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Effect of Urease on HeLa Cell Vacuolation Induced by *Helicobacter pylori* Cytotoxin

TIMOTHY L. COVER,[1,2†*] WONDER PURYEAR,[2] GUILLERMO I. PEREZ-PEREZ,[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,[3] Vanderbilt University School of Medicine, Nashville, Tennessee 37232

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Concentrated broth culture supernatants from 50 to 60% of *Helicobacter pylori* strains induce eukaryotic cell vacuolation in vitro. A quantitative assay for cell vacuolation was developed on the basis of the rapid uptake of neutral red dye into intracellular vacuoles. The neutral red dye uptake (NRU) of visibly vacuolated HeLa cells was significantly greater than that of nonvacuolated cells. By using the rapid NRU assay, we sought to determine the roles of *H. pylori* cytotoxin, urease, and ammonia in the vacuolation of HeLa cells. The NRU of HeLa cells incubated in medium containing ammonium chloride or ammonium sulfate was significantly greater than that of cells incubated in medium alone. In addition, ammonium salts augmented the NRU induced by *H. pylori* supernatants. The NRU induced by jack bean urease was augmented by the addition of urea to cell culture medium; this suggests that urease-mediated NRU occurs via the generation of ammonia. Acetohydroxamic acid blocked the NRU induced by jack bean urease and urea but failed to block the uptake induced by *H. pylori* supernatants. Supernatant from a non-urease-producing *H. pylori* mutant strain induced NRU identical to that of the urease-positive parental strain. These observations indicate that the vacuolating activity in *H. pylori* supernatants is not mediated solely by urease activity but that it may be potentiated by urease-mediated ammonia production.

Helicobacter pylori infection has been strongly associated with type B gastritis and peptic ulcer disease, but the mechanisms whereby infection may lead to tissue inflammation and damage are poorly understood (3). Concentrated broth culture supernatants from 50 to 60% of *H. pylori* strains induce vacuolation of cells in vitro (5, 12), a phenomenon that has been attributed to cytotoxin activity. The vacuolating activity in supernatants is abolished by heating to 70°C or by incubation with proteases (12), and thus it is probable that vacuolation is mediated at least in part by a protein. Recently, the vacuolating activity in *H. pylori* culture supernatants has been attributed to urease activity rather than to a specific cytotoxin (25). The degradation of urea by urease liberates ammonia, which is known to induce cell injury (2, 22, 25); in addition, ammonia has long been known to induce cell vacuolation (6, 18, 20).

To study further the vacuolation of cells induced by *H. pylori* supernatants, we developed a quantitative assay for cell vacuolation on the basis of the rapid uptake of neutral red dye by cell vacuoles. By using this assay, we sought to clarify the roles of *H. pylori* cytotoxin, urease, and ammonia in the phenomenon of cell vacuolation.

MATERIALS AND METHODS

Bacterial strains. *H. pylori* 60190 and Tx30a, Tox^+ and Tox^-, respectively (5, 12), were used for most studies. These strains were cultured in brucella broth (BBL Microbiology Systems, Cockeysville, Md.) containing 5% fetal bovine serum for 48 h on a rotary shaker at 37°C in ambient air supplemented with 5% CO_2. *H. pylori* 88-3887 (urease positive) and 26U1 (a urease-negative mutant derived from strain 88-3887) (8) were obtained from K. Eaton, Ohio State University, Columbus, and were cultured under the same conditions. Twenty-eight additional *H. pylori* strains were cultured as previously described (5), and the supernatants were stored at −70°C for approximately 1 year.

Assay for HeLa cell vacuolation. Supernatants from *H. pylori* broth cultures were harvested, concentrated with a 100-kDa ultrafiltration membrane, and incubated with HeLa cells in 96-well microtiter plates for 24 h as previously described (5), except that the concentration of cells was 10^4 cells per well. Visible vacuolation was assessed by light microscopy; as previously described, vacuolation of more than 50% of cells was defined as a vacuolating effect (5, 12).

Staining of HeLa cells with neutral red. A stock solution of 0.5% purified grade neutral red (Sigma Chemical Co., St. Louis, Mo.) was prepared in 0.9% saline and filtered with Whatman no. 1 filter paper (17). Staining solutions were prepared before each experiment by diluting the stock solution 1:10 in Eagle medium containing 10% fetal bovine serum. After incubation with test samples for 24 h, the medium overlaying HeLa cells was removed and replaced with 100 μl of staining solution per well for 4 min. The cells were washed twice with 150 μl of 0.9% saline per well, and the neutral red was extracted from cells by the addition of 100 μl of acidified alcohol per well (16). The optical density (OD) at 540 nm of wells was determined by using an MR700 enzyme-linked immunosorbent assay reader (Dynatech, Alexandria, Va.). All assays were performed in triplicate. In all experiments, the mean OD of wells containing cells incubated with medium alone was less than 0.130 (mean, 0.101 ± 0.007); this background OD was subtracted from the OD of experimental wells to yield a net OD (see Fig. 2 to 8). Solutions of jack bean urease type III, urea, and acetohydroxamic acid (AHA) (Sigma) were freshly prepared for each experiment.

