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The GroES homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice

(heat shock proteins/*Helicobacter felis* model/vaccine/gastric cancer/urease)

RICHARD L. FERRERO*†, JEAN-MICHEL THIBERGE*, IMAD KANSAU*, NICOLE WUSCHER‡, MICHEL HUERRE‡, AND AGNES LABIGNE*

*Unité des Entérobactéries, Institut National de la Santé et de la Recherche Médicale U389, 28 Rue du Dr Roux, Paris 75724, France; and ‡Unité d'Histopathologie, Institut Pasteur, Paris 75724, France

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ABSTRACT **_Helicobacter pylori_ is an important etiologic agent of gastroduodenal disease. In common with other organisms, _H. pylori_ bacteria express heat shock proteins that share homologies with the GroES–GroEL class of proteins from _Escherichia coli_. We have assessed the heat shock proteins of _H. pylori_ as potential protective antigens in a murine model of gastric _Helicobacter_ infection. Orogastric immunization of mice with recombinant _H. pylori_ GroES- and GroEL-like proteins protected 80% (n = 20) and 70% (n = 10) of animals, respectively, from a challenge dose of 10^4 _Helicobacter felis_ bacteria (compared to control mice, P = 0.0042 and P = 0.0904, respectively). All mice (n = 19) that were immunized with a dual antigen preparation, consisting of _H. pylori_ GroES-like protein and the B subunit of _H. pylori_ urease, were protected against infection. This represented a level of protection equivalent to that provided by a sonicated _Helicobacter_ extract (P = 0.955). Antibodies directed against the recombinant _H. pylori_ antigens were predominantly of the IgG1 class, suggesting that a type 2 T-helper cell response was involved in protection. This work reports a protein belonging to the GroES class of heat shock proteins that was shown to induce protective immunity. In conclusion, GroES-like and urease B-subunit proteins have been identified as potential components of a future _H. pylori_ subunit vaccine.**

Helicobacter pylori is an etiologic agent of chronic gastritis and peptic ulceration (1, 2). While a significant proportion of the population is infected by *H. pylori* bacteria, many infected individuals remain asymptomatic (3). Long-term infection with *H. pylori* is thought to promote the formation of premalignant lesions, such as antral intestinal metaplasia (4). Recent investigations have established a causal relationship between *H. pylori* and carcinogenesis (5–8), which has led the World Health Organization/International Agency for Research on Cancer to classify *H. pylori* as a "definite human carcinogen." It is therefore feasible to suggest that prophylaxis against *H. pylori* infection, as well as reducing the incidence of peptic ulcer disease, may also reduce the risks of developing gastric neoplasia. We believe that for such a strategy to succeed it will be necessary to target properties that are shared by all isolates of *H. pylori*.

Urease activity is a property common to all *H. pylori* isolates and is essential for colonization of the gastric mucosa (9, 10). *H. pylori* urease is composed of two subunits (UreA and UreB), which form a high molecular weight complex with nickel ions (11–13). These subunits are immunodominant antigens and are highly conserved between the different gastric *Helicobacter* species, including *Helicobacter felis* (14, 15). Various investigators noted a physical association between urease holoenzyme and a protein that had a calculated molecular mass of 54–62 kDa (16–18). This protein was identified to be a homolog of the class of heat shock proteins (HSPs), to which *Escherichia coli* GroEL belongs (16–18); the gene encoding the 54-kDa protein was designated *hspB* (19). Analysis of the DNA region upstream of *hspB* revealed a second open reading frame (designated *hspA*) that encoded a protein with a deduced molecular mass of 13 kDa (19). This protein (HspA) was found to share homology at the amino acid level with the GroES family of proteins. A remarkable feature of *H. pylori* HspA is the presence of a series of histidine and cysteine residues at the C-terminal domain of the polypeptide (19). Preliminary data suggest that this domain may be involved in the binding of nickel ions to the urease complex (20).

It has previously been shown that vaccination with GroEL proteins induces protective immunity against intracellular pathogens, such as *Mycobacterium tuberculosis* and *Legionella pneumophila* (21–23). Much less, however, is known regarding the potential of GroES proteins to serve as protective immunogens. The physical association between *H. pylori* HSP and urease, together with the unique structure of the GroES homolog from *H. pylori* (HspA), led us to speculate whether recombinant *H. pylori* HspA and HspB might be able to induce mucosal immunity in a murine infection model.

