Role of glutamine and arginase in protection against ammonia-induced cell death in gastric epithelial cells

EIJI NAKAMURA AND SUSAN J. HAGEN
Department of Surgery, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215
Received 17 June 2002; accepted in final form 20 August 2002

Nakamura, Eiji, and Susan J. Hagen. Role of glutamine and arginase in protection against ammonia-induced cell death in gastric epithelial cells. Am J Physiol Gastrointest Liver Physiol 283: G1264–G1275, 2002. First published August 28, 2002; 10.1152/ajpgi.00235.2002.—Ammonia is a cytotoxic factor produced during Helicobacter pylori infection that may reduce the survival of surface epithelial cells. Here we examine whether ammonia kills cells and whether l-glutamine (l-Gln) protects against cell death by stimulating ammonia detoxification pathways. Cell viability and vacuolation were quantified in rat gastric epithelial (RGM1) cells incubated with ammonium chloride at pH 7.4 in the presence or absence of l-Gln. Incubation of RGM1 cells with ammonium chloride caused a dose-dependent increase in cell death and vacuolation, which were both inhibited by l-Gln. We show that RGM1 cells metabolize ammonia to urea via arginase, a process that is stimulated by l-Gln and results in reduced ammonia cytotoxicity. l-Gln also inhibits the uptake and facilitates the extrusion of ammonia from cells. Blockade of glutamine synthetase did not reduce the survival of RGM1 cells, demonstrating that the conversion of l-glutamate and ammonia to l-Gln is not involved in ammonia detoxification. Thus our data support a role for l-Gln and arginase in protection against ammonia-induced cell death in gastric epithelial cells.

Helicobacter pylori; rat; rat gastric epithelial 1 cells; NH₄Cl

INFECTION OF THE STOMACH by Helicobacter pylori causes chronic-active gastritis and peptic/duodenal ulcer disease in humans and in many animal models. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling staining of the mucosa has shown that apoptosis increases significantly during H. pylori infection and is most prevalent in surface epithelial cells (42, 51). Although apoptosis occurs, it is not clear what factors associated with H. pylori infection cause injury and death of surface epithelial cells. It has been reported that H. pylori produce many deleterious factors for gastric epithelial cells, such as vacuolating cytotoxin (VacA), gene products of the Cag pathogenicity island (PAI), and urease. Although VacA was initially thought to be cytotoxic to gastric epithelial cells, compelling evidence against this contentions was recently shown in the gerbil model of infection by targeted deletion of the VacA gene in H. pylori, in which severe gastritis and mucosal injury were present (36, 53). In addition, H. felis, a related gastric Helicobacter that does not possess VacA or the Cag PAI but has potent urease activity (33), induces severe gastritis and injury to gastric epithelial cells in several animal models (12, 30). These combined results suggest that injury to epithelial cells during H. pylori infection may occur from urease-derived ammonia (NH₃) rather than from VacA or gene products of the Cag PAI. In fact, the severity of gastric injury during H. pylori infection is correlated with the concentration of NH₃ in the gastric juice (47) or the urease activity of H. pylori (26). Patients with H. pylori infection show a significant increase in gastric juice NH₃ compared with uninfected control patients (13, 14, 23, 24, 27, 32, 34, 35, 46, 50, 58).

A number of studies recently showed that NH₃ affects the gastric mucosa in vivo and gastric epithelial cells in vitro. NH₃, at a concentration below that detected in H. pylori-infected patients, inhibits oxygen consumption (48), cell proliferation (31), and acid secretion (17, 18, 57). In addition, NH₃ kills parietal and chief cells in isolated gastric glands by necrosis and apoptosis, respectively (17). NH₃, generated by using ammonium chloride (NH₄Cl) or urea/urease, kills gastric MKN 45 cells alone and in combination with cytokines, such as tumor necrosis factor-α or interferon-γ (21). Furthermore, NH₃ retards restitution of the injured gastric mucosa (43), leading to impaired barrier function. Thus NH₃ may significantly impair mucosal homeostasis, resulting in injury and death of gastric epithelial cells during H. pylori infection.

Although gastric surface epithelial cells are exposed to high levels of NH₃ during H. pylori infection, it is not established whether these cells are injured by NH₃ or whether they possess any mechanism(s) to protect against NH₃-induced injury. In the liver, systemic NH₃ detoxification occurs in metabolic zones, where periporal and perivenous hepatocytes have unique enzymatic pathways for the production of nontoxic NH₃ metabolites such as urea and glutamine, respectively (20, 59). Brain glial cells also produce glutamine from glutamate and NH₃ to protect neurons from NH₃-induced cytotoxicity (9, 59). Thus it is possible that gastric epithelial cells have the ability to process NH₃, the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: S. J. Hagen, Dept. of Surgery, Dana 805, Beth Israel Deaconess Medical Center, 330 Brookline Ave., Boston MA 02215 (E-mail: shagen@caregroup.harvard.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
either by facilitating the production of urea from NH₃ or by converting glutamate and NH₂ to glutamine. Either detoxification pathway would be beneficial to protect surface epithelial cells against the cytotoxic effects of NH₃ in the gastric lumen, in general, and during H. pylori infection, in particular.