Determination of urease activity and ammonia concentration. The urease activity of *H. pylori* cells and supernatants was determined by using a quantitative spectrophotometric

* Corresponding author.
† Present address: Division of Infectious Diseases, A3310 Medical Center North, Vanderbilt University, Nashville, TN 37232.

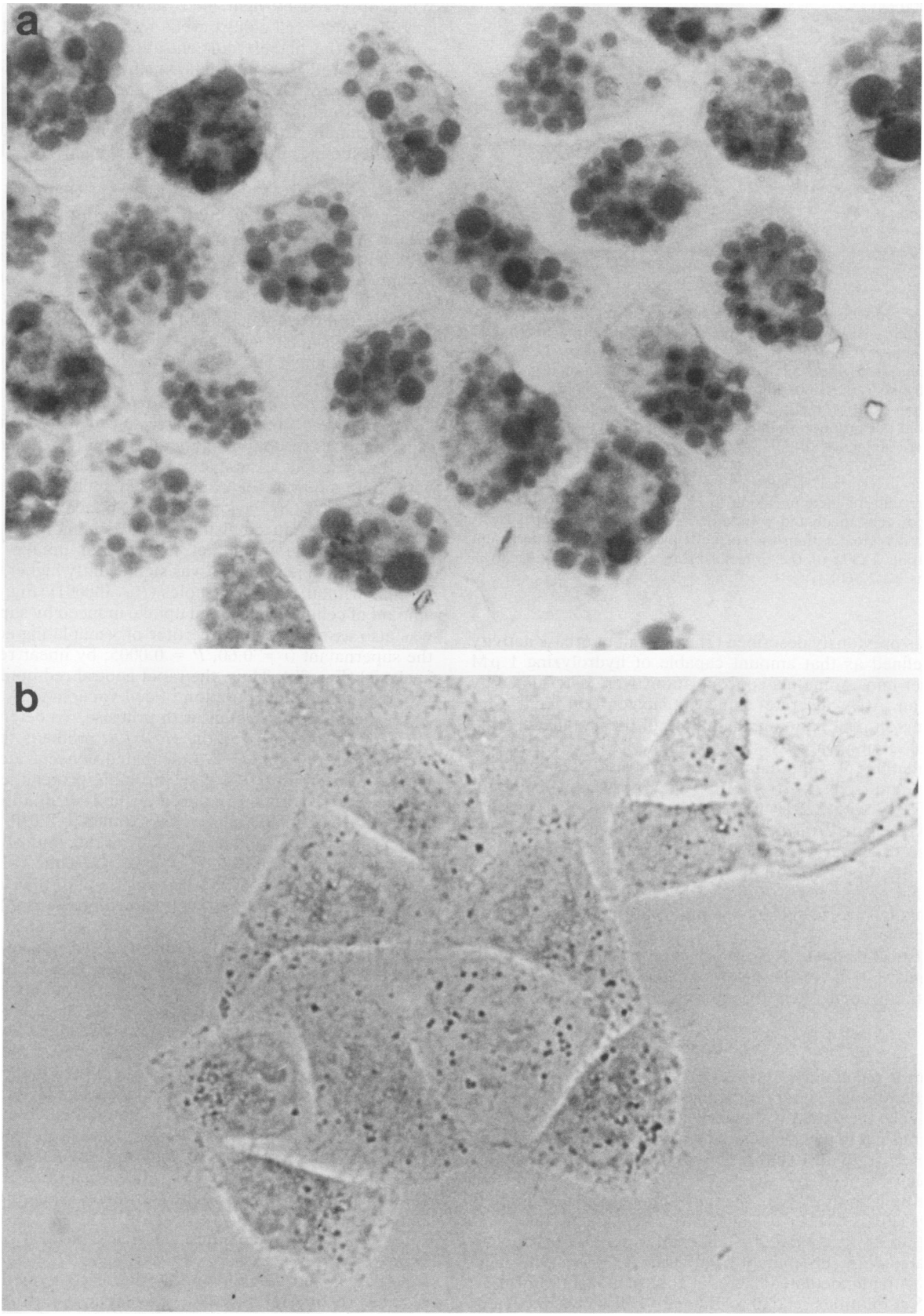

FIG. 1. Neutral red uptake by HeLa cells. HeLa cells were incubated for 18 h with concentrated supernatant from *H. pylori* 60190 (a) or with medium alone (b). The cells were stained for 4 min with 0.05% neutral red and washed. The vacuoles visible in the cells incubated with *H. pylori* supernatant accumulated neutral red dye, whereas the control cells did not. Original magnification, ×200.

