

Surface-Associated Heat Shock Proteins of *Legionella pneumophila* and *Helicobacter pylori*: Roles in Pathogenesis and Immunity

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ABSTRACT

Bacterial heat shock proteins (Hsps) are abundantly produced during the course of most microbial infections and are often targeted by the mammalian immune response. While Hsps have been well characterized for their roles in protein folding and secretion activities, little attention has been given to their participation in pathogenesis. In the case of *Legionella pneumophila*, an aquatic intracellular parasite of protozoa and cause of Legionnaires' disease, Hsp60 is uniquely located in the periplasm and on the bacterial surface. Surface-associated Hsp60 promotes attachment and invasion in a HeLa cell model and may alter an early step associated with the fusion of phagosomes with lysosomes. Avirulent strains of *L. pneumophila* containing defined mutations in several *dot/icm* genes are defective in localizing Hsp60 onto their surface and are reduced approximately 1000-fold in their invasiveness towards HeLa cells. For the ulcer-causing bacterium *Helicobacter pylori*, surface-associated Hsp60 and Hsp70 mediate attachment to gastric epithelial cells. The increased expression of these Hsps following acid shock correlates with both increased association with and inflammation of the gastric mucosa. A role for Hsps in colonization, mucosal infection and in promoting inflammation is discussed. *Infect. Dis. Obstet. Gynecol.* 7:58–63, 1999. © 1999 Wiley-Liss, Inc.

KEY WORDS

Legionella pneumophila; *Helicobacter pylori*; heat shock proteins; cellular immunity; adhesion

Heat shock proteins (Hsps, also known as stress or chaperone proteins), particularly the members of the Hsp60 and Hsp70 families, have been recognized for years as immunodominant antigens of many microbial pathogens.^{1–3} Moreover, immune responses against bacterial Hsps have often been implicated in certain autoimmune diseases^{2,3} and chronic infections.^{4,5} Yet, autoimmune disease can be prevented in several animal model systems by vaccination with Hsps,^{6,7} indicating that the immune response to Hsps is both complex and can be modulated by extrinsic factors. Generally, immu-

nodominant proteins are thought of as those that are either secreted or expressed on the surface of microbes, so studies indicating that the Hsp60 of *Escherichia coli* (GroEL) is exclusively located in the cytoplasm seemed contradictory. In addition, none of the heat shock or stress regulated genes studied to date encode proteins with signal sequences or any other known secretion-specific motif to suggest these proteins are secreted. Therefore, early reports implicating Hsps in pathogenesis were not taken too seriously or were thought to be too preliminary to support a strong case. To begin

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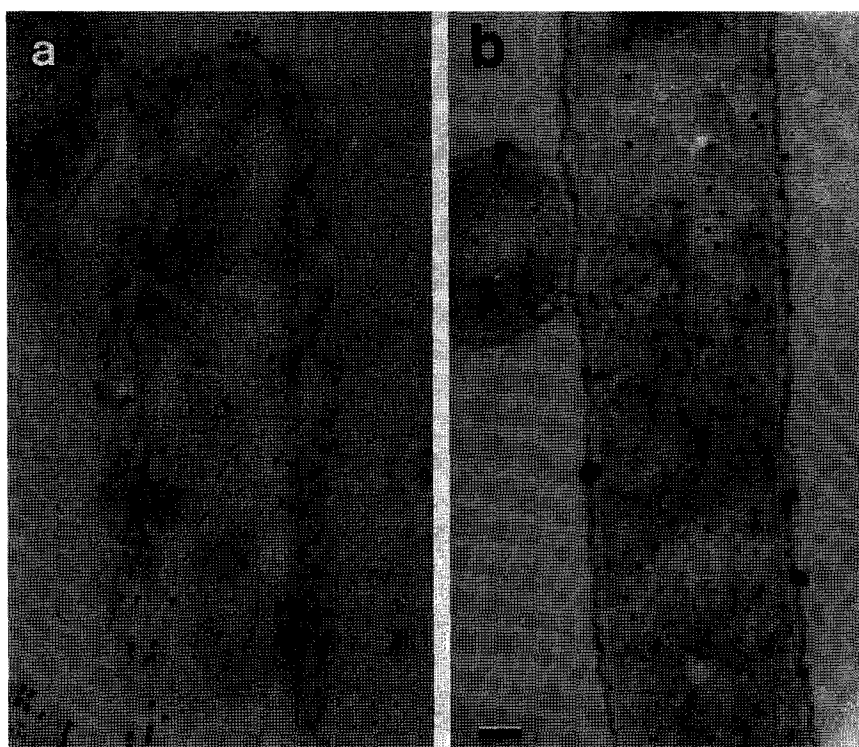