Thus the purpose of this study was to determine whether NH₃ affects the survival of gastric surface epithelial cells and, if so, to determine whether glutamine protects surface epithelial cells from injury by facilitating NH₃ detoxification. To accomplish this, we measured cell viability and the degree of vacuolation in rat gastric epithelial (RGM1) cells that were exposed to NH₄Cl, producing NH₃ and ammonium, with or without L-glutamine (L-Gln). Our results indicate that NH₄Cl significantly reduces the viability of RGM1 cells and that L-Gln and L-glutamate (L-Glu) both protect RGM1 cells against NH₄Cl-induced cell death. Our results establish that RGM1 cells metabolize NH₃ to urea, that L-Gln protects by decreasing the intracellular accumulation of NH₃ and increasing NH₃ metabolism, and that the conversion of NH₃ and L-Glu to L-Gln via glutamine synthetase does not protect RGM1 cells. Because L-Gln completely reverses the cytotoxic effects of NH₃ in our study, it is proposed that L-Gln supplementation may be beneficial to reduce mucosal injury during H. pylori infection.

MATERIALS AND METHODS

Preparation of RGM1 cell cultures. Rat gastric epithelial cell line, RGM1 cells, established by Dr. H. Matsui, Institute of Physical and Chemical Science (RIKEN) Cell Bank and Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Japan (28), are nontransformed gastric surface epithelial cells. RGM1 cells were cultured in DMEM-F12 (1:1) supplemented with heat-inactivated 10% fetal bovine serum (FBS; GIBCO/BRL, Gaithersburg, MD), 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 µg/ml amphotericin B. Confluent monolayers of RGM1 cells were starved for 24 h in culture medium without FBS (DMEM-F12 containing 15 mM HEPES at pH 7.4) at 37°C under 5% CO₂ in air and then used for experiments. All experiments were performed in standard (STD) buffer at pH 7.4 in the presence and/or absence of reagent(s) without FBS.

Treatment of RGM1 cells with NH₄Cl or methylamine with or without L-Gln or L-Glu. Starved RGM1 cells were transferred to STD buffer containing (in mM) 147 Na⁺, 5.0 K⁺, 131 Cl⁻, 1.3 Mg²⁺, 1.3 SO₄²⁻, 2 Ca²⁺, 25 HCO₃⁻, 15 HEPES, and 20 p-glucose at pH 7.4 and incubated at pH 7.4 with 0–100 mM NH₄Cl or 0–30 mM methylamine hydrochloride (MeNH₂HCl) in the presence or absence of 0–20 mM L-Gln. NH₄Cl was used as the source of NH₃, a primary amine weak base. Because NH₃ is in equilibrium with its protonated form (NH₄⁺) in a pH-dependent manner, 30 mM NH₄Cl (pKₓ is 9.24) results in 0.44 mM NH₃ (1.4%) and 29.56 mM NH₄⁺ (98.6%) at pH 7.4. Measurement of cell viability. The viability of RGM1 cells was evaluated by a colorimetric assay by using crystal violet (25), a cytochemical stain that binds to chromatin. For this assay, RGM1 cells were washed once with PBS to remove dead cells, fixed with methanol for 15 min, and then air-dried. The dried cells were stained with 0.1% crystal violet for 5 min at room temperature, washed twice with PBS, and then air-dried. Stained cells were solubilized with 0.5% SDS for 30 min with slight agitation. Lysates were diluted with 0.5% SDS, and the absorbance was measured at 590 nm by using a microplate reader. Crystal violet stain was purchased from Sigma (St. Louis, MO).

Measurement of cell vacuolation. Intracellular acidic vacuoles, containing H⁺ generated by the vacuolar ATPase, expand in the presence of a weak base (in a concentration-dependent manner) because the unprotonated weak base freely partitions into the acidic space, is protonated by H⁺, and cannot freely exit (10). The resulting loss of H⁺ alkalizes the vacuole and initiates further H⁺ generation by the vacuolar ATPase, which is followed by water movement into the vacuole and vacuole expansion (10). Because vacuolation is an indicator of intracellular weak base concentration, we evaluated the intracellular concentration of NH₃ or MeNH₂ by quantifying vacuolation.

To quantify vacuolation in RGM1 cells, uptake of neutral red into vacuoles was determined as described by Cover et al. (7, 8), with slight modification. In brief, RGM1 cells were incubated for 10 min at 37°C with 0.005% neutral red in STD buffer and then washed twice with PBS containing 0.3% BSA. The dye was extracted with isopropyl alcohol containing 0.04 M HCl. The extract was diluted, and the absorbance was measured at a test wavelength of 540 nm and a reference wavelength of 650 nm by using a microplate reader.

Assay for the extrusion of NH₄Cl or MeNH₂ from vacuoles. After the induction of vacuoles for 6 h with 30 mM NH₄Cl or 3 mM MeNH₂, RGM1 cells were incubated for 1 h in STD buffer with or without 0–20 mM L-Gln, in the absence of NH₄Cl or MeNH₂. Vaccumulation was quantified as described above.

Measurement of MeNH₂ accumulation in RGM1 cells. Intracellular accumulation of [¹⁴C]MeNH₂ was measured in RGM1 cells that were incubated for 3 h, at 37°C, with 3 mM MeNH₂ containing 0.5 µCi of [¹⁴C]MeNH₂-HCl (NEN Life Science Products, Boston, MA) and 0–20 mM L-Gln. Washing the cells with ice-cold PBS terminated the reaction. The cells were solubilized with 0.1 M NaOH, and the radioactivity was
measured by liquid scintillation (Packard Instruments, Downers Grove, IL).

