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Urease-Specific Monoclonal Antibodies Prevent *Helicobacter felis* Infection in Mice

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Experiments were performed to determine the antigenic specificity of a monoclonal antibody (immunoglobulin A [IgA] 71) previously demonstrated to neutralize the ability of *Helicobacter felis* to colonize mice. Immunoprecipitation of radiolabeled *H. felis* outer membrane proteins with IgA 71 revealed specificity for a 62-kDa protein. Another of our monoclonal antibodies, IgG 40, precipitated a protein of similar molecular weight. IgA 71 but not IgG 40 also precipitated purified recombinant *H. pylori* urease. The antigenic specificity of both antibodies was confirmed to be urease by the ability of each to select *Escherichia coli* clones expressing the *H. felis* urease genes. The two antibodies were shown to bind nonoverlapping epitopes in a competition enzyme-linked immunosorbent assay. Both IgA 71 and IgG 40 could effectively neutralize *H. felis* infectivity by incubating the bacteria with the antibodies prior to oral administration to naive mice. The mechanism of protection does not appear to be inhibition of urease activity, as IgA 71 does not inhibit the conversion of urea to ammonia by *H. pylori* urease in vitro. These results support a protective role for the secretory humoral immune response in *Helicobacter* immunity and provide further evidence that the urease enzyme can serve as a protective antigen.

Helicobacter pylori is a small, spiral, microaerophilic, gram-negative bacterium that colonizes the human upper gastrointestinal tract, primarily the stomach (27). It is now recognized as a significant gastrointestinal pathogen for its etiologic role in nonautoimmune type B gastritis, peptic ulcer disease (21), and mucosa-associated lymphoid tissue gastric lymphoma (13, 29). Additionally, there is an epidemiological association with gastric carcinoma incidence (7).

Current strategies aimed at eradication of *H. pylori* from patients with duodenal ulcers rely on triple antimicrobial therapy which includes a bismuth salt in combination with two antibiotics or omeprazole plus an antibiotic. These treatments must be administered multiple times a day for up to a month. However, despite the susceptibility of *H. pylori* to many antibiotics in vitro, antimicrobial treatment in vivo can be ineffective and even when successful may not prevent reinfection. Therefore, we have been investigating immunization and immunotherapy as a means of prevention and treatment of infection with *H. pylori* and its sequelae.

It has been demonstrated that specific secretory immunoglobulin A (sIgA) can prevent colonization by bacterial pathogens or infection by a viral pathogen at mucosal surfaces (18, 20, 28). Such an sIgA response has been generated at the gastric epithelia of both mice and ferrets. We have demonstrated in both mice and ferrets that multiple oral immunizations with *H. pylori* lysates, in combination with the mucosal adjuvant cholera toxin, generate an *H. pylori*-specific sIgA response in the gastrointestinal tract (4). Subsequently, we have described a similar vaccination protocol using *H. felis* lysates in a germ-free mouse model that results in protection from challenge with infectious *H. felis* (3). In the same study, *H. felis* preincubated with a specific monoclonal antibody (MAb), IgA 71, did not colonize mice. The identification of the antigen(s) recognized by this and other protective MAbs and elucidation of the mechanism(s) by which antibody neutralizes the bacteria would facilitate the development of a subunit vaccine for *Helicobacter* infections. Therefore, the objectives of this study were to define the antigenic specificity of this protective MAb and to analyze the protective potential of other *H. felis*-specific MAbs.

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MATERIALS AND METHODS

Mice. Six- to eight-week-old, germ-free, outbred Swiss Webster mice purchased from Taconic (Germantown, N.Y.) were housed in microisolator cages under germ-free conditions throughout the immunization protocol. Animals were fed autoclaved laboratory chow and water ad libitum. The animals were maintained in a germ-free state, as judged from our inability to culture any bacteria other than those experimentally introduced. The Case Western Reserve University animal facility is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Bacteria. A bacterial strain isolated originally from a feline gastric biopsy specimen was identified as *H. felis* on the basis of colony morphology, Gram stain, and the production of urease, catalase, and oxidase. *H. felis* was grown on Columbia agar (Difco, Detroit, Mich.) containing 7% horse blood under microaerophilic conditions (5% O_2, 10% CO_2) at 37°C for 96 h. Bacteria were stored at −70°C in 0.1 M phosphate (pH 7.2)–0.9% phosphate-buffered saline (PBS) with 25% glycerol and 25% heat-inactivated fetal calf serum.

