INFECTION AND IMMUNITY, Apr. 1993, p. 1427–1431
0019-9567/93/041427-05$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 61, No. 4

Effects of ATPase Inhibitors on the Response of HeLa Cells to *Helicobacter pylori* Vacuolating Toxin

TIMOTHY L. COVER,[1,2]* LAKSHMI Y. REDDY,[2] AND MARTIN J. BLASER[1,2,3]

Infectious Disease Section, Department of Veterans Affairs Medical Center,[1] and Departments of Medicine[2] and Microbiology and Immunology,[3] Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received 5 November 1992/Accepted 14 January 1993

Approximately 50% of *Helicobacter pylori* strains produce a toxin in vitro that induces vacuolation of eukaryotic cells. To determine whether ion transport pathways are important in the formation of toxin-induced vacuoles, HeLa cells were incubated with *H. pylori* toxin in the presence of nine different inhibitors of ion-transporting ATPases. Oligomycin, an inhibitor of predominantly F_1F_0-type ATPases, had no effect on toxin activity. Inhibitors of predominantly V-type ATPases, exemplified by bafilomycin A1, inhibited the formation of vacuoles in response to the *H. pylori* toxin and reversed the vacuolation induced by the toxin. In contrast, at concentrations of ≥100 nM, ouabain and digoxin, inhibitors of the Na^+-K^+ ATPase, potentiated the activity of *H. pylori* toxin. The inhibitory effects of bafilomycin A1 could not be overcome by the potentiating effects of ouabain. These data suggest that intact activity of the vacuolar ATPase of eukaryotic cells is a critical requirement in the pathogenesis of cell vacuolation induced by *H. pylori* toxin and that vacuole formation by this toxin is associated with altered cation transport within eukaryotic cells.

Helicobacter pylori infection is the principal cause of chronic gastritis in humans, and infection with this agent is a significant risk factor for peptic ulceration and gastric cancer (7, 22, 25). The pathophysiologic mechanisms by which *H. pylori* causes human disease are not yet well understood, but a vacuolating toxin produced by the organism may be an important virulence factor. This toxin is produced in vitro by approximately 50% of *H. pylori* isolates (10, 13, 18). The presence of neutralizing antibodies to the toxin in sera from infected persons indicates that the toxin also is produced in vivo (9). The vacuolating toxin recently has been purified, and migrates as an 87-kDa protein under denaturing conditions (8).

The mechanisms by which the *H. pylori* toxin induces vacuolation of eukaryotic cells are not yet known. Analysis of the N-terminal amino acid sequence of the purified toxin indicates partial homology with a variety of ion transport proteins (8), and therefore, we have speculated that the toxin may induce vacuole formation by initiating aberrant transport of ions within eukaryotic cells. The maintenance of transmembrane ion gradients within eukaryotic cells is mediated to a large extent by ion-transporting ATPases, including the Na^+-K^+ ATPase within the plasma membrane (27) and the vacuolar ATPase within acidic intracellular organelles (2, 21). Thus, we hypothesized that inhibitors of ion-transporting ATPases might alter the interaction between *H. pylori* toxin and eukaryotic cells. This study was conducted to determine the effects of ATPase inhibitors on *H. pylori* toxin-induced vacuolation of eukaryotic cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* 60190, a well-characterized strain that produces the vacuolating toxin, was cultured for 48 h at 37°C in brucella broth supplemented with 5% fetal bovine serum in a 5% CO_2 atmosphere (10). After centrifugation of the culture, the supernatant was concentrated 30-fold by ultrafiltration and passaged through a 0.2-μm-pore-size filter (10). Supernatants were stored at −70°C prior to testing in tissue culture assays. Purified toxin was prepared from *H. pylori* 60190 as previously described (8), except that gel filtration chromatography was performed with a Superose 6 HR 10/50 column instead of a Superose 12 HR 10/50 column.

Chemicals and enzymes. *N*-Ethylmaleimide (NEM), 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD chloride), *N*,*N*′-dicyclohexylcarbodiimide pentachlorophenol complex (DCCD), sodium nitrate, ouabain, digoxin, sodium orthovanadate, and oligomycin were from Sigma Chemical Co., St. Louis, Mo. Bafilomycin A1 was generously provided by Gary Dean, University of Cincinnati, Cincinnati, Ohio. Omeprazole was obtained from Astra Pharmaceuticals, Goteborg, Sweden.

