Catalase, a Novel Antigen for Helicobacter pylori Vaccination

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The efficacy of an orogastric vaccine comprised of purified Helicobacter pylori catalase plus the mucosal adjuvant cholera toxin (CT) was examined with both the Helicobacter felis and H. pylori mouse models with BALB/c mice. Native H. pylori catalase (200 μg) plus CT was initially used as a vaccine antigen in the H. felis mouse model and protected 80% (8 of 10) of the challenged animals, while all control animals were infected (20 of 20). In a follow-up experiment, recombinant H. pylori catalase plus CT was used for immunization, and groups of mice were challenged with the Sydney strain of H. pylori. Immunization with recombinant catalase protected a significant proportion (9 of 10) of the mice from H. pylori challenge, indicating that this enzyme should be considered as a candidate for a future vaccine. This study provides the first available data on the efficacy of protective immunization with the new Sydney strain of H. pylori in a mouse model. These data also provide indirect evidence that proteins which are normally intracellular, such as catalase, may be present on the surface of H. pylori and thus may provide targets for immunization.

Helicobacter pylori is a spiral-shaped microaerophilic bacterium which colonizes the gastric mucosa of humans. Infection with H. pylori has been associated with gastritis and peptic ulcer disease (14, 24), and the bacterium was recently categorized as a class I carcinogen (12). Natural symptomatic infection with H. pylori initially causes an acute inflammatory response (9, 21), followed by the development of a specific cellular and humoral immune response which is generally ineffectual at clearing the infection (5).

The acute inflammatory response to H. pylori infection consists chiefly of polymorphonuclear leucocytes, which through their oxidative burst are cytotoxic for bacteria. To combat this, H. pylori and many other bacteria produce enzymes such as catalase and superoxide dismutase (SOD) to catalyze the elimination of toxic oxygen species (2). The importance of catalase to survival, particularly in microaerophilic environments, has been demonstrated with enzyme-negative mutants of Campylobacter spp. and H. pylori (10, 11, 14). Genetic analysis of H. pylori SOD has indicated that the enzyme is similar to those of facultative intracellular pathogenic microorganisms and that it is either surface associated or actively secreted (25). This supports a role for the enzyme in protection against oxidative damage from processes external to the cell. Thus, it appears that both of these enzymes have an essential function in the pathogenesis of H. pylori, allowing the organism to resist attack from the host’s armory of inflammatory cells.

Characterization of H. pylori catalase (EC 1.11.1.6) has indicated that the enzyme is highly expressed and has a number of unique properties which may enable the bacterium to survive in an environment rich in toxic oxygen species (11). The enzyme consists of four subunits with an estimated Mr of ~50,000, and there is evidence that H. pylori catalase may be located both throughout the cytosol and in the periplasmic space (11). Other putative virulence determinants, such as urease (3, 6, 19, 22), the heat shock proteins associated with urease (8), and a vacuolating cytotoxin, VacA (18), are all protective in murine models of Helicobacter infection. This study was undertaken to determine if immunization with catalase could induce a protective immune response in rodents.

The murine model that has been used to evaluate most vaccine candidate antigens is the Helicobacter felis mouse model. H. felis was isolated from feline gastric mucosa, and in some strains of mice, H. felis causes pathology similar to that seen in humans infected with H. pylori (15). The model has been used to demonstrate that antibiotic regimens used against human H. pylori infection could be screened in an animal system (7) and was later found to be an effective tool in immunization studies (4–6). The use of this model to test the protective efficacy of H. pylori proteins may have the advantage of selecting for antigens which are conserved throughout the genus and which are not restricted to a subset of H. pylori strains.

Until fairly recently, a mouse model of H. pylori infection has not been available; however, it has now been shown that some clinical isolates of H. pylori can colonize the mouse stomach if passed through a series of mice (18). We have now established an H. pylori mouse model by using the same technique. Our mouse-adapted H. pylori isolate, referred to as the Sydney strain (SS1), has been shown to colonize mice with high infection levels, specific adhesion to gastric epithelial cells, and a pathology similar to that seen in humans (17).

