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HELICOBACTER PYLORI VACUOLATING TOXIN ACCUMULATES WITHIN THE ENDOSOMAL-VACUOLAR COMPARTMENT OF CULTURED GASTRIC CELLS AND POTENTIATES THE VACUOLATING ACTIVITY OF AMMONIA

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SUMMARY

This study explored the relationship between vacuolating toxin and ammonia in the genesis of *Helicobacter pylori*-induced vacuolation in cultured human gastric cells and investigated the intracellular sites of toxin accumulation. Neutral red dye uptake and electron microscopy were used in the investigation of the respective roles of, and of the reciprocal interaction between, toxin and ammonia in cell vacuolation and ultrastructural immunocytochemistry was used for the identification of the intracellular sites of internalized toxin. Toxin was found to cause an expansion of the endosomal compartment, where it accumulates after cellular internalization. However, toxin does not form large, neutral red-positive vacuoles unless combined with ammonia, whose moderate vacuolating activity is markedly potentiated by the toxin. It is concluded that the toxin accumulated within the endosomal compartment alters the morphology and function of this organelle and plays a permissive role towards cell vacuolation, possibly by increasing the accumulation of protonated ammonia within endosomes. In turn, ammonia induces excessive dilatation of the endosomes with reciprocal fusion of their membranes, thus causing cytoplasmic vacuolation. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Helicobacter pylori is a Gram-negative, curved or spiral bacterium which colonizes the gastric epithelium of approximately 50 per cent of the world's population.[1,2] *H. pylori* is now recognized as the major causative agent of chronic gastritis and of peptic ulcer disease.[3–5] Mounting evidence suggests that infection with *H. pylori* increases the risk of gastric cancer,[6,7] and *H. pylori* has recently been designated as a class I carcinogen by the World Health Organization.[8]

The mechanisms whereby *H. pylori* exerts its pathogenetic action are not yet well understood. Biopsies of *H. pylori*-colonized human gastric epithelium exhibit cellular swelling, cytoplasmic vacuolation, and expansion of endosomal compartments.[9] *H. pylori* bacterial extracts induce cytoplasmic vacuolar degeneration in epithelial cells in culture.[10–15] Two main *H. pylori* virulence factors are claimed to be involved in this cell damage: the so-called 'vacuolating toxin' and urease. Vacuolating toxin (VacA), encoded by the *vacA* gene, is produced by 50–60 per cent of *H. pylori* strains and migrates as an 87 kD protein under denaturing conditions.[15,16] Urease, which is common to all *H. pylori* clinical isolates, catalyses the hydrolysis of urea to carbon dioxide and ammonia; ammonia is known to cause cell vacuolation.[15,16]

The specific roles of toxin and ammonia in the generation of cell vacuolation remain largely unclear. In a previous study, our ultrastructural findings suggested that toxin- and ammonia-dependent cell vacuolation shared an endosomal origin.[14] Using a panel of markers for varying intracellular compartments, Papini *et al.*[15] confirmed our data and demonstrated that *H. pylori*-induced vacuoles originate from late endosomes. The mechanism by which ammonia and other weak bases cause vacuolation is well known:[17–19] such bases cross cell membranes in an uncharged state and are trapped by protonation within intracellular acidic compartments (endosomes, lysosomes, trans-Golgi vesicles), where they induce osmotic swelling. The mechanism of toxin action is not yet clear. It has been proposed that toxin may alter the functionality of cell compartments by acting on the function of ion-motive ATPases[13,20–22] and/or on intracellular membrane traffic.[15,16] Recently, toxin has been found to bind to the plasma membrane of HeLa cells *in vitro* and to be internalized into an unknown compartment.[23]

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The present study used a combined biochemical and electron microscopic approach to investigate the respective roles of, and the reciprocal interaction between, toxin and ammonia in the *H. pylori*-induced vacuolation of human gastric cells in culture and to identify the intracellular sites of internalized toxin.