Using this model, workers had previously shown that immunization with whole bacterial sonicates could provide immunity against a gastric *H. felis* infection (24, 25). More recently, antigenically defined antigens composed of *H. pylori* urease apoenzyme (26) or UreB subunit (26, 27) were reported to induce protective immunity in mice, although these antigens were less protective than whole-cell sonicates of *H. felis*. Presented here are data showing that coadministration of two defined antigens, *H. pylori* UreB and HspA, was able to confer a level of protection equivalent to that induced by a whole-cell preparation.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth. *H. pylori* (85P) was a clinical isolate (12). *H. felis* (ATCC 49179) was originally isolated from cat gastric mucosa (28). *Helicobacter* were grown on a blood agar medium, containing an antibiotic mixture, and incubated under microaerobic conditions at 37°C (14). *E. coli* MC1061 cells were grown routinely at 37°C on solid or in liquid Luria medium.

Production of Recombinant *H. pylori* Antigens. The genes encoding *H. pylori* urease B subunit and HSP polypeptides (*ureB*, *hspA*, and *hspB*, respectively) were cloned into the expression vector pMAL-C2 (New England Biolabs) as described (27). Recombinant *H. pylori* proteins were expressed as MalE fusions (i.e., MalE–HspA, MalE–HspB, and MalE–UreB). *E. coli* MC1061 cells harboring the recombinant plas-

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Abbreviations: HSP, heat shock protein; Th, T-helper cell.

†To whom reprint requests should be addressed.

mids were induced with isopropyl β-D-thiogalactopyranoside, and the fusion proteins were purified from culture lysates by affinity and anion-exchange chromatography. The purity of recombinant protein preparations was analyzed by SDS/PAGE and by immunoblotting.

SDS/PAGE and Immunoblotting Techniques. Solubilized protein preparations were analyzed on slab gels, comprising a 4.5% acrylamide stacking gel and a 12.5% resolving gel, according to the procedure of Laemmli. Proteins were transferred to nitrocellulose membranes in a mini Trans-Blot transfer cell (Bio-Rad). Immunoreactants were detected by chemiluminescence (ECL system; Amersham) (27).

Protein concentrations were determined by the Bradford assay (Sigma).

Animal Experimentation. Four- to 6-wk-old Swiss specific-pathogen-free mice (Centre d'Elevage R. Janvier, Le-Genest-St-Isle, France) were fed a commercial pellet diet with water ad libitum. These mice were previously shown to be free of the murine *Helicobacter* sp. *Helicobacter muridarum* (27). Aliquots (0.1 ml) containing 10^4 *H. felis* bacteria prepared from a low subculture stock suspension of *H. felis* were administered orogastrically to mice as described (27). Antigen extracts (50 μg of protein) containing 5 μg of cholera toxin (Sigma) were prepared in 0.1 M sodium bicarbonate before delivery to mice. After sacrifice, stomachs were removed and sera were collected.

H. felis colonization was assessed by the biopsy urease test and histological techniques. Portions of gastric antrum and body were placed on the surfaces of individual agar plates (1 × 1 cm) containing a modified Christensen's urea medium, to which had been added a *Helicobacter*-selective antibiotic mixture. The plates were observed for up to 48 h. The remaining two-thirds of each stomach were dissected into longitudinal segments (width, ≈2 mm), which were processed for histopathology (27).

To eliminate observer bias, Giemsa-stained sections were coded prior to histological assessment (by R.L.F.). For each stomach, all the available tissue (representing up to two-thirds of the stomach) was scrutinized. Protection from *H. felis* colonization was defined as the absence of *H. felis* bacteria from the totality of sections representing each stomach. The severity of gastritis was assessed on the basis of both the degree of mononuclear cell infiltration as well as the distribution of the cell infiltrates. Thus, gastritis was scored according to the following scale: 0, no significant infiltration; 1, infiltration of low numbers of lymphocytes limited to the muscularis mucosa and the submucosa; 2, infiltration of moderate numbers of lymphocytes in the submucosa, with variable numbers extending into the mucosa; and 3, infiltration of large numbers of lymphocytes in the mucosa, leading to the formation of several aggregates or even nodular structures.

