INFECTION AND IMMUNITY, Apr. 1997, p. 1181–1188 Vol. 65, No. 4
0019-9567/97/$04.00+0

Localization of *Helicobacter pylori* Urease and Heat Shock Protein in Human Gastric Biopsies

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Received 14 October 1996/Returned for modification 23 December 1996/Accepted 9 January 1997

***Helicobacter pylori* is a spiral, gram-negative bacterium which causes chronic gastritis and plays a critical role in peptic ulcer disease, gastric carcinoma, and gastric lymphoma. *H. pylori* expresses significant urease activity which is an essential virulence factor. Since a significant fraction of urease activity is located on the surface of the bacterium, the urease molecule is a logical choice as an antigen for a vaccine; currently recombinant urease apoenzyme is being tested as a vaccine in phase II clinical trials. We have recently demonstrated that urease and HspB (a homolog of the GroEL heat shock protein) become associated with the surface of *H. pylori* in vitro in a novel manner: these cytoplasmic proteins are released by bacterial autolysis and become adsorbed to the surface of intact bacteria, reflecting the unique characteristics of the outer membrane. To determine if similar mechanisms are operative in vivo, we determined the ultrastructural locations of urease and HspB within bacteria present in human gastric biopsies. Our results demonstrate that both urease and HspB are located within the cytoplasm of all bacteria examined in human gastric biopsies. Interestingly, a significant proportion of the bacteria examined also possessed variable amounts of surface-associated urease and HspB antigen (from 5 to 50% of the total antigenic material), indicating that in vivo, *H. pylori* has surface characteristics which enable it to adsorb cytoplasmic proteins. This is consistent with our altruistic autolysis model in which *H. pylori* uses genetically programmed bacterial autolysis to release urease and other cytoplasmic proteins which are subsequently adsorbed onto the surface of neighboring viable bacteria. These observations have important implications regarding pathogenesis and development of vaccines for *H. pylori*.**

Helicobacter pylori is a gram-negative bacterium which causes chronic gastritis and plays a pivotal role in peptic ulcer disease, gastric carcinoma, and gastric lymphoma (2, 25, 27).

All fresh isolates of *H. pylori* express significant urease activity which appears essential to survival and pathogenesis of the bacterium (10, 27). It is thought that within the gastric lumen hydrolysis of urea generates ammonia to counterbalance gastric acid, presumably forming a neutral microenvironment surrounding *H. pylori*. Consistent with this proposal, isogenic urease-negative mutants fail to colonize gastric mucosa. It has been shown by several different groups using diverse techniques that much of the urease activity is located on the surface of the bacteria (8, 9, 11, 22). Immunization with *H. pylori* urease has been shown to confer protection against challenge from *H. felis*, a bacterium closely related to *H. pylori*, in the mouse model (5, 14, 19). *H. pylori* urease has been proposed as a vaccine candidate and is currently undergoing human clinical trials (23).

We have recently demonstrated in vitro that urease and HspB become associated with the surface of *H. pylori* in a novel manner: these cytoplasmic proteins are released by bacterial autolysis and become adsorbed to the surface of intact bacteria due to unique characteristics of the outer membrane (28). In this communication, we report that similar mechanisms appear to occur in vivo.

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MATERIALS AND METHODS

Gastric antral biopsies were obtained from human subjects undergoing endoscopy for dyspeptic symptoms by using standard biopsy forceps. Informed consent was obtained from all patients enrolled in the study, based on a research protocol approved by the Human Subjects Committee of Sinai Samaritan Medical Center, Milwaukee, Wis.

Three gastric biopsies were obtained from each patient. The first biopsy was used to perform a rapid slide urease (Campylobacter-like organism [CLO]) test (Delta West Ltd., Bentley, New South Wales, Australia). A second biopsy was collected in sterile 20% glucose and used to isolate *H. pylori* under microaerobic conditions, using selective media (7). A third biopsy was processed for immunocytochemistry (see below).