MATERIALS AND METHODS

Bacterial strains and filtrate production

Two *H. pylori* strains were used (1) the toxin-producing CCUG 17874 strain (from Culture Collection University of Göteborg, Göteborg, Sweden) and (2) the toxin-negative G21 strain (a kind gift from N. Figura, Siena, Italy).[24] Bacteria were grown in *Brucella* broth, supplemented with 5 per cent fetal calf serum (FCS) (Gibco, Grand Island, NY, U.S.A.), for 24–36 h at 37°C in a thermostatic shaker under micro-aerophilic 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, U.S.A.) to obtain the broth culture filtrates (BCFs).[25,26] Uninoculated broth filtrate served as a control. To remove the ammonia content, control and BCFs were dialysed against Hanks's balanced salt solution (HBSS) for 36 h in dialysis tubing with a 12 kD molecular mass cut-off (Sigma, St Louis, MO, U.S.A.). The presence and the absence of the vacuolating toxin in the respective BCFs from CCUG 17874 and G21 *H. pylori* strains (respectively referred to as Tox^+ BCF and Tox^- BCF) were assessed by means of sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-toxin serum (αpKH C3 polyclonal rabbit antiserum).[20,27] The αpKH C3 antiserum (kindly given by J. L. Telford, IRIS, Siena, Italy) was raised against the carboxy-terminal portion of the vacuolating toxin expressed as a recombinant fragment in *Escherichia coli*.[27] To inactivate the toxin (which is known to be heat-labile[28]), we incubated aliquots of Tox^+ BCF in boiling water for 15 min.[22] The toxin was also selectively inactivated through the incubation of aliquots of Tox^+ BCF with an anti-toxin serum that neutralizes the toxin activity,[20] at a dilution of 1:20. The neutralizing anti-toxin serum (kindly given by T. L. Cover and M. J. Blaser, Nashville, TN, U.S.A.), was supplied from New Zealand White rabbits which were immunized with the chromatographically purified toxin of *H. pylori*.[20]

Gastric epithelial cells

We used the MKN 28 cell line, which derives from a human gastric tubular adenocarcinoma and shows moderate gastric-type differentiation.[29–31] The MKN 28 cells were grown as monolayers in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12, supplemented with 10 per cent FCS and 1 per cent antibiotic–antimycotic solution (both from Gibco) in 35 mm plastic Petri dishes (Corning Glass Works, Corning, NY, U.S.A.) at 37°C in a humidified atmosphere of 5 per cent CO_2 in air.

Cell incubation

Subconfluent cell cultures were washed twice with HBSS and then incubated at 37°C in a humidified atmosphere of 5 per cent CO_2 in air for 16 h with (1) several dilutions (in HBSS) of either control or *H. pylori* BCFs in the absence of ammonia and (2) defined concentrations of ammonium chloride (dissolved in HBSS), both alone and added to either control or *H. pylori* BCFs (both diluted 1:3 in HBSS). In other experiments, after an initial 16 h incubation with one or other of the control, Tox^+ BCF (supplemented or not with 4 mM ammonium chloride), and 4 mM ammonium chloride alone (step 1), cells were incubated for 5 h with HBSS (step 2) and then for 16 h with one or other of the control, 4 mM ammonium chloride alone, and Tox^+ BCF (without addition of ammonium chloride) (step 3). After each step, the incubation medium was completely removed and the cells were extensively washed before the addition of new reagents. After each step, the degree of cell vacuolation was assayed for each condition. In contrast with the practice of previous investigations,[10,11,13,15,20,21,32] we did not add FCS to the cell incubation medium, so as to avoid any interference by ammonia present and/or produced (from urea by *H. pylori* urease activity) in a medium supplemented with FCS.

Neutral red dye uptake assay

At the end of incubation, the degree of cell vacuolation was assayed by means of neutral red dye uptake in accordance with Cover *et al.*,[10] and was expressed as μg of neutral red per μg cell protein.[14] The protein content of cell monolayers was measured in accordance with Lowry *et al.*[33] Neutral red is an acidotropic, membrane-permeant amine that accumulates in the vacuolar lumen.[10,18] Neutral red uptake is a widely accepted *in vitro* assay for *H. pylori*-induced cell vacuolation.[10–15]

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 per cent glutaraldehyde and 2 per cent 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, post-fixed in 1 per cent osmium tetroxide, and then embedded in Epon-Araldite mixture.[14] Uranyl–lead-stained ultrathin sections were viewed with a Zeiss EM 902 electron microscope (Oberkochen, Germany).

