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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 M_r 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 passaged 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.*

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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* (clinical strains 921023 and RU1, provided by Hazel Mitchell, University 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 mg of amphotericin B per liter (Fungizone; Squibb, Princetown, N.J.), 5 mg of trimethoprim (Sigma, St. Louis, Mo.), 1,250 IU of polymyxin B per liter (Sigma), and 10 mg of vancomycin per liter (Eli Lilly, West Ryde, Australia). Plates were incubated in 10% CO_2 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 BR38; Oxoid) with anaerobe 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 BR56; Oxoid) with anaerobe jars. Bacteria were grown for 48 h at 37°C prior to being harvested in BHI broth.

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 sonicate was stored at −20°C until use. Purified catalase was obtained by the method of Hazell et al. (11). Briefly, *H. pylori* cells were harvested with 0.1 M sodium phosphate buffer (pH 7.2), centrifuged, and then resuspended in the buffer. Cells were disrupted by sonication, cellular material was removed by centrifugation (5 min, 10,000 × *g*), and then the supernatant was collected and filtered (0.22-μm-pore-size filter). Extracts were kept on ice throughout these processes. The filtrate was loaded onto a K26/100 gel filtration column of Sephacryl S-300 HR (Pharmacia Biotec Inc., Piscataway, N.J.) and eluted with sodium phosphate buffer at a flow rate of 1.0 ml min^{-1}. Catalase-positive fractions were selected by checking for oxygen-reducing activity in 3% H_2O_2, diluted 1/10 in deionized distilled water, and applied to a Millipore (Bedford, Mass.) MEMSEP 1000-CM ion-exchange capsule which had been equilibrated with 0.01 M sodium phosphate buffer. Catalase was eluted by the creation of a gradient with 1 M NaCl in 0.01 M sodium phosphate buffer. The purified catalase was then filter sterilized, stored at 4°C, and protected from light.

Construction and purification of recombinant *H. pylori* catalase. To obtain the catalase gene from *H. pylori*, genomic DNA from *H. pylori* RU1 was extracted, partially digested with *Sau*3A, and cloned into the ZAP Express vector (Stratagene). This genomic library was probed with a 710-bp fragment of the *H. pylori* catalase open reading frame which was generated by PCR and labelled with digoxigenin-dUTP (Boehringer Mannheim, Mannheim, Germany). Catalase-positive clones were excised into the phagemid form and introduced into *E. coli* XLOLR. Clones which produced a functional catalase were identified by placing the cells into 3% H_2O_2 and checking for the rapid formation of oxygen.

Recombinant catalase was purified by the method described for purification of native *H. pylori* catalase.

Characterization of native and recombinant *H. pylori* catalases. Catalase was initially identified by its ability to reduce 3% H_2O_2. The purity of the catalase preparations was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [12% polyacrylamide]) (13). Broad-molecular-mass standards (Bio-Rad Laboratories, Hercules, Calif.) were run in conjunction with the catalase to estimate M_r. Proteins were visualized by staining with Coomassie brilliant blue, and M_r was verified with the Bioimage whole-band analyzer (Millipore).

Further confirmation of the identity of the purified protein was obtained by Western blotting (26) with a monoclonal antibody against *H. pylori* catalase (provided by Diane Newell, Weybridge, United Kingdom). Protein samples were separated by SDS-PAGE and transferred electrophoretically to Immobilon-P membrane (Millipore) with a Trans-blot SD semidry transfer cell (Bio-Rad). The membrane was then blocked with 3% (wt/vol) gelatin in 20 mM Tris-buffered saline (pH 7.5 [TBS]), washed in 0.05% (vol/vol) Tween-TBS (TTBS), and placed in a Milliblot-MP membrane processor (Millipore). The anti-*H. pylori* catalase monoclonal antibody was diluted 1:100 in antibody buffer (1% [wt/vol] gelatin, 0.05% [vol/vol] Tween 20 in TBS) and incubated with the membrane for 1 h at 37°C. After being washed with TTBS, the membrane was incubated with goat anti-mouse alkaline phosphatase-conjugated immunoglobulin G (IgG) (Gibco-BRL, Life Technologies, Inc., Gaithersburg, Md.) for 1 h at 37°C. After further washes in TTBS, TBS, and distilled water, the Bio-Rad alkaline phosphatase development kit was used to develop the membranes. The molecular masses of the visualized bands were later calculated with the Bioimage whole-band analyzer (Millipore).

