Published in final edited form as:

J Leukoc Biol. 2006 June ; 79(6): 1214–1225.

Role of urease in megasome formation and *Helicobacter pylori* survival in macrophages

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Abstract

Previous studies have demonstrated that *Helicobacter pylori* (Hp) delays its entry into macrophages and persists inside megasomes, which are poorly acidified and accumulate early endosome autoantigen 1. Herein, we explored the role of Hp urease in bacterial survival in murine peritoneal macrophages and J774 cells. Plasmid-free mutagenesis was used to replace *ureA* and *ureB* with *cat* in Hp Strains 11637 and 11916. *ureAB* null Hp lacked detectable urease activity and did not express UreA or UreB as judged by immunoblotting. Deletion of *ureAB* had no effect on Hp binding to macrophages or the rate or extent of phagocytosis. However, intracellular survival of mutant organisms was impaired significantly. Immunofluorescence microscopy demonstrated that (in contrast to parental organisms) mutant Hp resided in single phagosomes, which were acidic and accumulated the lysosome marker lysosome-associated membrane protein-1 but not early endosome autoantigen 1. A similar phenotype was observed for spontaneous urease mutants derived from Hp Strain 60190. Treatment of macrophages with bafilomycin A1, NH₄Cl, or chloroquine prevented acidification of phagosomes containing mutant Hp. However, only ammonium chloride enhanced bacterial viability significantly. Rescue of *ureAB* null organisms was also achieved by surface adsorption of active urease. Altogether, our data indicate a role for urease and urease-derived ammonia in megasome formation and Hp survival.

Keywords

phagocytosis; phagosome maturation; ammonia

INTRODUCTION

Helicobacter pylori (Hp) is a microaerophilic, Gram-negative bacterium that colonizes the gastric mucosa of ~50% of all humans [1]. Chronic infection with this organism causes a broad spectrum of disease, which includes gastritis, peptic ulceration, and gastric cancer [1]. Virulence factors that modulate Hp survival in the hostile environment of the stomach include urease, which generates ammonia and is essential for colonization; flagella, which propel bacteria through gastric mucus; the *cag* pathogenicity island, which encodes a type IV secretion apparatus; and VacA, a secreted cytotoxin that damages the epithelium and impairs lymphocyte function [1].

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Phagocytosis is an element of the innate immune response, important for killing invading microbes [2,3]. A characteristic feature of Hp-induced inflammation is the robust accumulation of phagocytes in the gastric mucosa. Although the ability of Hp to thrive in a phagocyte-rich environment is well-documented, how bacteria evade elimination by the innate immune response is only partially understood [4–6]. The results of in vivo and in vitro studies indicate that unopsonized Hp are engulfed by macrophages and neutrophils, but only ~50% of ingested organisms are killed [4,5,7–14]. We have shown previously that the ability of Hp to escape killing by macrophages is associated directly with the ability of these organisms to activate atypical protein kinase C- ζ (PKC ζ) and delay phagocytosis [4,5,9]. Thereafter, Hp phagosomes undergo clustering and homotypic fusion, and bacteria survive inside the resulting megasomes for at least 24 h [4]. At the same time, Hp inhibit phagosome maturation, and megasomes accumulate coronin and early endosome autoantigen 1 (EEA1) but not acidotropic dyes or the late endosome/lysosome marker lysosome-associated membrane protein-1 (lamp-1) [10]. VacA regulates, in part, blockade of phagosome-lysosome fusion [10]. In contrast, the virulence factors that regulate megasome formation are unknown. Ultimately, infected macrophages undergo apoptosis and viable bacteria are liberated [15,16]. In view of these data, it has been suggested that Hp occupy a protected intra-cellular niche that favors bacterial persistence and contributes to treatment failure [4,8,17].

In addition to its role in colonization, urease regulates Hp-macrophage interactions. This enzyme is a chemotactic agent that recruits macrophages to the infected stomach [18]. Urease also regulates phagocytosis via its ability to retard opsonization [19]. Whether urease affects intracellular survival of Hp is unknown. In this study, we created *ureAB* null strains of Hp and tested the hypothesis that urease is essential for Hp survival inside macrophages.

MATERIALS AND METHODS

Macrophage cultivation

Resident peritoneal macrophages obtained from female CD-1 mice (Charles River Laboratories, Wilmington, MA) and cells of the murine macrophage cell line J774 [obtained from the American Type Culture Collection (ATCC), Manassas, VA] were cultured as described [4]. Where indicated, the culture medium was supplemented with 10 mM NH₄Cl, 10 μ M chloroquine, or 100 nM bafilomycin A1 (obtained from Sigma-Aldrich, St. Louis, MO) 15 min prior to infection.