Marked catalase activity is a distinctive characteristic of H. pylori. The enzyme has an important role in protecting these bacteria from oxidative damage in the gastric environment. H. pylori catalase is found in both the cytosol and the periplasmic space (11); additionally, it has been suggested that catalase may be surface expressed (23). Based on these observations, catalase was selected as a potential target for vaccination. This study reports the efficacy of purified native and recombinant H. pylori catalases to stimulate protective immunity with the H. felis mouse model and the Sydney strain of H. pylori.

MATERIALS AND METHODS

Mice. Female specific-pathogen-free (SPF) BALB/c mice were obtained from Combined Universities Lab Animal Services Pty Ltd., Sydney, Australia, and used at 6 to 8 weeks of age. The animals were housed in the School of Micro-
biology and Immunology Animal House, University of New South Wales, and fed on autoclaved food pellets (Clarkel Holdings, Sydney, Australia), and sterile water was given ad libitum.

**Bacteria.** *H. pylori* (clonal strains 921023 and RU1, provided by Hazel Mitchell, Univ. of New South Wales) and *Campylobacter jejuni* (strain 23, provided by Jani O’Rourke, University of New South Wales) were grown on blood agar base no. 2 (Oxoid, Basingstoke, United Kingdom) with 5% (vol/vol) defibrinated horse blood (Oxoid) containing 2.5 g of hemin, 0.5 mg of thiamin (Sigma, St. Louis, Mo.), 1.25 IU of polynym B per liter (Sigma), and 10 mg of vancomycine per liter (Eli Lilly, West Ryde, Australia). Plates were incubated in 10% CO2 at 95% humidity for 48 h at 37°C. The XLOLR strain of *Escherichia coli* (Stratagene, La Jolla, Calif.), which contains the gene for *H. pylori* catalase (EC 1.11.1.6), was cultured at 37°C in Luria broth containing 50 mg of kanamycin sulfate per liter (Gibco BRL, Grand Island, N.Y.).

A mouse-adapted *H. pylori* strain (SS1) was grown in brain heart infusion (BHI) broth (Oxoid) containing the same antibiotics as described above for blood agar plates, plus 5% (vol/vol) horse serum (Oxoid). The organism was grown microaerophilically (gas generating kit, anaerobic system BRS3; Oxoid) with anaerobic jars for 48 h at 37°C. The broth was then centrifuged, and the *H. pylori* cells were resuspended in BHI broth for the challenge of the mice. When *H. felis* (ATCC 49179) (16) was required for the challenge of the mice, the organism was grown on blood agar plates in a microaerophilic atmosphere (gas generating kit, campylobacter system BRS6; Oxoid) with anaerobic jars. Bacteria were grown for 48 h at 37°C prior to being used.

**Preparation of H. pylori sonicate and native H. pylori catalase.** When required for use as a vaccine antigen, *H. pylori* was harvested from plates with 0.1 M phosphate-buffered saline (PBS), pelleted by centrifugation, and disrupted by sonication. The method of Mitchell and colleagues (20) was followed. Flat-bottom 96-well plates (Linbro/Titterick; ICN Flow, Horsham, Pa.) were coated with 10 μg of catalase and incubated overnight at 4°C. The next day, positive clones were excised into the phagemid form and introduced into coding sequences. Each stomach section was coded and examined blind for the presence of *H. felis* or *H. pylori* by light microscopy (oil immersion, ×1,000).

**Grading of Helicobacter colonization.** (i) *H. felis.* All stomach sections were scanned from above the antrum-body border through to the end of the antrum. Each field of view examined was given a score based on the number of *H. felis* organisms observed; 0, no organisms; 1, 1 to 10 organisms; 2, 11 to 20 organisms; 3, 21 to 30 organisms; 4, 31 to 40 organisms; 5, 41 to 50 organisms. Animals were considered to be infected with *H. felis* even if only one organism was visible by this method.