Assessment of vacuolating toxin activity. HeLa cells were cultured in Eagle's modified minimal essential medium with Earle's salts containing 10% fetal bovine serum and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.2) in a 5% CO_2 atmosphere, as described previously (10). In experiments involving purified *H. pylori* toxin, the medium was supplemented with 10 mM ammonium chloride to potentiate activity (11). After preincubation of cells with ATPase inhibitors for 1 h, concentrated culture supernatant or purified toxin from *H. pylori* 60190 was added and cells were incubated for an additional 18 h at 37°C. Vacuolation was assessed visually by inverted light microscopy (×200 magnification) or quantitated by a neutral red uptake assay (10). In the microscopic assay, inhibition of *H. pylori* vacuolating toxin activity was defined by the stringent criterion of visible vacuoles in <10% of cells.

RESULTS

Inhibition of vacuolation induced by *H. pylori* toxin. To determine whether inhibitors of ATPase activity altered the effects of *H. pylori* toxin on eukaryotic cells, we tested nine agents. Since the *H. pylori* toxin induced vacuolation of

* Corresponding author.

TABLE 1. Inhibition of *H. pylori* toxin-induced vacuolation by ATPase inhibitors

Inhibitor	MIC for *H. pylori* toxin[a]	Predominant class of ATPase inhibited	IC_{50} for eukaryotic vacuolar ATPase[b] (reference)
Bafilomycin	25 nM	V type	2–10 nM (20, 28, 34)
N-Ethylmaleimide (NEM)	25 μM	V type	1–13 μM (12, 14, 16, 30)
NBD-Cl[c]	50 μM	V type	2.5 μM (12)
DCCD[d]	25 μM	V type, F_1F_0	5–20 μM (12, 14, 30)
Sodium nitrate	100 mM	V type	30–100 mM (29, 30)
Ouabain	>100 μM	P type	>2,000 μM (30)
Vanadate	>100 μM	P type	>100 μM (12, 30)
Omeprazole	200 μM	Gastric	≥100 μM (20)
Oligomycin	>50 μM	F_1F_0	50 μM (30)

[a] The minimum concentration required to inhibit vacuole formation in >90% of HeLa cells incubated with a 1:10 dilution of concentrated supernatant from *H. pylori* 60190; result shown is median of three experiments.
[b] The concentration of agent required for half-maximal inhibition of vacuolar proton transport in cell-free systems.
[c] 7-Chloro-4-nitrobenz-2-oxa-1,3-diazole.
[d] *N,N'*-dicyclohexylcarbodiimide.

cells, we first examined agents that inhibit vacuolar ATPase activity. Bafilomycin, NEM, NBD-Cl, DCCD, and sodium nitrate each inhibited the formation of vacuoles in response to the *H. pylori* vacuolating toxin (Table 1). At the concentrations that inhibited *H. pylori* toxin-induced vacuolation, each of these agents is known to inhibit the vacuolar ATPase-mediated acidification of intracellular organelles (3, 12, 14, 16, 20, 28, 29, 30, 34). Of the agents tested, bafilomycin was the most potent inhibitor of toxin-induced vacuolation. In contrast, in concentrations known to inhibit the Na^+-K^+ ATPase of eukaryotic cells (27, 32), ouabain and vanadate did not inhibit *H. pylori* toxin activity (Table 1). Omeprazole, a potent inhibitor of gastric parietal cell H^+-K^+ ATPase under acidic conditions (20), also had no effect except at high (200 μM) concentrations. Similarly, oligomycin did not inhibit *H. pylori* toxin activity when tested in a concentration known to inhibit F_1F_0-type (mitochondrial) ATPase (31). Thus, inhibitors of the eukaryotic vacuolar ATPase inhibited the vacuolation of HeLa cells induced by the *H. pylori* toxin, whereas agents that primarily inhibited other classes of ATPases had little or no effect.

Effects of bafilomycin on *H. pylori* toxin-induced vacuolation. Bafilomycin is known to be a relatively specific, potent inhibitor of vacuolar ATPases (4, 28, 34), and therefore, the inhibitory effects of bafilomycin were characterized further in the next series of studies. First, since the crude concentrated supernatant from *H. pylori* 60190 contains an incompletely defined mixture of components, the effect of bafilomycin on vacuolation induced by the purified *H. pylori* toxin was tested. Bafilomycin (25 nM) inhibited the activity of the purified *H. pylori* vacuolating toxin in the same manner as that observed when crude supernatant was tested (Fig. 1). Bafilomycin also inhibited neutral red uptake by cells incubated with *H. pylori* toxin (Fig. 2); this phenomenon was attributed both to the inhibition of vacuole formation in these cells and to the known inhibitory effect of bafilomycin upon the uptake of weak bases related to neutral red into acidic cell compartments (28, 34).