Measurement of urea production in RGM1 cells. Urea concentration in the culture supernatant was measured in two ways. First, by using a commercially available assay kit (Sigma), which follows the procedure of Ormsby (37), and second, by measuring the conversion of L-[guanido-14C]arginine into 14C]urea as described below for the measurement of arginase activity. For measurement of urea by the Sigma assay kit, RGM1 cells were cultured in 100-mm dishes to obtain 4 × 10⁶ cells/dish. After starvation for 24 h, the cells were incubated with or without 20 mM L-Gln in the presence or absence of 30 mM NH₄Cl for 6 h. The culture supernatant was collected from four dishes, combined into one sample, and lyophilized. The lyophilized sample was solubilized in PBS and used for the urea assay, where the absorbance at 540 nm of hydroxylamine generated by the reaction of urea with diacetylmonoxime was measured. This assay is not affected by other nitrogen compounds such as NH₃ or nitrogen oxides (37). The urea concentration was determined from a standard curve by using urea purchased from Sigma.

Measurement of arginase activity in RGM1 cells. Starved RGM1 cells were incubated at 37°C for 6 h with STD buffer. The cells were solubilized and sonicated in lysis buffer containing 0.01% Triton X-100, 2 mg/ml BSA, 10 mM MnCl₂, and 12 mM Na maleate (pH 7.5). After centrifugation at 1,000 g at 4°C, arginase activity was determined in the supernatant by measuring the conversion of L-[guanido-14C]arginine to [14C]urea (6, 39, 40). In brief, the supernatant was added to reaction buffer (100 mM glycine, pH 7.4) in the presence or absence of 30 mM NH₄Cl, 20 mM L-Gln, and 1 mM nor-NOHA, and the reaction was started by the addition of 250 mM L-arginine containing 0.05 μCi of L-[guanido-14C]arginine. After 90 min at 37°C, the reaction was terminated by the addition of 0.8 ml of stop buffer containing 250 mM acetic acid, 100 mM urea, 10 mM L-arginine (pH 4.5), and a 50% suspension of Dowex 50-WX8 resin (H⁺ form). After centrifugation, the supernatant containing [14C]urea (500 μl) was measured by liquid scintillation. Under these conditions, the resin removed 99.8% of the arginine substrate and 99.0% of the converted ornithine. Arginase activity in the supernatant was extrapolated from a standard curve by using purified arginase (Sigma).

Morphological analysis of cell cultures. Cell morphology was evaluated in cultured RGM1 cells at 6 and 24 h in STD buffer or in STD buffer containing 30 mM NH₄Cl or 3 mM MeNH₂ with or without 20 mM L-Gln. Cells were photographed with a Nikon TE300 microscope (MicroVideo Instruments, Avon, MA) outfitted with an Orca charge-coupled device camera (Hamamatsu Photonics) and IP laboratory software (Scanalytics, Fairfax, VA).

Statistical analysis. The data represent means ± SE for four wells of RGM1 cells from three different experiments. Statistical differences were evaluated by using Dunnett’s multiple comparison test and Student’s t-test, with a value of P < 0.05 regarded as significant.

RESULTS

NH₄Cl and MeNH₂ reduce the viability of cultured RGM1 cells. Treatment of RGM1 cells with NH₄Cl or MeNH₂ at pH 7.4 reduced viability in a concentration-dependent manner (Fig. 1). The viability of RGM1 cells was significantly reduced with 3, 10, 30, and 100 mM NH₄Cl (Fig. 1A). Similarly, the viability of RGM1 cells was significantly reduced with 1, 3, 10, and 30 mM MeNH₂ (Fig. 1B). For all further experiments, we used 30 mM NH₄Cl, which reduced viability to 37.6 ± 1.1% of control, and 3 mM MeNH₂, which reduced viability to 54.6 ± 0.5% of control. It should be noted that a significantly greater concentration of NH₄Cl was required to produce the same reduction in viability compared with MeNH₂.

Time course studies showed that the viability of RGM1 cells decreased over time in the presence of 30 mM NH₄Cl or 3 mM MeNH₂ (Fig. 2). The viability of RGM1 cells was significantly reduced at 12, 24, and 36 h, when incubated with 30 mM NH₄Cl or 3 mM MeNH₂.

Fig. 1. Effect of NH₄Cl (A) or MeNH₂ (B) on cell viability. Rat gastric epithelial (RGM1) cells were incubated with 1–100 mM NH₄Cl (A) or 0.3–30 mM MeNH₂ (B) for 24 h, and then the number of viable cells was quantified by the crystal violet assay. The data show that both NH₄Cl and MeNH₂ kill RGM1 cells in a dose-dependent manner. Values are means ± SE of 4 wells from 3 different experiments and are expressed as the percentage of control cells that were incubated with standard (STD) buffer alone. *Significant decrease in viability compared with control cells, P < 0.05.