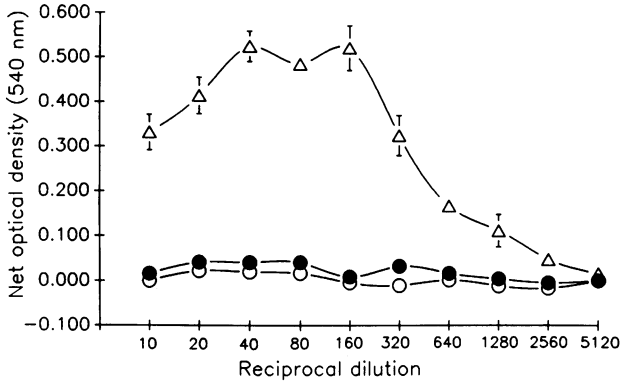

FIG. 2. Induction of HeLa cell neutral red uptake by *H. pylori* supernatants. HeLa cells were incubated for 24 h with concentrated culture supernatant from *H. pylori* 60190 (△), *H. pylori* Tx30a (●), or concentrated, uninoculated bacterial culture medium (○). Supernatant from strain 60190, diluted 1:160, produced visible vacuoles in more than 50% of HeLa cells, whereas supernatant from strain Tx30a did not produce visible vacuoles at any dilution tested. The cells were stained with 0.05% neutral red dye for 4 min, and after washing, the dye was extracted with acid alcohol. The net OD of wells was determined by subtracting the mean OD of cells incubated in tissue culture medium alone (0.117 ± 0.009). The neutral red uptake of cells incubated with supernatant from strain 60190 was significantly greater than that of cells incubated with supernatant from strain Tx30a or the brucella broth control ($P < 0.05$ at all dilutions ≤1:2,560).

assay as previously described (7). One unit of urease activity was defined as that amount capable of hydrolyzing 1 μM urea per min. Ammonia concentrations were determined by using an ACA discrete clinical analyzer (Du Pont Co., Wilmington, Del.) and an adaptation of the glutamate dehydrogenase enzymatic assay (24).

Incubation of supernatant with protease. Concentrated supernatant from *H. pylori* 60190 (125 μl) was incubated for 5 h at 37°C with 2.5 U of insoluble proteinase K (protease type XI-A from *Tritirachium album*) attached to 4% beaded agarose (Sigma). As a control, supernatant was incubated under the same conditions without added protease. The insoluble protease was removed by centrifugation, and the supernatant was tested for the ability to induce neutral red dye uptake.

Statistical methods. Results are expressed as the mean OD ± standard error of the mean. Analysis was performed by using the Student *t* test for independent variables.

RESULTS

Neutral red uptake by HeLa cell vacuoles. As previously described (5, 12), large intracellular vacuoles developed in HeLa cells incubated with supernatant from Tox$^+$ *H. pylori* 60190 but not in cells incubated with supernatant from Tox$^-$ *H. pylori* Tx30a. Localization of neutral red within the vacuoles induced by supernatant from strain 60190 was visible by light microscopy within 4 min after the addition of the dye (Fig. 1). HeLa cells incubated with supernatant from strain 60190 accumulated significantly more dye than cells incubated with supernatant from strain Tx30a or cells incubated with uninoculated bacterial culture medium (Fig. 2) ($P < 0.05$ at dilutions of 1:10 to 1:2,560). HeLa cells incubated with 1:10 and 1:20 dilutions of supernatant from strain 60190 showed less apparent neutral red uptake than did cells incubated with greater dilutions of this supernatant (Fig. 2). When observed by light microscopy, the number of cells adherent to the cell culture wells was decreased in the wells containing the highest concentrations of this supernatant. Thus, impaired growth or detachment of cells exposed to the highest concentrations of supernatant may have resulted in diminished neutral red uptake; the same phenomenon was observed under a variety of other experimental conditions at the highest concentrations of the tested agents (see Fig. 4, 5, and 7). Vacuoles were microscopically detectable in more than 50% of cells incubated with a 1:160 dilution of supernatant from strain 60190, but further dilutions of supernatant did not produce visible vacuoles. In contrast, cells incubated with dilutions ≤1:2,560 of supernatant from strain 60190 accumulated significantly more neutral red than controls ($P < 0.05$ in all cases). These experiments demonstrated that rapid neutral red uptake could be used to quantitate vacuolation of HeLa cells and suggested that this assay was more sensitive than light microscopic examination for detection of cell vacuoles.

Correlation between light microscopic evidence of vacuoles and neutral red uptake. To determine if the results of the rapid neutral red uptake assay consistently correlated with light microscopic examination, we incubated HeLa cells with 1:10 dilutions of concentrated culture supernatants from each of 30 *H. pylori* strains. Sixteen (53%) of these supernatants produced visible vacuoles in more than 50% of HeLa cells. The mean net neutral red uptake by cells containing visible vacuoles was significantly higher than that by cells without visible vacuoles ($P < 0.0001$) (Fig. 3a). The amount of cellular neutral red uptake induced by supernatant was also associated with the titer of vacuolating activity in the supernatant ($r = 0.60$, $P = 0.0005$; by linear regression analysis) (Fig. 3b). Thus, the rapid neutral red uptake assay provided reliable quantitation of cell vacuolation.