Reversibility of toxin-induced vacuolation. To determine whether toxin-induced vacuolation was reversible by the addition of bafilomycin, we induced cell vacuolation by incubating HeLa cells with concentrated *H. pylori* culture supernatant for 24 h; bafilomycin (final concentration, 25 nM) or medium alone then was added to these wells. After 18 h of further incubation, vacuolation was visualized in 3.0% ± 1.4% of cells to which bafilomycin was added, whereas 94.8% ± 1.8% of control cells remained vacuolated ($P < 0.0001$). Thus, bafilomycin not only inhibited the vacuolation induced by *H. pylori* toxin, but also reversed the effects of the toxin.

Potentiation of *H. pylori* vacuolating toxin activity by ouabain. During the foregoing experiments, it was noted that high concentrations (100 μM) of ouabain did not inhibit *H. pylori* vacuolating toxin activity but that low concentrations of ouabain potentiated the vacuolation induced by the toxin. To quantitate and characterize further this phenomenon, cells were incubated for 18 h with concentrations of *H. pylori* vacuolating toxin below that required to induce vacuolation, in the presence of various concentrations of ouabain (Table 2). Significantly greater cell vacuolation occurred in the presence of ouabain (≥100 nM) than when ouabain was absent ($P \leq 0.0002$), an observation that was confirmed by the neutral red assay ($P < 0.05$ for ≥10 nM ouabain versus 0 nM ouabain). Digoxin, a closely related compound, also potentiated toxin activity (Table 2). When tested independently, neither ouabain nor digoxin induced visible vacuolation of HeLa cells or increased neutral red uptake by the cells. In contrast to ouabain and digoxin, vanadate, another inhibitor of Na^+-K^+ ATPase, failed to potentiate *H. pylori* toxin activity. The failure of vanadate to potentiate toxin activity was attributed to its inability to inhibit Na^+-K^+ ATPase activity from the extracellular side of the cytoplasmic membrane (5).

Effect of the combination of bafilomycin and ouabain on *H. pylori* toxin-induced vacuolation. To determine whether inhibition by bafilomycin or potentiation by ouabain is the dominant effect on toxin-induced vacuolation, we incubated HeLa cells with *H. pylori* toxin in the presence of 1 μM ouabain plus 25 nM bafilomycin. *H. pylori* toxin activity was completely inhibited by this combination, despite the use of ouabain in a concentration 10-fold higher than that required for potentiation (Table 3). Thus, inhibition of toxin-induced vacuolation by bafilomycin could not be overcome by the potentiating effect of ouabain.

DISCUSSION

These studies indicate that inhibitors of the eukaryotic vacuolar ATPase block the cell vacuolation induced by *H. pylori* toxin. Bafilomycin A1, the most specific known inhibitor of the vacuolar ATPase (4), was the most potent inhibitor of toxin-induced vacuolation. The concentrations of bafilomycin that inhibited toxin-induced vacuolation were similar to the concentrations required to inhibit vacuolar acidification in intact cells (28, 34). Although the inhibition of *H. pylori* toxin-induced vacuolation by bafilomycin and other V-type ATPase inhibitors may be explained entirely by the inhibitory effects of these agents on the vacuolar ATPases in eukaryotic cells, at present we are unable to exclude the possibility that these agents also may interact directly with the *H. pylori* toxin.