Hp strains and cultivation

Hp strains 11637 [20], 11916 [21], 60190 [20], and a spontaneous urease-deficient mutant derived from 60190 (Strain 4 [22]) were obtained from ATCC. Bacteria were grown under microaerophilic conditions on pH 7.2 trypticase soy agar plates containing defibrinated sheep blood as described [4]. Bacteria were harvested into cold phosphate-buffered saline (PBS), washed twice, and then resuspended in buffer or tissue culture medium. To obtain growth curves, Hp were cultured in brain heart infusion broth containing 5% fetal bovine serum, and the optical density at 600 nm was monitored. Bacterial motility was assessed qualitatively using light microscopy.

Generation of *ureAB* null mutants

The plasmid-free, allelic replacement mutagenesis method of Tan and Berg [23] was used to create Hp mutants in which *ureA* and nearly all of *ureB* of Hp strains 11637 and 11916 were deleted and replaced with *cat*. DNA encoding the chloramphenicol (Cm)-resistance cassette was the generous gift of Dr. Daniel Berg (Washington University Medical School, St. Louis, MO). Oligo-nucleotides for homologous recombination were generated using polymerase chain reaction (PCR) and ligated to the *cat* cassette exactly as described [23]. After purification

and DNA sequencing, the allelic replacement construct was introduced into Hp 11637 and 11916 by natural transformation [24]. Thereafter, transformants were selected on blood agar plates containing 20 µg/ml Cm. Single colony isolates of Cm-resistant Hp were expanded on selective media. Diagnostic PCR analysis demonstrated that *ureAB* had been replaced with *cat* in all Cm-resistant clones.

Immunoblotting

Bacteria were lysed in radio immunoprecipitation assay (RIPA) buffer containing protease inhibitors [9], and samples were normalized for protein content (Pierce Coomassie Plus kit, Pierce, Rockford, IL). Proteins in each lysate were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes, and probed for UreA, UreB, CagA, or VacA using antibodies obtained from Austral Biologicals (San Ramon, CA). Secondary antibodies were conjugated to horseradish peroxidase, and bands were visualized using enhanced chemiluminescence reagents (Pierce) [9].

Urease activity assays

Urease activity in bacterial lysates was quantified using the Berthelot assay [25,26]. Urease activity in whole bacteria was measured using a modification of the phenol red method of Nagata et al. [27]. Specifically, bacterial suspensions were adjusted to 1×10^7 – 1×10^9 Hp/ml in PBS. Each suspension (50 µL) was mixed with 50 µl phenol red test solution (300 mM urea and 0.025% phenol red in PBS containing Ca^{++} and Mg^{++} , pH 7.2) and incubated at 37°C. Urease activity was observed by monitoring the change in absorbance at 550 nm over 70 min. Postive controls contained Jack bean urease (Fluka, 102 U/mg), and negative controls contained boiled organisms or boiled lysates.

To assess the capacity of mutant bacteria to adsorb active urease, wild-type or mutant Hp were suspended in brain heart infusion broth containing protease inhibitors [9] and disrupted by sonication. Each sonicate was clarified by centrifugation (8000 rpm, 3 min, 4°C) and then sterilized by passage through 0.2 µm filters. Thereafter, mutant organisms were incubated with filtered wild-type (urease-positive) or mutant (urease-negative) sonicates or Jack bean urease at 37°C for 60–90 min and then washed three times with PBS or tissue culture medium. Urease adsorption was assessed by immunoblotting with anti-UreB antibodies, and enzyme activity was measured as described above.

Phagocytosis and intracellular killing

Phagocytosis of Hp by macrophages attached to glass coverslips was synchronized using centrifugation as we described [4,9]. Bacteria were added to macrophages at a ratio of 10:1–25:1. Bacterial binding and rate of phagocytosis were determined using differential staining [9]. Phagocytic killing was quantified by enumeration of colony-forming units (CFU) [4,9].

Immunofluorescence microscopy

Infected macrophages were processed for microscopy using our established methods [4,9, 28]. Hp were detected using rabbit anti-Hp and goat anti-Hp polyclonal antibodies (pAb; Accurate Chemical and Scientific Corp., Hicksville, NY, and Kirkegaard and Perry Laboratories, Gaithersburg, MD, respectively) or mouse anti-Hp lipopolysaccharide monoclonal antibodies (mAb; BioSource International, Camarillo, CA). Rat anti-lamp-1 mAb (Clone 1D4B) were from the Developmental Studies Hybridoma Bank at the University of Iowa (Coralville). pAb to EEA1 were from Santa Cruz Biotechnology (CA). Secondary antibodies conjugated to fluorescein isothiocyanate or rhodamine were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Coverslips were mounted to glass slides in

gelvatol, and samples were examined using an Axioplan2 fluorescence microscope or using an LSM-510 laser-scanning confocal microscope (both from Carl Zeiss Inc., Thornwood, NY). The profile and thresholding functions of the LSM-510 software were used to quantify the intensity of EEA1 and lamp-1 staining on endosomes or phagosomes for 35–50 samples in each category in triplicate as we described previously [29].