Treatment of supernatant with protease. To determine the effect of a protease upon the *H. pylori* products that mediated neutral red uptake, we incubated concentrated supernatant from strain 60190 with insoluble proteinase K. The net neutral red uptake induced by a 1:40 dilution of the protease-treated supernatant was −0.008 ± 0.030, whereas the net neutral red uptake induced by the control supernatant was 0.514 ± 0.037 ($P = 0.0004$). Thus, a protease abolished the activity in the supernatant that mediated neutral red uptake, as has been shown previously for vacuolation (5, 12).

Effect of ammonium salts on neutral red uptake by HeLa cells. Ammonium chloride is known to induce vacuolation in cultured cells (6, 18, 20). To examine the effects of ammonium salts on neutral red uptake by HeLa cells, we incubated HeLa cells in medium containing added ammonium chloride or ammonium sulfate; four other salts were tested as controls. At concentrations ≥1.56 mM, both ammonium chloride and ammonium sulfate induced significantly greater neutral red uptake by HeLa cells than did medium alone ($P < 0.05$ for each point), an effect not observed with sodium chloride, sodium acetate, sodium sulfate, or calcium chloride (Fig. 4). Thus, ammonium salts, when present in tissue culture medium, induced net neutral red uptake by HeLa cells.

Potentiation by ammonium chloride of neutral red uptake induced by *H. pylori* supernatant. Since *H. pylori* culture supernatant and ammonium chloride each induced neutral red uptake by HeLa cells, we compared the neutral red uptake induced by the agents individually with that induced by the two agents simultaneously. The neutral red uptake of cells incubated with supernatant from strain 60190 plus 2 to

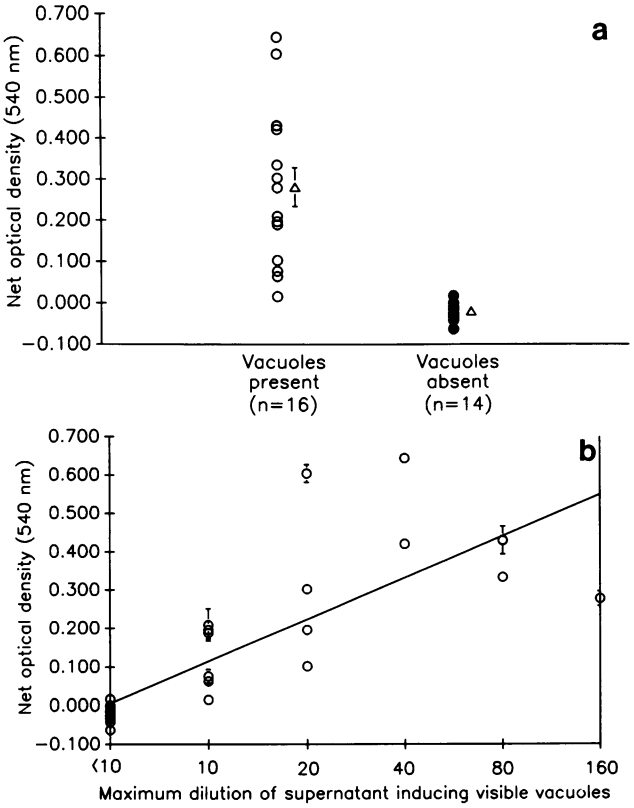

FIG. 3. Neutral red uptake by HeLa cells incubated with each of 30 *H. pylori* supernatants. HeLa cells were incubated with 1:10 dilutions of concentrated supernatant from each of 30 *H. pylori* strains. Sixteen supernatants produced visible vacuoles in >50% of cells when tested at this dilution, whereas the remaining supernatants did not (Fig. 3a). The mean net neutral red uptake (△) induced by the supernatants that caused visible vacuolation was significantly higher than that induced by the remaining supernatants (0.280 ± 0.047 versus −0.021 ± 0.005, $P < 0.0001$). Under these conditions, the titer of vacuolating activity in the supernatant was associated with the magnitude of neutral red uptake (Fig. 3b) ($r = 0.60$, $P = 0.0005$ by linear regression analysis).

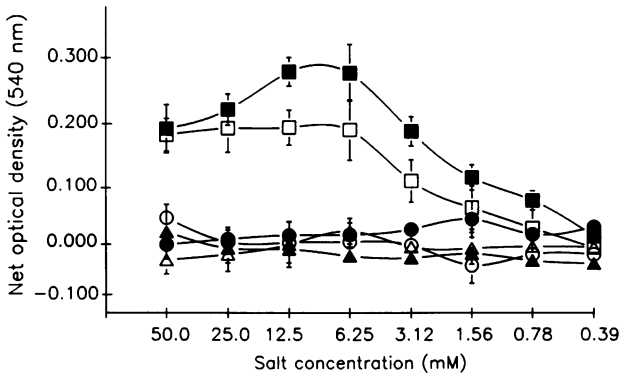

FIG. 4. Induction of HeLa cell neutral red uptake by ammonium salts. HeLa cells were incubated for 24 h in medium containing added salts in the concentrations shown. Ammonium sulfate (■) and ammonium chloride (□) induced net neutral red uptake by HeLa cells, whereas sodium chloride (▲), sodium acetate (△), sodium sulfate (●), and calcium chloride (○) did not.