The inhibition of toxin activity by vacuolar ATPase inhibitors yields insight into the pathogenesis of *H. pylori* toxin-induced cell vacuolation. From these data, we can conclude that acidification of intracellular organelles by vacuolar

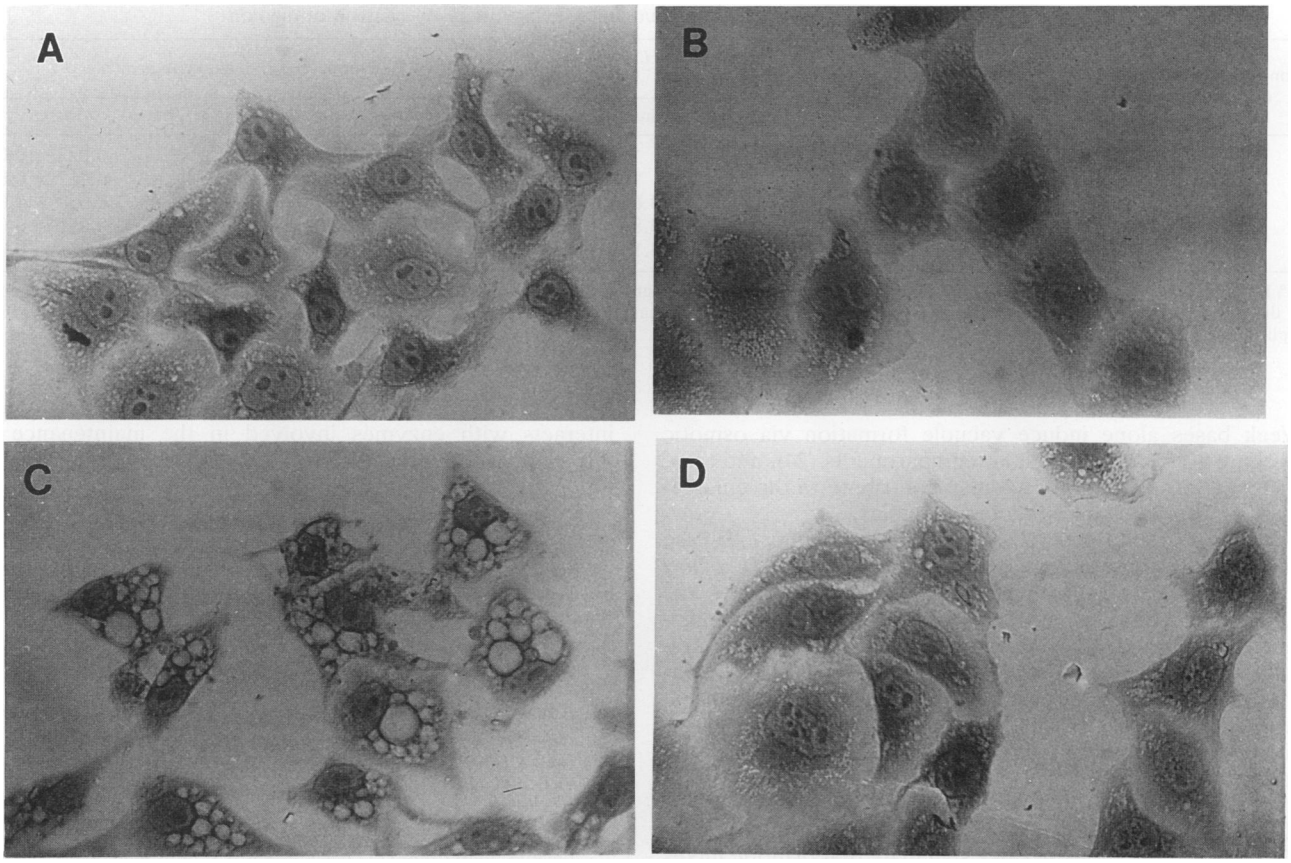

FIG. 1. Inhibition of *H. pylori* toxin activity by bafilomycin A1. HeLa cells were incubated for 18 h at 37°C with medium containing no additives (A), 25 nM bafilomycin (B), purified *H. pylori* toxin (1 μg/ml) (C); or purified toxin (1 μg/ml) plus 25 nM bafilomycin (D). In each case, medium was supplemented with 10 mM ammonium chloride. Cells were stained for 20 min with crystal violet (0.1%) in methanol. At the concentrations shown, bafilomycin completely inhibited the vacuolating activity of *H. pylori* toxin (original magnification, ×200).

ATPases is essential for the formation of vacuoles in response to the toxin. One hypothesis to explain this observation is that intracellular acidification may be required for intracellular processing of the toxin. For example, processing of diphtheria toxin in acidic pH is believed necessary prior to the entry of this toxin into the cytoplasm (28). However, the reversal of *H. pylori* toxin-induced vacuolation by the addition of bafilomycin suggests instead that proton transport is essential for the maintenance of the vacuolar structure.