AJPGI-Gastrointest Liver Physiol • VOL 283 • DECEMBER 2002 • www.ajpgi.org
L-Gln improves the viability of RGM1 cells in the presence of NH₄Cl and MeNH₂. Treatment of RGM1 cells with L-Gln prevented the reduction in cell viability induced by 30 mM NH₄Cl or 3 mM MeNH₂ in a concentration-dependent manner (Fig. 3). In the presence of NH₄Cl, significant protection occurred with 0.2, 2, and 20 mM L-Gln (Fig. 3A). In fact, 20 mM L-Gln completely (100.3 ± 1.1% of the initial value) protected RGM1 cells that were incubated with 30 mM NH₄Cl (Fig. 3A). Similarly, significant protection occurred with 0.2, 2, and 20 mM L-Gln in RGM1 cells that were incubated with 3 mM MeNH₂. Like with NH₄Cl, 20 mM L-Gln completely (99.1 ± 1.1%) protected RGM1 cells that were incubated with 3 mM MeNH₂ (Fig. 3B). L-Gln had no effect on the viability of RGM1 cells in the absence of NH₄Cl or MeNH₂ at 24 h (104.2 ± 3.6% viability with L-Gln vs. 100.0 ± 4.1% with STD buffer alone). In addition, treatment with 20 mM mannitol, used to control for osmotic changes produced by 20 mM L-Gln, had no effect on the viability of RGM1 cells that were treated with 30 mM NH₄Cl (42.2 ± 1.9% viability with mannitol and NH₄Cl vs. 45.0 ± 2.0% with NH₄Cl alone).

NH₄Cl or MeNH₂ causes vacuolation of RGM1 cells that is reduced by L-Gln. Incubation of RGM1 cells for 6 h with NH₄Cl or MeNH₂ resulted in the vacuolation of RGM1 cells in a concentration-dependent manner.

Fig. 2. Time course analysis of cell viability in the presence of NH₄Cl (A) or MeNH₂ (B). RGM1 cells were incubated for 36 h in the presence or absence of 30 mM NH₄Cl (A) or 3 mM MeNH₂ (B), and the number of viable cells was quantified by the crystal violet assay. These data demonstrate that NH₄Cl and MeNH₂ significantly reduce the viability of RGM1 cells over time. Values are means ± SE of 4 wells from 3 different experiments and are expressed as percentage of control cells that were incubated with STD buffer alone. *Significant decrease in viability compared with control cells, P < 0.05. †Significant increase in viability compared with cells treated with NH₄Cl (A) or MeNH₂ (B), P < 0.05.

Fig. 3. Effect of L-glutamine (L-Gln) on the viability of RGM1 cells incubated with NH₄Cl (A) or MeNH₂ (B). RGM1 cells were incubated with 0.02–20 mM L-Gln in the absence or presence of 30 mM NH₄Cl (A) or 3 mM MeNH₂ (B) for 24 h, and then cell viability was quantified by the crystal violet assay. The data show that L-Gln significantly improves the viability of RGM1 cells in a dose-dependent manner. Values are means ± SE of 4 wells from 3 different experiments and are expressed as percentage of control cells that were incubated with STD buffer alone. *Significant decrease in viability compared with control cells, P < 0.05. †Significant increase in viability compared with cells treated with NH₄Cl (A) or MeNH₂ (B), P < 0.05.
Vacuolation increased significantly in the presence of 0.3–30 mM NH₄Cl, resulting in a maximum increase of 86.4 ± 4.5% compared with control cells treated with buffer alone (Table 1). Similarly, vacuolation increased significantly in the presence of 0.3–10 mM MeNH₂, resulting in a maximum increase of 181.6 ± 9.1% compared with control cells treated with buffer alone (Table 1). In all cases, the percentage of vacuolation induced by NH₄Cl was significantly less than with an equal concentration of MeNH₂ (Table 1).

When RGM1 cells were treated with L-Gln in the presence of 30 mM NH₄Cl for 6 h, vacuolation was reduced in a concentration-dependent manner (Fig. 4A). The vacuolation induced by NH₄Cl decreased significantly with 0.02, 0.2, 2, and 20 mM L-Gln (Fig. 4A). Treatment with L-Gln also resulted in a decrease in vacuolation induced by 3 mM MeNH₂ (Fig. 4B). L-Gln significantly reduced MeNH₂-induced vacuolation at 0.2, 2, and 20 mM (Fig. 4B).

Time course experiments with 3 mM MeNH₂ showed that vacuolation increased rapidly for the first hour and then increased slowly 2–6 h thereafter (Fig. 5A). Treatment of RGM1 cells with L-Gln in the presence of MeNH₂ significantly reduced vacuolation (by > 60%) in RGM1 cells (Fig. 5A). However, some vacuolation was always present with MeNH₂ and L-Gln, compared with control cells incubated with buffer alone (Fig. 5A).

Time course experiments revealed that the formation of vacuoles induced by 30 mM NH₄Cl was slower than with MeNH₂, whereby vacuoles increased rapidly for the first 2 h and then increased slowly for 2–6 h thereafter (Fig. 5B). When RGM1 cells were treated with 30 mM NH₄Cl in the presence of 20 mM L-Gln, the response to L-Gln was significantly different than described in Fig. 5A for MeNH₂ and L-Gln (L-Gln-dependent response). For the first 2 h, vacuole formation with 30 mM NH₄Cl was significantly reduced by 20 mM L-Gln (Fig. 5B) and would be a “predicted response” if the L-Gln-dependent response were to continue through the 6-h experiment. However, there was an actual decline in vacuole formation 2–6 h after the addition of 20 mM L-Gln to cells that were treated with 30 mM NH₄Cl. In fact, if the decline in vacuolation were to be extrapolated to the x-axis in Fig. 5B, these data would suggest that, after 10 h of incubation with NH₄Cl and L-Gln, vacuolation would be the same as in control cells treated with buffer alone.