16 mM ammonium chloride was significantly greater than that of cells incubated with supernatant alone or ammonium chloride alone (Fig. 5a). This effect was evident only with greater than 160-fold dilutions of supernatant ($P < 0.05$) and is best illustrated by the data from the 1:320 dilution (Fig. 5b). The plateau in neutral red uptake at higher supernatant concentrations suggested that the maximum uptake had been attained. Concordant with the results of the neutral red assay, by light microscopy the titer of vacuolation induced by supernatant plus ammonium chloride was higher than that induced by supernatant alone (data not shown). Thus, ammonium chloride potentiated the vacuolating activity present in *H. pylori* culture supernatant.

Effect of jack bean urease on neutral red uptake by HeLa cells. We next sought to determine whether urease was able to induce neutral red uptake by HeLa cells. Jack bean urease, tested in a range of final concentrations from 0.039 to 10 U/ml, induced small but significant increases in neutral red uptake by HeLa cells compared with medium alone (Fig. 6). The addition of acetohydroxamic acid (AHA), a urease inhibitor, at a concentration previously shown to inhibit *H. pylori* urease (500 μg/ml) abolished the net neutral red uptake induced by jack bean urease. When cells were incubated with jack bean urease plus 5 mM urea, approximating the maximum concentrations of urea in gastric juice (13), the net neutral red uptake was greater than that induced by jack bean urease alone; this effect was largely blocked by the addition to cell culture medium of AHA. AHA failed to completely abolish net neutral red uptake when cells were incubated with urea plus the highest concentrations of urease tested. Neither urea alone nor AHA alone induced net increases or decreases in neutral red uptake by HeLa cells (data not shown). The augmentation by urea of urease-mediated neutral red uptake and blocking by AHA suggests that the neutral red uptake induced by urease is mediated by ammonia.

Urease activity of vacuolating and nonvacuolating strains. To determine whether the vacuolating activity in *H. pylori* supernatants was related to urease activity, we compared the urease activities of Tox^+ strain 60190 and Tox^- strain Tx30a. The urease activities of 60190 and Tx30a whole cells harvested from broth culture were 36.5 ± 4.3 and 32.9 ± 5.07 U/mg of protein, respectively ($P = 0.601$). The urease activity in concentrated supernatant from Tox^+ strain 60190 was 33.3 ± 19.6 U/ml (1.05 ± 0.63 U/mg of protein), and the activity in concentrated supernatant from Tox^- strain Tx30a was 28.2 ± 18.8 U/ml (0.81 ± 0.50 U/mg of protein) ($P = 0.86$). Thus, supernatants with and without vacuolating activity did not differ significantly in urease activity. When diluted in the tissue culture assay, these concentrations were similar to the concentrations of jack bean urease tested (Fig. 6) and were within a range that could be neutralized by AHA (500 μg/ml).

Effect of AHA on neutral red uptake induced by *H. pylori* supernatant. We then further investigated the role of urease in the vacuolation induced by *H. pylori* supernatants. In these experiments, concentrated supernatants from Tox^+ strain 60190 and Tox^- strain Tx30a were diluted as required with tissue culture medium so that the urease activity in each sample was 20 U/ml. Despite identical urease activities in the two samples, only supernatant from strain 60190 induced significant neutral red uptake (Fig. 7). The neutral red uptake by cells incubated with supernatant from strain 60190 plus 5 mM urea was greater than that by cells incubated with supernatant alone ($P = 0.013$ at 1:640 dilution) (Fig. 7a). When cells were incubated with strain 60190 supernatant, urea, and AHA simultaneously, the neutral red uptake