OMPs. *H. felis* from 200 confluent plates was harvested in 1 ml of 50 mM Tris (pH 7.8)–1 mM EDTA. One milligram each of DNase and RNase (Sigma Chemical Co., St. Louis, Mo.) were added immediately prior to sonication. Iced bacteria were sonicated in four 30-s bursts with 30-s intervals. Unbroken cells were removed by centrifugation using a Sorvall SS-34 rotor at 9,000 rpm and 4°C for 30 min. Outer membrane proteins (OMPs) were recovered by centrifugation of the supernatant in a Beckman 50Ti rotor at 45,000 rpm for 1 h at 4°C. The pellet was resuspended in 2 ml of 2% *n*-lauroyl sarcosine (Sigma) and allowed to sit at room temperature for 20 min. The solution was spun again at 40,000 rpm for 1 h at 4°C using the Beckman 50Ti rotor, and the pellet was washed three times by resuspension in 1% *n*-lauroyl sarcosine and spinning at 40,000 rpm for 1 h at 4°C using the Beckman 50Ti rotor. The final pellet was resuspended in 50 mM phosphate buffer (pH 7.0) and frozen at −70°C. The concentration of the final OMP solution was determined by the Lowry assay (15).

Urease. Recombinant *H. pylori* urease apoenzyme was purified from *Escherichia coli* ORV154 as previously described (12, 14). Briefly, whole bacteria were harvested by centrifugation, lysed, and subjected to ultrafiltration. The filtrate was absorbed onto DEAE-Sepharose (Pharmacia Biotec Inc., Piscataway, N.J.).

Urease was eluted from DEAE with 150 mM NaCl and used for immunoprecipitations (see below). Native *H. pylori* urease for the enzyme inhibition assay was purified by harvesting bacteria from blood agar plates, lysis, and DEAE binding. Eluted urease was applied to Sephacryl 300 (Pharmacia). Fractions containing urease activity were pooled, bound to Mono Q resin (Pharmacia), and eluted with a 0 to 1 M NaCl gradient.

MAb production. *H. felis*-specific MAbs were prepared as previously described (3) by a modification of the procedure described by Mazanec et al. (18). Briefly, BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were immunized by gastric intubation four times over a 6-week period with 2 mg of *H. felis* sonic extract. The first three doses included 10 μg of cholera toxin (Sigma), and the final dose was given without cholera toxin but was accompanied by an intravenous boost of 2 mg of *H. felis* sonic extract. Three days following the final immunization, mice were sacrificed, and their spleens cells were hybridized to SP2/0 myeloma cells. Clones were obtained by limiting dilution and screened for anti-*H. felis*-specific antibodies by enzyme-linked immunosorbent assay (ELISA) using *H. felis* OMP antigen. Stable antibody secretors were injected intraperitoneally into pristane-primed BALB/c mice, and the ascitic fluid was harvested and clarified.

ELISA. Ascites fluids were assayed for anti-*H. felis* OMP specificity as follows. Microtiter plates containing, per well, 100 μl of *H. felis* OMP (10 μg/ml) in PBS were placed in humid chambers at 4°C overnight. Plates were emptied by inversion and blocked for 1 h at room temperature with 200 μl of 1% bovine serum albumin (BSA) per well. Plates were washed three times between each subsequent step with 0.1% BSA in PBS. Ascites fluids (100 μl per well) in half-log dilutions were added and incubated for 1 h at room temperature. Antibodies specific to OMP were detected by incubating either a goat anti-mouse IgA (Southern Biotechnology Associates, Inc., Birmingham, Ala.)- or goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc., West Grove, Pa.)-alkaline phosphatase conjugate (100 μl per well) for a further hour at room temperature. After the plates were developed with 100 μl of *p*-nitrophenylphosphate (1 mg/ml) per well, the change in optical density at 410 nm was measured and recorded on an MR 700 microtiter plate reader (Dynatech Laboratories, Inc., Chantilly, Va.).