**Table 1. Vacuolation of RGM1 cells by NH₄Cl or MeNH₂**

<table>
<thead>
<tr>
<th>Concentration, mM</th>
<th>NH₄Cl</th>
<th>MeNH₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0)</td>
<td>0.0 ± 3.2</td>
<td>0.0 ± 2.7</td>
</tr>
<tr>
<td>0.3</td>
<td>4.3 ± 2.4</td>
<td>11.5 ± 1.0*</td>
</tr>
<tr>
<td>1</td>
<td>06.6 ± 3.7</td>
<td>19.9 ± 2.0*</td>
</tr>
<tr>
<td>3</td>
<td>14.3 ± 4.4*</td>
<td>102.6 ± 2.7*</td>
</tr>
<tr>
<td>10</td>
<td>42.8 ± 5.6*</td>
<td>181.6 ± 9.1†</td>
</tr>
<tr>
<td>30</td>
<td>86.4 ± 4.5*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. *Significant increase in vacuolation compared with control cells incubated with buffer alone, P < 0.05. †Significant increase in vacuolation compared with control cells, P < 0.05. ††Significant reduction in vacuolation compared with cells incubated with NH₄Cl (A) or MeNH₂ (B) alone, P < 0.05.
that was unchanged by incubation with STD buffer for 6 h (Fig. 6A). By 24 h after the addition of STD buffer, some cell death occurred, as demonstrated by cell rounding and loss of attachment to the culture dish (Fig. 6B). Cultures incubated for 6 and 24 h in STD buffer containing 20 mM L-Gln were nearly identical to cultures incubated in STD buffer alone (Fig. 6, C and D). In contrast, RGM1 cells incubated with 30 mM NH₄Cl or 3 mM MeNH₂ showed significant vacuolation by 6 h (Fig. 6, E and G). By 24 h in NH₄Cl or MeNH₂, >70 and 60%, respectively, of cells were rounded and/or detached from the culture plate (Fig. 6, F and H). In cultures incubated with NH₄Cl or MeNH₂ containing L-Gln for 6 h, vacuolation was significantly reduced (Fig. 6, I and K). In cultures incubated with NH₄Cl or MeNH₂ containing L-Gln for 24 h, cultures were confluent with cell rounding and detachment

Fig. 5. Time course analysis of MeNH₂- (A) and NH₄Cl-induced vacuolation (B) of RGM1 cells in the presence or absence of L-Gln. RGM1 cells were incubated with 20 mM L-Gln in the presence of 3 mM MeNH₂ (A), 30 mM NH₄Cl (B), or STD buffer alone (control) for 6 h, and the rate of vacuolation was quantified by the neutral red uptake assay. Values are means ± SE of 4 wells from 3 different experiments, and the data are expressed as the change in optical density (OD) compared with the initial value at 0 h. The results demonstrate that L-Gln significantly decreases the rate of vacuolation in RGM1 cells. In addition, the data in B show that, when RGM1 cells are incubated with NH₄Cl and L-Gln, the L-Gln-dependent response, identified in A, is greater than the "predicted response" to L-Gln. Thus there is a significant difference in the response of MeNH₂ and NH₄Cl to L-Gln. *Significant decrease in vacuolation compared with cells treated with MeNH₂ (A) or NH₄Cl (B) alone, P < 0.05.

Fig. 6. Phase-contrast images of RGM1 cells that were incubated with or without L-Gln in the absence or presence of NH₄Cl or MeNH₂. A and B: RGM1 cells maintained a confluent monolayer in STD buffer for 6 and 24 h, respectively. At 24 h, some dying cells (arrows) were present in the monolayer from control cells. C and D: L-Gln alone resulted in no change in the monolayer of cells for 6 and 24 h, respectively. Like in control cells, some dying cells (arrows) were present in the monolayer at 24 h.

E and G: addition of 30 mM NH₄Cl or 3 mM MeNH₂, respectively, for 6 h resulted in the vacuolation of RGM1 cells (arrowheads). F and H: by 24 h, the number of adherent cells decreased significantly in the presence of 30 mM NH₄Cl or 3 mM MeNH₂, respectively. Many cells were rounded (arrows), and the few remaining attached cells (arrowheads) had large vacuoles. I and K: L-Gln at 20 mM protected RGM1 cells against NH₄Cl- or MeNH₂-induced vacuolation, respectively, at 6 h. The number of rounded and detached cells (arrows) with L-Gln was not different from that of control cells in B. Bar = 20 µm.
from the culture dish not significantly different from that of control cultures (Fig. 6, J and L).

Identification of the mechanism(s) by which L-Gln protects RGM1 cells in the presence of NH₄Cl and MeNH₂. To determine the mechanism by which L-Gln improves viability and decreases the vacuolation of RGM1 cells, we studied two potential pathways. First, we investigated whether L-Gln inhibits the intracellular accumulation of MeNH₂ in RGM1 cells. The intracellular accumulation of MeNH₂, and not NH₃, was done because radiolabeled [¹⁵N]NH₃ is not commercially available. In addition, MeNH₂ is a weak base with no potential for entry into an intracellular metabolic pathway, so that intracellular reduction in weak base concentration by metabolism is not a factor in the experiment. Second, we determined whether RGM1 cells, like liver or brain cells, utilize the urea cycle and/or glutamine synthetase, with or without L-Gln, as potential NH₃ detoxification pathways. NH₃ detoxification would reduce the intracellular concentration of NH₃ in cells, resulting in less cell death and vacuolation.