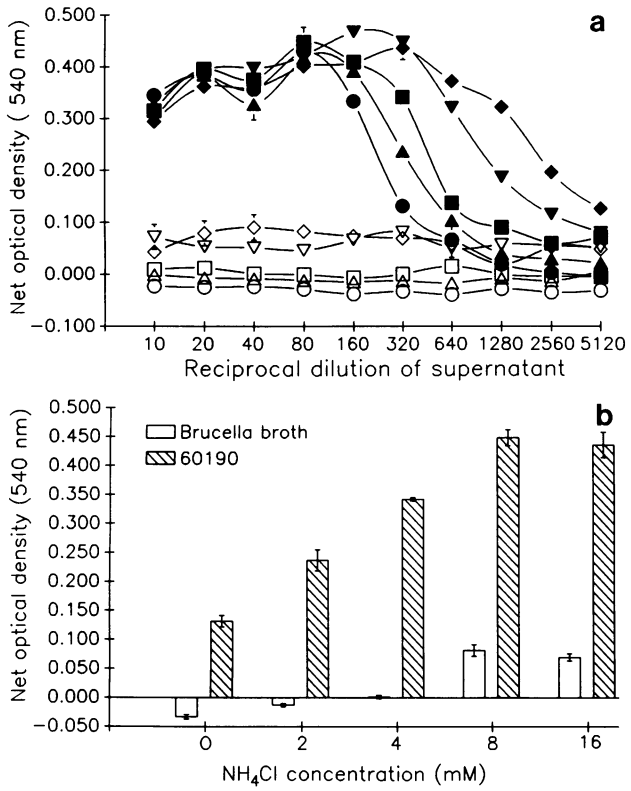

FIG. 5. Potentiation by ammonium chloride of HeLa cell neutral red uptake induced by *H. pylori* supernatant. (a) Dilutions of concentrated supernatant from Tox$^+$ *H. pylori* 60190 were incubated with HeLa cells in medium containing 0 to 16 mM ammonium chloride (closed symbols), and dilutions of concentrated, uninoculated bacterial culture medium were tested as controls (open symbols). Ammonium chloride concentrations were 0 (○), 2 (△), 4 (□), 8 (▽), and 16 (◇) mM. Together, supernatant and ammonium chloride induced greater neutral red uptake by cells than did either agent alone. (b) The potentiating effect of ammonium chloride is best demonstrated by comparing 1:320 dilutions of the supernatant from *H. pylori* 60190 (hatched bars) and the brucella broth control (open bars).

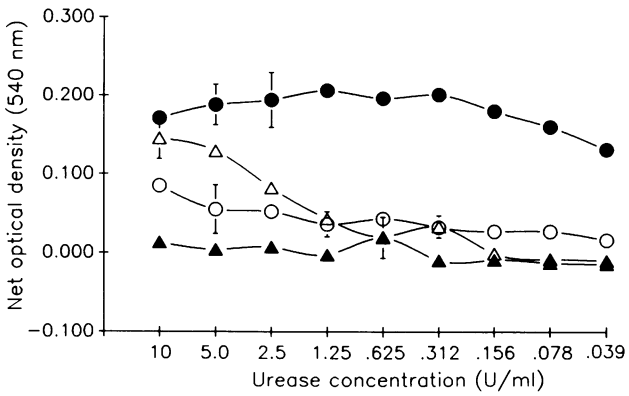

FIG. 6. HeLa cell neutral red uptake induced by jack bean urease. HeLa cells were incubated with jack bean urease in medium containing 5 mM urea (●) or in medium alone (○). The neutral red uptake induced by urease alone was significantly augmented by added urea ($P < 0.05$ at all dilutions tested). Urea alone did not induce net neutral red uptake (data not shown). AHA (500 μg/ml) inhibited the neutral red uptake induced by urease alone (▲) and by urease plus urea (△), except when the highest concentrations of urease (2.5 to 10 U/ml) were used.

decreased to the level observed with supernatant alone. The same phenomenon was observed with supernatant from the Tox$^-$ strain Tx30a (Fig. 7b). The failure of AHA to completely abolish the neutral red uptake induced by *H. pylori* supernatant indicated that vacuolation was not mediated solely by urease activity.

Measurement of ammonia concentration in supernatants. Because ammonium salts potentiated the neutral red uptake induced by *H. pylori* supernatants, we determined whether supernatants from Tox$^+$ strain 60190 and Tox$^-$ strain Tx30a differed in ammonia concentration. The ammonia concentration of uninoculated brucella broth containing 5% fetal bovine serum was 3.9 ± 0.1 mM. The ammonia concentrations of unconcentrated supernatants from strain 60190 and strain Tx30a were 21.9 ± 1.5 and 21.9 ± 0.7 mM, respectively. Thus, the ammonia concentrations of supernatants with and without vacuolating activity were not significantly different ($P = 0.970$).

Neutral red uptake induced by supernatants from a urease-positive and a urease-negative strain. To study further the possible relationship between urease and vacuolating activity, we tested culture supernatants from *H. pylori* strain 88-3887 (urease positive) and strain 26U1 (urease negative), a mutant derived from strain 88-3887 (8). The urease activities of the cells harvested from the two broth cultures were 67 and 0.34 U/mg of protein, respectively. Concentrated culture supernatants from each of the two strains produced visible vacuolation of HeLa cells when diluted 1:20, and the two supernatants induced similar neutral red uptake by HeLa cells (Fig. 8). The neutral red uptake induced by the urease-positive supernatant was augmented by the addition of urea to the cell culture medium ($P < 0.05$ at all dilutions tested), and the addition of AHA decreased the neutral red uptake to the level induced by the urease-positive supernatant alone (Fig. 8a). The addition of urea did not augment the neutral red uptake induced by the urease-negative supernatant (Fig. 8b). Thus, urease activity potentiated the neutral red uptake induced by *H. pylori* supernatant, but neutral red uptake and cell vacuolation were not dependent upon the presence of urease activity.