For ultrastructural localization of urease, gastric biopsies from five *H. pylori* culture- and CLO test-positive and two *H. pylori* culture- and CLO test-negative patients were placed within 10 s after collection into fixative containing 1% glutaraldehyde and 4% formaldehyde. Biopsies were dehydrated in a graded series of ethanols and embedded in LR Gold (Polysciences, Niles, Ill.) as described previously (30). Samples were polymerized with UV light for 24 h at 4°C. Thin sections (60 to 100 nm) were labeled with affinity-purified rabbit antiserum against *H. pylori* urease or against HspB (28), diluted 1:200 to 1:800, with a labeling protocol as described by Schneider and Papermaster (31). Bound antibodies were detected by using goat anti-rabbit immunoglobulin G conjugated to 10-nm colloidal gold (Amersham, Chicago, Ill.). As a control for specificity of labeling, a preimmune serum for the antiurease antibody was substituted for the primary antibody in labeling the *H. pylori*-positive cases. Gastric biopsies negative for *H. pylori* were labeled with antiurease and anti-HspB as controls to assess the specificity of urease and HspB antibodies.

Quantitation of immunocytochemical labeling was performed as follows. A transparent acetate sheet bearing parallel lines was dropped randomly across micrographs. The intersection of the lines with surface of the bacterium was taken as a point of reference, and a tangential line was drawn on the micrographs

at the point of reference. A line perpendicular to the tangential line at the point of reference on the surface of the bacterium formed the side of a sampling square 1 cm long (corresponding to 0.31 μm long) which was placed on either side of the bacterial outer membrane. Gold particles were counted within these two compartments (extracellular and intracellular) for every intersection of the reference line with a bacterial membrane. In this fashion, we quantitated 12 micrographs of labeling experiments using antiurease and 6 micrographs using anti-HspB, examining six intersections per micrograph. Three different biopsies were examined. Results were expressed for the extracellular label as the percentage of the intracellular label.

RESULTS

Colloidal gold particles representing the localization of urease were numerous in bacteria having the morphology and location (adjacent to the luminal aspect of gastric epithelial cells) of *H. pylori* within *H. pylori*-positive individuals. The majority of urease antigen was located within the cytoplasm (Fig. 1). However, variable amounts of antigenic urease were associated with the outer membrane of most of the bacteria examined (Fig. 1). Discrete foci of extracellular antigen were observed in some instances. Occasional bacteria, or portions thereof, which contained significant amounts of antigenic urease within the cytoplasm, but little or no surface-associated antigen, were observed (Fig. 1b). Immunolocalization of HspB revealed results similar to those obtained with urease except that the number of colloidal gold particles appeared to be fewer in both the cytoplasmic and surface-associated compartments (Fig. 2).

To express the labeling densities obtained with antiurease in the intracellular and extracellular compartments of the bacteria, we counted gold grains by random sampling in these two compartments. The portion of the bacterium shown in Fig. 1a has 28% of the labeling density present in the extracellular compartment. The presence of extracellular antigenic urease, as detected by the labeling density of the gold particles, varied for bacteria or portions thereof in Fig. 1b and c from 5 to 50%. The amount of extracellular material present for HspB varied from 44 and 66% in Fig. 2a and b, respectively. The analysis of several electron micrographs from three different biopsies revealed that for both antigens, the extracellular labeling density varied from 5 to 70% of the intracellular labeling density.

There was very little labeling of gastric mucosal tissue from biopsies infected with *H. pylori*, with either antiurease or anti-HspB, indicating that very little antigen is present on the mucosal surface. The amounts of urease and HspB antibodies bound to gastric mucosal tissue were not significantly different in biopsies from *H. pylori*-infected individuals and those from uninfected individuals (Fig. 1 to 3).

H. pylori-positive biopsies incubated with preimmune serum showed no significant labeling (Fig. 3). Neither of the *H. pylori*-negative biopsies showed significant staining of gastric tissues with either the urease or HspB antibody, indicating that there was no significant immune cross-reactivity between gastric tissues and these antibodies.

DISCUSSION

Prior to performing localization studies, we recognized three possible subcellular locations of urease and HspB in *H. pylori* in human gastric biopsies.

The first possibility was that urease and HspB are located strictly within the cytoplasmic compartment as we have observed within fresh subcultures of *H. pylori* in vitro (28). Such results would imply that *H. pylori* growing in vivo does not undergo significant autolysis, resulting in subsequent surface adsorption of these two proteins as observed in vitro (28), suggesting that the occurrence of such phenomena may be restricted to in vitro growth conditions.