For the ultrastructural immunolocalization of *H. pylori* vacuolating toxin, we used the colloidal gold technique. Briefly, ultrathin sections collected on 300-mesh nickel grids were pretreated with a saturated water solution of sodium metaperiodate for 10 min, washed with buffer A [0·45 M NaCl, 1 per cent 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 non-specific binding of immunoglobulins. The sections were then incubated at 4°C overnight with

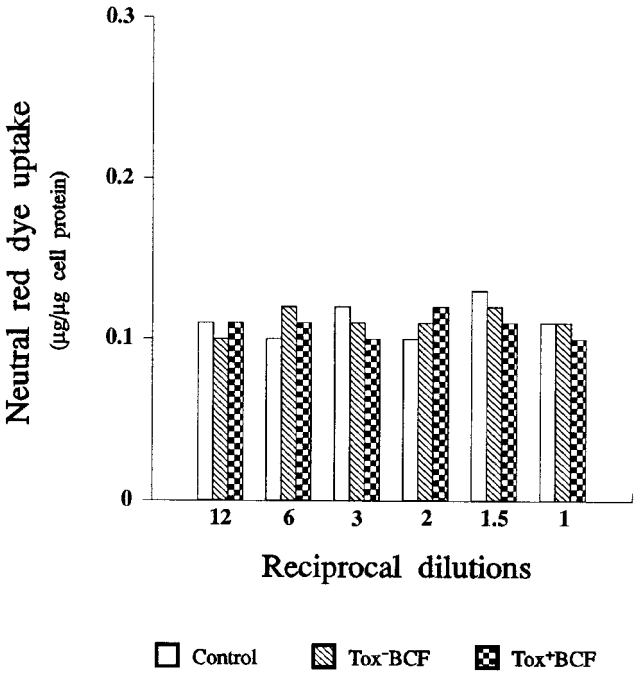

Fig. 1—Neutral red dye uptake induced in MKN 28 cells by several dilutions of *H. pylori* broth culture filtrates in the absence of ammonia. Cells were incubated with (1) uninoculated broth filtrate (control), (2) broth culture filtrate from the toxin-producing CCUG 17874 strain (Tox^+ BCF), and (3) broth culture filtrate from the toxin-negative G21 strain (Tox^- BCF). All the displayed data are the means of five experiments and each SEM was less than 10 per cent of the respective mean. No statistically significant differences were found

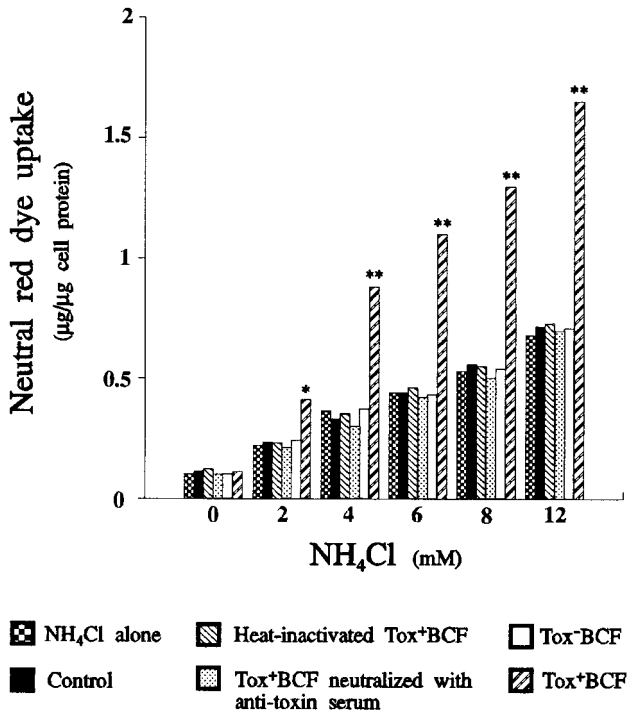

Fig. 2—Neutral red dye uptake induced in MKN 28 cells by defined concentrations of ammonium chloride, alone or added to (1) uninoculated broth filtrate (control), (2) broth culture filtrate from the toxin-producing CCUG 17874 strain (Tox^+ BCF), (3) heat-inactivated Tox^+ BCF, (4) Tox^+ BCF neutralized with anti-toxin serum, and (5) broth culture filtrate from the toxin-negative G21 strain (Tox^- BCF). Control and BCFs were diluted 1:3 in HBSS. All the displayed data are the means of five experiments and each SEM was less than 10 per cent of the respective mean. $*P<0{\cdot}05$ and $**P<0{\cdot}001$ versus all other conditions