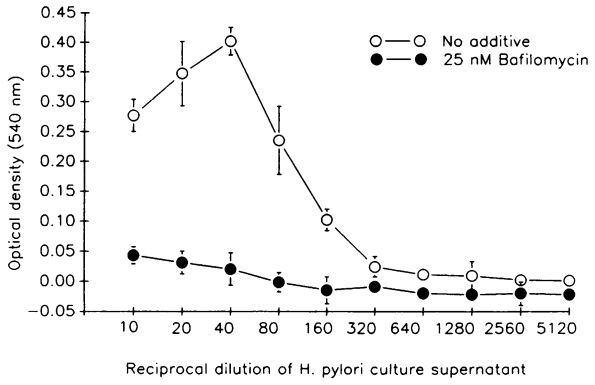

FIG. 2. Effect of bafilomycin on *H. pylori* toxin-induced neutral red uptake. HeLa cells were incubated for 18 h at 37°C with serial dilutions of concentrated supernatant from *H. pylori* 60190, with or without added bafilomycin (25 nM). Neutral red uptake was quantitated as previously described (10); diminished neutral red uptake with the highest concentrations of toxin was due to cell detachment (10). Bafilomycin inhibited the neutral red uptake induced by *H. pylori* toxin.

By inhibiting vacuolar ATPase activity, agents such as bafilomycin induce an increase in intralysosomal pH (28, 34). Similarly, weak bases are known to increase intralysosomal pH by accumulation within acidic cell compartments (23). Bafilomycin and weak bases have been shown previously to modulate the activity of various toxins in an identical manner; for example, diphtheria toxin activity is inhibited by both bafilomycin and weak bases (28), and ricin activity is potentiated by each of these agents (33). Therefore, it is notable that bafilomycin inhibits *H. pylori* vacuolating toxin activity, whereas weak bases potentiate the activity of this toxin (11). There are several hypotheses that could explain these observations. (i) Bafilomycin inactivates the vacuolar ATPase, whereas weak bases neutralize or partially neutralize the acidifying effects of this enzyme; inhibition of *H. pylori* toxin-induced vacuolation by only the former agent suggests that functional vacuolar ATPase activity may be required for the formation and maintenance of vacuoles. (ii) Potentiation of *H. pylori* toxin activity by weak bases but not bafilomycin may be related to the effect of weak bases upon cytoplasmic pH rather than intralysosomal pH (26). (iii)

TABLE 2. Potentiation of *H. pylori* vacuolating toxin activity by ouabain or digoxin

Concn of ouabain or digoxin	% of cells vacuolated in response to *H. pylori* supernatant in presence of[a]:		Net neutral red uptake in presence of:	
	Ouabain	Digoxin	Ouabain	Digoxin
0	0	0	−0.017 ± 0.005	−0.023 ± 0.008
10 nM	0	0	0.004 ± 0.003	−0.011 ± 0.009
100 nM	65 ± 4.8	30 ± 4.6	0.074 ± 0.025	0.038 ± 0.012
1 μM	83 ± 3.3	79 ± 3.8	0.093 ± 0.009	0.051 ± 0.018
10 μM	87 ± 3.3	81 ± 3.1	0.083 ± 0.007	0.055 ± 0.006

[a] HeLa cells were incubated with a 1:320 dilution of concentrated supernatant from *H. pylori* 60190 in the presence of the indicated concentrations of ouabain or digoxin. Cell vacuolation was assessed visually (×200 magnification) and quantitated by neutral red uptake (10). Results shown are mean ± SEM for three replicate wells.

Weak bases alone induce vacuole formation via osmotic swelling of acidic intracellular compartments (24), and thus, accumulation of weak bases may contribute to the enlargement of *H. pylori* toxin-induced vacuoles.

In contrast to the inhibitory effects of V-type ATPase inhibitors, ouabain potentiated the activity of the *H. pylori* toxin. Ouabain is an agent that specifically inhibits the eukaryotic cell Na^+-K^+ ATPase (32), an enzyme that maintains high intracellular concentrations of K^+ and low intracellular concentrations of Na^+ relative to the extracellular environment (27). Exposure of cells to ouabain results in several consequences, including increased intracellular sodium concentration (1, 15), inhibitory effects on cellular volume regulation (6, 19) decreased intracellular pH (17), and increased cytosolic free Ca^{2+} (17). It is not clear which of these phenomena may be most important in potentiation of toxin activity. However, we speculate that osmotic fluxes and changes in cell volume accompanying the increase in intracellular sodium concentration produced by ouabain may be the critical event. It is notable that inhibition of vacuole formation by bafilomycin could not be overcome by potentiating effects of ouabain. This suggests that ouabain and bafilomycin interact with different targets, both of which are related to toxin-induced cell vacuolation.