The second possibility was that urease and HspB are confined strictly to the outer membrane and/or extracellular compartments. If so, based on our previous observations that both cytoplasmic and surface-associated urease molecules are enzymatically active in vitro (28), *H. pylori* would appear capable of assembling active urease in both the periplasmic and cytoplasmic compartments. However, if this possibility were validated, urease assembly within the periplasmic compartment would appear to be favored in vivo whereas assembly within the cytoplasmic compartment would appear to be preferred in vitro, which to our knowledge would be an unprecedented occurrence in bacterial physiology. Furthermore, similar distinct assembly processes would have to be invoked for HspB as well.

The third possibility was that urease and HspB are located within the cytoplasm and associated with the outer membrane, as occurs in late-log-phase *H. pylori* in vitro (28). Such results would strongly support the hypothesis that autolysis occurs in vivo and is responsible for surface association of proteins originally assembled within the cytoplasm. Based on this reasoning, it might be predicted that the distribution of urease and HspB on the surface of individual bacteria would be irregular; some bacterial cells might not adsorb significant amounts of urease or HspB on the surface, depending on the proximity of neighboring bacteria undergoing autolysis. These three possibilities are outlined in Fig. 4.

In this communication, we report for the first time that significant fractions of urease and HspB appear to be present both within the cytoplasm and associated with the bacterial outer membrane in vivo. These results are consistent with the hypothesis that urease and HspB released by autolysis of *H. pylori* are subsequently adsorbed to the surface of intact bacteria, as we have recently demonstrated in vitro (28). We believe that our observations in vivo are consistent with a fourth mechanism of surface localization of proteins in bacteria in addition to the general secretory (29), ABC (12), and type III (17) pathways.

It is important to note that the results reported here cannot be explained by invoking one or more known bacterial protein export mechanisms. Since both cytoplasmic and surface-associated forms of urease are enzymatically active in vitro (28), the presumed export mechanism(s) must have the capacity to export a large, fully assembled active enzyme. Further, it would appear necessary to postulate that two distinct populations of urease molecules are generated within the cytoplasm. One form is exported, while the other remains within the cytoplasmic compartment. Urease is a nickel-dependent, hexameric molecule with a molecular mass of approximately 540 kDa consisting of equal numbers of large (UreB, approximately 62 kDa) and small (UreA, approximately 30 kDa) subunits (8, 9, 11, 16). The structural genes *ureA* and *ureB* are present in an operon containing a variety of additional genes essential for expression of enzymatic activity (4, 13). To date, only a single form of the active enzyme has been recognized by biochemical (6, 9, 11, 16) and genetic (4, 6, 22) analyses. Alternatively, *H. pylori* has the capacity to assemble active urease in both the cytoplasmic and periplasmic space/surface compartments. In the face of strong evidence supporting autolysis and surface adsorption (28), the occurrence of protein export in *H. pylori* by the hypothetical events outlined above appears extremely unlikely.

Our results help explain another long-standing puzzle about *Helicobacter* pathogenesis: in studies using the mouse-*H. felis* model, three different antigens prepared from *H. pylori* (ure-