DISCUSSION

Concentrated culture supernatants from 50 to 60% of *H. pylori* strains induce visible cell vacuolation in vitro (5, 10, 12). The following lines of evidence suggest that the cell vacuolation observed in vitro may also be relevant in vivo. (i) Epithelial vacuoles have been observed in the gastric mucosa of humans infected with *H. pylori* (19, 23). (ii) Vacuoles have been observed in the mucosa of gnotobiotic piglets experimentally infected with vacuolating strains of *H. pylori* (9). (iii) Serum samples from humans infected with *H. pylori* neutralize vacuolating activity more frequently than serum samples from persons not infected with *H. pylori* (11).

To facilitate further study of the cell vacuolation induced by *H. pylori* supernatants, we developed a quantitative assay for cell vacuolation on the basis of the rapid uptake of neutral red into intracellular vacuoles. We have demonstrated that neutral red uptake by HeLa cells is correlated with the presence of visible vacuoles within the cells. However, in comparison with visual detection of vacuoles, the neutral red uptake assay is simple, quantitative, objective, and more sensitive. Neutral red staining of cells for

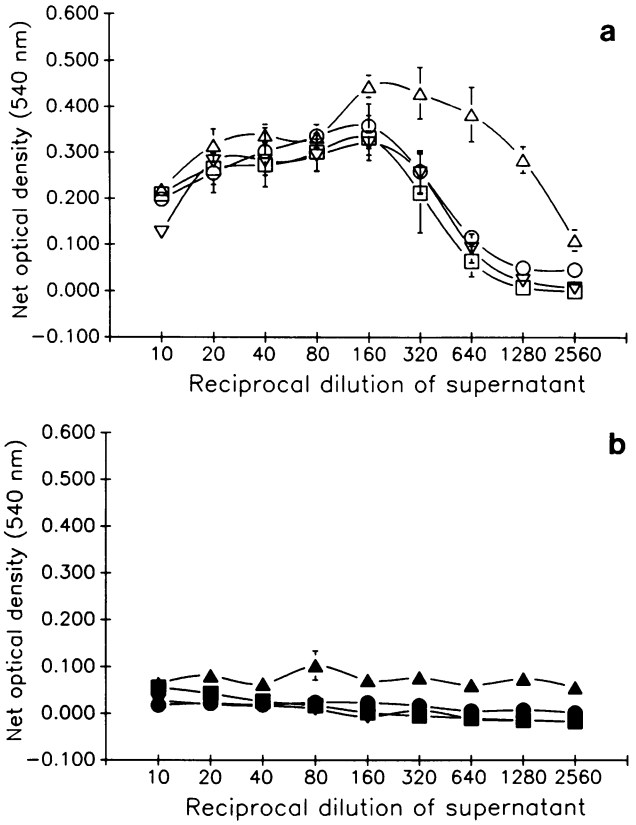

FIG. 7. Effect of urea and AHA on HeLa cell neutral red uptake induced by *H. pylori* supernatants. HeLa cells were incubated with concentrated supernatant from Tox$^+$ *H. pylori* 60190 (a; open figures) or Tox$^-$ *H. pylori* Tx30a (b; closed figures), each containing 20 U of urease activity per ml. The supernatants were diluted in medium alone (○) or in medium containing 5 mM urea (△), 500 μg of AHA per ml (▽), or both 5 mM urea and 500 μg of AHA per ml (□). Neither urea alone nor AHA alone induced net increases or decreases in neutral red uptake by HeLa cells (data not shown). Supernatant from strain 60190 induced net neutral red uptake by HeLa cells, whereas supernatant from strain Tx30a did not. However, the neutral red uptake of cells incubated with supernatant from either strain in the presence of urea was significantly greater than that of cells incubated with supernatant alone. For each strain, the addition of AHA reduced the neutral red uptake to the level observed with supernatant alone.