αpKH C3 polyclonal rabbit antiserum diluted 1:600 in buffer B [0·45 M NaCl, 1 per cent bovine serum albumin, 0·5 per cent sodium azide, 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, U.K.) diluted 1:20 in buffer B. The sections were stained with uranyl and lead before electron microscopy. The evaluation of gold labelling and of endosomal areas was performed by means of an IBAS 2 image analyser (Zeiss) and expressed as mean ± SEM ($n=20$) labelling, both per total endosomal area of the cell and per μm^2 of endosomal area.

Statistics

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.[34]

RESULTS

Neutral red dye uptake assay

No increase in neutral red uptake by MKN 28 cells was found when cells were incubated in ammonia-free medium supplemented with either Tox^+ or Tox^- BCF (Fig. 1). We observed that ammonium chloride alone increased neutral red uptake in a dose-dependent manner (Fig. 2). Both when ammonium chloride was added to the uninoculated broth filtrate (control) and when it was added to the Tox^- BCF, the degree of neutral red uptake was virtually identical to that of ammonium chloride alone. When ammonium chloride was added to Tox^+ BCF, we observed a dose-dependent neutral red uptake which was about 2·5-fold higher than that induced by ammonium chloride alone. When the toxin was inactivated by heating or by neutralization with anti-toxin serum, the amount of neutral red uptake was virtually identical to the amounts found for ammonium chloride alone and for Tox^- BCF (Fig. 2). To investigate further the respective roles of VacA toxin and ammonia, we compared the neutral red uptake of MKN 28 cells as variously exposed: initially, to one or other of Tox^+ BCF alone, ammonium chloride alone, and Tox^+ BCF plus ammonium chloride; finally, after extensive washing and 5 h incubation with HBSS, to one or other of ammonium chloride alone and Tox^+ BCF alone. As shown in Fig. 3, treatment of cells with ammonium chloride after exposure to the toxin induced a neutral red uptake that was identical to that of cells treated with the two agents simultaneously. In contrast, treatment with Tox^+ BCF alone after initial exposure to ammonium chloride failed to induce significant neutral red uptake (Fig. 3). Interestingly, the massive neutral red uptake induced by Tox^+ BCF plus ammonium chloride at the end of step 1 was completely reversed by subsequent 5 h cell incubation with HBSS (Fig. 3).