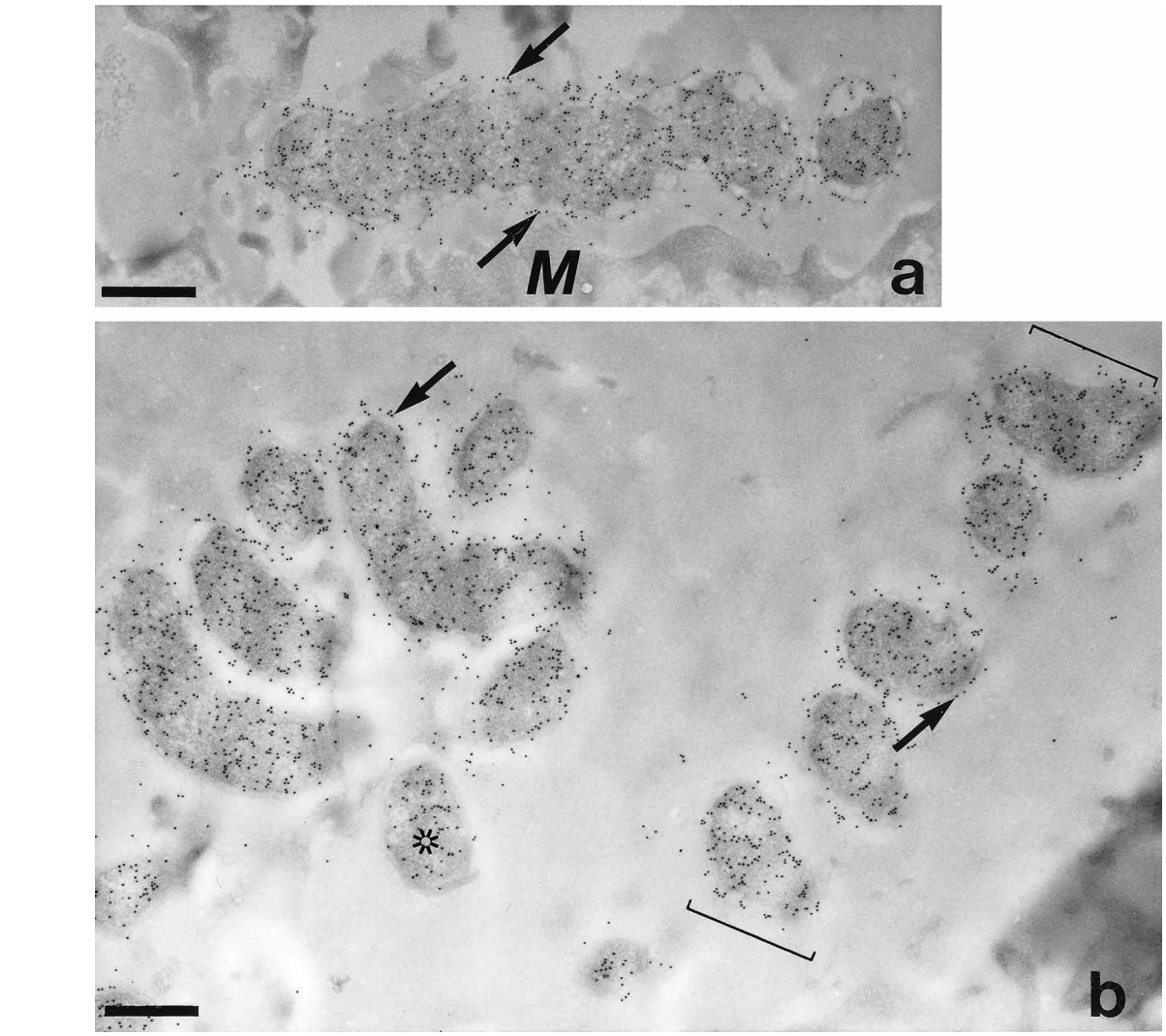

FIG. 1. Immunolocalization of *H. pylori* urease in situ in human gastric biopsies. In all panels, arrows denote surface-associated antigen. (a) Single spiral bacterium with prominent surface-associated urease in juxtaposition with the apical fragment of a gastric mucosal cell (M) (bar = 0.5 μm). (b) Multiple portions of *H. pylori* with surface-associated urease antigen. The portions included within the brackets are thought to represent a single bacterium, parts of which are not included in the thin resin section due to the spiral nature of the microorganism (compare with panel a). A bacterial portion showing cytoplasmic antigen almost exclusively is denoted by the asterisk (bar = 0.5 μm). (c) Three portions of bacteria showing a moderate amount of surface-associated urease. The bacteria are located above a fragment of a gastric mucosal cell (M) (bar = 0.5 μm).

ase, HspB, and catalase), administered either separately or in combination with appropriate adjuvants, have been shown to be effective in resisting challenge with *H. felis* (14, 23). How are typical cytoplasmic proteins like urease, HspB, and catalase effective in preventing infection in a mouse-*H. felis* model? It is thought that *H. felis* possesses most of the virulence and colonization properties of *H. pylori* and therefore is a reasonable animal model with which to study *H. pylori* pathogenesis. The fact that typical cytoplasmic proteins such as urease, HspB, and catalase are successful as vaccine antigens suggests that antibodies against them must have direct access to these proteins and are bactericidal, leading to clearance of infection. It is likely that autolysis followed by surface adsorption occur in *H. felis* both in vitro and in vivo, similar to events in *H. pylori*,