Immunization. The protein concentrations of the purified catalase preparations and the *H. pylori* sonicates were determined with the Bio-Rad DC protein assay with bovine serum albumin as a standard.

In experiment 1, groups of mice were dosed orogastrically on days 0, 7, 14, and 21 with 200 μg of purified native *H. pylori* catalase plus 10 μg of cholera toxin (CT; Sigma), 10 μg of CT alone, or PBS buffer alone. Three weeks after the last immunization dose, all groups were given two orogastric challenges with live *H. felis* cells (~10^7 organisms/dose) 2 days apart to ensure all animals were infected. After a further 3 weeks, the animals were killed and assessed for *H. felis* infection.

In experiment 2, groups of mice were dosed orogastrically on days 0, 7, 14, and 21 with 200 μg 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 or were left unimmunized and unchallenged. One week after the last immunization dose, animals from the catalase plus CT and untreated groups were bled to obtain prechallenge sera. Two weeks after the last immunization dose, mice were challenged with three orogastric doses of live *H. pylori* SS1 cells (~10^7 organisms/dose) 2 days apart to ensure all animals were infected. After a further 2 weeks, the animals were killed and assessed for *H. pylori* infection. After each immunization in experiments 1 and 2, the catalase preparation was checked for activity and for any signs of degradation by SDS-PAGE and Coomassie blue staining.

Sample collection. At the end point of both experiments 1 and 2, mice were anesthetized by intraperitoneal injection with 50 mg of both ketamine and xylazine per kg of body weight (Parnell Laboratories, New South Wales, Australia). Blood was then taken from the aortic arch, and the animals were killed by cervical dislocation. Blood samples were centrifuged in Microvette CB 1000 S tubes (Sarstedt, Germany) to obtain serum, which was stored at −20°C. Stomach tissue was also collected. Half of the antral portion of the stomach was used for the rapid urease test (10), which detects the presence of *Helicobacter* spp. The other half of the stomach was placed in 10% formalin and processed for histology, and 4-μm-thick sections were stained with May-Grünwald-Giemsa stain. 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 described 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 anti-*H. pylori* serum IgG or salivary IgA antibody in mice immunized with catalase plus CT or control preparations. The method of Mitchell and colleagues (20) was followed. Flat-bottom 96-well plates (Linbro/Titertek; ICN Flow, Horsham, Pa.) coated with 10 μg of *H. pylori* antigen per well were washed several times in buffer (0.85% [wt/vol] NaCl, 0.02% [wt/vol] NaN_3, 0.05% [vol/vol] Tween 20) and then blocked (0.1 M phosphate buffer [pH 7.2], 0.02% [wt/vol] NaN_3, 1.0 mg of gelatin per ml) for 1.5 h at 37°C. After the blocking step, plates were washed once before the addition of samples and then incubated at 37°C for 1.5 h.

For the IgG ELISA, standard positive sera (from mice immunized four times with *H. pylori* plus CT) were diluted 1/100 and then titrated in duplicate as a series of doubling dilutions to form a standard curve. Serum samples were added in duplicate at dilutions of 1/100 or 1/200 for immunized mice or 1/25 and 1/50 for normal and infected control animals, respectively. For the IgA ELISA, standard positive saliva (from mice immunized four times with *H. pylori* plus CT) and negative saliva (from normal untreated, unchallenged mice) were diluted 1/10 and added in duplicate to each ELISA plate. Single aliquots of the test samples were added diluted 1/4 in blocking buffer.