L-Gln reduces the intracellular accumulation of MeNH₂ in RGM1 cells. When RGM1 cells were incubated with 3 mM MeNH₂, containing 0.5 μCi of [¹⁴C]MeNH₂, accumulation of MeNH₂ was 0.28 ± 0.13 μmol·5 × 10⁵ cells/well (Fig. 7). When RGM1 cells were treated with 3 mM MeNH₂ containing L-Gln, [¹⁴C]MeNH₂ accumulation was reduced in a concentration-dependent manner (Fig. 7). Treatment with L-Gln resulted in a significant reduction in the accumulation of [¹⁴C]MeNH₂ by 9.8 ± 2.7, 30.0 ± 3.6, and 45.8 ± 3.2% for 0.2, 2.0, and 20 mM L-Gln, respectively, compared with 3 mM MeNH₂ alone. Incubation with STD buffer containing 0.5 μCi [¹⁴C]MeNH₂ and no additional L-Gln resulted in little accumulation of [¹⁴C]MeNH₂ (0.021 ± 0.001 μmol·5 × 10⁵ cells/well) or vacuolation (data not shown) in RGM1 cells.

The intracellular concentration of a weak base is regulated by the equilibrium between entry (uptake) and extrusion. We showed in Fig. 7 that L-Gln reduced the accumulation of MeNH₂ in RGM1 cells. However, these results cannot distinguish between inhibited uptake or facilitated extrusion. To examine the role of L-Gln in facilitated extrusion of weak bases, we used RGM1 cells preloaded with NH₄Cl or MeNH₂.

Preloading RGM1 cells for 6 h with 30 mM NH₄Cl or 3 mM MeNH₂ caused a significant increase in vacuolation (195.6 ± 2.8 and 213.6 ± 2.2%, respectively) compared with control cells incubated with STD buffer alone (Fig. 8, A and B, respectively). When NH₄Cl or MeNH₂ preloaded cells were incubated for 1 h with STD buffer alone, vacuolation decreased by 64.5% in NH₄Cl-treated cells and 78.6% in MeNH₂-treated cells (Fig. 8, A and B, respectively). This reduction in vacuolation reflects a decrease in the intracellular concentration of NH₄Cl or MeNH₂ due to diffusion into the culture medium (10), resulting in smaller vacuoles. When the preloaded RGM1 cells were incubated with STD buffer containing 0.2–20 mM L-Gln, vacuolation was significantly reduced in a concentration-dependent manner (Fig. 8). In both NH₄Cl and MeNH₂ preloaded cells, vacuolation was significantly reduced by 0.2, 2, and 20 mM L-Gln (Fig. 8, A and B, respectively). Substitution of 20 mM mannitol for 20 mM L-Gln, to control for the osmotic effects of L-Gln, resulted in no reduction in the size of vacuoles compared with STD buffer alone (20 mM mannitol is 34.8 ± 5.0% vs. STD buffer of 35.0 ± 1.0%). Thus these results demonstrate that L-Gln stimulates the extrusion of both NH₄Cl and MeNH₂ from RGM1 cells.

Production of urea contributes to L-Gln-induced protection against NH₄Cl but not MeNH₂ in RGM1 cells. To determine whether gastric epithelial cells are protected from NH₃ (but not methylamine) cytotoxicity by utilizing NH₃ to form urea, we blocked arginase activity, a key enzyme in the urea synthetic pathway, with nor-NOHA. In RGM1 cells treated with 30 mM NH₄Cl, cell viability was reduced significantly in the presence of 0.01–1 mM nor-NOHA (Fig. 9A). In fact, cell viability was reduced to 5.5 ± 0.8% in the presence of 1 mM nor-NOHA, a concentration that did not affect cell viability in the absence of NH₄Cl (Fig. 9A). When RGM1 cells were treated with NH₄Cl in the presence of 20 mM L-Gln and nor-NOHA, protection induced by L-Gln was abolished (Fig. 9B). In contrast, nor-NOHA (at 1 mM) had no effect on viability in the presence of MeNH₂ (Fig. 9C) or on L-Gln-induced protection against MeNH₂ (Fig. 9D). RGM1 cells in STD buffer had arginase activity (87.2 ± 3.1 μU/ml) that increased significantly in the presence of NH₄Cl (112.9 ±
nor-NOHA blocked arginase activity in a dose-dependent manner by 39.9, 63.5, and 98.0% at 0.01, 0.1, and 1 mM, respectively, in the presence of NH₄Cl, and by 38.3, 71.6, and 100.3% at 0.01, 0.1, and 1 mM, respectively, in the presence of NH₄Cl and L-Gln.

**L-Glu improves the viability of RGM1 cells in the presence of NH₄Cl but does not improve viability by the conversion of L-Glu and NH₃ to L-Gln.** Treatment of RGM1 cells with L-Glu prevented the reduction in cell viability induced by 30 mM NH₄Cl in a concentration-dependent manner (Fig. 10A). In the presence of NH₄Cl, significant protection occurred with 0.02, 0.2, 2, and 20 mM L-Glu (Fig. 10A). In fact, 20 mM L-Glu completely (100.3 ± 1.1% of the initial value) protected RGM1 cells that were incubated with 30 mM NH₄Cl (Fig. 10A).