(ii) *H. pylori.* All stomach sections were scanned from the end of the antrum through to the body-corpus region, because we were unsure which regions of the stomach the organisms would colonize, if any, after immunization. Because *H. pylori* SS1 does not infect BALB/c mice to the same extent as *H. felis,* colonization levels were determined in a different manner. The number of *H. pylori* organisms visible in each field of view was counted and recorded. Sections were then scored as follows: uninfected, no *H. pylori* organisms present; minimal infection, <10 organisms evident in an entire section; and infected, >10 organisms present in the section.

**Quantitation of the anti-Helicobacter antibody response.** An enzyme-linked immunosorbent assay (ELISA) was used to detect IgG and IgA antibody to recombinant catalase. Germ-free mice were immunized subcutaneously with 10 μg of CT; Sigma), 10 g of purified recombinant catalase plus 10 μg of CT, 1 mg of *H. pylori* 921023 sonicate plus 10 μg of CT, 1 mg of *E. coli* XLOLR sonicate plus 10 μg of CT, or PBS alone and were left unimmunized and unchallenged. One week after the last immunization dose, animals from the catalase plus CT and untreated groups were bled for the ELISA. For the catalase plus CT immunization, mice were challenged with three orogastric doses of live *H. pylori* SS1 cells (~105 organisms/dose) 2 days apart to ensure all animals were infected. After a further 2 weeks, the animals were killed and assessed for *H. felis* infection.

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The sample absorbance as a proportion of that of the positive control. All samples which fell below the absorbance score of the negative control were given a zero value.

**Qualitative analysis of the anti-**H. pylori** antibody response.** Western blotting of sera was used to confirm that the immunized mice had antibodies against H. pylori catalase. The procedures already outlined above for characterization of H. pylori catalase were followed, except that serum samples diluted 1:100 in antibody buffer were used in the primary incubation step.

Some serum samples were also adsorbed with a whole-cell sonicate of C. jejuni in order to reduce the amount of cross-reacting IgG antibody to H. pylori SSI antigen evident in normal serum. The C. jejuni sonicate was prepared by the same protocol as the one already described earlier in this paper for H. pylori vaccine antigen. Serum samples were diluted 1:100 in TTBS containing 10% (wt/vol) skim milk and 50% (vol/vol) C. jejuni sonicate at 40 mg/ml and then were incubated at room temperature for 4 h with frequent mixing. The adsorbed serum was used for Western blotting as already described.

**Statistics.** The significance of the difference in H. felis infection levels between mice immunized with H. pylori catalase plus CT and the control mice was assessed by the χ² test. The statistical significance of differences in IgG or IgA antibody levels was calculated by the Mann-Whitney U test. Both sets of statistics were performed with the Primer of Biostatistics program (version 3.01 for the Macintosh; McGraw-Hill).

**RESULTS**

**Catalase purification and stability.** Native and recombinant H. pylori catalases were purified and checked for contaminants by electrophoresis of Coomassie blue-stained gels for additional proteins. With this system, catalase was found to be the dominant protein, with an M₀ of approximately 53,000. There were also two minor bands with approximate Mₛ of 47,000 and 45,000, respectively (Fig. 1). The identity of the major protein band was confirmed by immunoblotting with a monoclonal antibody against H. pylori catalase (Fig. 1). In the case of native H. pylori catalase, the two minor bands also reacted with the monoclonal antibody, indicating that they are probably catalase degradation products. In the recombinant catalase preparation, several additional bands also reacted with the catalase monoclonal antibody. By overloading the gel, the recombinant catalase protein detected by the monoclonal antibody formed a large band. The gel was so loaded to verify that the bands evident below both the native and recombinant catalase protein were in fact degradation products. Both the native and recombinant catalase preparations had high levels of H₂O₂-reducing activity after purification.

The stability of the purified catalase was assessed throughout the duration of the immunization schedule (days 0, 7, 14, and 21). Both the native and recombinant H. pylori catalases retained high levels of H₂O₂-reducing activity, and no additional bands or breakdown products were visible on Coomassie blue-stained gels (data not shown).