Fig. 1. Immunolocalization of Hsp60 in *L. pneumophila* (a) and *E. coli* expressing recombinant *L. pneumophila* Hsp60 from pSH16 (b). Bar indicates 0.1 μ m for both panels.

to understand what roles Hsps might play in pathogenesis and host immune response, we have been investigating *Legionella pneumophila*, an aquatic microbe that has not evolved with humans, and *Helicobacter pylori*, an organism highly evolved and specialized to the human gastric mucosa. While both pathogens express Hsps on their surface, the pathogenesis role for these Hsps differs. Our studies suggest that the diversity of function attributed to Hsps, and in particular to pathogenesis mechanisms, is largely determined by cellular location.

LOCALIZATION OF HSPTS

Immunolocalization techniques have been successfully used to determine the cellular location of Hsps. Surface localization can be determined with whole bacterial cells by either immunofluorescence (light microscopy) or by immunogold electron microscopy using monoclonal (MAb) or polyclonal (PAb) antibodies raised against the particular Hsp. Performing immunolocalization with sectioned bacteria permits further localization of these proteins among internal compartments. When immunogold electron microscopy was performed on thin

sections of *L. pneumophila*, using antibodies specific for Hsp60 and gold-conjugated anti-immunoglobulin (MAb or PAb), the gold particles were found to surround the periphery of the bacteria (see Fig. 1a), indicating both a periplasmic and a surface location.^{8,9} As a control for these experiments, recombinant *L. pneumophila* Hsp60 was produced from a clone in *E. coli*. As can be seen in Figure 1b, the *E. coli* bacteria accumulated Hsp60 in the cytoplasm, with little or no gold spheres located in other compartments or on the surface. Comparative immunogold studies with antibodies raised to well-known surface associated antigens (LPS, OmpS) gave results similar to those observed with Hsp60 antiserum,⁸ supporting the case that Hsp60 is a legitimate surface antigen in *L. pneumophila*. Several studies employing immunogold labeling of whole *H. pylori* cells showed that Hsp60 was located on the bacterial surface.¹⁰⁻¹² Further, immunogold labeling of biopsy samples from patients infected with *H. pylori* also revealed Hsp60 associated with inflamed tissue.¹³ These studies indicate that Hsp60 is one of the dominant proteins expressed in situ by *H. pylori*.