To determine whether the conversion of L-Glu and NH₃ to L-Gln contributes to protection by L-Glu, we blocked this conversion with MS (Fig. 10B), a potent inhibitor of glutamine synthetase activity (52). If the conversion of L-Glu and NH₃ to L-Gln is involved in NH₃ detoxification, blockade of the pathway with MS would reduce viability in the presence of L-Glu. However, we found that there was no significant difference in survival with 30 mM NH₄Cl and 20 mM L-Glu containing 0.1–10 mM MS compared with RGM1 cells incubated with 30 mM NH₄Cl and 20 mM L-Glu alone (Fig. 10B). These results demonstrate that RGM1 cells do not convert L-Glu and NH₃ to L-Gln to protect against NH₃-induced cell death.

**DISCUSSION**

The present study shows that apical exposure of gastric surface epithelial (RGM1) cells to NH₃ significantly reduces cell viability within 24 h. The mean concentration of NH₃ (measured as NH₄⁺) in the gastric juice of *H. pylori*-infected patients is from 3.4 to 22.8 mM (13, 23, 24, 27, 32, 34, 35, 46, 50, 58) but may be much higher next to surface epithelial cells, because most *H. pylori* colonize the adherent mucous layer in vivo. Our study demonstrates that surface epithelial cells possess an active NH₃ detoxification pathway that provides some level of protection against NH₃-induced cytotoxicity. When the luminal NH₃ concentration increases, our results demonstrate that L-Gln facilitates NH₃ detoxification and improves cell survival.

Although our work and other reports conclude that a high concentration of NH₃ is cytotoxic to cells in vitro, several findings that address the role of NH₃ or NH₄Cl in vivo are not consistent with these results. For instance, intragastric administration of urea (6%)/urease (100 units) or concentrations of NH₄Cl up to 3% (560 mM, pH 4.8 or pH 8.0) for 1 h in vivo caused no damage to epithelial or other cells in the stomach (44). Tsuji et al. (49) showed that 187.5–250 mM of NH₃ decreased oxygen consumption, energy charge, and the survival of isolated mucosal cells, but the same concentrations of NH₄Cl at pH 7.4 in vivo did not (48). From these results, it is tempting to conclude that NH₃ plays no role in gastric epithelial injury under physiological conditions. However, we propose that the intact mucosa must be incubated with NH₄Cl for many hours before cell death is evident. To support this contention, the present study shows that RGM1 cells must be incubated with a high concentration of NH₄Cl (from the luminal surface) for at least 12 h before NH₃ initiates cell death. Although it is not known why it
takes NH₄Cl so long to kill cells from the luminal surface, parietal and chief cells in gastric glands have a permeability barrier to NH₃ (3) that may also occur in surface epithelial cells. Thus, with the slow paracellular flux of a weak base that occurs in gastric tissues (18), NH₃ may move from the lumen to the basolateral compartment and kill cells after entry from the basolateral surface. Alternatively, our results suggest that gastric surface cells are protected from NH₃ by the metabolic elimination of NH₃ to urea, via arginase activity. The rate at which arginase metabolizes NH₃ may determine ultimate cell fate.

That gastric surface epithelial cells can metabolize NH₃ to urea is a concept demonstrated, for the first time, in the present study. The use of an intracellular detoxification pathway in RGM1 cells was suggested in our study because cell death occurs with ~10-fold higher concentrations of NH₄Cl than MeNH₂, a primary amine weak base with similar properties to NH₄Cl. In addition, NH₄Cl and MeNH₂ should cause the same degree of vacuolation due to similar properties as weak bases, but it requires 10-fold higher concentrations of NH₄Cl to cause the same degree of vacuolation as with MeNH₂. These results are even more significant if the weak base concentration is taken into consideration, where the NH₃ concentration in 30 mM NH₄Cl (pH 7.4) is 0.44 and the MeNH₂ (weak base) concentration in 3 mM MeNH₂ (pH 7.4) is 0.0017 mM. Because NH₃ can enter intracellular metabolic pathways and MeNH₂ cannot, we suggest that the metabolism of NH₃ to urea must lower the effective concentration of NH₃ in cells, causing less vacuolation and cytotoxicity.

The results presented here demonstrate that gastric RGM1 cells have arginase activity that is inhibitable by nor-NOHA, a selective arginase inhibitor (15, 45). Arginase, an enzyme that catalyzes the hydrolysis of L-arginine to urea and L-ornithine, is a key enzyme in NH₃ detoxification via the urea cycle (22). Arginase exists in two isoforms. Arginase I, a cytosolic enzyme, is expressed exclusively in liver as a component of the urea cycle (16, 22, 38). In contrast, arginase II is a mitochondrial enzyme that is expressed in many tissues, including the stomach (16, 38). Compared with that in the intestine and liver, arginase activity is extremely low in the stomach, and the glandular stomach (as a whole) produces very little urea (19). We also found this to be true in our study, because urea production by RGM1 cells, even in the presence of NH₄Cl, was below detectable levels using the commercial urea
assay kit (Sigma). This finding was not surprising, because the urea kit measures between 1,650 and 3,300 μM of urea (37), a concentration that can easily be measured in blood, urine, and liver, a tissue that produces urea at a rate of 158 μmol·min⁻¹·g⁻¹ of tissue (19). Because the RGM1 cells in our study produced urea at a rate of 1 nmol·min⁻¹·g⁻¹ of cells, it would take 27.5 h to generate enough urea to measure using the commercial urea assay kit, which would not be possible in the presence of NH₄Cl. Thus it was necessary to use a radioactive procedure, developed by Ruegg and Russell (39), to measure urea that is produced (by arginase activity) by the conversion of L-[guanido-¹⁴C]arginine to [¹⁴C]urea. Byrne et al. (4) showed that arginase activity in the stomach is found predominantly in a low-density fraction that contains 84 ± 2% parietal cells. Our study shows that arginase II activity must be present in surface epithelial cells and that arginase II activity may increase in the presence of L-Gln or other amino acids that regulate urea cycle activity. In the liver, there are five urea cycle enzymes that contribute to the synthesis of urea for NH₃ detoxification (38). Because no other urea cycle intermediates have been described in gastric tissues, further studies will be necessary to complete our understanding of the active components of the urea cycle in gastric mucosal cells.