**Helicobacter colonization.** (i) **Experiment 1.** Colonization with H. felis was determined with the rapid urease assay and verified by histology. The urease assay indicated that immunization with native catalase plus CT completely prevented H. felis colonization (0 of 10 animals infected [Table 1]). All animals immunized with PBS (10 of 10) and 7 of 10 of the mice immunized with CT alone were infected with H. felis. However, some of the mice which were negative by urease assay were actually found to be infected with small numbers of H. felis when stomach sections were examined by light microscopy (Table 1). When H. felis colonization was determined by this method, detection of even one H. felis isolate in a section was scored as an infection. The urease assay is not sensitive enough to detect this very low grade of infection.

Two of the 10 (20%) mice immunized with the purified catalase plus CT were colonized by H. felis. In comparison, all of the animals immunized with PBS or CT alone were infected with H. felis (20 of 20) (Table 1). The two infected mice in the catalase plus CT group had less than three organisms visible in an entire section—a minimal infection. Of the three urease-negative mice in the group immunized with CT alone, two actually had very small numbers of H. felis organisms (two and three organisms, respectively), while the other mouse had a very low grade of colonization with H. felis. The difference in H. felis colonization levels between the mice immunized with catalase plus CT and the control animals (CT alone and PBS alone) was statistically significant (χ² test; P < 0.001).

(ii) **Experiment 2.** H. pylori colonization. Assessment of infection in H. pylori-challenged mice by gastric urease assay indicated that immunization with recombinant catalase plus CT or H. pylori plus CT prevented H. pylori colonization—0 of 10 and 1 of 10 mice were infected, respectively (Table 2). In

![Fig. 1](http://iai.asm.org/)
contrast, most mice immunized with E. coli plus CT or PBS alone were colonized with H. pylori — 9 of 10 and 8 of 10 mice were infected, respectively. An examination by light microscopy of May-Grünwald-Giemsa-stained stomach sections for colonization was again more sensitive than the urease assay. The colonization counts revealed that immunization with catalase plus CT protected 9 of 10 (90%) of animals from H. pylori challenge (Table 3). This result was comparable to the level of protection stimulated in the control group, which was immunized with H. pylori sonicate plus CT (8 of 10 [80%]). A significantly reduced proportion of mice immunized with E. coli plus CT or PBS were protected from H. pylori challenge (only 10 to 20% of the mice reduced or cleared the infection (χ² test; P < 0.01).

**Quantitative antibody response to H. pylori antigen. (i) Experiment 1.** The anti-H. pylori serum IgG and salivary IgA antibody responses were measured by ELISA in order to compare the anti-H. pylori antibody responses in the different experimental groups. It was found that immunization with purified native catalase plus CT produced a significantly higher anti-H. pylori IgG antibody response than that stimulated by immunization with either PBS or CT alone (Fig. 2; Mann-Whitney U test; P < 0.001). This result was mirrored in the saliva samples, which indicated that the pooled (10 mice per group) salivary IgA antibody response in SPF BALB/c mice immunized with purified native H. pylori catalase plus CT (0.564 ELISA units) and challenged with live H. felis cells was greater than that seen in either the CT-alone (0.19 ELISA units) or PBS-alone (0.203 ELISA units) groups. Note that the ELISA was performed with pooled samples because there was not enough saliva for individual assays. Sample values were expressed as a ratio of a standard positive control (saliva from mice immunized with H. pylori plus CT on days 0, 7, 14, and 21). It appears that neither of the control groups (PBS or CT alone) had developed a cross-reactive IgG or IgA antibody response to H. pylori 3 weeks after H. felis challenge.

