Immune Response to an 18-Kilodalton Outer Membrane Antigen Identifies Lipoprotein 20 as a \textit{Helicobacter pylori} Vaccine Candidate

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Experiments were performed using the standardized murine model of \textit{Helicobacter pylori} infection to determine the immunogenicity of \textit{H. pylori} outer membrane vesicles in immune protection. These vesicles, which are naturally shed from the surface of the bacterium, induce a protective response when administered intragastrically to mice in the presence of cholera holotoxin, despite the absence of the urease enzyme and associated Hcp54 chaperonin. Immunoblotting identified a specific serum immunoglobulin G (IgG) response to an 18-kDa outer membrane protein in a significant number of immunized animals. This commonly expressed, immunodominant protein was subsequently identified as lipoprotein 20 (Lpp20). Hybridoma backpacks secreting an IgG1 subclass monoclonal antibody to Lpp20 were generated in \textit{H. pylori}-infected mice and were found to significantly reduce bacterial numbers, providing evidence that this surface-exposed antigen is a true vaccine candidate and not merely an antigenic marker for successful, protective immunization.

\textit{Helicobacter pylori}, a bacterium which is estimated to infect more than half the world’s population, is associated with peptic ulcer disease (4) and the development of gastric cancer (32). Immunization against this bacterium represents a cost-effective strategy to reduce global gastric cancer rates (5) and would also have a major impact on \textit{H. pylori}-related peptic ulcer disease. \textit{H. pylori} vaccine candidates identified to date include the urease enzyme (20, 40, 51, 55) and the urease enzyme chaperonin heat shock protein A (21). Mice immunized with purified VacA cytotoxin are also protected from challenge with a Tox\textsuperscript{+} strain of \textit{H. pylori} (48). A common factor among these three vaccine candidates is their reported association with the outer membrane of \textit{H. pylori} (1, 16, 17, 27, 36, 52, 57). The potential of catalase as an \textit{H. pylori} vaccine candidate has also been identified (58). This enzyme, which is found in both the cytosol and the periplasmic space of \textit{H. pylori} (28), is also thought to be surface exposed (57). More recently, the screening of recombinant \textit{H. pylori} antigens (30) has identified another five potential \textit{H. pylori} vaccine candidates. These include Lpp20, a conserved \textit{H. pylori} lipoprotein that is membrane associated but not surface exposed (38).

In our search for candidate \textit{H. pylori} vaccine antigens, we have focused on the outer membrane of the bacterium. Like many other gram-negative bacteria (reviewed in reference 25), \textit{H. pylori} and \textit{Helicobacter felis} shed part of their outer membrane as vesicles when grown under certain conditions (34). These outer membrane vesicles (OMV) are thought to be formed when the outer membrane of the bacterium expands faster than the underlying peptidoglycan layer, resulting in portions of the membrane blebbing off the surface of growing cells (44). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis reveals that the protein and lipopolysaccharide content of these OMV closely resembles that of a Sarkosyl-insoluble outer membrane preparation of the parent bacterium (J. Keenan, unpublished observation).

We found that 70% of BALB/c mice were protected from infectious challenge with \textit{H. felis} following intragastric immunization with \textit{H. felis} OMV and cholera toxin (CT) (Keenan, unpublished). Furthermore, protection from infectious challenge in these animals correlates with marked serum immunoglobulin G (IgG) antibody responsiveness to an 18-kDa antigen present in \textit{H. felis} OMV (35). \textit{H. pylori} outer membranes are also immunogenic in mice (14). We found that intragastric immunization with \textit{H. pylori} OMV in conjunction with CT as an adjuvant elicits a serum IgG response to a similarly sized immunodominant outer membrane antigen (35) which is commonly expressed by \textit{H. pylori} strains (34).

In this study, we used the recently developed standardized murine model of \textit{H. pylori} infection (39) and confirmed the immunogenicity of \textit{H. pylori} OMV in immune protection. As with the \textit{H. felis} model, antibodies to the 18-kDa outer membrane antigen were a marker for protective immunity in mice. A monoclonal antibody (MAb) to the \textit{H. pylori} antigen, used to screen an \textit{H. pylori} genomic expression library, identified this outer membrane antigen as Lpp20. In vivo passive-protection experiments with mice confirmed that Lpp20 is a candidate vaccine antigen and not merely an antigenic marker for successful, protective immunization. In addition, we used immunolabeling studies to show that Lpp20 is surface exposed, not only on \textit{H. pylori} but also when expressed as a recombinant protein by \textit{Escherichia coli}.