In summary, inhibitors of eukaryotic ion-transporting ATPases have been shown to alter significantly the interaction between *H. pylori* toxin and HeLa cells. These observations support the concept that vacuole formation in response to the *H. pylori* toxin is related to altered cation transport within eukaryotic cells. Further investigation is required to determine whether the toxin has specific ion transport or ion channel properties, or whether the toxin interacts with enzymes involved in the maintenance of intracellular ion gradients.

TABLE 3. Effects of ouabain and bafilomycin on *H. pylori* toxin-induced vacuolation of HeLa cells

Ouabain concn (μM)	Bafilomycin concn (nM)	% of cells vacuolated in response to indicated dilution of *H. pylori* supernatant[a]	
		1:40	1:320
0	0	100	0
1	0	100	68.8 ± 6.7
0	25	0	0
1	25	0	0

[a] HeLa cells were incubated for 18 h at 37°C with 1:40 or 1:320 dilutions of concentrated supernatant from *H. pylori* 60190. Cell vacuolation was assessed visually (×200 magnification). Results shown are mean ± SEM for three replicate wells.

ACKNOWLEDGMENTS

This study was supported in part by the Medical Research Service, Department of Veterans Affairs, and a grant from Merck, Sharp & Dohme.

REFERENCES

1. **Adler, S.** 1975. Nondependence of cell pH on sodium transport in rat diaphragm muscle. Metabolism **24:**183–188.
2. **Al-Awqati, Q.** 1986. Proton-translocating ATPases. Annu. Rev. Cell Biol. **2:**179–199.
3. **Bowman, E. J.** 1983. Comparison of the vacuolar membrane ATPase of *Neurospora crassa* with the mitochondrial and plasma membrane ATPases. J. Biol. Chem. **258:**15238–15244.
4. **Bowman, E. J., A. Siebers, and K. Altendorf.** 1988. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc. Natl. Acad. Sci. USA **85:**7972–7976.
5. **Cantley, L. C., Jr., M. D. Resh, and G. Guidotti.** 1978. Vanadate inhibits the red cell (Na^+, K^+) ATPase from the cytoplasmic side. Nature (London) **272:**552–554.
6. **Cooke, K. R.** 1978. Ouabain and regulation of cellular volume in freshly prepared slices of rabbit renal cortex. J. Physiol. **279:** 361–374.
7. **Cover, T. L., and M. J. Blaser.** 1992. *Helicobacter pylori* and gastroduodenal disease. Annu. Rev. Med. **43:**135–145.
8. **Cover, T. L., and M. J. Blaser.** 1992. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. J. Biol. Chem. **267:**10570–10575.
9. **Cover, T. L., P. Cao, U. K. Murthy, M. S. Sipple, and M. J. Blaser.** 1992. Serum neutralizing antibody response to the vacuolating cytotoxin of *Helicobacter pylori*. J. Clin. Invest. **90:**913–918.
10. **Cover, T. L., W. Puryear, G. I. Perez-Perez, and M. J. Blaser.** 1991. Effect of urease on HeLa cell vacuolation induced by *Helicobacter pylori* cytotoxin. Infect. Immun. **59:**1264–1270.
11. **Cover, T. L., S. G. Vaughn, P. Cao, and M. J. Blaser.** 1992. Potentiation of *Helicobacter pylori* vacuolating toxin activity by nicotine and other weak bases. J. Infect. Dis. **166:**1073–1078.
12. **Dean, G. E., H. Fishkes, P. J. Nelson, and G. Rudnick.** 1984. The hydrogen ion-pumping adenosine triphosphatase of platelet dense granule membrane. J. Biol. Chem. **259:**9569–9574.
13. **Figura, N., P. Guglielmetti, A. Rossolini, et al.** 1989. Cytotoxin production by *Campylobacter pylori* strains isolated from patients with peptic ulcers and from patients with chronic gastritis only. J. Clin. Microbiol. **27:**225–226.
14. **Flatmark, T., M. Gronberg, E. Husebye, Jr., and S. V. Berge.** 1982. Inhibition by *N*-ethylmaleimide of the MgATP-driven proton pump of the chromaffin granules. FEBS Lett. **149:**71–74.
15. **Harootunian, A. T., J. P. Y. Kao, B. K. Eckert, and R. Y. Tsien.** 1989. Fluorescence ratio imaging of cytosolic free Na^+ in