ELISA. Seric IgG antibodies in immunized mice were detected by ELISA (19). Briefly, 96-well plates (Nunc Maxisorb) were coated with a sonicated extract of *H. pylori* (25 μg of protein per well). Bound IgG was detected with biotinylated goat anti-mouse antibodies (Amersham) and streptavidin–peroxidase conjugate. Immune complexes were detected by reaction with a solution containing *o*-phenylenediamine dihydrochloride (Sigma) and hydrogen peroxide. Optical densities were read at 492 nm in an ELISA plate reader (Titertek, Flow Laboratories).

Statistics. Data were analyzed by χ^2 and χ_c^2 (with Yate's correction) tests as appropriate (29), using the STATVIEW 512^+ computer software package (Brain Power, Calabasas, CA).

RESULTS

Determination of the Minimum Infectious Dose for *H. felis* in the Mouse. The *H. felis*-infected mouse has become the model of choice for trials aimed at identifying antigens that may serve in a future *H. pylori* vaccine. Thus far, the size of the *H. felis* inoculum used to challenge immunized animals has not been reconciled with the low *H. pylori* bacterial load that a vaccinated, noninfected individual would be expected to encounter when exposed to *H. pylori*-infected persons. To this end, we have determined the minimum infectious dose required to colonize Swiss mice with *H. felis* (under the conditions in our laboratory). Groups of five mice were thus colonized with inocula prepared from virulent *H. felis* bacteria, which varied from 10^1 to 10^5 bacteria (Table 1). While an inoculum containing ≈10^1 bacteria was found to be insufficient to colonize mice, gastric infection in mice was achieved with inocula containing at least 10^2 bacteria (minimum infectious dose). A challenge inoculum equivalent to 100 times the minimum infectious dose (i.e., 10^4 bacteria) was subsequently chosen for all immunoprotection studies.

Table 1. Determination of the minimum infectious dose for *H. felis* in mice

Inoculum dose, no. of bacteria	Identification of *H. felis* infection in mice at 2 wk postinoculation	
	Urease activity	Culture
10^1	0/5	0/5
10^2	4/5	3/5
10^3	5/5	4/5
10^4	5/5	3/5
10^5	4/5	4/5

To determine cell density, various dilutions of a stock *H. felis* culture (which contained predominantly helical-shaped forms) were prepared. Viable *H. felis* bacteria were then enumerated under phase-contrast microscopy (×400) with a Malassez chamber. Mice were inoculated orogastrically with 0.1 ml of the appropriate inoculum containing virulent *H. felis* bacteria. Urease activity was detected in murine gastric biopsies. *H. felis* bacteria were isolated from gastric tissue biopsies after incubation on blood agar plates under microaerobic conditions for 5–7 days at 37°C.

Protection Against *H. felis* Infection in Mice by Immunization with Recombinant HSPs from *H. pylori*. To demonstrate the presence of HSP homologs in *H. felis*, whole-cell extracts of the organism were immunoblotted and then reacted with hyperimmune rabbit antisera raised against *H. pylori* MalE–HspA and MalE–HspB fusions. Cross-reactive antigens were detected in the *H. felis* extract; the denatured antigens had approximate molecular masses of 15 and 58 kDa, respectively,

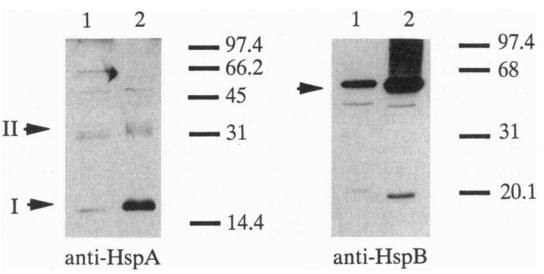

FIG. 1. Immunoblot analyses of total cell extracts of *H. felis* (lane 1) and *H. pylori* (lane 2) using rabbit antisera raised against recombinant *H. pylori* HspA and HspB antigens (dilution, 1:5000). Arrows refer to cross-reactive proteins: monomeric (I) and dimeric (II) forms of HspA antibody-reactive proteins are indicated. Protein standards (kDa) are indicated on the right. Immunoreactants on the anti-HspA-blotted membrane were revealed directly with a peroxidase-labeled secondary antibody, while antigens on the membrane reacted with anti-HspB antibody were detected by a biotinylated secondary antibody/streptavidin–peroxidase procedure. The latter was found to give higher background staining and, when used to detect immunoreactants on membranes blotted with the anti-HspA antibody, produced very weak signals.