After the incubation, the plates were washed several times before alkaline phosphatase-conjugated goat anti-mouse IgG or IgA antibody (Gibco-BRL, Life Technologies, Inc., and Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) diluted 1/2,000 or 1/500 in blocking buffer was added to each well for 1.5 h at 37°C. Following this, the plates were washed in wash buffer and distilled water before 100 μl of substrate was added to each well, and then they were incubated in the dark for 30 min at 30°C. The substrate contained diethanolamine buffer at pH 9.8 (9.6% [vol/vol] diethanolamine, 5% [wt/vol] $MgCl_2 \cdot 6H_2O$) plus one disodium *p*-nitrophenyl phosphate tablet for every 8.3 ml of solution (Sigma Diagnostics, St. Louis, Mo.). The reaction was stopped by addition of 100 μl of 3 M NaOH to each well, and A_{405}s were read on a microplate reader (model 3550; Bio-Rad Laboratories, Richmond, Calif.).

Anti-*H. pylori* IgG ELISA units were calculated with sample values that fell within the linear region of the standard curve, which was calculated by the Microplate Manager program (Bio-Rad Laboratories) by absorbance versus log (concentration). Absorbance units for IgA were standardized by expression of

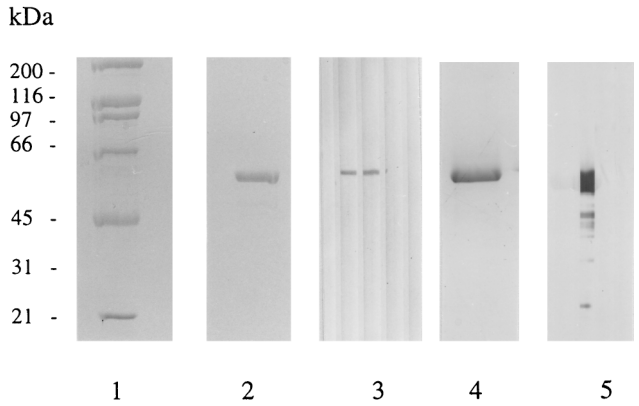

FIG. 1. Confirmation of the identity of the purified native and recombinant protein as *H. pylori* catalase. Lanes: 1, broad-molecular-mass standards; 2, SDS-PAGE of the purified native *H. pylori* catalase preparation; 3, immunoblot of the native catalase against an anticatalase monoclonal antibody; 4, SDS-PAGE of the purified recombinant *H. pylori* catalase preparation; 5, immunoblot of the recombinant *H. pylori* catalase against an anticatalase monoclonal antibody.

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* SS1 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 χ^2 test. The statistical significance of differences in IgG or IgA antibody levels was calculated by the Mann-Whitney U test. Both sets of statistical analyses 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 examination of Coomassie blue-stained gels for additional proteins. With this system, catalase was found to be the dominant protein, with an M_r of approximately 53,000. There were also two minor bands with approximate M_rs 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 proteins were in fact degradation products. Both the native and recombinant catalase preparations had high levels of H_2O_2-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 H_2O_2-reducing activity, and no additional bands or breakdown products were visible on Coomassie blue-stained gels (data not shown).

TABLE 1. *H. felis* infection in immunized mice challenged with *H. felis*[a]

Group	No. of mice with *H. felis* infection/ no. in group (%)	
	Urease assay	Histology
Catalase + CT	0/10 (0)	2/10 (20)[b]
CT alone	7/10 (70)	10/10 (100)
PBS alone	10/10 (100)	10/10 (100)

[a] Infection was determined by gastric urease assay and light microscopy of May-Grünwald-Giemsa-stained stomach sections from mice immunized with purified native *H. pylori* catalase plus CT, CT alone, or PBS alone.

[b] The two infected mice had <3 organisms each in the entire stomach section examined.

***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 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 (χ^2 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

TABLE 2. *H. pylori* infection determined by gastric urease assay in immunized mice challenged with the Sydney strain of *H. pylori*[a]

Group	No. of mice with stomach infection/ no. in group (%)
Catalase + CT	0/10 (0)
H. pylori + CT	1/10 (10)
E. coli + CT	9/10 (90)
PBS	8/10 (80)
Normal	0/8 (0)

[a] Mice were immunized with purified recombinant *H. pylori* catalase plus CT, *H. pylori* sonicate plus CT, *E. coli* sonicate plus CT, or PBS. An untreated and unchallenged (normal) group was also included in the experiment.

