, the observation that VacA binding to the surface of HeLa cells at acidic pH is higher than that at neutral pH (29) should rule out the alternative hypothesis that reduced VacA internalization at acidic pH may result from reduced cell binding of the toxin. Acid-mediated suppression of endocytosis has been also shown to operate at the luminal membrane of pancreatic acini (18) and polarized kidney cells (30) under conditions (pH 6, preserved cell viability) comparable with those of our experiments. It may represent a general mechanism ensuring homeostasis of luminal epithelia. In the case of gastric mucosa, acid modulation of endocytosis is likely to be

FIG. 2. MKN 28 cells incubated at pH 7.4 with $VacA^+$ BCF in the presence of 4 mM NH_4Cl, fixed in aldehyde–osmium; ultrathin sections stained with immunogold procedure using anti-VacA serum. (A) Polarized cell with luminal differentiation of the upper, free cellular membrane (top) while the membrane lying on plastic support remains smooth, undifferentiated (bottom). Dilated endosomal cisternae and related vacuoles are present in the cytoplasm and show immunogold particles, which are also seen adherent to the luminal membrane. ×20,000. (B) Intense VacA immunogold reactivity of fused vacuoles. ×33,000. (C) VacA immunoreactive bacterial OMVs are found in a vacuole, in addition to free VacA immunoreactivity; the latter is also seen in dilated endosomal cisternae and in an invagination of the luminal membrane (top). ×33,000. Inset: Enlargement of one of the intravacuolar OMVs to show the double membrane characteristic of bacterial OMVs. ×75,000.