Table 2. Immunization of mice against *H. felis* infection using *H. pylori* antigens

Antigen(s)	*H. felis* infectious status of mice: Infected, no.	*H. felis* infectious status of mice: Not infected, %	Grade of gastritis: Infected	Grade of gastritis: Not infected
MalE (M)	14/20	30	2.57 ± 0.65 (14)	1.0 ± 0 (6)
Sonicate*	1/17	94	3 (1)	1.31 ± 0.79 (16)
M-HspA**	4/20	80	3 (4)	1.19 ± 0.83 (16)
M-HspB***	3/10	70	3 (3)	1.0 ± 0.82 (7)
M-UreB****	3/21	86	2.3 (3)	1.17 ± 0.38 (18)
M-HspA/UreB*****	0/19	100	—	1.53 ± 0.70 (19)
			2.68 ± 0.56 (25)†	1.28 ± 0.71 (82)†

Mice were immunized once per wk (wk 0–3) with 50 μg of antigen (or 1 mg of *H. felis* whole-cell sonicate) and 5 μg of cholera toxin. At wk 5, the mice were challenged with an inoculum containing ≈10^4 *H. felis* bacteria. At wk 7, the mice were sacrificed. *, $P = 0.0003$; **, $P = 0.0042$; ***, $P = 0.0904$; ****, $P = 0.001$; *****, P, 0.0001 compared with the MalE group of animals. Mice were considered not infected when the biopsy urease test was negative, and no *H. felis* bacteria were detected in coded histological sections. Gastritis was scored from 0 to 3. Mean scores ± SD are presented. Numbers in parentheses refer to numbers of animals per group. —, No mice from this group were infected.
†Comparison of score frequencies between immunized animals that became infected and those that were protected ($P = 0.0001$).

which corresponded to those of the *H. pylori* HSPs (Fig. 1). Interestingly, it appeared that the HspA homologs of both *H. pylori* and *H. felis* exist in dimeric forms and these multimeric forms appeared to be resistant to the denaturing effects of SDS.

Recombinant *H. pylori* HSP antigens were assessed for their potential to induce protective mucosal responses in the *H. felis* mouse model. Immunization with MalE–HspA or MalE–HspB fusions protected 80% and 70%, respectively, of mice against *H. felis* infection (Table 2). In comparison, 30% of MalE-immunized control mice did not become infected when challenged with the *H. felis* inoculum ($P = 0.0042$ and $P = 0.0904$, respectively).

Coadministration of recombinant *H. pylori* MalE–UreB and MalE–HspA antigens to mice resulted in 100% protection, which compared with a protection rate of 86% in those animals that had received the MalE–UreB antigen alone (Table 2). The level of protection afforded by coadministration of MalE–UreB and MalE–HspA was equivalent to that obtained in the group of *H. felis* sonicate-immunized animals ($P = 0.955$; Table 2).

Serological Responses After Immunization with Recombinant HSPs and Urease Polypeptides. Measurement of *H. pylori*-specific IgG antibodies in the serum of immunized mice demonstrated that virtually all of the animals developed strong humoral responses to the administered *H. pylori* urease and heat shock antigens. As would be predicted of a mucosal immune response (30), serum antibodies directed against these antigens appeared to be primarily of the IgG1 isotype (Fig. 2). This finding was indicative of a predominantly type 2 T-helper cell (Th2) response. Consistent with this, serum levels of *H. pylori*-specific IgG2a antibodies, which are normally associated with Th1-type responses (30), were relatively low and varied depending on the antigen administered; MalE–HspA appeared to induce particularly weak IgG2a serum responses (Fig. 2). These differences were considered to be specific to the *H. pylori* antigenic components of the recombinant proteins, since approximately equivalent levels of IgG1 and IgG2a antibody isotypes were detected when MalE-specific antibodies were measured (unpublished data). No qualitative or quantitative differences could be found between IgG serum responses and the infectious status of the mice at sacrifice.

Cellular Responses Induced in Mice After Immunization. Histological assessment of gastric mucosa tissue from the immunized mice revealed low levels of mononuclear cells (mean inflammation score, 1.28 ± 0.71) for those mice that were protected from an *H. felis* infection (Table 2). In contrast, those immunized animals that became infected tended to have a significantly more severe form of lymphocytic gastritis in which lymphoid follicular structures were often observed (mean score, 2.68 ± 0.56; $P = 0.0001$). Large numbers of mononuclear cells were also observed in the gastric tissue of *H. felis*-colonized mice from the MalE-immunized group.