TABLE 3. *H. pylori* infection determined by light microscopy of May-Grünwald-Giemsa-stained stomach sections from immunized mice challenged with the Sydney strain of *H. pylori*[a]

Group	No. of mice with result/no. in group (%) by histology		
	No colonization	Minimal colonization[b]	Colonization
Catalase + CT	9/10 (90)		1/10 (0)
H. pylori + CT	8/10 (80)		2/10 (20)
E. coli + CT	1/10 (10)	1/10 (10)	8/10 (80)
PBS		2/10 (20)	8/10 (80)
No treatment	8/8		

[a] Mice were immunized with purified recombinant *H. pylori* catalase plus CT, *H. pylori* sonicate plus CT, *E. coli* sonicate plus CT, or PBS. An untreated and unchallenged group was also included in the experiment.
[b] Minimal colonization, <10 organisms visible in an entire stomach section.

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 (χ^2 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

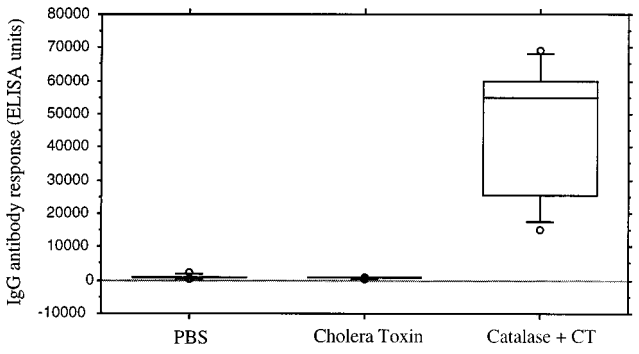

FIG. 2. Anti-*H. pylori* serum IgG antibody response in SPF BALB/c mice immunized with PBS, CT, or native catalase plus CT and challenged with live *H. felis* cells. Serum from each mouse (10 mice in each group) was assayed individually. ELISA units were calculated from a standard curve of serum from mice immunized four times with *H. pylori* sonicate plus CT. The box plots represent the range of data points within each group (10 mice in each group), with the outlying points being represented by circles. The line across the middle of the box plot represents the mean data point.

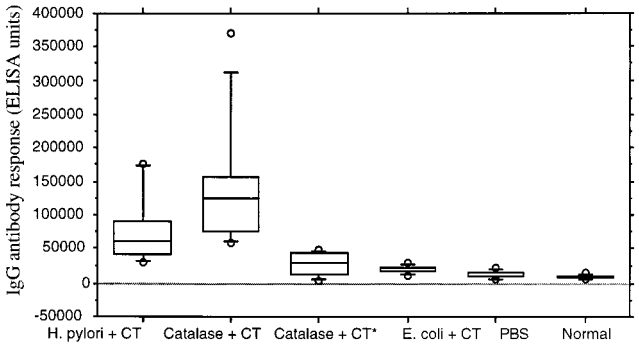

FIG. 3. Anti-*H. pylori* serum IgG antibody response in SPF BALB/c mice immunized with purified recombinant *H. pylori* catalase, *H. pylori* sonicate plus CT, *E. coli* sonicate plus CT, or PBS and then challenged with the Sydney strain of *H. pylori*. The catalase + CT* group is comprised of sera taken from these mice prechallenge. A control group of normal (untreated and unchallenged) mice from the same experiment were also included. The IgG antibody response was determined with a standard curve of titrated positive control sera (serum from mice immunized with *H. pylori* plus CT on days 0, 7, 14, and 21). Sample IgG ELISA units were calculated from the linear portion of the standard curve. The box plots represent the range of data points within each group (10 mice in each immunized group, 6 mice in the normal group), with the outlying points being represented by circles. The line across the middle of the box plot represents the mean data point.