Bacteria and acidic endosomes of macrophages were visualized using acridine orange (AO; Molecular Probes, Eugene, OR). Hp were stained green by incubation in medium containing 1 µg/ml AO for 10 min at 22°C. Washed bacteria were used to infect macrophages whose acidic endosomal compartments were stained red by incubation in medium containing 2 µg/ml AO for 20 min. Alternatively, macrophages were labeled with LysoTracker Red (LT; Molecular Probes) for 90 min at 37°C and counter-stained with 0.3 µM 4',6-diamidino-2-phenylindole (Molecular Probes) after infection.

Detection of inducible nitric oxide synthase (iNOS)

J774 cells were left untreated or stimulated with live Hp [multiplicity of infection (MOI) 20:1] or an equivalent amount of bacterial sonicate for 1–24 h at 37°C. Macrophages were washed twice with cold PBS and then disrupted in RIPA lysis buffer as described above. Samples were separated by SDS-PAGE, transferred to PVDF membranes, and processed for immunoblotting. Rabbit antibodies directed against iNOS were from BD PharMingen (San Diego, CA).

Statistical analysis

Paired samples were compared by Student's *t*-test, and groups were compared by ANOVA. $P < 0.05$ was considered significant.

RESULTS

Generation of ureAB null Hp mutants

Plasmid-free allelic exchange mutagenesis [24] was used to create Hp mutants, in which *ureA* and *ureB* were replaced with *cat* [23]. DNA was introduced into Hp 11637 and 11916 by natural transformation, and resistant organisms were selected on plates containing Cm. Four independent transformants obtained from Hp 11637 (designated M1–M4) and three transformants isolated from Hp 11916 (M9–M11) were analyzed. Immunoblotting demonstrated that wild-type bacteria contained high levels of both urease structural subunits (Fig. 1A). By contrast, neither UreA nor UreB was detected in the transformants (Fig. 1A). The urease activity of Hp 11637 and 11916 was 11.04 ± 2.65 µmol/min/mg and 9.94 ± 3.12 µmol/min/mg, respectively. However, the urease activity of the mutants was reduced at least 20-fold and was undetectable by the Berthelot assay. As judged by the phenol red assay (Fig. 1B), the urease activity of whole Hp 11637 was dose- and time-dependent and compared favorably with Jack bean urease (Fig. 1C). In contrast, urease activity was undetectable in mutants M1–M4, even when the number of organisms included in the reaction mixtures was increased tenfold (Fig. 1B). Comparable data were obtained for strain 11916 and mutants M9–M11 (not illustrated). None of the mutants exhibited defects in growth or motility, and production of other virulence factors including CagA and VacA was not affected (Fig. 1A).

Urease mutants exhibit impaired survival inside macrophages

Having established that our Cm-resistant bacteria were urease-deficient, we asked whether loss of this virulence factor affected intracellular survival. Hp binding and uptake were measured using differential staining [9], and intramacrophage survival was quantified by enumeration of CFU [4,9]. All urease null mutants adhered normally to macrophages, and the rate and extent of phagocytosis were indistinguishable from wild-type bacteria (not illustrated). By contrast,

intracellular survival of the mutants was impaired significantly (Fig. 1D). Within 4 h of infection with M1 or M4, macrophages contained many degraded bacterial fragments and few intact organisms (Fig. 1E). Similar data were obtained for the other mutants. As all the mutants had a similar phenotype, additional analyses used only Hp 11637 and M1 or M4.

Urease mutants cannot prevent phagosome maturation and are not found in megasomes

Shortly after engulfment, Hp phagosomes undergo clustering and homotypic fusion [4]. Only live, metabolically active Hp trigger megasome formation, and residence in these organelles correlates directly with survival [4]. In addition, Hp disrupts phagosome maturation, and megasomes accumulate the early endosome marker EEA1 but not acidotropic dyes or the late endosome/lysosome marker lamp-1 [10]. As urease null Hp were killed by macrophages, we predicted that these organisms may have lost the ability to block phagosome maturation. To test this hypothesis, we used confocal microscopy to compare the composition of phagosomes containing wild-type and mutant bacteria. To analyze phagosomes containing mutant bacteria prior to significant bacterial digestion (as shown in Fig. 1E), microscopy analyses used macrophages infected for 30–90 min.

Phagosome acidification was assessed by staining live cells with AO or LT. Concordant with published data [10], fewer than 10% of Hp 11637 phagosomes were acidic (Fig. 2A). Conversely, $66.3 \pm 4.2\%$ of phagosomes containing M4 (Fig. 2A) and $68.4 \pm 5.1\%$ of phagosomes containing M1 accumulated AO 90 min after uptake ($P \leq 0.010$ vs. 11637). Comparable data were obtained using LT (not illustrated).