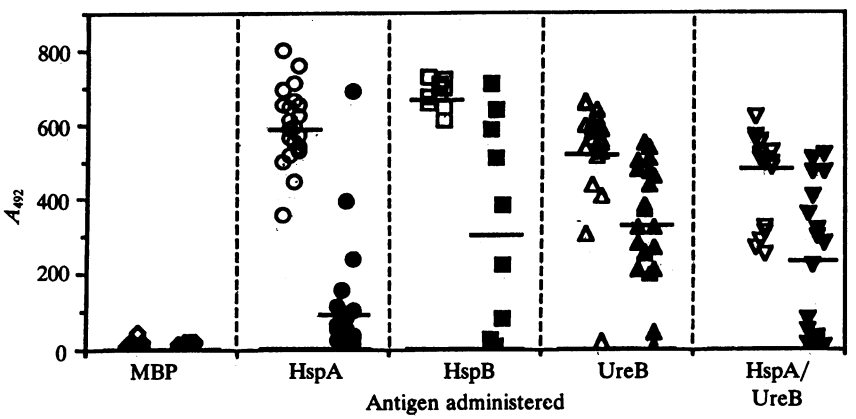

FIG. 2. Measurement by ELISA of serum antibodies [IgG1 (open symbols) and IgG2a (solid symbols) isotypes] in mice immunized with recombinant *H. pylori* antigens. A_{492} values for individual serum samples (diluted 1:100) are shown. Horizontal lines represent mean A_{492} values for each set of data. MBP, maltose-binding protein.

DISCUSSION

HSPs represent conserved families of proteins, the synthesis of which increases in response to environmental stresses, such as heat shock. These families of proteins, of which *E. coli* GroES and GroEL are the archetypes, are involved in chaperone functions in the cell (31). We have shown that the HSP homologs of *H. pylori*, designated HspA and HspB, respectively, are able to confer immunity to a gastric mucosal infection. Preliminary findings suggest that this protection, in common with immunization against other mucosal infections, is mediated by a predominantly Th2-type immune response.

In previous studies, promising results were presented regarding the use of recombinant urease proteins as antigens. Nonetheless, the protective efficacies of these recombinant antigens could not, for the most part, be directly compared with that of the "gold standard": a sonicated whole-cell extract of *H. felis*. In this study, we tested an antigenic preparation consisting of two recombinant proteins, *H. pylori* UreB and HspA, and showed that, under identical experimental conditions, it was as effective as a whole-cell extract of *H. felis* in protecting against *H. felis* infection in mice.

The infectious dose of a given pathogen is an important parameter in the testing of any vaccine candidate. In the case of *H. pylori*, volunteer studies (32, 33) and experimental models (34, 35) (which used very high bacterial doses, usually delivered to hypochlorhydric stomachs) have provided limited information in this regard. There is indirect evidence which suggests that the infectious dose for *H. pylori* may in fact be quite low. *H. pylori* infection appears to be transmitted by close person-to-person contact, possibly via the oral–oral route (36). Yet, despite this, *H. pylori* bacteria cannot easily be detected in the saliva and feces (37) of infected individuals. Environmental sources of infection seem unlikely as the organism does not survive for long once outside the human body.

If the *H. felis*-infected mouse is to serve in the initial testing of potential *H. pylori* vaccines, it is necessary that mice be challenged with doses of *H. felis* bacteria that do not overwhelm the host's immunity and mimic those that a vaccinated individual might encounter. By passaging *H. felis* cultures *in vivo*, we have selected for virulence and, as a consequence, were able to show that a single dose of 10^2 virulent bacteria resulted in colonization of the mouse (Table 1). At a dose of 10^4 bacteria, 70% of animals that had been immunized with MalE and cholera toxin became infected (Table 2); the remaining 30%, we suggest, were able to resist infection. It may be that the natural resistance of the mice was increased due to the stimulation by cholera toxin of a nonspecific mucosal response. Alternatively, it is tempting to speculate that this resistance may be analogous to that of certain individuals who presumably, given the ubiquitous nature of *H. pylori* infection within the population, come into contact with *H. pylori* bacteria but somehow do not become infected. Further studies aimed at unraveling the complex relationship between infectious dose, genetic differences between hosts, and differences in bacterial virulence should help to elucidate the processes involved in *H. pylori* infection.