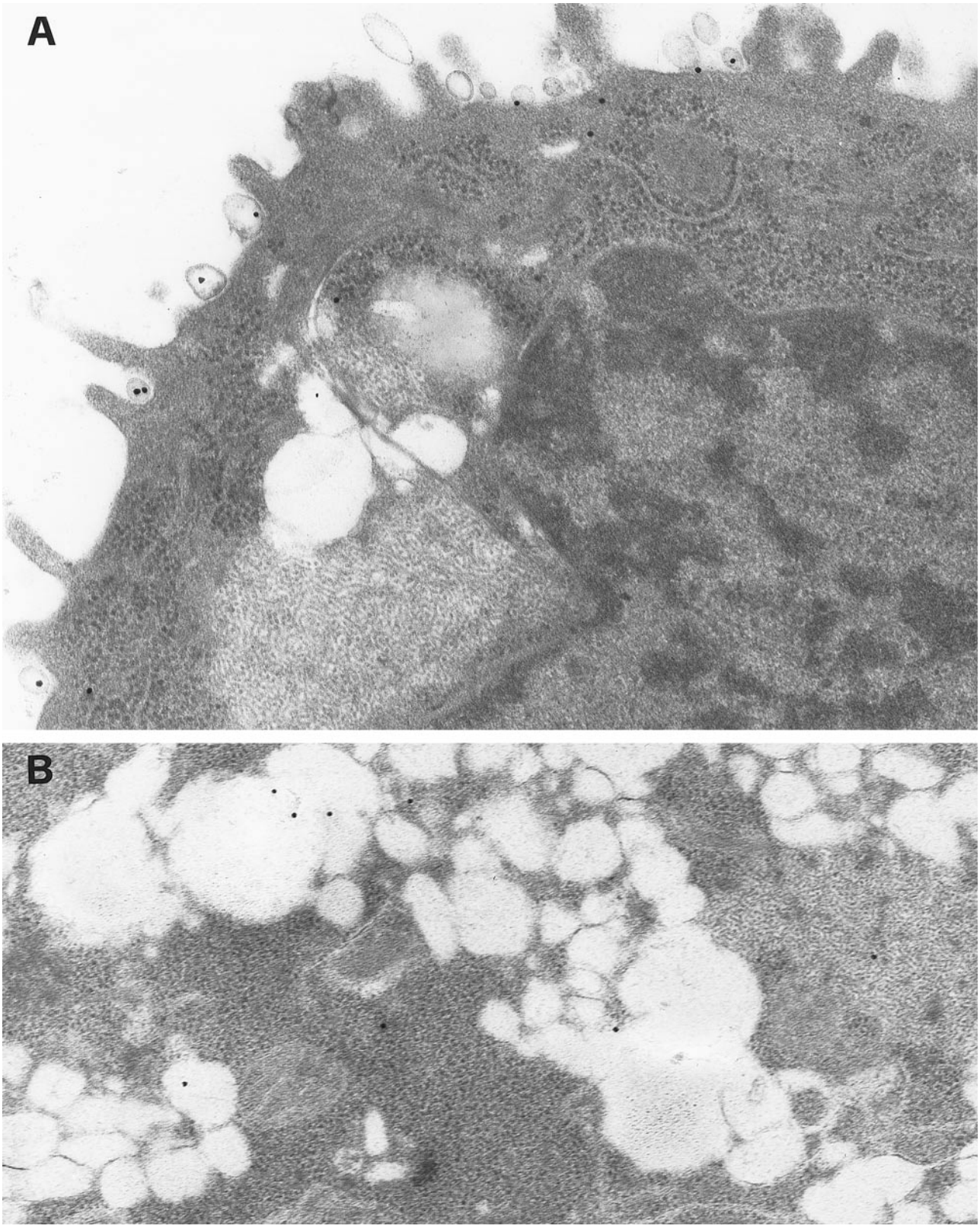

FIG. 3. MKN 28 cells incubated at pH 6.2 with $VacA^+$ BCF in the presence of 4 mM NH_4Cl and processed as in Fig. 2. (A) A cell with scarce intracellular vacuolation and VacA internalization and with lack of intracellular OMVs shows abundant extracellular OMVs adhering to the free luminal type membrane. ×45,000. (B) Another cell shows marked dilation and expansion of endosomes with limited fusion to form vacuoles; the scarce VacA immunostaining is mostly restricted to vacuoles. ×33,000.

important in preventing acid/peptic damage to the epithelium, especially when acid hypersecretion, *H. pylori*-induced gastritis or other mucosal pathologies disrupt or dissipate the protective mucous-bicarbonate gel layer covering gastric surface epithelium (20, 21, 31).

Ammonia production by *H. pylori* urease is likely to increase gastric surface pH (32), at least at sites of *H. pylori* colonization. While preventing possible acid-mediated damage, this pH change might activate epithelial endocytosis, thus enhancing the internalization and intracellular transport of VacA and other bacterial products and luminal contents. Evidence for increased transcytosis of undegraded exogenous proteins has been obtained when urease-positive (but not urease-negative) *H. pylori* were placed in the apical compartment of confluent monolayers formed by polarized intestinal cells (33). In fact, VacA and other *H. pylori* antigens have been detected in expanded endosomal vesicles of *H. pylori*-colonized gastric epithelium as well as in mesenchimal cells of immediately underlying lamina propria, a pattern suggestive of active antigens transcytosis through the epithelium, which may be crucial for the activation of mucosal immune-inflammatory response (16, 34).

Activation of endocytosis by interruption of an acid-mediated inhibitory mechanism might contribute to the hitherto unexplained worsening of gastritis found in *H. pylori*-colonized oxyntic mucosa when luminal acid is suppressed by antisecretory drugs or antacids (35). Indeed, suppression of luminal acid, by enhancing endocytosis, should increase epithelial internalization of *H. pylori* virulence factors and antigens as well as their transport to underlying lamina propria. In addition, the increased concentration of unprotonated ammonia which should result from the increased luminal pH is likely to enhance its transmembrane diffusion and cytotoxicity (5, 36, 37) as confirmed by our finding of pH-dependent ammonia-related cell vacuolation. Both mechanisms may contribute to the increased cytotoxic damage to gastric epithelium and increased severity and activity of inflammation which have been consistently reported in *H. pylori*-positive patients under antisecretory drug treatment (38, 39). Further *in vivo* studies are needed to test whether an altered luminal endocytosis may significantly contribute to gastric mucosa pathology.

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