An examination of Hsp60 sequences from many

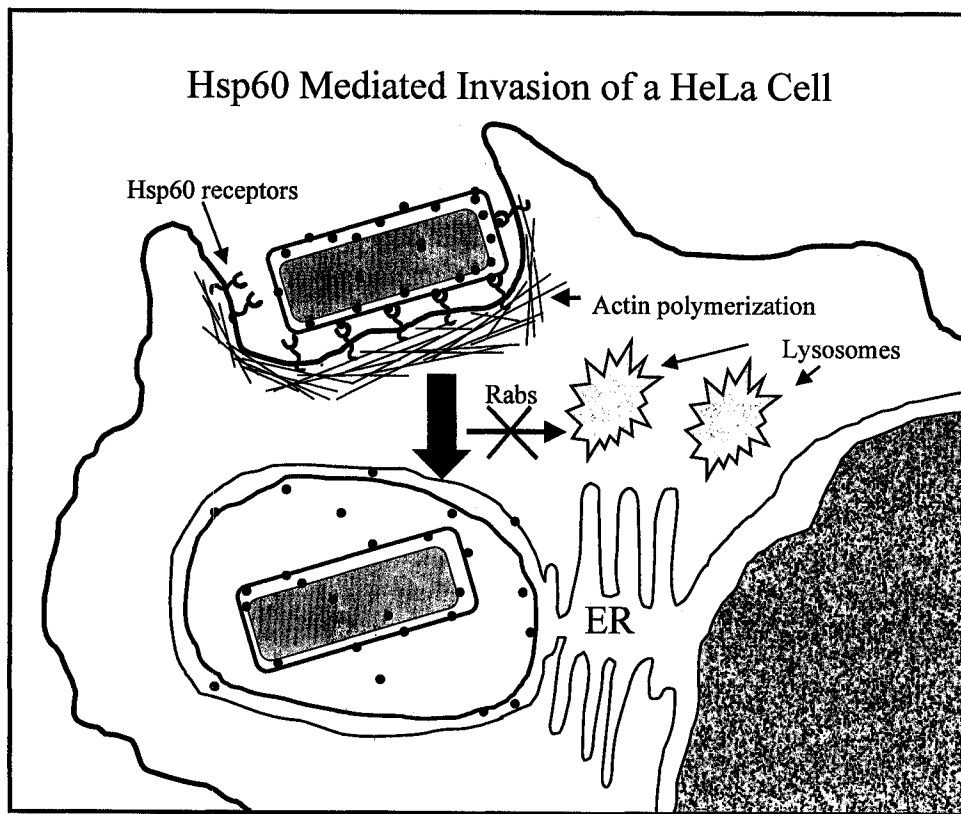


Fig. 2. Parasite-directed receptor-mediated endocytosis of *L. pneumophila* by HeLa cells. The illustration indicates that Hsp60 binds to specific receptors on the HeLa cells, initiating actin polymerization and endocytosis of the bacterium into an early endosome. We propose that Hsp60, interacting with the phagosome membrane, alters signal transduction networks that would ordinarily traffic the bac-

teria-laden endosome to secondary lysosomes. A feature of the specialized endosome in which *L. pneumophila* replicates is its envelopment by the endoplasmic reticulum. "Rabs" indicates several families of GTP-proteins associated with trafficking activities. GTP = guanosine triphosphate; ER = endoplasmic reticulum.

microbial pathogens deposited in Genbank reveals no obvious secretion motifs in this family of highly conserved proteins. Therefore, it has been proposed that the observed surface location of Hsps is achieved by mechanisms that do not involve secretion. For instance, it has been proposed that in *H. pylori*, Hsp60 released from naturally lysed bacteria binds to the surface of neighboring bacteria,¹¹ but this is not the mechanism by which Hsp60 reaches the surface of *L. pneumophila*.⁸ Specific studies addressing the secretion of Hsp60 by *L. pneumophila* have not been conducted. However, the observation that *E. coli* expressing the *L. pneumophila* gene for Hsp60 does not secrete the protein would imply that *E. coli* lacks the machinery used by *L. pneumophila* and perhaps other bacteria to mobilize Hsp60 to the periplasm or cell surface.⁸

Avirulent mutants of *L. pneumophila* can be iso-

lated in the laboratory by selection for tolerance to 0.65% sodium chloride. Mutations conferring sodium tolerance map to several regions of the chromosome containing the *dot/icm* genes involved in facilitating DNA or protein transport.^{14,15} These genes seem to be important in facilitating the surface localization of Hsp60 since avirulent mutants appear unable to efficiently localize Hsp60 to the surface.⁹ Several lines of evidence outlined in the following sections support the hypothesis that surface-exposed Hsp60 is utilized by *L. pneumophila* as an invasin that additionally may alter early events associated with organelle trafficking and phagolysosomal fusion.