Next, phagosome maturation was assessed using antibodies to EEA1 and lamp-1. Shortly after bacterial engulfment, ~85% of all phagosomes were EEA1-positive (Fig. 2, B and C). However, compartments containing Hp 11637 accumulated significantly more of this protein than did compartments containing *ureAB* mutants (Fig. 2D). As infection progressed, $77.6 \pm 4.5\%$ of wild-type Hp phagosomes retained EEA1, but compartments containing mutant bacteria did not (Fig. 2, B–D). By 90 min, limited amounts of EEA1 were detected on only 22–29% of M1 and M4 phagosomes ($P \leq 0.010$, Fig. 2, C and D).

Coincident with loss of EEA1, phagosomes containing mutant Hp accumulated large amounts of lamp-1 and in this manner, resembled model phagosomes containing latex beads (Fig. 3A). Conversely, wild-type Hp phagosomes acquired only trace/moderate amounts of this late endosome/lysosome marker (Fig. 3A). Specifically, lamp-1 fluorescence was 2.4-fold greater on M1 or latex bead phagosomes than on Hp 11637 compartments and as such, was comparable in intensity with the signal associated with late endosomes and lysosomes (Fig. 3B, $P \leq 0.045$). The microscopy data also indicated that $55.1 \pm 7.3\%$ of macrophages infected with wild-type Hp contained at least one megasome. Conversely, these structures were detected in only 3–4% of cells containing M1 or M4 ($P < 0.010$) and were not present in cells containing latex beads (Fig. 3A). Thus, ablation of urease activity favored phagosome maturation and reduced homotypic phagosome fusion tenfold.

In contrast to our data (Fig. 3A), Zheng and Jones [10] did not detect lamp-1 on wild-type Hp megasomes. This discordance is likely a result of differences in experimental design. For our microscopy experiments, we used cold acetone to permeabilize macrophages, whereas Zheng and Jones [10] used detergent. In our hands, acetone permeabilization enhanced the sensitivity of lamp-1 detection. Moreover, we used resident peritoneal macrophages, but Zheng and Jones [10] used RAW 264.7 cells; lamp-1 may differ in abundance or be distributed somewhat differently in primary macrophages and transformed cell lines. Therefore, we performed additional experiments using the macrophage cell line J774. As shown in Figure 3, C and D, more than 90% of J774 phagosomes containing mutant bacteria acquired lamp-1, but phagosomes containing wild-type Hp did not. Therefore, our data define subtle differences in

the composition of wild-type Hp megasomes in peritoneal macrophages as compared with macrophage cell lines. In either system, deletion of urease-enhanced phagosome maturation and mutant Hp were found in acidic phagolysosomes that were distinct from megasomes containing wild-type bacteria.

Surface adsorption of active urease rescues mutant bacteria

A distinctive feature of Hp urease is the localization of this enzyme to the bacterial surface as well as the cytoplasm [30–32]. Surface urease is acquired by adsorption following “altruistic autolysis” of neighboring organisms, and analysis of bacteria in biopsy samples indicates that 5–62% of this enzyme is surface-associated [30]. Sequential subculturing of Hp increases surface urease activity in vitro and enhances acid resistance [31,32]. In view of these data, we reasoned that adsorption of active urease onto *ureAB* null Hp might confer functional complementation and thereby impact phagosome maturation. To test this notion, we assessed the ability of mutant Hp to adsorb urease from sterile-filtered sonicates prepared from wild-type bacteria. As shown in Figure 4A, M1 acquired active urease from wild-type Hp sonicates as indicated by immunoblotting and the phenol red assay. The amount urease activity adsorbed by M1 was ~2% of the total amount in Hp 11637, and vital staining demonstrated that >98% of bacteria remained viable. Next, we infected J774 cells with these organisms and assessed phagosome composition. After 90 min at 37°C, 94 ± 3% of untreated M1 were found in acidic phagolysosomes (Fig. 4, B–D). Following urease adsorption, only ~27% of M1 phagosomes retained this phenotype (Fig. 4, B–D, “+WT sup” panels). By contrast, 23 ± 3% of M1 compartments resembled wild-type Hp phagosomes, as indicated by accumulation of EEA1 (Fig. 4B) but not AO (Fig. 4C) or lamp-1 (Fig. 4D, arrowheads). The remaining M1 phagosomes (~50%) were acidic and EEA1-negative but acquired only moderate amounts of lamp-1 (pixel intensity 70–189 fluorescence units; Fig. 4D, double arrowheads). Thus, surface adsorption of urease was sufficient to inhibit maturation of ~70% of M1 phagosomes in J774 cells; one-third of these organelles resembled wild-type Hp compartments, and the remainder had a distinct composition that differed from phagosomes containing wild-type or mutant bacteria. Specificity for active urease is indicated by our finding that incubation of M1 with urease-negative bacterial sonicates (Fig. 4, “+M1 sup” panels) was without effect.

Consistent with its impact on phagosome maturation, urease supplementation enhanced intracellular survival of M1 during the first 6 h after phagocytosis (Fig. 4E). Conversely, urease-negative bacterial components (which may have been adsorbed from M1 sonicates) did not confer protection. Nevertheless, the effects of urease supplementation were transient, and bacteria were degraded in phagolysosomes by 24 h. Therefore, adsorbed urease supports significant but transient rescue of mutant Hp.