Competition ELISAs were performed between IgA 71 and IgG 40 to determine if IgG 40 bound to epitopes distinct from or overlapping with IgA 71. Preliminary ELISAs showed that a 10^3 dilution of IgG 40 ascites fluid resulted in subsaturation levels of alkaline phosphatase activity. Therefore, this dilution was chosen for use in the competition. One hundred microliters of IgG 40 ascites fluid diluted 10^3 was combined with an equal volume of IgA 71 ascites fluid diluted in half-log increments prior to the 1-h incubation in *H. felis* OMP-coated microtiter wells. The remainder of the assay was performed as described above, using an anti-IgG-alkaline phosphatase conjugate. In a control experiment, IgG 40 was placed in competition with an irrelevant IgA ascites fluid, Sv255 (specific for Sendai virus), for binding to OMP. In the reciprocal competition, a 10^3 dilution of IgA 71 was combined with various dilutions of IgG 40. In this case, binding of IgA 71 was measured by using an IgA-specific conjugate. Results of the competition ELISA were compared with the activities of the MAbs tested in the absence of a competing antibody.

Urease inhibition assay. Inhibition of urease activity by MAb IgA 71 was determined by comparison of the rate of release of ammonia from urea in the presence of various concentrations of IgA 71. Purified *H. pylori* urease (0.1 μg) was incubated at 37°C for 30 min in the presence of 0, 0.1, 1.0, or 10 μg of IgA 71 in 96-well microtiter plates. Rapid urea test broth (100 μl) was added to each mixture, and the incubation continued at room temperature. The color development was monitored at 5-min intervals at 550 nm, using a microtiter plate reader.

Radiolabeling of proteins. *H. felis* OMP and purified *H. pylori* urease were radiolabeled with $Na^{125}I$ by using lactoperoxidase (26). Briefly, 10 μg of protein was combined with 30 mU of lactoperoxidase, 1.0 mCi of $Na^{125}I$, and 10 μl of 0.00042% H_2O_2 for 30 s at room temperature. The reaction was stopped by the addition of 100 μl of 0.1% BSA in PBS with 0.05% NaN_3. Labeled proteins were separated from carrier-free ^{125}I by fractionation on a 10-ml Sephadex G-50 (Pharmacia) column. Aliquots from individual fractions were counted in a Beckman 5000 γ counter (Beckman Instruments, Inc., Fullerton, Calif.) and subsequently precipitated with 20% trichloroacetic acid. The fractions corresponding to the aliquots of which greater than 98% of the activity could be precipitated were pooled and saved for use in immunoprecipitation assays.

Coupling antibodies to Sepharose 4B. Sepharose 4B (Pharmacia) was activated according to the manufacturer's instructions. Briefly, 1 g of Sepharose 4B was swollen in 10 ml of H_2O, and the resulting slurry was washed extensively with H_2O. Five grams of the slurry was placed in a beaker on ice and combined with 5 ml of 10% CNBr solution. One molar NaOH was added to the slurry while stirring, and the pH was monitored until a reading of 11 to 11.5 could be maintained for at least 10 min. The activated Sepharose was then washed extensively with 10 volumes of cold 0.1 M $NaHCO_3$. Five milliliters of a 1-mg/ml solution of affinity-pure goat anti-mouse IgA antibodies (Southern Biotechnology) was dialyzed against 0.1 M $NaHCO_3$, combined with 2 g of the activated Sepharose 4B, and then rocked overnight at 4°C. An aliquot of the supernatant was assayed for protein content (15) to determine the efficacy of binding.