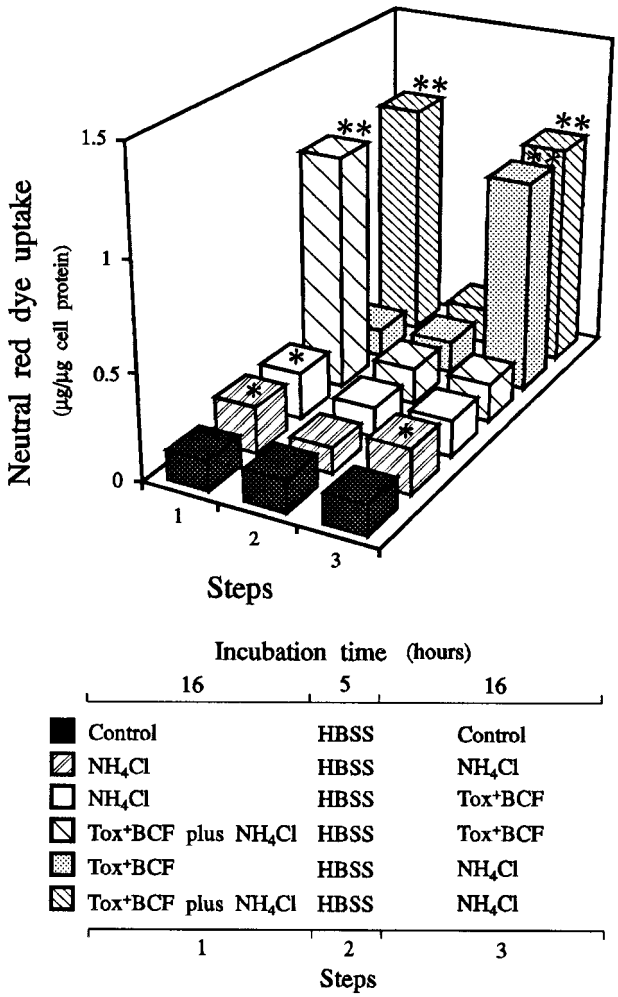

Fig. 3—Neutral red dye uptake by MKN 28 cells incubated (step 1) with one or other of uninoculated broth filtrate (control), broth culture filtrate from the toxin-producing CCUC 17874 strain (Tox^+ BCF) alone, 4 mM ammonium chloride alone, and Tox^+ BCF plus 4 mM ammonium chloride; each followed by incubation (step 2) with HBSS; and then (step 3) with one or other of uninoculated broth filtrate (control), 4 mM ammonium chloride alone, and Tox^+ BCF alone. Neutral red dye uptake was evaluated at the end of each incubation step. All the displayed data are the means of five experiments and each SEM was less than 10 per cent of the respective mean. $*P<0{\cdot}05$ and $**P<0{\cdot}001$ versus the control at the same step

Nevertheless, further incubation (step 3) with ammonium chloride alone of cells treated in step 1 with Tox^+ BCF plus ammonium chloride induced massive neutral red uptake, similar to that produced at the end of step 1 (Fig. 3).

Electron microscopy

Conventional electron microscopy of MKN 28 cells incubated for 16 h in uninoculated, ammonia-free broth filtrate showed an absolute prevalence of normal vacuole-free cells with very few small, membrane-bound, clear vesicles or tubules scattered in their cytoplasm, while cells with dilated tubulovesicles were only an occasional finding (Fig. 4a). Addition of Tox^+ BCF to the ammonia-free medium caused a marked increase in more or less dilated vesicles, which were mostly scattered in the cytoplasm or, less frequently, concentrated in the perinuclear area, and showed the morphology of endocytic-endosomal tubulovesicles (Fig. 4b). No consistent evidence of endosomal fusion was found. Simultaneous incubation of MKN 28 cells with both Tox^+ BCF and 4 mM ammonium chloride gave rise extensively to large vacuoles, probably as a result of fusion and the subsequent rupture of dilated vacuoles (Figs 4c and 4d). Parallel immunogold tests with *a*pKH C3 serum showed internalization of VacA toxin from Tox^+ BCF into the accumulated tubulovesicles, as well as into the large vacuoles (Figs 5a and 5b). No significant difference in immunolabelling was found for toxin-incubated cells, irrespective of the addition of ammonium chloride. The total number of gold particles deposited over the whole endosomal-vacuolar area of the cell (mean ± SEM of 20 cells) was $18{\cdot}25 \pm 2{\cdot}14$ without and $14{\cdot}21 \pm 1{\cdot}71$ with ammonia ($P = 0{\cdot}151$). When the mean labelling per μm^2 of endosomal-vacuolar area was evaluated we found $3{\cdot}36 \pm 0{\cdot}32$ gold particles per μm^2 in the absence and $1{\cdot}48 \pm 0{\cdot}13$ in the presence of ammonia ($P<0{\cdot}001$). The reduction in toxin concentration caused by ammonia was counterbalanced by a two-fold expansion in the endosomal-vacuolar area ($10{\cdot}71 \pm 1{\cdot}49\ \mu m^2$ per cell with ammonia as opposed to $5{\cdot}48 \pm 0{\cdot}54\ \mu m^2$ per cell without ammonia, $P<0{\cdot}01$). In addition, ultrastructural findings showed partial polarization of MKN 28 cells, with luminal-type differentiation (microvilli, cell web) of the free surface and basolateral-type differentiation (phylopodes) of the remaining surface, which was partly attached to the plastic Petri dish. Interestingly, toxin immunolabelling was also found in a few tubulovesicles located immediately below the luminal-type surface, which showed sparse plasma membrane invaginations.