MATERIALS AND METHODS

Mice. Specific-pathogen-free, female BALB/c mice were housed according to Health Research Council of New Zealand guidelines and were allowed free access to food and water.

\textit{Bacteria}. A well-characterized, \textit{Tox}\textsuperscript{+} strain, \textit{H. pylori} 60190 (41), produced the OMV used to immunize the mice. Mice were subsequently challenged with the SS1 (Sydney) strain of \textit{H. pylori} (39). Both strains were grown in 2.8\% (wt/vol) brucella broth base (Difco, Detroit, Mich.), supplemented with 5% fetal calf serum (Gibco BRL, Auckland, New Zealand). Cultures were incubated at 37°C in a microaerobic environment (10% hydrogen, 10% carbon dioxide, and 80% nitrogen) and were shaken at 120 rpm. \textit{E. coli} strains were routinely grown in...
Luria-Bertani (LB) broth or on LB plates (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract [Difco], 0.5% [wt/vol] NaCl [pH 7.0]) at 37°C under aerobic conditions with aeration at 200 rpm. Recombinant E. coli organisms were grown in LB medium containing 100 μg of ampicillin ml⁻¹, which were selected by ampicillin resistance. H. pylori OMV. Whole bacteria were harvested from 48- to 72-h broth cultures by two centrifugations (10,000 × g, 15 min, 4°C). The spent-culture supernatants were ultracentrifuged (100,000 × g, 2 h, 4°C), and the resulting pellet of OMV was washed three times with phosphate-buffered saline (PBS) (100,000 × g, 2 h, 4°C) (35). The absence of whole bacteria and flagella in the preparation was confirmed by electron microscopy. The protein concentration of the OMV fraction was assayed (49) prior to storage of the fraction at −20°C until use.

Polyethylene glycol precipitation and challenge of mice. Six to eight-week-old mice were immunized four times by gastric intubation at weekly intervals. Each dose consisted of 50 μg of H. pylori (60190) OMV protein and 10 μg of CT (Sigma Chemical Co., St. Louis, Mo.) (33). Age-matched control mice were not immunized. Mice were challenged with a single dose of 10⁶ H. pylori (SS1) organisms 7 days after the last immunization.

Assessment of protection. Twenty-eight days after challenge, the mice were killed by cervical dislocation. The stomach of each animal was removed, bisected longitudinally, and pinned out. Full-thickness tissue (5 by 5 mm) was taken from the antrum-body area of one-half of each stomach and placed in 0.2 ml of urease test medium (29). Urease activity in the samples, identified by a distinctive color change in the medium, was assessed after 24 h of incubation at room temperature (RT). The remainder of the stomach was fixed in 10% buffered formalin and embedded in paraffin. Longitudinal sections, stained with a modified May-Grünwald Giemsa stain, were scanned full length using light microscopy (oil immersion lens). H. pylori cells per longitudinal section were counted and scored as follows: 0, no cells; 1, less than 10 cells; 2, 10 to 50 bacteria; 3, 50 to 100 bacteria; or 4 (+) >100 bacteria. Mice with scores of 0 or 1 were considered protected (40).

Immunoblot analysis of antibody response to immunization. Serum antibody specificity was determined by immunoblotting following electrophoretic transfer of SDS-PAGE-separated (12.5% acrylamide) H. pylori OMV to 0.45-μm-pore size nitrocellulose (NC) membranes. Following a 30-min wash in Tris-saline blotting buffer, antigen-impregnated NC strips (5 μg of protein) were incubated with individual sera for 2 h at RT. After a washing, bound murine antibodies were detected by incubation of the strips in alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma) for 1 h at RT. Secondary antibody binding was detected by reaction with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium substrates (12).