Ammonium chloride supplementation restores megasome formation and enhances intracellular survival of urease mutants

It has been known for some time that acidification parallels phagosome maturation [33–35], and treatment of macrophages with the lysosomotropic agent NH₄Cl or the vacuolar proton ATPase inhibitor bafilomycin A1 neutralizes endosomal compartments and blocks phagosome-lysosome fusion [34,36,37]. Our data suggest that urease null Hp were killed in acidic phagolysosomes. Therefore, we examined whether artificial neutralization of phagosome pH affected intracellular survival of mutant organisms.

As shown in Figure 5, NH₄Cl ablated phagosome acidification (Fig. 5A), supported phagosome retention of EEA1 (Fig. 5B), and enhanced survival of ingested M1 (Fig. 5D). In addition, NH₄Cl increased megasome formation significantly (Fig. 5C). After 90 min at 37°C, 25.1 ± 8.6% of infected macrophages contained at least one megasome (an eightfold increase relative to cells infected with M1 alone, *P*=0.039). This effect of NH₄Cl was specific for Hp, and

homotypic phagosome fusion did not occur in cells containing latex beads. Like wild-type Hp compartments, NH₄Cl-induced M1 mega-somes were lamp-1-negative in J774 cells and acquired only moderate amounts of lamp-1 in peritoneal macrophages (Fig. 5C, arrowheads). Although bafilomycin A1 also elevated phagosome pH (Fig. 5A) and interfered with phagosome maturation (Fig. 5C, 40–50% reduction in lamp-1-positive phagosomes), this direct blockade of vacuolar proton ATPase activity did not support phagosome retention of EEA1 (Fig. 5B) or megasome formation (Fig. 5C) and did not enhance bacterial survival (Fig. 5D).

To explore this issue in more detail, we performed additional experiments using macrophages pretreated with chloroquine. Like the primary amine NH₄Cl, lipophilic secondary and tertiary amines (such as chloroquine) accumulate inside lysosomes and elevate organelle pH [37,38]. However, chloroquine accelerates maturation of yeast phagosomes and overrides the block in phagosome-lysosome fusion induced by *Mycobacterium tuberculosis* [37,38]. In agreement with these data, we now show that chloroquine neutralized M1 and M4 phagosomes (Fig. 5A) and supported robust accumulation of lamp-1 on these organelles (Fig. 5C). These structures were likely mature phagolysosomes, as they lacked EEA1 (Fig. 5B) and within 90 min of bacterial uptake, contained digested bacterial fragments (Fig. 5C, double arrowheads). Quantitation of CFU confirmed that these organisms were not viable (Fig. 5D). In view of these data, we also assessed the effect of chloroquine on wild-type Hp phagosomes in J774 cells. As shown in Figure 5E, chloroquine markedly increased phagosome acquisition of lamp-1 and favored bacterial fragmentation. Altogether, our data demonstrate that phagosome neutralization per se is not sufficient for Hp survival in macrophages and further suggest a specific role for urease-derived ammonia in megasome formation.

Spontaneous urease mutants do not survive in macrophages

The Hp mutants generated for this study did not survive in macrophages, and in each case, *ureAB* was replaced with *cat*. Our ability to rescue the mutants by surface adsorption of urease or medium supplementation with NH₄Cl suggested that loss of urease activity accounts for the mutant phenotype. Nevertheless, it is possible that insertion of *cat* into the genome impaired Hp virulence by mechanisms unrelated to urease disruption. Therefore, we characterized the phenotype of a spontaneous urease mutant derived from Hp 60190 [22]. This mutant (referred to here as Ure) produces CagA and VacA but not UreA or UreB (Fig. 6A and ref. [22]). Consequently, urease activity was undetectable by the phenol red (Fig. 6B) and Bethelot assays. More importantly, intramacrophage survival of Ure was compromised beginning 1 h after uptake; and nearly all ingested bacteria were eliminated within 4 h (Fig. 6C). Confocal microscopy demonstrated that Ure phagosomes accumulated lamp-1 at the expense of EEA1 (Fig. 6D), and pretreatment of macrophages with NH₄Cl (but not bafilomycin A1 or chloroquine) increased Ure survival fourfold (Fig. 6E). Thus, Ure (which was generated without use of antibiotic-resistance cassettes) and our *ureAB* null mutants have a similar phenotype.

Deletion of *ureAB* does not prevent synthesis of iNOS

Intact Hp and bacterial extracts induce macrophage iNOS, and urease has been implicated in this process [39,40]. To assess the effect of urease deletion on iNOS synthesis, J774 cells were infected with wild-type or mutant Hp at a MOI of 20:1 or treated with an equivalent amount of filtered bacterial sonicate. As shown in Figure 7, deletion of *ureAB* did not prevent iNOS synthesis in response to either stimulus.