Immunoprecipitation. Microcentrifuge tubes were precoated with dilution buffer (0.1% Triton X-100, 0.1% BSA, 0.01 M Tris [pH 8.0], 0.14 M NaCl) for 10 min at room temperature. The tubes were emptied, 10 μl (approximately 10^6 cpm) of ^{125}I-labeled *H. felis* OMP or *H. pylori* urease was aliquoted to each tube, and the volume was brought up to 200 μl with dilution buffer. Ten microliters of ascites fluid was added to each tube, and the contents were rocked for 1.5 h at 4°C. Fifty microliters of goat anti-mouse IgA–Sepharose 4B conjugate (prepared as described above) or protein G-Sepharose (Pharmacia) mixed 1:1 with dilution buffer was added, and the contents were rocked for 1.5 h at 4°C. The tubes were spun at 200 × *g* to pellet the beads and washed twice with dilution buffer, once with 0.01 M Tris (pH 8.0)–0.14 M NaCl, and once with 0.05 M Tris (pH 6.8). Fifty microliters of sodium dodecyl sulfate sample buffer was added to the pellet and heated for 5 min at 100°C. The tubes were gently agitated and spun at 200 × *g* for 2 min, and the supernatants (50 μl) were loaded onto 10% polyacrylamide gels for polyacrylamide gel electrophoresis. Gels were dried on filter paper under vacuum and developed by autoradiography. Controls included tubes without antibody, as well as IgA Sv255 and IgG Sv271 MAbs specific for Sendai virus glycoproteins. Anti-Sendai virus MAbs were a gift from Mary Mazanec, Case Western Reserve University, Cleveland, Ohio.

Passive oral immunization. *H. felis* was harvested from a fresh plate and brought to a concentration of 5×10^6 CFU/ml in PBS. Two-milliliter aliquots were combined with 2 ml of ascites fluid containing MAb (IgA 71 anti-*H. felis* OMP, IgG 40 anti-*H. felis* OMP, or IgA 255 anti-Sendai virus) and incubated at 37°C for 30 min. Four hundred microliters (10^6 organisms) was administered to each mouse by gastric intubation. All mice received an additional 200 μl of ascites fluid at 4, 8, and 24 h. Mice were necropsied on day 9, and the gastric tissue was examined for *H. felis* colonization by the urea broth assay and culture methods described below.

Urease detection assay. Biopsy specimens (2 by 2 mm) were taken from the gastric antrum, placed in 0.5 ml of Stuart's urease test broth (25), and incubated at room temperature. *H. felis* colonization was confirmed by a change in broth color from orange to red. Tubes were observed over a 24-h period.

Culture. Gastric antral biopsy specimens were homogenized in 200 μl of Columbia broth and plated on Columbia blood agar with 7% horse blood. Plates were incubated at 37°C for 96 h under microaerophilic conditions. Colonies were confirmed as *H. felis* on the basis of morphology, Gram stain, and the production of urease, catalase, and oxidase.

Antibody screening of *H. felis* expression library. Genomic DNA was isolated from *H. felis* CS1 (a gift from James Fox, Massachusetts Institute of Technology, Cambridge) by the Marmur technique (16). This DNA was sent to Stratagene (La Jolla, Calif.), where it was used to construct an *H. felis* genomic DNA expression library containing 6- to 10-kb inserts in lambda ZAP II. Five thousand plaques from five 150-mm-diameter plates were replicated onto duplicate nitrocellulose filters soaked in 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Replicate filters were screened for antigen-producing recombinants by immunoblotting using MAbs as probes. Briefly, filters were blocked in 1% BSA in 20 mM Tris-buffered saline (pH 7.5) at 4°C overnight and then incubated with either MAb IgA 71 or MAb IgG 40 diluted 1:2,000 in blocking buffer for 1 h at room temperature. After filters were washed in Tris-buffered saline with 0.05% Tween 20 (Sigma), antibody binding was detected by soaking the filters for 1 h at room temperature in alkaline phosphatase-conjugated goat anti-mouse isotype antibodies (see ELISA methods) diluted 1:2,000 in blocking buffer. After a further wash, all phosphatase activity was detected with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (Bio-Rad Laboratories, Hercules, Calif.) as a precipitating substrate. Positive plaques were picked and stored in 500 μl of SM buffer (0.1 M NaCl, 5 mM $MgSO_4$, 50 mM Tris [pH 7.5]) and 50 μl of chloroform.