Production and screening of an anti-H. pylori 18-kDa outer membrane antigen hybridoma. H. pylori 18-kDa outer membrane antigen-specific MAbs were produced following subcutaneous immunization of a BALB/c mouse, as described previously (35). Briefly, the 18-kDa antigen was identified following its separation from other outer membrane components by preparative SDS-PAGE and immunoblotting to NC. The band was excised and implanted under the dorsal skin of the mouse. Twenty-one days later, the animal was injected intraperitoneally with H. pylori (60190) OMV (50 μg of protein). Ten syngeneic lymphocytes were fused with FOXY mouse myeloma cells at a ratio of 5:1, 7 days after the second immunization (42). Clones were obtained by limiting dilution and were screened with FOX-NY mouse myeloma cells at a ratio of 5:1, 7 days after the second immunization. Mice were challenged with a single dose of 10⁶ H. pylori (SS1) organisms 7 days after the last immunization.

In vivo passive-protection experiment. Eight- to ten-week-old naive BALB/c mice were infected with H. pylori SS1 by intragastric intubation. A total of 10⁹ viable bacteria were given over two consecutive days. Hybridoma cells producing anti-H. pylori Lpp20 (MAb 6A8) or anti-tetanus toxoid (CMRF-82) were grown in RPMI medium, harvested, and washed twice in PBS. CMRF-82, an IgG1 subclass antibody against a tetanus toxin component, fails to display reactivity against H. pylori OMV antigens (see Fig. 2D) and was used in this experiment as a control. At day 4, 10⁶ hybridoma cells were injected subcutaneously between the scapulae of each mouse to generate IgG1-secreting hybridoma tumors (50, 64). When the experiment was concluded at day 20, every mouse was carrying a large backpack tumor. Immunoblotting of sera from these mice confirmed the presence of circulating MAb. The ability of MAb 6A8 to protect these mice from H. pylori infection was assessed by quantitative culture (22) as well as biopsy urease and histological analyses (see above) of antrum-body tissue samples. To perform quantitative bacterial counts, tissue segments were homogenized in 500 μl PBS containing 0.1% Triton X-100. The homogenate was serially diluted and plated onto selective medium. Bacterial counts were expressed as the mean number of CFU per gram of tissue.

Immunolabeling of whole bacteria with MAB 6A8. MAB 6A8 was used to immunolabel the surface of H. pylori 60190, as described previously (35). In a similar experiment, the same technique was used to immunolabel E. coli expressing recombinant Lpp20. Briefly, whole bacteria were washed twice with low-salt phosphate, overlaid onto carbon-collodion-coated mesh grids, and blocked with 0.1% bovine serum albumin (in low-salt phosphate) for 15 min before being incubated in MAB 6A8 (diluted in blocking buffer) for 1 h at RT. Gold (10 nm)-labeled goat anti-mouse IgG was used to detect murine antibody binding. The grids were then negatively stained with 1% aqueous phosphotungstic acid (pH 7.0) prior to examination.

Statistical analysis. Fisher’s exact test was used to evaluate the presence or absence of experimental infection in test and control animals as well as the anti-18-kDa outer membrane antigen response to immunization. P values were determined by the InStat software program (GraphPad, San Diego, Calif.).

RESULTS

Immunization of mice with H. pylori OMV correlates with serum reactivity against an immunodominant 18-kDa major antigen. Intragastic immunization with H. pylori (60190) OMV and CT conferred immune protection against H. pylori (SS1) challenge in 10 of 10 mice (100%). In contrast, 4 of 5 (80%) naive control animals were infected with H. pylori (Fig.
1). These differences were statistically significant ($P < 0.0013$). Protection from infectious challenge correlated with serum antibody reactivity to an *H. pylori* 60190 OMV antigen with an apparent molecular mass of 18 kDa in 8 of the 10 mice following intragastric immunization (Fig. 2b). Similar reactivity, absent in sera collected from these same animals prior to immunization (Fig. 2a), was seen when these same sera were immunoblotted against OMV from *H. pylori* SS1 (results not shown). Statistical analysis revealed that this specific antibody response to immunization was significant ($P < 0.0007$). Sera from mice sham immunized with PBS and CT (35) failed to display similar immune responsiveness (results not shown), indicating that this response is specific to animals immunized with *H. pylori* OMV.