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

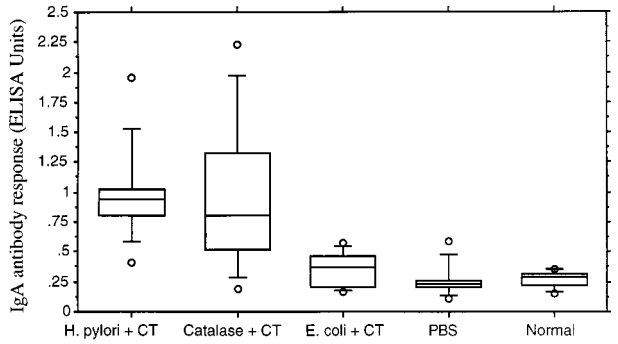

FIG. 4. Anti-*H. pylori* salivary IgA antibody response in SPF BALB/c mice immunized with purified recombinant *H. pylori* catalase, *H. pylori* sonicate plus CT, *E. coli* sonicate plus CT, or PBS and then challenged with the Sydney strain of *H. pylori*. A control group of normal (untreated and unchallenged) mice from the same experiment were also included. The IgA antibody response was determined by measuring absorbance, and then the data were standardized by expression of the absorbance as a proportion of that of a positive control (saliva from mice immunized with *H. pylori* plus CT on days 0, 7, 14, and 21). The box plots represent the range of data points within each group (10 mice in each immunized group, 6 mice in the normal group), with the outlying points being represented by circles. The line across the middle of the box plot represents the mean data point.

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_r of ~66 kDa, possibly the B subunit of the urease enzyme of *H. pylori*. In

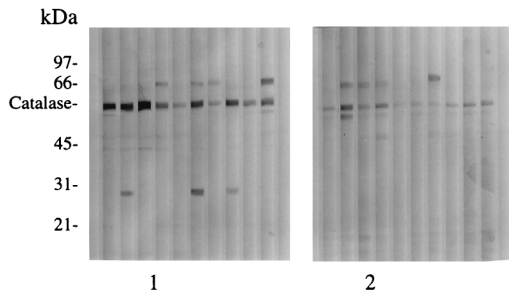

FIG. 5. Determination of the serum IgG antibody response to specific *H. pylori* antigens by immunoblotting. Samples were from SPF BALB/c mice immunized with 200 μg of native *H. pylori* catalase plus 10 μg of CT (panel 1) or saline (panel 2) after *H. felis* challenge.

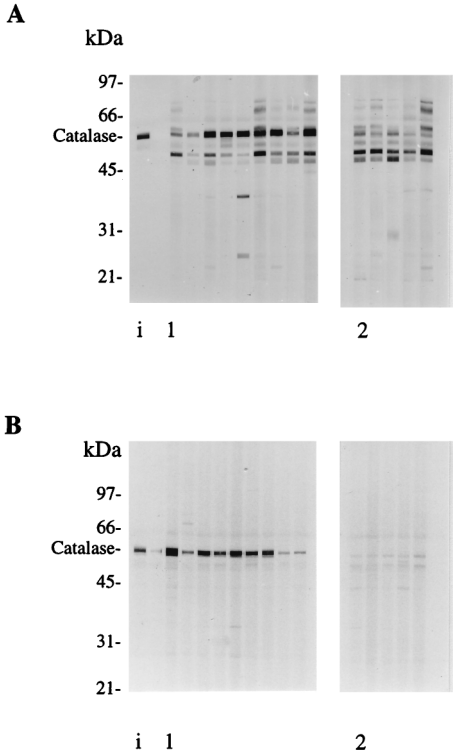

FIG. 6. Determination of the prechallenge serum IgG antibody response to *H. pylori* SS1 catalase by immunoblotting. An anti-*H. pylori* catalase monoclonal antibody was included to confirm the location of the anticatalase antibodies (i). Samples were from SPF BALB/c mice immunized with 200 μg of recombinant *H. pylori* catalase plus 10 μg of CT (panel 1) or left untreated (normal) (panel 2). (A) *H. pylori* antigen immunoblotted with untreated serum. (B) *H. pylori* antigen immunoblotted with serum adsorbed with *C. jejuni* whole-cell sonicate to remove nonspecific cross-reacting antibodies.