DISCUSSION

Urease plays a central role in Hp pathogenesis. This enzyme is essential for bacterial colonization and generates a local ammonia cloud that buffers Hp as they pass through the

gastric lumen [1]. Urease is also a potent macrophage chemoattractant [18], which modulates phagocytosis via its ability to retard opsonization [19]. In this study, we used plasmid-free mutagenesis to delete *ureAB* in Hp strains 11637 and 11916. Loss of urease had no effect on bacterial growth or motility and did not alter expression of other virulence factors. In contrast, we show for the first time that urease plays a key role in modulating phagosome pH and megasome formation and as such, is essential for Hp survival in macrophages.

Targeted deletion or spontaneous mutation of urease in three Hp strains ablated enzyme activity, and ingested organisms were killed rapidly and efficiently, as judged by measurement of CFU and the presence of bacterial debris in the macrophage cytoplasm. Wild-type Hp megasomes resist acidification and accumulate EEA1 [10] (and in this study, Figs. 2 and 6). Conversely, urease mutants were unable to disrupt phagosome maturation and were trafficked to compartments with lysosomal characteristics. Four lines of evidence indicate that loss of urease activity accounts for the mutant phenotype. First, all urease mutants had a similar fate in macrophages, regardless of Hp strain or the presence or absence of *cat* in the genome. Second, mutation of urease had no effect on expression of CagA or VacA. Third, surface adsorption of active urease by mutant organisms was sufficient to prevent phagosome maturation and impair intracellular killing for at least 6 h after uptake. Fourth, sustained rescue of mutant Hp could be achieved by treating macrophages with NH₄Cl. Moreover, the transient rescue achieved by urease adsorption is consistent with the fact that sustained urease synthesis is required to maintain Hp infection. Whether the adsorbed enzyme is ultimately shed or inactivated is unknown.

Phagosome acidification is an important element of host defense. Consequently, artificial elevation of phagosome pH (using NH₄Cl, chloroquine, or bafilomycin A1) impairs killing of *Aspergillus fumigatus* [41], *Staphylococcus aureus* [42], and *Bordetella pertussis* [43]. Conversely, growth and survival of pathogens, which replicate in acidic compartments (such as *Legionella pneumophila*, *Coxiella burnetii*, *Cryptococcus neoformans*, *Leishmania* promastigotes, *Brucella*, and *Salmonella*), are impaired [44–50]. In these studies, all phagosome-neutralizing agents had similar effects on microbe survival [43–48]. By contrast, the results of our work indicate that NH₄Cl prevented intracellular killing of urease null Hp, but bafilomycin A1 and chloroquine did not; as such, our data indicate that phagosome neutralization per se cannot prevent elimination of Hp. Rather, our findings suggest that urease-derived ammonia modulates the intraphagosome environment in a manner that supports Hp survival. Concordant with this hypothesis, chloroquine impaired intramacrophage survival of wild-type Hp, but NH₄Cl did not (Fig. 5E and our unpublished data).

The source of urea used by intracellular Hp has not been identified, but several scenarios can be envisioned. Urea may be synthesized by Hp arginase (RocF) [51,52]. Alternatively, Hp may obtain urea from the culture medium or scavenge urea generated by macrophage arginase II [15]. In addition to its role as an essential Hp nutrient [53], L-arginine is also a substrate of iNOS [51]. We show here that iNOS is synthesized by macrophages 4 h after infection. Nevertheless, wild-type Hp are protected from reactive nitrogen intermediates, as the concerted action of Hp arginase and urease limits NO synthesis via substrate depletion [51]. As our mutants induced iNOS but lacked urease, they may be subjected to higher concentrations of NO than wild-type Hp. However, the fact that nearly all mutant bacteria were killed prior to iNOS synthesis (≤ 4 h) suggests that urease-deficient Hp can be controlled by NO-independent mechanisms. At the same time, we demonstrated previously that urease is not required for diversion of reduced nicotinamide adenine dinucleotide phosphate oxidase components and superoxide away from Hp phagosomes [29].

VacA and urease are the only Hp virulence determinants known to influence phagosome-lysosome fusion in macrophages (ref. [10] and this study). However, neither of these factors

is sufficient, and disruption of *ureAB* or *vacA* enhances phagosome maturation. Therefore, it is tempting to speculate that these proteins act in concert to disrupt membrane trafficking. We show here that urease-derived ammonia elevates phagosome pH and retards acquisition of late endosome markers. VacA has a similar effect [10], but neither its targets nor its effects on macrophage membrane trafficking have been described. Nearly all studies of this cytotoxin have used epithelial cell lines. In this system, purified VacA oligomers are ingested by receptor-mediated endocytosis [54,55], and several hours later, cells accumulate large acidic vacuoles derived from late endosomes [56,57]. In all cases, concomitant exposure of epithelial cells to NH_4Cl is essential for vacuolation [55]. Conversely, the effects of this toxin are inhibited by bafilomycin A1 [58]. Thus, in the context of macrophage infection with whole bacteria, it is likely that urease generates the ammonia required to enhance VacA function. This notion is supported by our finding that intracellular survival of urease null Hp is enhanced by NH_4Cl but not by bafilomycin A1. At present, neither the kinetics of VacA secretion nor its fate in macrophages has been defined.