DISCUSSION

Our data indicate that, in the absence of ammonia, both Tox^+ and Tox^- BCF fail to induce a significant increase in neutral red uptake by cultured MKN 28 cells. Ammonium chloride alone, at concentrations similar to those present in the gastric juice,[35,36] increased neutral red uptake in a dose-dependent manner. The simultaneous presence of ammonium chloride and Tox^+ BCF produced about 2·5 times the neutral red uptake induced by ammonium chloride alone. This potentiating effect of Tox^+ BCF on ammonium chloride-induced cell vacuolation was completely reversed by toxin inactivation as achieved both by heating and, more selectively, by neutralization with anti-toxin serum. Taken together, these results would suggest that whereas toxin does not induce neutral red-positive cell vacuolation by itself, it does increase the vacuolating activity of ammonia, an interpretation at variance with the hitherto generally held concept that ammonia potentiates the vacuolating activity of toxin.[10,11] Another important observation is that previous incubation with toxin enhanced the neutral red uptake caused by subsequent ammonia treatment, whereas no synergistic effect was obtained when ammonia preceded treatment with the toxin. This suggests that the damage caused by toxin, unlike that

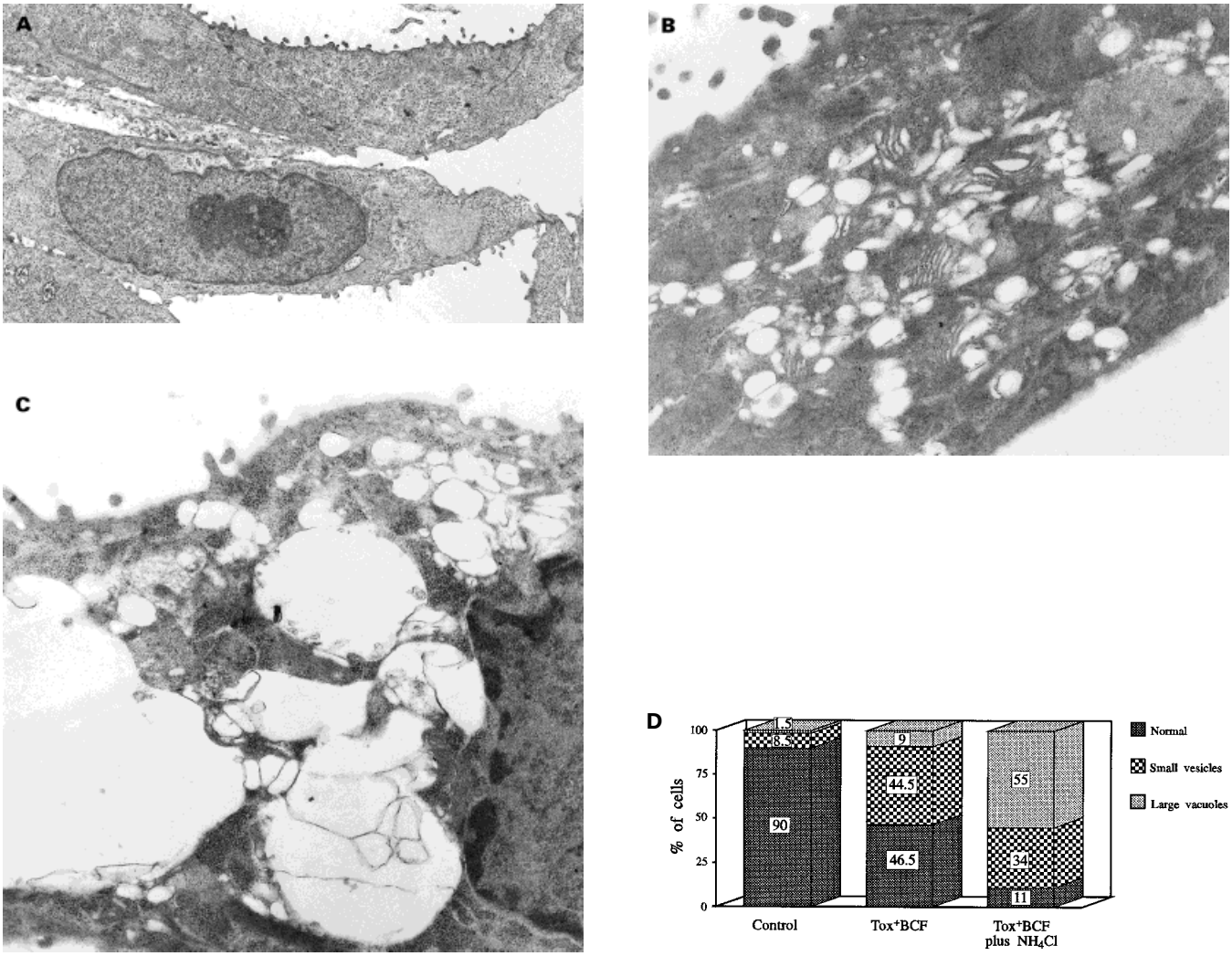

Fig. 4—Ultrastructural features of MKN 28 cells incubated for 16 h with uninoculated broth filtrate (a: × 3600), Tox$^+$ BCF (b: × 14400), and Tox$^+$ BCF plus 4 mM ammonium chloride (c: × 14400). (a) Normal, vacuole-free cells predominate in a, in contrast with the abundance of small dilated tubulovesicles in b (note also the moderate dilation of a few Golgi cisternae) and with the extensive development of large vacuoles in c. Large vacuoles seem to result from rupture and fusion of dilated vesicles. The mean percentages of normal vacuole-free, small vesicle-rich, and large vacuole-rich cells found in each experimental condition are shown in the graph (d). The data derive from the evaluation of 200 cells for each experimental condition