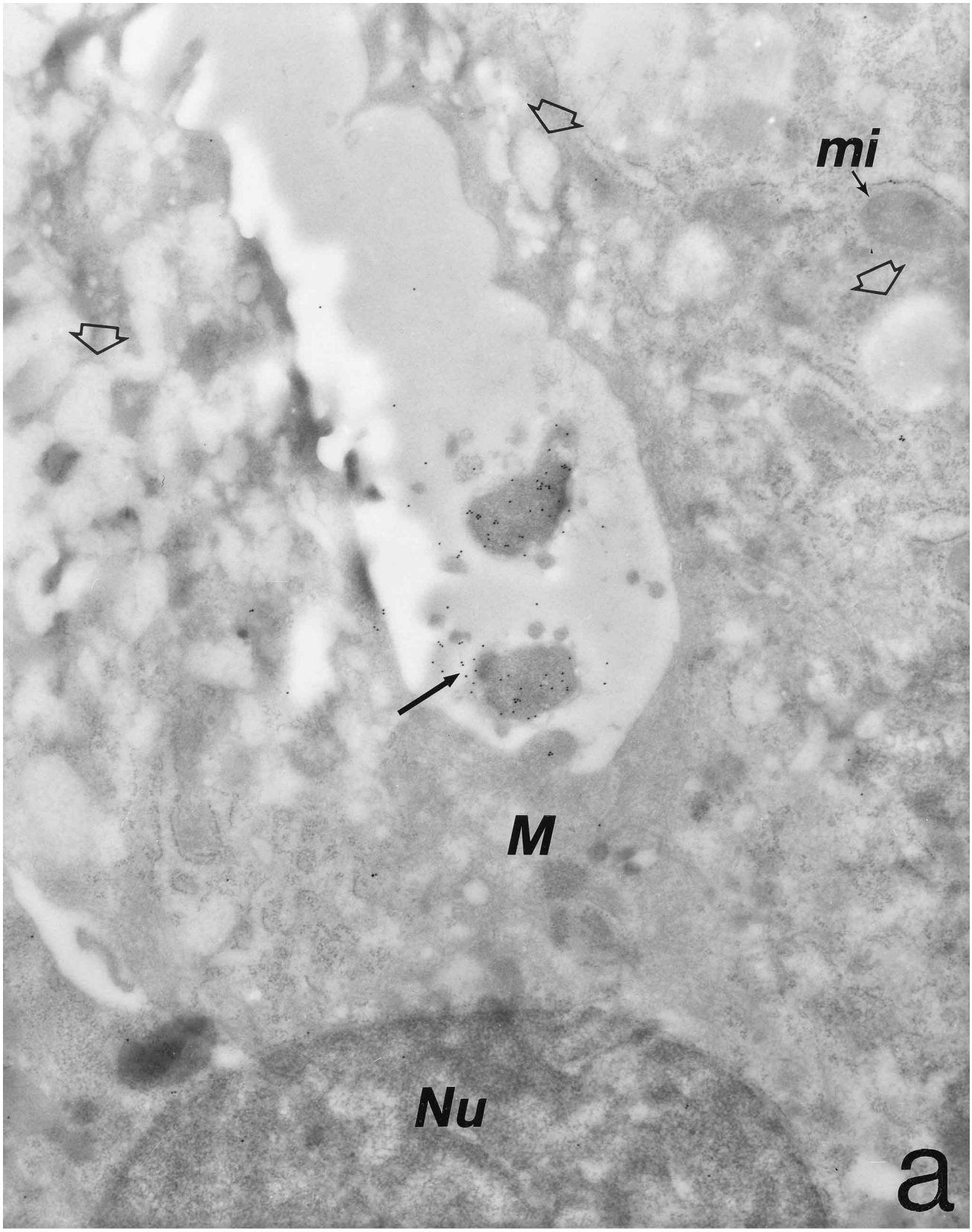

FIG. 2. Immunolocalization of *H. pylori* HspB in situ in human gastric biopsies. In both panels, arrows denote surface-associated antigen. (a) Two isolated bacteria lie adjacent to the gastric epithelial cell. The human gastric epithelial cell (M) surrounding the bacterium is a columnar mucus-secreting cell. Mucus granules (open arrowheads), nuclei (Nu), and mitochondria (mi) are depicted. The area surrounding *H. pylori* in both panels is likely encased in mucus, although it appears empty due to its electron transparent nature. Magnification, ×28,710. (b) *H. pylori* next to a gastric epithelial cell (M). The microvilli of gastric epithelial cells (closed arrowheads) are indicated. Magnification, ×28,710.