Aside from a requirement for bacterial protein synthesis and microtubules [4], the mechanism of megasome formation is unknown. Thus, it is of interest that loss of urease impaired phagosome clustering and fusion (Fig. 3), but deletion of *vacA* does not [10]. In 1991, Hart and Young [34] demonstrated that NH_4Cl increases phagosome-endosome fusion in macrophages. Recent studies have shown that EEA1 is a tethering molecule required for endosome clustering and homotypic fusion [59,60]. Taken together, these data suggest that urease-derived ammonia may enhance fusion of nascent Hp phagosomes with EEA1-positive endosomes, and phagosome accumulation of EEA1 may, in turn, facilitate the phagosome clustering and fusion required for megasome formation. In addition to its role as a regulator of endosome size, EEA1 is also required for delivery of newly synthesized lysosomal membrane glycoproteins to early endosomes from the trans-Golgi network [61,62]. Thus, retention of EEA1 on wild-type Hp phagosomes may allow these compartments to acquire small amounts of lamp-1 directly from the biosynthetic pathway in peritoneal macrophages without concomitant phagosome acidification or acquisition of other late endosome/lysosome markers. Conversely, J774 and RAW264.7 cells may resemble HeLa cells, wherein newly synthesized lamp-1 is targeted directly to late endosomes [61]. Regardless of the mechanism, the absence of lamp-1 on Hp megasomes in macrophage cell lines facilitated analysis of the mutant phenotype.

Altogether, the available data suggest the following model: Unopsonized Hp bind tightly to macrophages and activate phosphoinositide 3-kinase and atypical PKC ζ [9,63]. Local actin polymerization ensues after a lag of several minutes, and bacteria are internalized [4,9]. Nascent phagosomes retain coronin and associate with microtubules [4,10]. The concerted actions of arginase and urease allow intraphagosomal Hp to generate ammonia, which elevates the pH of these compartments and sustains phagosome-endosome fusion (refs. [34,51,52] and this study). Phagosome accumulation of EEA1 and the association of these organelles with the cytoskeleton facilitate clustering and homotypic fusion (refs. [4,10] and this study). The early inhibition of phagosome-lysosome fusion conferred by ammonia and phagosome retention of coronin [10,34,63] allows ingested Hp to synthesize and secrete VacA. Urease-derived ammonia enhances VacA activity [55], and this cytotoxin disrupts membrane trafficking at the late endosome stage, thereby preventing phagosome maturation [10]. After 24 – 48 h, viable bacilli are released upon macrophage apoptosis [15,16].

Acknowledgements

We thank Dr. Daniel Berg for providing the chloramphenicol-resistance cassette and for advice regarding plasmid-free mutagenesis. This work was supported by funds from the National Institutes of Health (R01 AI43617) and the Veteran's Administration (Merit Review Grant) to L-A. H. A.

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Fig. 1. Deletion of urease impairs intramacrophage survival. (A) Immunoblots of Hp lysates probed with antibodies to UreA, UreB, CagA, or VacA. Wild-type Hp (11637 and 11916) contain UreA and UreB, but mutants (M1–M4 and M9–M11) do not. (B) Urease activity of whole bacteria measured using the phenol red assay. One experiment representative of four independent determinations is shown. (C) Activity of Jack bean urease. (D) Macrophages were infected with Hp 11637 or M1 at a ratio of 25:1, and the viability of intracellular bacteria was scored at 0.5–24 h. Data are the mean $\pm$ SE of three independent experiments performed in triplicate. *, $P < 0.050$; **, $P < 0.010$. (E) Anti-Hp pAb detect whole M4 in macrophages infected for 1 h and digested bacterial fragments at 4 h.

Fig. 2.

Phagosomes containing urease mutants are acidified and do not retain EEA1. (A) Macrophages containing Hp 11637 or M4 stained with AO. All bacteria are green (arrows), but only mutant organisms are in acidic (red) phagosomes. Percentage of organisms in acidic phagosomes is indicated (mean $\pm$ SE, n=3). N, macrophage nucleus. (B and C) Time course of EEA1 association with phagosomes containing Hp 11637 (B) or M4 (C). Macrophages infected for 30 or 90 min were fixed and stained to show EEA1 (green) and Hp (red). Arrows indicate phagosomes. (D) Phagosomal EEA1 measured as pixel intensity. Data are the mean $\pm$ SE for three experiments performed in triplicate. *, $P \leq 0.016$, versus wild-type.

Fig. 3.