caused by ammonia, is permanent and predisposes to vacuolation.

By means of conventional electron microscopy, we tried to clarify the genesis of *H. pylori*-induced vacuoles and the interaction between toxin and ammonia in this phenomenon. Ultrastructural data confirmed that *H. pylori* toxin gives rise extensively to large vacuoles only when ammonium chloride is present in the incubation medium. However, incubation with Tox$^+$ BCF in the absence of ammonia affected the endocytic-endosomal compartment of the cell by causing a substantial increase in, and dilatation of, tubulovesicles, which are poorly represented in normal MKN 28 gastric cells. Together with neutral red uptake tests, our ultrastructural findings show that (1) toxin-dependent expansion and dilatation of tubules and vesicles of the endocytic-endosomal compartment may precede large vacuole formation, (2) ammonia- or ammonia plus toxin-promoted coalescence and fusion with subsequent rupture of the membranes of accumulating tubulovesicles is an important process in vacuole formation, and (3) only vacuoles seem to concentrate neutral red significantly, while tubulovesicles seem to be relatively inactive.

Telford *et al.*[16] proposed that *H. pylori* toxin interferes with the processes that control the structure of late endosomes and/or their cycling to lysosomes and the trans-Golgi network. In such a model, late endosomes would presumably accumulate, fuse with each other, and then swell to form large vacuoles when weak bases are present. By conventional electron microscopy, we investigated whether MKN 28 cells treated with Tox$^+$ BCF in the absence of ammonia exhibited such ultrastructural features as would suggest endosomal fusion. While we found an expansion of the endocytic-endosomal compartment, we obtained no substantial evidence of endosomal fusion. When ammonium chloride and Tox$^+$ BCF were simultaneously added to the medium, most MKN 28 cells showed heavy cytoplasmic vacuolation, with