In this study, we show that L-Gln protects RGM1 cells against NH₄Cl-induced cell death. It is noteworthy that protection was observed even at very low concentrations of L-Gln (0.2 mM), as shown in Fig. 3A. Because the plasma level of L-Gln is 0.5–0.8 mM (52), physiological concentrations of L-Gln may protect against the cytotoxic effects of NH₃ in daily life. In general, L-Gln is involved in a wide variety of metabolic processes, such as the synthesis of proteins and nucleotides, and in energy metabolism (41, 59). L-Gln plays an essential role in intestinal mucosal protection in many animal models of critical illness, including burns, trauma, obstruction, radiation damage, cytotoxic chemotherapy, and sepsis (5, 11, 28). Cellular ATP levels are maintained in the presence of L-Gln, which protects mitochondria from damage and partially protects α-ketoglutarate dehydrogenase activity in the TCA cycle (1). L-Gln also induces heat shock protein expression to protect cells against injury (54, 56). Furthermore, L-Gln reduces the expression of proinflammatory cytokines (55), which may reduce inflammatory cell-induced mucosal damage in vivo. Although it is not known how L-Gln protects against the cytotoxic effects of NH₄Cl, our study suggests that it inhibits the uptake and/or facilitates the extrusion of NH₃ from cells and increases cellular metabolism of NH₃ via arginase. Although L-Glu does not protect RGM1 by NH₃ detoxification via glutamine synthetase activity per se, it may act to increase cellular metabolism and ATP production, inhibit uptake and/or increase extrusion, or facilitate some other process that facilitates protection against NH₃.

The results presented here clearly show that L-Gln protects cells differently in the presence of NH₄Cl than in the presence of MeNH₂. Our data suggest that L-Gln, in some way, accelerates cellular NH₃ metabolism to reduce both the intracellular concentration of NH₃ and NH₃ cytotoxicity. Our data in Fig. 5B suggest that

Fig. 10. Effect of L-glutamate (L-Glu) with or without methionine sulfoximine (MS) on cell viability in the presence of NH₄Cl. A: RGM1 cells were incubated with 30 mM NH₄Cl with or without 0.02–20 mM L-Glu for 24 h, and the number of viable cells was quantified by the crystal violet assay. The data show that L-Glu protects RGM1 cell viability in the presence of NH₄Cl in a dose-dependent manner. B: RGM1 cells were incubated with 30 mM NH₄Cl and 20 mM L-Glu in the presence of 0.1–10 mM MS for 24 h, and the number of viable cells was quantified by the crystal violet assay. The data show that there was no difference in viability with NH₄Cl, L-Glu, and MS compared with RGM1 cells incubated in NH₄Cl and L-Glu alone. Values represent means ± SE of 4 wells from 3 different experiments and are expressed as the percentage of control cells that were incubated with STD buffer alone. *Significant decrease in viability compared with control cells, P < 0.05. †Significant increase in viability compared with cells incubated with 30 mM NH₄Cl without L-Glu, P < 0.05.
accelerated NH₃ metabolism occurs 2 h after the addition of NH₄Cl and L-Gln to RGM1 cells. In addition, blockade of urea production with nor-NOHA completely reversed the protective effect of L-Gln (against NH₄Cl-induced death), suggesting that NH₃ detoxification via arginase activity is paramount in protecting RGM1 cells against NH₄Cl-induced cell death. In contrast, even though L-Gln completely protects against the cytotoxic effects of MeNH₂, our data clearly show that urea cycle activity is not involved in L-Gln protection against MeNH₂. This is because nor-NOHA did not reverse the protective effect of L-Gln against MeNH₂-induced cell death. If exposure to L-Gln protected RGM1 cells solely by extrusion of weak base, increased expression of heat shock proteins, and/or by increased cellular ATP production, it is likely that protection would be similar with both NH₄Cl and MeNH₂. Thus the differential effect of weak bases on viability in RGM1 cells may lend important insights into the mechanism by which L-Gln protects against injury in gastric and other tissues.

In summary, we demonstrate that L-Gln and L-Glu protect gastric epithelial RGM1 cells against NH₄Cl-induced cell death. Because L-Gln administration is used routinely in human patients (2), it is possible that L-Gln alone or in combination with L-Glu would be effective as a therapeutic treatment for gastric epithelial damage induced by NH₃ during H·pylori infection.

The authors thank Sarah W. Morrison for technical help with cell culture and Marianne Smith and Dr. Kimito Tashima for critical evaluation of the manuscript. We are especially grateful to Dr. Koji Takeuchi for critical reading of the manuscript and for helpful discussions concerning the results of this study.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants R01-DK-15681 (to S. J. Hagen) and DK-34854 (to Harvard Digestive Diseases Center).

REFERENCES