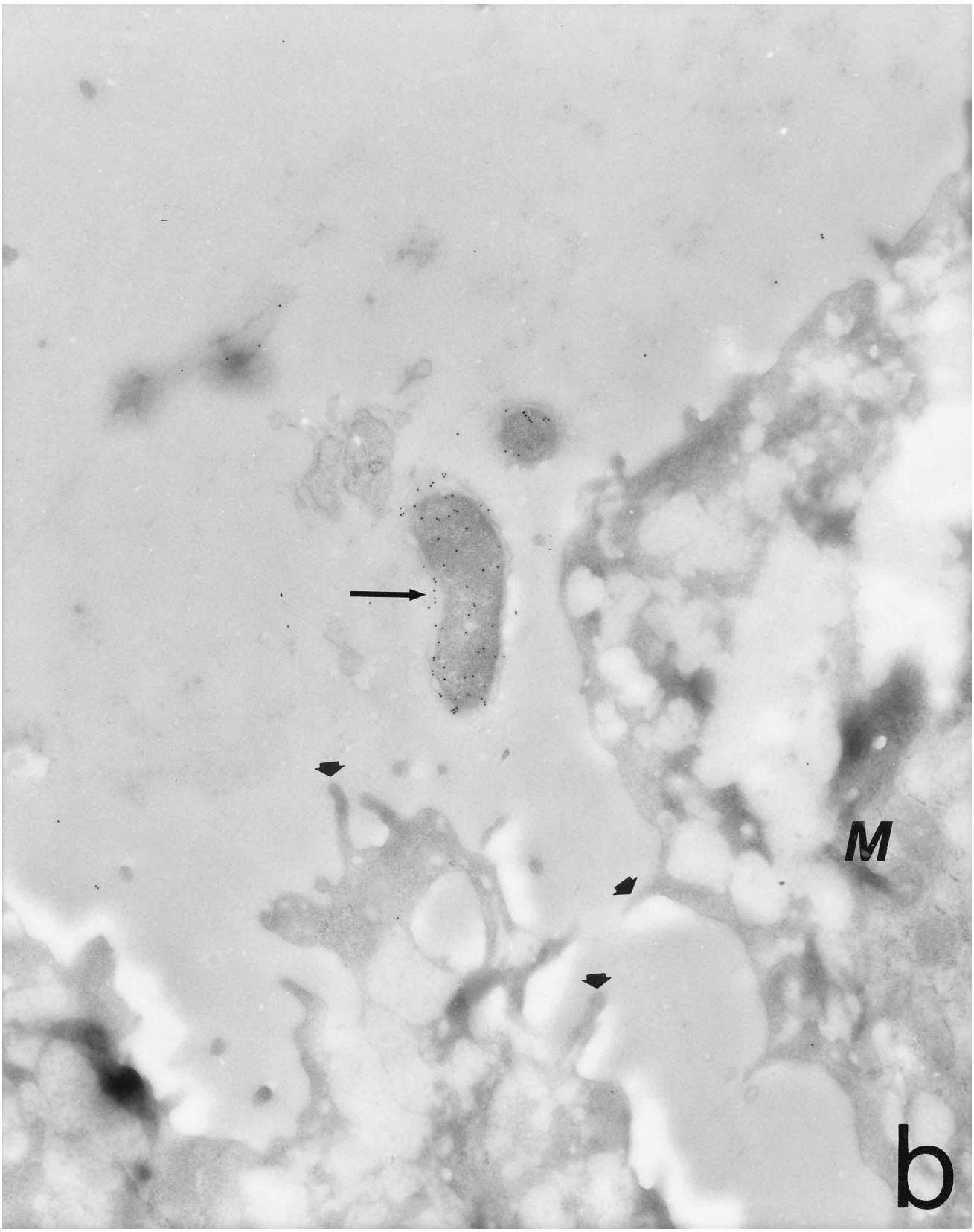

FIG. 2—*Continued.*

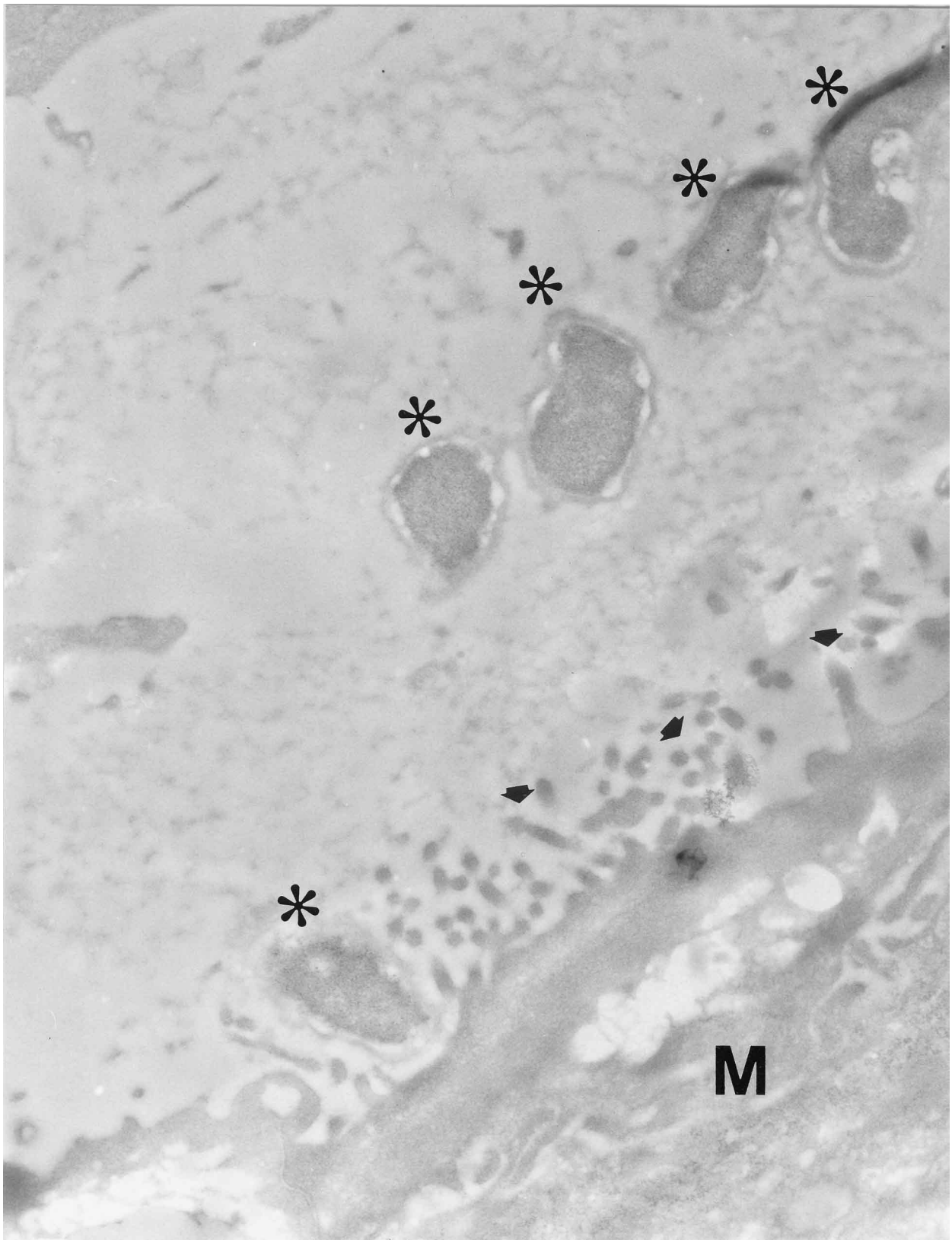

FIG. 3. An *H. pylori*-positive biopsy incubated with preimmune serum. There are few background gold particles due to nonspecific cross-reactivity. The *H. pylori* cells (asterisk) lie adjacent to the gastric epithelial cell (M) and its villi (arrowheads). Magnification, ×31,900.