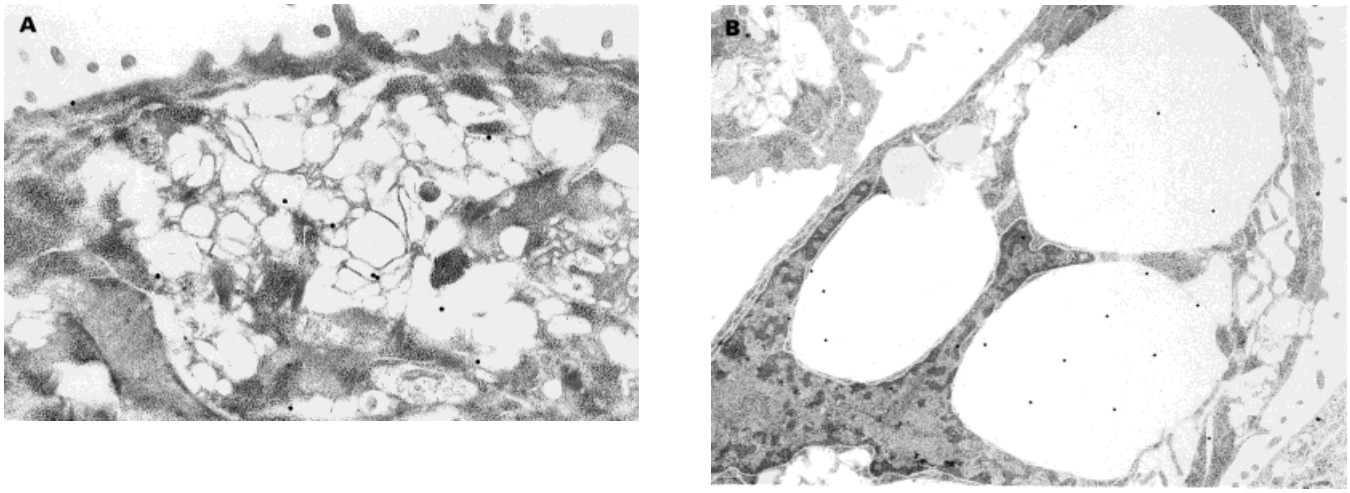

Fig. 5—Ultrastructural immunolocalization of *H. pylori* vacuolating toxin in MKN 28 cells by means of the colloidal gold technique. Cells were incubated for 16 h with broth culture filtrate from the toxin-producing CCUG 17874 strain either in the absence (a: × 14400) or in the presence (b: × 8400) of 4 mM ammonium chloride

plenty of large vacuoles exhibiting features that suggest membrane adhesion and fusion. Our ultrastructural data thus confirm the results of neutral red uptake tests, in that most toxin-promoted cell vacuolation requires ammonia and that the toxin, although it causes an increase in the amount and dilatation of endosomes, does not of itself cause the extensive fusion and rupture of endosomal membranes that is required for large vacuole formation.

By means of ultrastructural immunocytochemistry, we provide the first direct evidence that *H. pylori* VacA toxin localizes in the endocytic-endosomal compartment from which vacuoles originate. In keeping with previous findings by Garner and Cover,[23] we observed that the amount of toxin internalization appeared to be insensitive to the presence or absence of ammonia in the incubation medium. However, the presence of ammonia probably caused an increase in water uptake during vacuole formation, with consequent dilution of toxin in larger vacuoles. This might explain why the toxin concentration found in larger vacuoles, under light[23] as well as electron (this study) microscopy, was low. In any case, our finding of VacA toxin in both endosomal tubulovesicles and vacuoles further supports the hypothesis[9,14,15,37] that the origin of vacuoles is endosomal. Indeed, the observation of toxin immunolabelling in some tubulovesicles located immediately below the luminal-type surface of MKN 28 cells, which also showed plasma membrane invaginations, suggests that VacA is internalized by endocytosis. This hypothesis is in keeping with the fact that both the fluid-phase marker lucifer yellow[15] and toxin (this study) concentrate selectively in endosomal vacuoles. Interestingly, endocytic-endosomal vesicles, with or without accompanying vacuoles, have been observed in the juxtalumenal cytoplasm of *H. pylori*-colonized gastric foveolar-surface epithelium,[9,38] with the implication that the basic mechanisms described here *in vitro* also operate *in vivo* on human gastric mucosa.

As to the mechanism of VacA action, it seems possible that when internalized into the endosomal compartment, the toxin persistently alters the vacuolar-type ATPase activity of late endosomes (possibly by altering cell membrane trafficking at this level)[37] and induces an increased production of H^+ in response to ammonia entry into the endosomal lumen. Ammonia, whether delivered simultaneously or subsequently to the cell, is substantially protonated; the consequent accumulation is accompanied by osmotic swelling of late endosomes. In contrast, only a scanty accumulation of protonated ammonia will occur in the lumen of endosomes not previously or simultaneously exposed to the toxin.

In conclusion, the present study shows that *H. pylori* vacuolating toxin causes an expansion of the endosomal compartment, where it accumulates after cellular internalization. The toxin does not form large, neutral red-positive vacuoles unless combined with ammonia. On the other hand, a persisting change in the endosomal compartment caused by the toxin is required for simultaneous or subsequent development of prominent cell vacuolation by incubation with ammonia or other weak bases.

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