**Immunoscreening of the *H. pylori* expression library with MAB 6A8.** Immunoscreening of the expression library with MAB 6A8 revealed eight strongly positive plaques. After secondary screening to confirm their reactivity, the *H. pylori* DNA was excised from each and recircularized to form a phagemid containing the *H. pylori* insert DNA. Restriction digests of these phagemids revealed that six contained an identical insert of approximately 2 kb, while the remaining two contained inserts of approximately 2.5 kb (data not shown). Sequence analysis of these phagemid inserts revealed that they all mapped to the same region of the *H. pylori* genome, encompassing the promoter and start codons of three genes (HP 1455, HP 1456, and HP 1457). Each of these three genes contains a putative signal peptide and therefore is a candidate to encode an OMV protein. Two of these genes (HP 1456 and HP 1457), when translated from the putative cleavage point, produce proteins of approximately 19.11 kDa (HP 1456) and 23.31 kDa (HP 1457). Although the predicted size of the gene product indicates that the HP 1456 ORF is likely to encode the 18-kDa antigen detected by the antibody, further experiments were required to confirm this.

**Identification of Lpp20 as the immunogenic 18-kDa antigen.** To investigate which ORF (HP 1456 or HP 1457) encoded the 18-kDa antigen, the ORFs of each were cloned and the protein was expressed as a recombinant fusion protein. Oligonucleotide primers were designed to amplify a truncated ORF devoid of the signal sequence. Expression of the recombinant fusion protein was maximally induced in pPROex HTb over 4h by the addition of 0.5 mM IPTG (results not shown). Expression of HP 1456 and HP 1457 in this vector resulted in the production of fusion proteins of 23.6 and 27.8 kDa, respectively, with the N-terminus being replaced by glutathione S-transferase (GST) and a recognition site for the protease...
PreScission. Total protein was prepared from cultures of cells containing each construct, separated on an SDS–12.5% PAGE gel, and transferred to PVDF for Western blotting with MAb 6A8. A positive reaction was seen with both HP 1456 fusion proteins, but no reaction was observed with either HP 1457 protein (Fig. 4). The HP 1456 gene codes for H. pylori Lpp20, which is released from the surface of the bacterium during growth in broth culture, we were able to protect 100% of mice from H. pylori SS1 challenge following oral immunization with H. pylori 60190 OMV with CT as a mucosal adjuvant.

Two well-documented vaccine candidates are associated with the surface of Helicobacter (20, 21, 40, 51, 55) and could have contributed to the protective effect seen in these studies. However, both functional and immunological assays used to screen for the presence of the urease enzyme and its associated Hsp54 chaperonin failed to detect either of these antigens in the OMV fraction of H. pylori (36). Our ability to protect mice from infectious challenge in the absence of both urease and associated Hsp suggested the presence of a new vaccine candidate in the outer membrane fraction. Immunoblotting demonstrated specific serum IgG immunoreactivity to an OMV component with an apparent molecular mass of 18 kDa in immunized and protected mice. Using a MAb, we subsequently identified Lpp20 as the potential vaccine candidate.