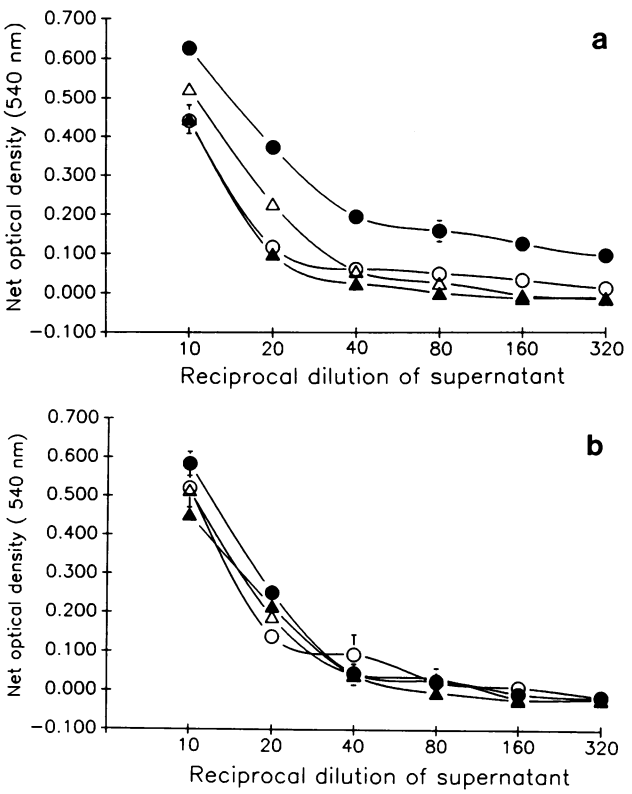

FIG. 8. HeLa cell neutral red uptake induced by urease-positive and urease-negative *H. pylori* culture supernatants. HeLa cells were incubated with concentrated supernatant from urease-positive *H. pylori* 88-3887 (a) or urease-negative *H. pylori* 26U1 (b). The supernatants were diluted in medium alone (○) or in medium containing 5 mM urea (●), 500 μg of AHA per ml (▲), or both 5 mM urea and 500 μg of AHA per ml (△). The two supernatants induced similar neutral red uptake by HeLa cells. The neutral red uptake by HeLa cells induced by supernatant from strain 88-3887 was augmented by the addition of urea ($P < 0.05$ for all dilutions tested), but this effect was absent for supernatant from strain 26U1. The addition of AHA reduced the neutral red uptake to the level observed with strain 88-3887 supernatant alone.

periods of 1 to 3 h has been used previously to quantitate viable cells (16, 22). The use of a 4-min staining time in our assay resulted in minimal uptake by control HeLa cells incubated in media alone but high uptake by cells containing visible intracellular vacuoles.

The vacuoles that form within cells in response to *H. pylori* supernatants have not yet been characterized in detail. Electron microscopic studies indicate that the vacuoles are membrane bound and contain electron-lucent reticular material and small membranous inclusions (12). Neutral red is known to localize rapidly within the lysosomes of cells (1, 4, 18, 21); hence, the rapid incorporation of neutral red into *H. pylori*-induced vacuoles suggests that these structures may represent enlarged lysosomes or analogous compartments with acidic pH. Ammonia is another lysosomotropic agent, capable of inducing lysosomal swelling of cells in vitro (6, 18, 20). The additive effect of *H. pylori* supernatant and ammonium salts on neutral red uptake by HeLa cells suggests that lysosomes may be the organelle upon which both agents have activity.

The mechanisms whereby *H. pylori* supernatants induce cell vacuolation are poorly understood. Leunk et al. initially ascribed the vacuolating activity in supernatants to the activity of a cytotoxin (12), but more recently, vacuolation has been attributed to urease activity (25). By using the neutral red assay, we sought to clarify the role of urease in cell vacuolation. We studied the effects of a commercially available preparation of jack bean urease, which served as a model for the activity of *H. pylori* urease (14, 15, 22). The neutral red uptake induced by jack bean urease was significantly greater in the presence of added urea than without added urea and was blocked by AHA, an inhibitor of urease activity. This suggests that urease causes neutral red uptake by HeLa cells via the generation of ammonia from urea rather than by a direct, antigenic effect. These findings are consistent with the data reported by Xu et al., who noted *H. pylori* urease-mediated vacuolation of Vero cells by *H. pylori* cell washes only when urea was added to the cell culture medium (25). The small but detectable neutral red uptake induced by jack bean urease without added urea may

result from the activity of the enzyme on urea in the tissue culture medium. In several previous investigations demonstrating urease-mediated cytotoxicity to cultured cells, the medium contained added urea (2, 22); thus, these effects also may have been attributable to ammonia production.

The following results of several experiments indicate that the vacuolating activity in *H. pylori* broth culture supernatants is not mediated solely by urease activity. (i) The urease activity and ammonia concentrations of supernatants with and without vacuolating activity were not significantly different. (ii) An *H. pylori* mutant without urease activity caused neutral red uptake and cell vacuolation indistinguishable from that of the parent strain. Finally, (iii) AHA failed to completely inhibit the neutral red uptake of HeLa cells induced by *H. pylori* culture supernatants. Thus, we have identified the following three features of *H. pylori* supernatants that contribute to the induction of neutral red uptake and cell vacuolation. (i) Urease induces neutral red uptake indirectly by metabolizing urea to ammonia. (ii) Ammonium chloride alone induces neutral red uptake and also potentiates the uptake induced by other agents. (iii) A third component, previously termed a cytotoxin (12), induces neutral red uptake independently from the activities of urease and ammonia. Because the ammonia and urease concentrations in supernatants from strains Tx30a and 60190 are similar, it is likely that the cytotoxin is important in mediating the vacuolation induced by the latter strain. Our demonstration that *H. pylori* supernatants may contain a vacuolating activity in addition to urease is now leading us to the purification and characterization of that activity.

It is interesting to speculate that the putative toxin and ammonia generated by *H. pylori* urease may contribute to cell vacuolation and injury in an additive fashion in vivo. In vivo, ammonium chloride is generated from the combination of ammonia with hydrochloric acid of parietal cell origin. The gastric juice ammonia concentrations in *H. pylori*-infected persons are 34 ± 16 mM (13). Thus, in vivo concentrations of ammonia are similar to the concentrations of ammonium chloride that potentiate *H. pylori*-induced cell vacuolation in the cell culture assay.

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