Recovery of plasmids from lambda phage vectors. Plasmids were rescued from positive lambda phage recombinants as recommended by Stratagene. Briefly, 200 μl of Stratagene *E. coli* SOLR cells (optical density at 600 nm of 1.0) was combined with 200 μl of recombinant phage stock (from SM buffer storage; see above) and 1 μl (10^{10}/ml) of Stratagene ExAssist helper phage. After incubation at 37°C for 15 min, 5 ml of 2× YT medium (16 g of Bacto Tryptone, 10 g of yeast extract, and 10 g of NaCl per liter) was added, and the culture was incubated at 37°C for 3 h with shaking. Cultures were heated at 70°C for 20 min to kill the bacteria. The phage were separated from particulate residue by centrifugation at 6,000 rpm in a Sorvall SS-34 rotor for 5 min at room temperature. Ten microliters of supernatant containing the rescued plasmid in filamentous phage particles was incubated with 200 μl of Stratagene *E. coli* XL1-Blue cells (optical density at 600 nm of 1.0) at 37°C for 15 min and plated on LB agar containing ampicillin (100 μg/ml) for selection of *E. coli* harboring the rescued plasmids.

DNA hybridization. Restriction digests of purified plasmids were resolved on 1% agarose gels. DNA was transferred to GeneScreen membranes (Du Pont NEN Research Products, Boston, Mass.) by the Southern method (24) and hybridized to *ure*B-specific fluorescein-labeled oligonucleotides at 42°C for 2 h. Hybridization was detected with Amersham's enhanced chemiluminescence system (Amersham Corp., Arlington Heights, Ill.). The same oligonucleotides were used as primers in the sequencing reactions listed below.

Sequencing. Sequencing was accomplished by the dideoxy-chain termination method (23), using Sequenase (U.S. Biochemicals, Cleveland, Ohio) according to the manufacturer's instructions. The oligonucleotides used as primers included a T3 promoter primer (5′ATTAACCCTCACTAAAG3′), a T7 promoter primer (5′AATACGACTCACTATAG3′), and an *H. pylori ureB* primer (HpURE1; 5′ATGAAAAAGATTAGCAGAAAAGAATATGTTTCTATG3′).

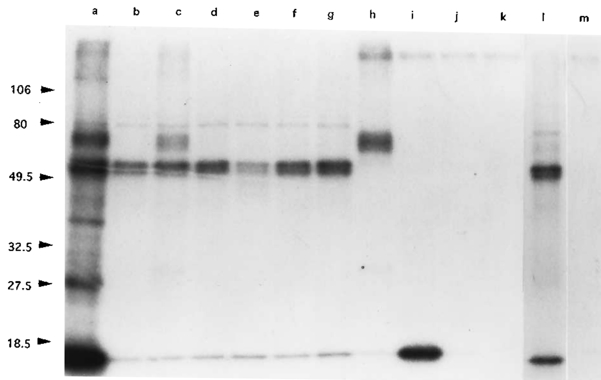

FIG. 1. Immunoprecipitation of radiolabeled *H. felis* OMPs with anti-*H. felis* MAbs. Goat anti-mouse IgA–Sepharose was used as a precipitant for lanes b to g and l. Protein G-Sepharose was used as a precipitant for lanes h to k and m. Precipitated antigens were resolved on a 10% polyacrylamide gel. Lanes: a, labeled OMPs; b, IgA 34; c, IgA 71; d, IgA 109; e, IgA 251; f, IgA 395; g, IgA 255 (anti-Sendai virus); h, IgG 40; i, IgG 50; j, IgG 84; k, IgG 271 (anti-Sendai virus); l, no antibody; m, no antibody. Sizes are indicated in kilodaltons.

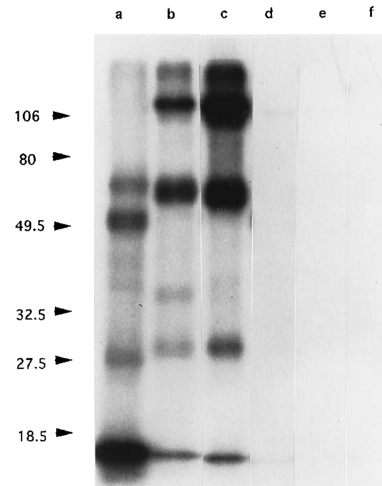

FIG. 2. Immunoprecipitation of radiolabeled *H. pylori* urease. Goat anti-mouse IgA–Sepharose was used as a precipitant for lanes c and d. Protein G-Sepharose was used as a precipitant for lanes e and f. Lanes: a, *H. felis* OMPs; b, *H. pylori* urease; c, IgA 71; d, IgA 255 (anti-Sendai virus); e, IgG 40; f, IgG 271 (anti-Sendai virus). Sizes are indicated in kilodaltons.