Lipoproteins are major antigens in a number of bacterial pathogens, including E. coli (33), Haemophilus influenzae (9), Pseudomonas aeruginosa (43), Borrelia burgdorferi (18), and Campylobacter jejuni (7). A number of unrelated studies have now identified Lpp20 as an immunodominant H. pylori antigen (8, 30, 34, 38). Moreover, it is likely that the immunoreactive species-specific 19-kDa H. pylori outer membrane protein described in an earlier study is also Lpp20 (15). H. pylori is noninvasive, and it is likely that outer membrane-associated Lpp20 (38), which is released from the surface of H. pylori during growth in vitro (8), is delivered to the gastric mucosa in the OMV shed from the surface of the bacterium in vivo (36). Despite the potential problems of qualitative and quantitative expression of outer membrane proteins, the Lpp20 antigen
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labeling studies of that bind significantly to homologous antigen only (34). Immunor
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and not merely an immunogenic marker for protection. Using mice (50, 64), to show that Lpp20 is a true vaccine candidate
based on the generation of hybridoma backpack tumors in molecules (26). We used an in vivo passive-protection model,
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Bacterial lipoproteins are well described, not only as vaccine
target candidates (23, 61, 62) but also as immunostimulatory molecules (26). We used an in vivo passive-protection model,
based on the generation of hybridoma backpack tumors in mice (50, 64), to show that Lpp20 is a true vaccine candidate and not merely an immunogenic marker for protection. Using this model, we demonstrated that an anti-Lpp20-secreting tu-
ror in vivo correlated with a significant decrease in H. pylori colonization of the murine gastric mucosa. A recently pub-
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An isogenic mutant, defective in the production of Lpp20, shows this H. pylori protein to be nonessential for growth in vitro (38). However, in the hybridoma backpack model, the reduction in gastric H. pylori levels correlated with expression of the Lpp20-specific MAb in the circulation of infected mice. This finding strongly suggests a role for antibody-mediated protection against this bacterium, despite recent evidence to the contrary (19, 56). We find that BALB/c mice recognize a similarly sized surface antigen following infection with H. pylori SS1. However, our preliminary evidence suggests that protection is not only related to the specificity but also reliant on the magnitude and subclass of the response (Keenan, unpublished).

However, a protective antibody response does not have to target a specific bacterial function (3). If protection is medi-
ated (at least in part) by specific antibody action, successful
immunization may be the result of sufficient antibody binding to Lpp20 epitopes, thereby cross-linking and agglutinating the bacteria and ultimately enhancing their removal by peristalsis (40). Support for this hypothesis comes from the observation that the most likely H. pylori vaccine candidates identified to date (urease, heat shock protein, and Lpp20) are all surface exposed (1, 16, 17, 27, 35, 52, 57).

Finally, electron microscopy of immunolabeled E. coli trans-
formants expressing recombinant Lpp20 showed the lipoprotein to be surface exposed, an observation which is also noted when the P. aeruginosa lipoprotein gene (opri) is cloned into E. coli (10). Live carriers are ideal vaccine delivery systems and are being increasingly used to express large amounts of protective recombinant antigens (11, 12, 23, 61, 62). We are cur-
cently investigating a role for a recombinant carrier such as Salmonella enterica serovar Typhimurium phoP(Con) (11) to provide a mucosal vaccine vector to deliver Lpp20 to antigen-presenting cells on mucosal surfaces.

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FIG. 6. Immunogold electron microscopy of H. pylori 60190 (a) and E. coli expressing recombinant Lpp20 (b) following incubation in 6A8, a MAb specific for Lpp20. A gold-labeled goat anti-mouse IgG antibody detected MAb binding to the outer membranes of both bacteria.

appears to be commonly expressed in all H. pylori strains ex-
amined so far (15, 34, 38). Furthermore, no cross-reaction is shown when antibodies (polyclonal and monoclonal) to H. pylori Lpp20 are used to immunoscreen closely related species of Helicobacter (15, 34, 38), Campylobacter (15, 38), or a di-
verse range of other bacteria (38). This supports a search of
data banks which shows the lpp20 gene to be unique to H. pylori (38).

The amino acid sequence of Lpp20 implies outer membrane localization of this protein based on the prediction of Yamagu-
chi et al. (65). This was confirmed with an anti-Lpp20 MAb. Furthermore, immunolabeling of H. pylori with gold-labeled anti-Lpp20 antibodies confirmed that the protein is expressed on the surface of the bacterium. In contrast, cross-reactivity with H. felis proteins was not demonstrated using MAb 6A8 (35). This supports an earlier observation that mice immunized with either H. pylori or H. felis OMV produce serum antibodies that bind significantly to homologous antigen only (34). Immuno-
labeling studies of H. pylori by Drouet et al. also find an immunogenic 19-kDa antigen to be surface exposed (15). The
failure, therefore, of Kostrzynska et al. to show Lpp20 on the surface of H. pylori may simply reflect conformational differ-
ces in the SDS-PAGE-denatured protein used to raise their rabbit polyclonal anti-H. pylori Lpp20 antisera (38).

PhoP of P. aeruginosa is cloned into E. coli (Con) (11) to

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