Differential recruitment of lamp-1 to Hp phagosomes in peritoneal macrophages and J774 cells. Primary macrophages (A and B) and J774 cells (C and D) were infected with bacteria or latex beads for 90 min. Cells were processed for microscopy and stained to detect Hp (red) and lamp-1 (green). (A) Confocal sections show 11637, M1, or latex beads phagosomes. Latex beads are also shown in bright field. (B) Intensity of lamp-1 staining associated with Hp 11637, M1, latex beads, or late endosomes and lysosomes (LE/L). Data are the mean $\pm$ SE, $n = 3$. *, $P < 0.050$, versus other organelles. (C) Merged confocal sections of J774 cells show lamp-1 (green) and Hp (red). Note that M4 phagosomes acquire lamp-1 (yellow in merged image), but wild-type phagosomes do not. (D) Percentage of phagosomes in J774 cells, which acquired lamp-1. Data are the mean $\pm$ SE of three experiments performed in triplicate. *, $P = 0.001$.

Fig. 4.

Surface adsorption of active urease rescues mutant bacteria. (A) Urease activity associated with mutant M1 (1×10^9 bacteria/ml) before and after incubation with filtered wild-type or mutant bacterial sonicates. (Inset) UreB immunoblot of M1 after incubation with mutant (–) or wild-type (+) sonicates. Data shown are from one experiment representative of three independent determinations. (B–D) J774 cells were infected with mutant M1 alone or M1, which had been incubated with wild-type (+WT sup) or mutant sonicates (+M1 sup) for 90 min. (B) Confocal sections show M1 (red) and EEA1 (green). Arrows indicate EEA1-positive phagosomes. (C) AO staining. Arrows indicate bacteria (green) in nonacidic phagosomes. (D) Recruitment of lamp-1 (green) to M1 phagosomes (red). Arrows indicate phagosomes with strong lamp-1 staining (yellow in merged images); arrowheads indicate lamp-1-negative phagosomes (red in merged images); and double arrowheads indicate phagosomes with moderate lamp-1 (orange in merged images). (E) Bacterial viability in J774 cells. Data are the mean $\pm$ SE from three experiments performed in triplicate. *, $P < 0.040$, versus other treatments. Mφ, Macrophage.

Fig. 5.

Effects of NH_4Cl , bafilomycin A1, and chloroquine on phagosome maturation and intracellular killing. (A–D) Peritoneal macrophages or J774 cells were left untreated or exposed to 10 mM NH_4Cl , 10 μM chloroquine (CLQ), or 100 nM bafilomycin A1 (Bafilo) prior to infection with M1. Phagosome composition was assessed after 90 min, and bacterial viability was scored at 4 h. (A) All three drugs impair phagosome acidification. Percentage of bacteria located in acidic (red) phagosomes (mean $\pm$ SE, $n=3$) is indicated. (B) Only NH_4Cl favors M1 retention of EEA1 (green). Arrows indicate phagosomes. Con, Confocal. (C) Effect of drugs on megasome formation. Merged confocal sections of peritoneal macrophages (PMφ) and J774 cells show M1 (red) and lamp-1 (green). Arrows indicate phagosomes with moderate or strong lamp-1;

arrowheads indicate lamp-1-negative phagosomes; and double arrowheads indicate digested bacterial fragments in chloroquine-treated cells. (D) Effect of NH_4Cl (+ NH_4), bafilomycin A1 (+Baf), and chloroquine on survival of M1 in peritoneal macrophages. Data are the mean $\pm$ SE of four determinations, *, $P < 0.001$. (E) Chloroquine enhances maturation of wild-type Hp phagosomes in J774 cells. Graph indicates the fraction of lamp-1-positive phagosomes (mean $\pm$ SD, $n=3$; *, $P=0.028$). Confocal section contains a single phagosome (arrow) and bacterial debris (arrowheads).

Fig. 6.

Spontaneous urease mutants do not survive in macrophages. (A) Immunoblots of Hp 60190 (WT) and Ure probed with antibodies to UreA, CagA, and VacA. (B) Urease activity of wild-type and mutant Hp. Data shown are from one experiment representative of four. (C) J774 cells were infected with Hp 60190 or Ure at a ratio of 25:1, and viable intracellular bacteria were scored after 0.5–4 h. Data are the mean $\pm$ SE of three independent experiments performed in triplicate. *, $P < 0.030$. (D) Merged confocal sections of infected J774 cells show EEA1 or lamp-1 (green) and Hp (red). Arrows indicate phagosomes. (E) Effect of NH₄Cl, bafilomycin A1, and chloroquine on intramacrophage (M ϕ) survival of Ure. Data are the mean $\pm$ SD of three determinations. *, $P = 0.027$, versus Ure.

Figure 7.

Urease is not required for induction of iNOS. J774 cells were left untreated or exposed to whole bacteria (Hp 11637 or M1) or bacterial sonicate for 1, 4, or 24 h. iNOS was detected by immunoblotting. Data shown are from one experiment representative of three independent determinations. Uninf., Uninfected cells; son., sonicate.