Plasmid analysis and subcloning. Restriction endonucleases and all other enzymes used for the manipulation of DNA were purchased from Gibco-BRL (Gaithersburg, Md.). All common DNA manipulations were performed as described by Sambrook et al. (22). Plasmid DNA was isolated from cells lysed by boiling (11), and transformations were performed by using a one-step transformation and storage solution (1a).

Antibody screening of bacterial colonies. Colonies of bacteria harboring plasmid pBluescript II SK(−) (Stratagene) or pTGB1 were grown on nitrocellulose filters and screened with MAbs for the production of antigen as described by Sambrook et al. (22). Briefly, the nitrocellulose filters were exposed to chloroform vapors for 15 min and then incubated in lysis buffer (0.1 M Tris [pH 7.8], 0.15 M NaCl, 5 mM $MgCl_2$, 1.5% BSA, 1 mg of pancreatic DNase I per ml, 400 μg of lysozyme per ml) while shaking on an orbital shaker overnight at room temperature. Membranes were washed three times for 30 min each in 10 mM Tris (pH 8.0)–0.15 M NaCl–0.05% Tween 20 (TNT) and were then blocked for 30 min in 20% fetal calf serum in TNT. Filters were subsequently incubated for 4 h in MAb ascites fluid diluted 1:2,000 in blocking buffer, washed three times for 10 min each in TNT containing 0.1% BSA, and then incubated in goat anti-mouse isotype-alkaline phosphatase conjugates (see procedures for library screening) before being washed again and developed with BCIP and nitroblue tetrazolium.

Statistical analysis. The presence or absence of experimental infection among groups of mice was evaluated by χ^2 analysis.

RESULTS

Immunoprecipitation of *H. felis* OMPs with MAbs. We previously reported that incubation of infectious *H. felis* with an anti-*H. felis* monoclonal IgA ascites fluid in vitro effectively neutralized the ability of the bacteria to colonize the stomachs of germ-free mice (3). The protective MAb IgA 71 failed to bind specifically to any *H. felis* proteins in Western blots (immunoblots) (not shown). Therefore, IgA 71 and seven other anti-*H. felis* MAbs developed in our laboratory were used to immunoprecipitate radiolabeled *H. felis* OMPs in a further attempt to determine their antigenic specificities. A protein of 62 kDa was precipitated by MAb IgA 71 (Fig. 1, lane c), and MAb IgG 40 precipitated a protein of similar molecular weight (lane h). None of the other six MAbs specifically precipitated any OMPs in this molecular weight range. Although other protein bands appeared in all lanes, these were attributed to association of OMPs with the goat anti-mouse IgA–Sepharose (lanes b to g and l) or protein G-Sepharose (lanes h to k and m) used to immunoprecipitate the immune complexes. This result was verified by the banding pattern achieved when radiolabeled OMPs were combined with goat anti-mouse IgA–Sepharose (lane l) or protein G Sepharose (lane m) in the absence of antibody.

Immunoprecipitation of *H. pylori* urease with MAb IgA 71. The results from the immunoprecipitation with *H. felis* OMPs suggested that both IgA 71 and IgG 40 bind to the large subunit of *H. felis* urease, which has a molecular mass of 62 kDa. Amino acid sequence analysis of the N terminus of the gel-purified protein was inconclusive. Since MAb IgA 71 binds to both *H. felis* and *H. pylori* OMPs in ELISA (not shown) and since the deduced *H. pylori* and *H. felis* urease amino acid sequences are highly homologous (8), we investigated whether IgA 71 and IgG 40 would immunoprecipitate recombinant *H. pylori* urease. Urease apoenzyme prepared under nondenaturing conditions was precipitated by IgA 71 (Fig. 2, lane c) but not by IgG 40 (Fig. 2, lane e). This result confirmed the recognition of *Helicobacter* urease proteins by IgA 71 and suggested that IgG 40 either recognizes a different *H. felis* OMP of approximately 62 kDa or may bind to an epitope of *H. felis* urease which is not shared by *H. pylori* urease.