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_rs 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

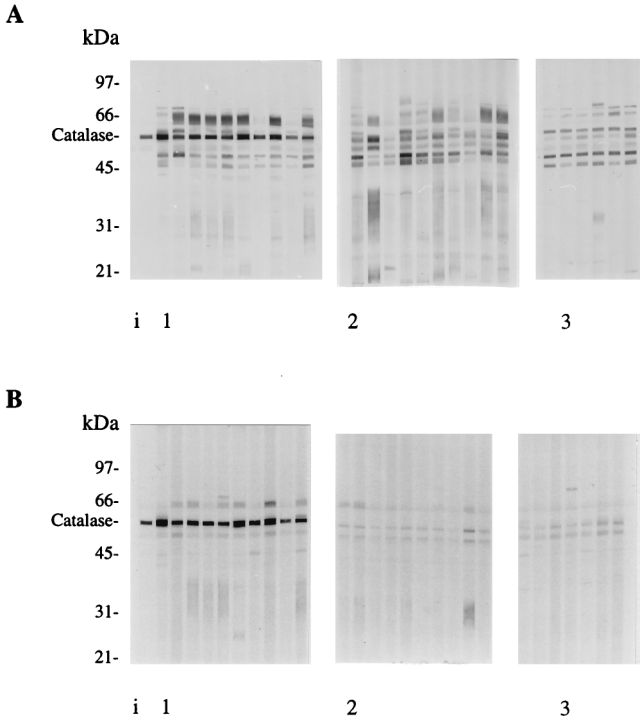

FIG. 7. Determination of the serum IgG antibody response to specific *H. pylori* SS1 antigens by immunoblotting. An anti-*H. pylori* catalase monoclonal antibody was included to confirm the location of the anticatalase antibodies (i). Samples were from *H. pylori* SS1-challenged SPF BALB/c mice immunized with 200 μg of recombinant *H. pylori* catalase plus 10 μg of CT (panel 1) or saline (panel 2) left untreated and unchallenged (panel 3). (A) *H. pylori* antigen immunoblotted with untreated serum. (B) *H. pylori* antigen immunoblotted with serum adsorbed with *C. jejuni* whole-cell sonicate to remove nonspecific cross-reacting antibodies.

antibody response to *H. pylori* catalase, and some of these animals also possessed antibodies to an ~66-kDa antigen, which is probably urease. In contrast, the PBS-immunized, *H. pylori*-challenged mice had a negligible IgG antibody response to *H. pylori* antigen, similar to the one exhibited by the normal control animals.

DISCUSSION

This study has demonstrated that mice orogastrically immunized with purified native *H. pylori* catalase were protected from *H. felis* challenge and that animals vaccinated with recombinant catalase developed effective immunity against *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 *H. pylori* model indicates that the enzyme may be effective in protecting against human *H. pylori* infection and that it should definitely be considered as a potential vaccine candidate. The anti-*H. pylori* serum IgG ELISA demonstrated that immunization with catalase stimulated a good pre- and postchallenge antibody response. An anti-*H. pylori* salivary IgA ELISA produced results which mirrored the IgG antibody data: immunization with *H. pylori* catalase produced anti-*H. pylori* antibody levels comparable to that produced by immunization with a whole-cell sonicate of *H. pylori*. Immunoblots indicated that immunization with recombinant catalase stimulated a good antibody response to the catalase expressed by *H. pylori* SS1 and that this enzyme is one of the main *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 *C. jejuni* antigen before its use as a primary antibody. This step substantially reduced the number of preexisting nonspecific antibodies to *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 *H. pylori* Sydney strain-infected mouse model (17). It builds on the immunization data produced in the *H. felis* mouse model with whole-cell sonicates of *H. felis* and *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 *H. pylori* mouse model. Our data indicate that whole-cell sonicates of *H. pylori* and our new vaccine candidate antigen, catalase, can stimulate protection against *H. pylori* challenge. Because *H. pylori* does infect slightly different areas of the stomach from *H. felis* and tends to adhere more closely to the gastric mucosae, this result offers more hope that an effective human vaccine against *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 *Helicobacter* species. Several proteins, including Hp54K, urease, and catalase have been found on the outer membrane of *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 *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 *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 *H. pylori* enzyme other than urease can act as a vaccine antigen. Like urease, catalase is highly conserved between *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 *H. pylori* offers further hope that an effective vaccine can be produced for human usage.

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