(ii) **Experiment 2.** Determination of the anti-H. pylori IgG and IgA antibody responses in mice immunized with purified recombinant H. pylori catalase or in the appropriate control animals revealed that the catalase preparation stimulated a substantial antibody response to H. pylori. These data indicated that immunization with purified recombinant catalase plus CT stimulated a significantly higher prechallenge anti-H. pylori antibody response than that seen in normal control animals (Fig. 3 [Mann-Whitney U test; P < 0.05]). After H. pylori challenge, the IgG antibody response was boosted substantially (Fig. 3 [Mann-Whitney U test; P < 0.05]) in this group of animals. The postchallenge anti-H. pylori antibody response in the catalase plus CT group was significantly higher than that in any other group, with the exception of the H. pylori-plus-CT-immunized animals (Mann-Whitney U test; P < 0.001). Again, these trends were reflected in the anti-H. pylori salivary IgA antibody ELISA. No prechallenge saliva samples were available for inclusion in the assay, but the postchallenge salivary IgA response in the catalase plus CT group was significantly higher than that seen in any other group, with the exception of the H. pylori-plus-CT-immunized animals (Fig. 4 [Mann-Whitney U test; P < 0.05]).

**Serum recognition of H. pylori catalase.** To confirm that H. pylori catalase was the protein which most of the catalase-plus-CT-immunized mice had an antibody response to, sera were immunoblotted against a whole-cell sonicate of H. pylori.

(i) **Experiment 1.** Sera from mice immunized with native H. pylori catalase plus CT or PBS alone and then challenged...
with *H. felis* were immunoblotted against a whole-cell sonicate of *H. pylori* 921023 (Fig. 5). All mice immunized with catalase had high levels of IgG antibodies against the enzyme, while a response to most other *H. pylori* antigens was lacking. In contrast, mice immunized with PBS alone had a reduced antibody response to all *H. pylori* antigens, including catalase, despite being infected with *H. felis*.

(ii) Experiment 2. Prechallenge sera from mice immunized with recombinant *H. pylori* catalase plus CT or left untreated (normal) were immunoblotted against *H. pylori* SS1 sonicate (Fig. 6A). Most of the catalase-immunized mice had a strong serum IgG antibody response to the *H. pylori* catalase, while the normal animals had a faint band in the region of the catalase protein, probably as a consequence of exposure to the catalases produced by the commensal bacteria colonizing the intestinal tract. The number of other cross-reacting antibodies to *H. pylori* was also quite high in normal, untreated mice.

An examination of the postchallenge serum IgG antibody response to *H. pylori* SS1 sonicate in recombinant catalase-plus-CT-immunized mice after *H. pylori* challenge indicated that there was a strong anticatalase response in all individuals (Fig. 7A). Seven of the immunized animals also developed a strong antibody response to an antigen with an *M*ₚ of ~66 kDa, possibly the B subunit of the urease enzyme of *H. pylori*. In contrast, the PBS-immunized mice had a weaker response to *H. pylori* catalase, little different from the normal response, despite being infected with live *H. pylori* cells (Fig. 7A). Some of these mice also developed an antibody response to the ~66-kDa antigen, but antibodies to other *Helicobacter* antigens were not evident. The normal antibody response was directed against proteins with sizes of between 45 and 66 kDa, which are presumably cross-reactive proteins produced by the commensal alimentary tract flora of these SPF mice.

(iii) Examination of the serum antibody response to *H. pylori* catalase after adsorption with *C. jejuni* sonicate. Because of the number of distracting bands evident in the normal serum IgG antibody response (Fig. 6A and 7A), serum from experiment 2 mice was adsorbed with a whole-cell sonicate of *C. jejuni*, a spiral-shaped organism related to *H. pylori*. Adsorption with *C. jejuni* sonicate effectively removed a large number of the cross-reacting antibodies present in the normal antibody response without interfering with the antibody response to *H. pylori* catalase. Figure 6B shows the prechallenge IgG antibody response to *H. pylori* SS1 sonicate in mice immunized with catalase plus CT or left untreated. The IgG antibody response to catalase was still dominant in the immunized mice, while the antibody response in normal animals was reduced to a faint response to antigens with *M*ₚs of ~66,000, 50,000, and 47,000, respectively. Figure 7B illustrates that adsorption with *C. jejuni* also substantially reduced the level of nonspecific antibody in immunized mice after *H. pylori* challenge. The catalase-plus-CT-immunized mice clearly had a marked IgG
antibody response to \textit{H. pylori} catalase, and some of these animals also possessed antibodies to an \textasciitilde{66}-kDa antigen, which is probably urease. In contrast, the PBS-immunized, \textit{H. pylori}-challenged mice had a negligible IgG antibody response to \textit{H. pylori} antigen, similar to the one exhibited by the normal control animals.