Screening of an *H. felis* expression library with MAbs. To clarify the antigenic specificities of IgA 71 and IgG 40, we next used these antibodies as probes to screen an *H. felis* lambda phage expression library. Ten plaques representing four distinct clones were identified when 5,000 plaques were probed with IgA 71. All IgA 71-positive plaques also reacted with IgG 40. The plasmids from all four positive clones were rescued by in vivo excision. Southern blot analysis revealed that all of the plasmids could be hybridized by a synthetic oligonucleotide corresponding to the first 36 nucleotides of the 5′ end of the *H. pylori ureB* gene (2) (data not shown). The predicted amino acid sequence derived from a partial nucleotide sequence from one of the clones, pTGB1, is shown in Fig. 3 and compared with the published sequences of both *H. pylori* and *H. felis ureB* (2, 8). The first 73 amino acids share 79.5% identity with the *H. pylori ureB* sequence and 97.3% identity with the recently reported sequence of *H. felis ureB* from strain ATCC 49179 (8).

pTGB1 contained an *Eco*RI insert of 5.6 kb which when further analyzed by restriction endonuclease digestion mapping and Southern blotting experiments was found to encode both *ureA* and *ureB*. The presence of restriction fragments identical to those mapped for *ureA* and *ureB* by Ferrero and Labigne (8) strengthened this conclusion. Immunoblots of col-

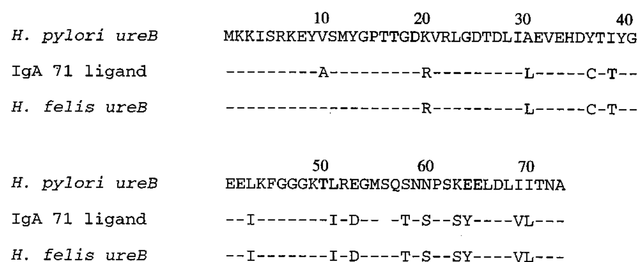

FIG. 3. Predicted amino acid sequence of DNA from an expression library clone selected with the protective MAb IgA 71 compared with the 5′ ends of the *ureB* genes from *H. pylori* and *H. felis*.

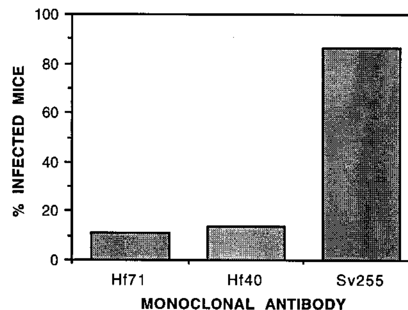

FIG. 5. Protection of germ-free mice from *H. felis* infection by incubation of bacteria with MAb ascites fluid prior to oral inoculation of mice. Groups of 9, 14, and 14 mice were used for MAbs IgA 71, IgG 40, and IgA Sv255, respectively.

onies performed with both IgA 71 and IgG 40 resulted in the recognition of *E. coli* containing pTGB1 but not to *E. coli* containing vector DNA with no insert. These results confirm that the ligand for both IgA 71 and IgG 40 is urease and when combined with the OMP immunoprecipitation data (Fig. 1) strongly suggest that the *H. felis ureB* protein contains the respective epitopes.

MAbs IgA 71 and IgG 40 bind distinct epitopes. To demonstrate that these two antibodies do indeed recognize distinct *H. felis* urease epitopes as suggested by the failure of IgG 40 to immunoprecipitate *H. pylori* urease, competitive ELISAs were performed. In these assays, we took advantage of the fact that each antibody could be recognized by a distinct isotype-specific second antibody, obviating the need to label one or the other of the primary MAbs. In this competitive ELISA, increasing doses of IgG 40 failed to displace a fixed, subsaturating amount of IgA 71 (Fig. 4). Similar results were obtained in the reciprocal assay in which a fixed amount of IgG 40 was placed in competition with increasing amounts of IgA 71 (data not shown).