ADHERENCE AND INVASION

Virulent as well as avirulent strains of *L. pneumophila* are phagocytized by professional phagocytes

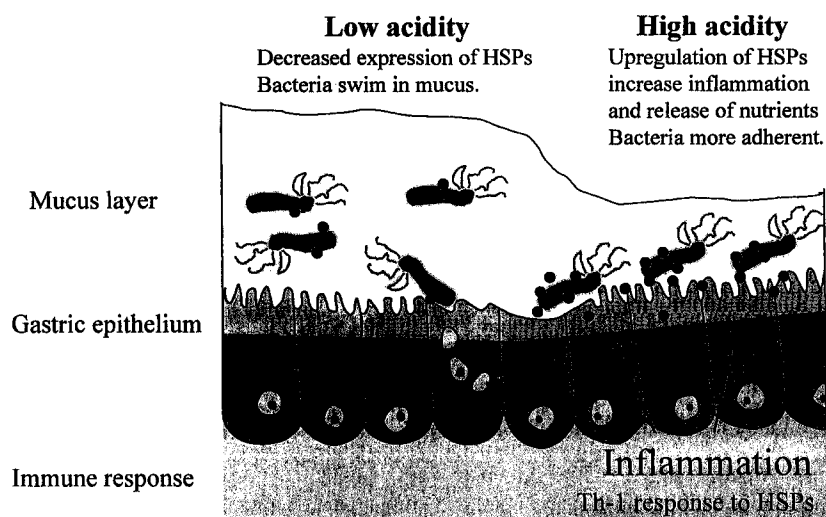


Fig. 3. Illustration of the interactions of *H. pylori* in the gastric mucosa. Under conditions of high acidity, such as might occur in a gastric or duodenal ulcer, *H. pylori* Hsps are upregulated and both localized on the surface and released. The increased surface-expressed Hsp60 and Hsp70 promote attachment to the epithelial cells, which might afford protection from the effects of high acid. Released Hsp60 and Hsp70 promote inflammation through the stimulation

of IL-1 β and IL-12 production by recruited macrophages and interferon- γ by activated Th-1 T-cells. Under conditions of lower acid (more alkaline pH), repression of Hsp production leads to less adherent bacteria that can be found throughout the mucus lining of the stomach. From this remote location, released Hsps can still promote inflammation, while the bacteria remain elusive to the mounting host immune response.

like macrophages, monocytes, neutrophils, and natural protozoan hosts. In all of these hosts, invasion cannot be easily dissected from abrogation of phagosome-lysosome fusion, since phagocytosis is host driven and the earliest distinguishable event in these cells occurs after internalization. In non-professional phagocytes, like epithelial cells (HeLa), invasion is generally parasite driven. Therefore, while virulent and avirulent strains of *L. pneumophila* are phagocytosed by macrophages with similar efficiencies, in HeLa, cells virulent strains of *L. pneumophila* are ~1000-fold more invasive than avirulent strains. Interestingly, avirulent strains of *L. pneumophila* were not impaired in their ability to adhere to HeLa cells.¹⁶ Using specific antibodies raised to Hsp60 or to the major outer membrane protein OmpS, we could demonstrate that while both anti-Hsp60 and anti-OmpS could block the association of virulent strains to HeLa cells, only the anti-OmpS serum blocked the association of avirulent *L. pneumophila* with HeLa cells.⁹ Collectively, these findings suggested that Hsp60 played a significant role in mediating invasion.

To further delineate the role of surface-associated Hsp60 in pathogenesis, competition

with purified Hsp60 or Hsp60 bound to latex beads was used to block attachment and invasion in the HeLa cell model.⁹ Additionally, treatment of virulent, but not avirulent, *L. pneumophila* strains with trypsin was shown to degrade surface-associated Hsp60 and diminish invasiveness for HeLa cells. Finally, Hsp60 bound to latex beads, but not bovine serum albumin- (BSA) beads, were efficiently taken up by HeLa cells, and the phagosomes containing Hsp60-beads did not traffic to or fuse with secondary lysosomes as was observed for BSA-beads, suggesting that Hsp60 may interfere with early signals required for fusion with lysosomes.⁹ The notion that a single protein (Hsp60) mediates both invasion and abrogation of phagosome-lysosome fusion is satisfying for three main reasons. First, previous studies had predicted that the invasion factor of *L. pneumophila* must be preexisting and not synthesized de novo, as invasion occurs in the presence of protein synthesis inhibitors.¹⁶ Second, phagosome-lysosome fusion is an early event (virtually coupled with invasion) inhibited by virulent *L. pneumophila* even in the presence of protein synthesis inhibitors. Third, avirulent *L. pneumophila* strains that are unable to abrogate phago-