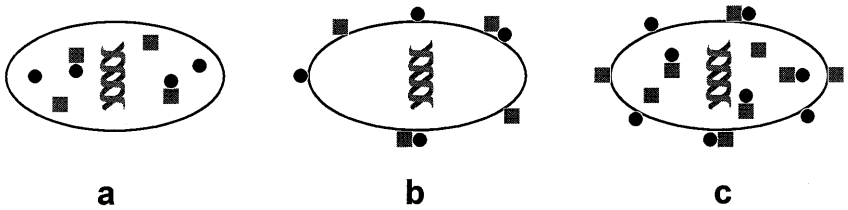

FIG. 4. Schematic diagram of three possible locations of urease and HspB, as described in the text. (a) Urease and HspB are located strictly within the cytoplasmic compartment; (b) urease and HspB are confined strictly to the outer membrane and/or extracellular compartments; (c) urease and HspB are located within the cytoplasm and associated with the outer membrane.

allowing antibodies specific for typical cytoplasmic proteins to prevent infection, although this has not been demonstrated directly. Our model, however, predicts that because the surface association of urease and HspB is variable and some forms (early-log-phase and coccodial forms) of bacteria do not have any surface-associated urease (28), a vaccine based on urease as an antigen, although effective against *H. felis* in a mouse model, may not be effective against *H. pylori.*

We feel it extremely unlikely that the surface localization of urease and HspB presented in this report results from artifacts occurring during the process of sample preparation for electron microscopy. Gastric biopsies containing bacteria are put in fixative within seconds (after endoscopy) in the endoscopy suite. Moreover, the surrounding gastric epithelial tissue does not reveal any evidence of poor fixation. Multiple biopsies fixed and processed independently showed the same labeling results. Although we cannot prove a negative (absence of an artifact), we feel that the labeling pattern observed in the human biopsies accurately reflects the localization of the antigens within and around *H. pylori* in the human gastric environment.

H. pylori is not alone among the pathogenic bacteria in exhibiting autolysis. For example, autolysis of *Streptococcus pneumoniae* occurs in vivo and in vitro and is regulated genetically (15, 26); inactivation of the autolysin gene decreases virulence of *S. pneumoniae* in an animal model (1). Autolysis of *Neisseria gonorrhoeae* leads to liberation of DNA which is taken up by viable cells. It has been postulated that this accounts for antigenic variation of pilin (18, 32).

Based on our previous observations in vitro (28) and the present observations that antigenic urease is present both within the cytoplasm and associated with the surface of *H. pylori* in vivo, it seems likely that urease is enzymatically active in both cytoplasmic and surface-associated compartments in vivo. We suggest that *H. pylori* urease probably serves at least two functions in vivo. First, surface-associated urease hydrolyzes urea to generate extracellular ammonia, which neutralizes gastric acid in the immediate vicinity of individual bacteria, thus promoting survival (20). Second, cytoplasmic urease functions in utilization of exogenous urea as a nitrogen source for amino acid synthesis (33). Of interest, the latter function of urease does not appear to be essential, since isogenic urease-negative mutants of *H. pylori* grow normally in vitro (13). The observed cytoplasmic and surface-associated locations of urease are consistent with this dual functional role. It remains to be determined whether ammonia generated from cytoplasmic urease is exported and is sufficient to permit survival of *H. pylori* in the gastric environment.

We postulate that urease originated as a strictly cytoplasmic enzyme in *H. pylori*; as the bacterium adapted to the acid milieu of the gastric lumen, altruistic autolysis, in which release of urease and other cytoplasmic contents of *H. pylori* results in surface adsorption of these proteins to neighboring intact bacteria, evolved as a protective mechanism. Such altruistic autolysis, in which autolysis of a fraction of the bacterial population presumably benefits the remaining viable bacteria, appears to be essential in understanding the pathogenesis of *H. pylori* and may help to explain (i) how vaccines against an archetypal cytoplasmic protein such as urease can be effective against related *Helicobacter* spp. in animal models, as described by a number of investigators (5, 19, 21, 24); (ii) how the integral membrane proteins of *H. pylori* evade immune detection, thus contributing to bacterial persistence in the face of humoral and cellular immune responses (3); and (iii) how the noninvasive bacterium *H. pylori* can present virulence factors and immunogens to the host. Our model also predicts, however, that vaccines utilizing purified urease alone will not be effective against early-log-phase *H. pylori* in which urease is located strictly within the cytoplasm (28).

Taken together, these observations define the fourth mechanism of surface localization of proteins in bacteria and may have implications regarding pathogenesis and development of vaccines for *H. pylori*. Studies are in progress in this laboratory to elucidate the mechanisms of *Helicobacter* autolysis.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA-67527 from the National Cancer Institute (B.E.D.), by a grant from Lederle-Praxis Biologicals (S.H.P.), and by a grant from the Schoenleber Foundation (B.E.D. and S.H.P.).

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Editor: A. O'Brien