The presence of high levels of *H. pylori*-specific IgG1 antibodies in the sera of mice that had been immunized with recombinant *H. pylori* antigens was indicative of a Th2-type immune response. No correlation, however, was found between the humoral response and the ability of mice to clear an *H. felis* infection. Mice produced variable IgG2a antibody responses depending on the *H. pylori* antigen administered; since this antibody subclass has a high complement-fixing activity (38), opsonization does not seem to be involved in the clearance of *H. felis* bacteria from the mouse. These findings tend to concur with those of other workers, who reported that the presence of antigen-specific secretory IgA in mucosal secretions was associated with protection against *H. felis* challenge in mice (25, 39). Such antibodies would probably be involved in the agglutination and hence removal of bacteria from the stomach.

It has previously been shown that certain bacterial GroEL proteins are able to induce protective immune responses (21, 22). The involvement of GroEL-reactive T lymphocytes in autoimmune disease has, however, led to controversy concerning the use of these proteins as components in vaccines (22, 40). Several workers have reported that the existence of T and B cells reactive to GroEL proteins in an individual may not necessarily be associated with disease (22). In the case of *H. pylori*, it has been speculated that its GroEL homolog (HspB) is an important proinflammatory factor and is believed to mediate the chronic inflammation seen in *H. pylori*-infected individuals (41, 42). Sharma *et al.* (43), however, found that T cells from *H. pylori*-infected subjects did not recognize autologous HSP, while lymphocytes reactive to human GroEL did not react with *H. pylori* HspB. Similarly, we observed in both this study and in an independent one in which immunized mice were not challenged with *H. felis* (unpublished data) that the administration of MalE-fused *H. pylori* HSPs did not appear to be associated with an unduly severe pathology.

The evidence to date suggests that a mild gastric inflammation may be a necessary prerequisite for a successful orogastric immunization (26, 27). Activation of a Th2 immune response is normally associated with the migration of both IgA-secreting B lymphocytes and Th lymphocytes to effector tissue sites (30). It is, therefore, perhaps not surprising that orogastric immunization of mice results in a mild degree of lymphocytic gastritis. Administration of cholera toxin may contribute to this inflammation; *in vitro* experiments showed that cholera toxin alone increased the proliferation of murine B and T lymphocytes (44). It is also likely that the antigenic load provided by the *H. felis* bacterial challenge exacerbates the inflammation; immunized mice that became infected with *H. felis* displayed a higher degree of gastritis than those immunized animals that were protected against *H. felis* infection. However, as this difference was also observed among the MalE-immunized group of mice, it is unlikely that cross-reactivity between the recombinant *H. pylori* antigens and the *H. felis* bacteria accounted for the severe pathology seen in those immunized mice that were not protected. Eaton and Krakowka (45) also observed that immunized piglets, which were not protected against *H. pylori* infection, developed severe gastritis.

H. pylori HspA is particularly appealing as a vaccine component because, in contrast to HspB, it possesses a unique domain at its C terminus that is absent from other known heat shock homologs, including those of eukaryotic organisms (19). The C terminus of *H. pylori* HspA consists of a series of 26 amino acids (of a total of 118 amino acids) and undoubtedly confers a unique conformational structure to this polypeptide. The capacity of *H. pylori* HspA to bind to nickel ions should facilitate the large-scale purification of this polypeptide by metal affinity chromatography.

Evidence from the immunoprotection studies and immunoblot analyses suggests that *H. felis* produces a GroES homolog. Whether this protein also contains the C-terminal nickel-binding domain remains to be determined. It is noteworthy that these *Helicobacter* GroES homologs seem to exist as dimeric forms, a feature that has also been described for other known nickel-binding proteins, such as the UreE proteins from *Proteus mirabilis* (46) and *Klebsiella aerogenes* (47).

While HSPs of the GroEL class have been successfully used as antigens to immunize against infections by legionellae and mycobacteria, GroES homologs have not previously been used for such a purpose. Recently, Launois *et al.* (48) proposed that the GroES homolog of *Mycobacterium leprae* may be a candidate antigen against lepromatous disease. Induction of protective immunity in mice after immunization with *H. pylori*

HspA provides an example of an instance in which a protein belonging to the GroES class has been shown to act as a protective antigen.

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