some-lysosome fusion do not efficiently express Hsp60 on the cell surface.¹⁷

Our studies suggest that HeLa cells must express surface receptors specific for Hsps. The nature of surface receptors has not been fully explored but may be generic to the family of Hsps, since we showed that Hsp60-beads, but not BSA-beads, when bound to the surface of cytochalasin D-treated macrophages (frozen for phagocytosis) initiated the production of interleukin (IL)-1b via a protein kinase C signal transduction pathway.¹⁸ Since both Hsp60 and Hsp70 protein families can induce IL-1b and IL-12 production by macrophages,^{18,19} from the host defense perspective, these proteins most likely play a key role in immune recognition of invading microbes via a Th-1 mediated inflammatory response. Since Legionnaires' disease is a disease of the elderly and immunocompromised, one can appreciate the key role of the Th-1 immune response in immunity against intracellular pathogens like *L. pneumophila*.

Several groups have extensively explored the role of surface-located Hsps in *H. pylori* pathogenesis. Yamaguchi et al. have shown that Hsp60 mediates adhesion of *H. pylori* to human gastric epithelial cells.¹² Treatment of *H. pylori* with an anti-Hsp60 monoclonal antibody dramatically decreased attachment of the bacteria to gastric epithelial cells. It should be noted that a number of adhesins have been described for *H. pylori*, and much more study is needed to delineate roles for these adhesins in *H. pylori* colonization and pathogenesis. Several groups have used immunogold electron microscopy to document the association of secreted *H. pylori* Hsp60 with inflamed gastric tissue.^{12,13} Studies in our group have shown that Hsp60 and Hsp70 can bind to glycosulfolipids.^{10,20} Recently, Hsp70 has been implicated in adhesion, and we suggested that Hsp70 might play a more important role in adhesion following acid stress.²⁰ Our studies suggest that under acid stress, *H. pylori* bacteria exhibit elevated synthesis of Hsps and may be more adherent to mucosal epithelial cells. Under more alkaline conditions, such as might be generated by the action of the constitutively expressed urease, the bacteria may lower expression of Hsps, thus becoming less adherent and permitting the bacteria to migrate in the mucus to areas of higher acidity.

CONCLUSIONS

Our studies of two very different bacterial pathogens have revealed a range of activities for Hsps in pathogenesis. In the case of *L. pneumophila*, a pathogen whose pathogenesis strategy involves invading and growing in freshwater protozoa, Hsp60 appears to promote infection, as well as to participate in mediating an endosymbiotic relationship with its host. Surface-associated Hsp60 promotes attachment to macrophages and HeLa cells (see Fig. 2) through Hsp60 specific receptors that in the former signal production of IL-1b and IL-12, two important initiators of the cell mediated Th-1 immune response. The production of interferon-g by the Th-1 immune response is critical to the resolution of the infection.¹⁸

H. pylori, a mucosal pathogen, has evolved a strategy of using Hsp60 and Hsp70 to promote attachment to gastric epithelial cells, particularly under conditions of high acidity (see Fig. 3). Liberated Hsps may also play a prominent role in promoting inflammation, since in a mouse model it has been shown that Hsps induce the production of interferon-g and tumor necrosis factor- α in gastric tissue infected with *H. pylori* (unpublished). We propose that the secretion of Hsps by the pathogen may be part of a colonization strategy and, by promoting inflammation, may provide a means for acquiring nutrients via the leakage of serum from inflamed tissue. Perhaps this strategy is plied by other mucosal pathogens, including *Neisseria gonorrhoea*,²¹ *Bordetella pertussis*,⁸ *Haemophilus ducreyi*,²² and *Coxiella burnetii*,²³ all of which seem to display Hsps on their cell surface. If indeed Hsps are "key signatures" for immune recognition of mucosal or intracellular pathogens, then it could be argued that normal flora like *E. coli* have learned to hide their Hsps (cytoplasmic location) from immune surveillance, and hence are tolerated ("no harm, no foul"). This knowledge may be helpful in the design of new vaccines or strategies to control autoimmune diseases.

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