\section*{DISCUSSION}

This study has demonstrated that mice orogastrically immunized with purified native \textit{H. pylori} catalase were protected from \textit{H. felis} challenge and that animals vaccinated with recombinant catalase developed effective immunity against \textit{H. pylori} challenge. Protection from helicobacter challenge was determined via two well-characterized methods: the rapid urease assay and histologic screening of stomach tissue. Histologic analysis of stomach sections provides a sensitive method of detecting small numbers of bacteria, as well as providing information on which region of the stomach the organisms colonize.

The success of immunization with this antigen in the \textit{H. pylori} model indicates that the enzyme may be effective in protecting against human \textit{H. pylori} infection and that it should definitely be considered as a potential vaccine candidate. The anti-\textit{H. pylori} serum IgG ELISA demonstrated that immunization with catalase stimulated a good pre- and postchallenge antibody response. An anti-\textit{H. pylori} salivary IgA ELISA produced results which mirrored the IgG antibody data: immunization with \textit{H. pylori} catalase produced anti-\textit{H. pylori} antibody levels comparable to that produced by immunization with a whole-cell sonicate of \textit{H. pylori}. Immunoblots indicated that immunization with recombinant catalase stimulated a good antibody response to the catalase expressed by \textit{H. pylori} SS1 and that this enzyme is one of the main \textit{H. pylori} antigens the antibody response was directed against in immunized mice. The clarity of these immunoblots was markedly improved by adsorption of sera with \textit{C. jejuni} antigen before its use as a primary antibody. This step substantially reduced the number of preexisting nonspecific antibodies to \textit{H. pylori} SS1 and also clearly demonstrated that in catalase-plus-CT-immunized animals, the pre- and postchallenge IgG antibody responses were predominantly directed to catalase, while unimmunized mice had a negligible response to this protein.

This study includes the first data on the efficacy of protective immunization with the \textit{H. pylori} Sydney strain-infected mouse model (17). It builds on the immunization data produced in the \textit{H. felis} mouse model with whole-cell sonicates of \textit{H. felis} and \textit{H. pylori} (4--6) or purified antigens such as urease (18, 19, 22) and HSP60 (8). The results from this experiment also support the study by Marchetti et al. (18), which provided the first indication that immunization may be feasible in an \textit{H. pylori} mouse model. Our data indicate that whole-cell sonicates of \textit{H. pylori} and our new vaccine candidate antigen, catalase, can stimulate protection against \textit{H. pylori} challenge. Because \textit{H. pylori} does infect slightly different areas of the stomach from \textit{H. felis} and tends to adhere more closely to the gastric mucosa, this result offers more hope that an effective human vaccine against \textit{H. pylori} can be produced.

The fact that protective immunity was acquired from immunization with catalase suggests that this enzyme may be either periplasmic or is exported to the cell surface by \textit{Helicobacter} species. Several proteins, including Hp54K, urease, and catalase have been found on the outer membrane of \textit{H. pylori} and it has been proposed that these proteins have reached this site via bacterial lysis (23). However, because many independent studies have indicated that \textit{H. pylori} requires enzymes such as urease to colonize the stomach, it could also be possible that the organism has an as yet unknown mechanism for exporting such an important enzyme to the cell surface. There is precedence for this in other organisms—virulent strains of \textit{Nocardia asteroides} selectively secrete SOD, which also protects bacteria from oxidative damage, and this enzyme becomes associated with the bacterial cell surface (1).

This is the first indication that an \textit{H. pylori} enzyme other than urease can act as a vaccine antigen. Like urease, catalase is highly conserved between \textit{Helicobacter} species and is almost certainly fundamental to the survival of the organism and thus provides an ideal target for a vaccine. The discovery of an additional protective antigen of \textit{H. pylori} offers further hope that an effective vaccine can be produced for human usage.

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