individual fibroblasts and lymphocytes. J. Biol. Chem. **264:** 19458–19467.
16. **Kaunitz, J. D., R. D. Gunther, and G. Sachs.** 1985. Characterization of an electrogenic ATP and chloride-dependent proton translocating pump from rat renal medulla. J. Biol. Chem. **260:**11567–11573.
17. **Kim, D., E. J. Cragoe, Jr., and T. W. Smith.** 1987. Relations among sodium pump inhibition, Na-Ca and Na-H exchange activities, and Ca-H interaction in cultured chick heart cells. Circ. Res. **60:**185–193.
18. **Leunk, R. D., P. T. Johnson, B. C. David, W. G. Kraft, and D. R. Morgan.** 1988. Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. J. Med. Microbiol. **26:**93–99.
19. **MacKnight, A. D. C., and A. Leaf.** 1977. Regulation of cellular volume. Physiol. Rev. **57:**510–573.
20. **Mattsson, J. P., K. Vaananen, B. Wallmark, and P. Lorentzon.** 1991. Omeprazole and bafilomycin, two proton pump inhibitors: differentiation of their effects on gastric, kidney and bone H^+-translocating ATPases. Biochim. Biophys. Acta **1065:**261–268.
21. **Mellman, I., R. Fuchs, and A. Helenius.** 1986. Acidification of the endocytic and exocytic pathways. Annu. Rev. Biochem. **55:**663–700.
22. **Nomura, A., G. N. Stemmermann, P. H. Chyou, I. Kato, G. I. Perez-Perez, and M. J. Blaser.** 1991. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. N. Engl. J. Med. **325:**1132–1136.
23. **Ohkuma, S., and B. Poole.** 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. USA **75:**3327–3331.
24. **Ohkuma, S., and B. Poole.** 1981. Cytoplasmic vacuolation of mouse peritoneal macrophages and the uptake into lysosomes of weakly basic substances. J. Cell Biol. **90:**656–664.
25. **Peterson, W. L.** 1991. *Helicobacter pylori* and peptic ulcer disease. N. Engl. J. Med. **324:**1043–1048.
26. **Roos, A., and W. F. Boron.** 1981. Intracellular pH. Physiol. Rev. **61:**296–434.
27. **Skou, J. C.** 1988. Overview: the Na,K-pump. Methods Enzymol. **156:**1–25.
28. **Umata, T., Y. Moriyama, M. Futai, and E. Mekada.** 1990. The cytotoxic action of diphtheria toxin and its degradation in intact vero cells are inhibited by bafilomycin A1, a specific inhibitor of vacuolar-type H^+-ATPase. J. Biol. Chem. **265:**21940–21945.
29. **Van Dyke, R. W.** 1986. Anion inhibition of the proton pump in rat liver multivesicular bodies. J. Biol. Chem. **261:**15941–15948.
30. **Van Dyke, R. W., C. A. Hornick, J. Belcher, B. F. Scharschmidt, and R. J. Havel.** 1985. Identification and characterization of ATP-dependent proton transport by rat liver multivesicular bodies. J. Biol. Chem. **260:**11021–11026.
31. **Van Dyke, R. W., C. J. Steer, and B. F. Scharschmidt.** 1984. Clathrin-coated vesicles from rat liver: enzymatic profile and characterization of ATP-dependent proton transport. Proc. Natl. Acad. Sci. USA **81:**3108–3112.
32. **Wallick, E. T., and A. Schwartz.** 1988. Interaction of cardiac glycosides with Na^+, K^+-ATPase. Methods Enzymol. **156:**201–213.
33. **Yoshida, T., C. Chen, M. Zhang, and H. C. Wu.** 1990. Increased cytotoxicity of ricin in a putative Golgi-defective mutant of Chinese hamster ovary cell. Exp. Cell. Res. **190:**11–16.
34. **Yoshimura, T., A. Yamamoto, Y. Moriyama, M. Futai, and Y. Tashiro.** 1991. Bafilomycin A_1, a specific inhibitor of vacuolar-type H^+-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. J. Biol. Chem. **266:**17707–17712.