Passive protection of mice from *H. felis* by MAbs specific for urease. To compare the effectiveness of MAb IgG 40 in neutralizing *H. felis* with that of MAb IgA 71, ascites fluid containing either IgG 40 or IgA 71 was incubated with 10^6 viable *H. felis* cells prior to oral administration to germ-free mice (see Materials and Methods). Control mice received *H. felis* preincubated with an irrelevant anti-Sendai virus IgA ascites fluid. The results of the experiment are shown in Fig. 5. Twelve of the fourteen mice (86%) receiving *H. felis* preincubated with irrelevant ascites fluid were colonized by *H. felis*, while only one of the nine mice (11%) receiving *H. felis* preincubated with IgA 71 was infected ($P = 0.002$). These results provide additional support to our previous findings that IgA 71 could protect mice from infection (3). Only two of the fourteen mice (14%) receiving *H. felis* in combination with IgG 40 were infected ($P = 0.0004$). Thus, both IgA 71 and IgG 40 were able to passively protect mice from *H. felis* infection.

MAb IgA 71 does not prevent colonization by inhibiting urease activity. Since IgA 71 precipitates *H. pylori* urease, we tested its ability to inhibit urease activity in a urea hydrolysis assay. Increasing amounts of IgA 71 failed to inhibit the conversion of urea to ammonia as determined in a colorimetric assay (Fig. 6). Thus, the mechanism by which IgA 71 prevents colonization of mice stomachs does not appear to be by inhibition of urease activity. Although urease activity is not affected by IgA 71, both IgA 71 and IgG 40 do agglutinate *H. felis* at the concentrations used for passive protection. This agglutination did not affect bacterial viability, as the agglutinated *H. felis* continued to grow well in culture.

DISCUSSION

This study provides further evidence that *H. felis*-specific antibodies can prevent colonization of mouse gastric tissue by *H. felis* and demonstrates that both an IgA and an IgG neutralizing MAb are specific for urease. Incubation of *H. felis* with either of our MAbs, IgA 71 or IgG 40, prior to oral administration to mice effectively prevents the ability of the bacteria to colonize the gastric mucosa. When combined with

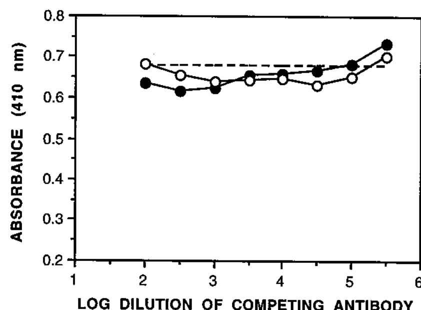

FIG. 4. Effect of IgG 40 on IgA 71 binding to *H. felis* OMPs. A predetermined dilution (10^{-3}) of IgA 71 ascites fluid was combined with serial half-log dilutions of IgG 40 to determine if the two MAbs were specific for overlapping epitopes. Solid circles represent the detectable IgA when IgG 40 is used as an inhibitor of IgA 71. Open circles represent the amount of IgA detected when an irrelevant IgG, Sv271, is used to compete with IgA 71. The line without symbols represents the signal generated by a 10^{-3} dilution of IgA 71 ascites fluid in the absence of an inhibitor.

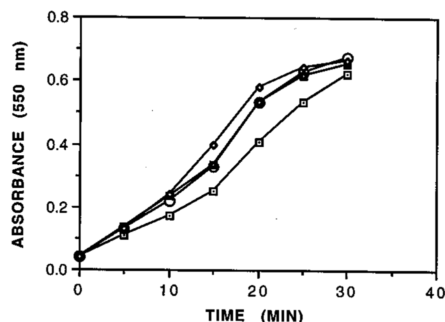

FIG. 6. *H. pylori* urease activity in the presence of MAb IgA 71. Urease holoenzyme (0.1 μg) was combined with 0.0 (open squares), 0.1 (open circles), 1.0 (closed squares), or 10 (open diamonds) μg of IgA 71 and combined with rapid urea broth for 30 min. Urease activity was monitored by increased A_{550}.

our previous report on the induction of protection by active oral immunization, these results indicate the importance of a mucosal, presumably sIgA, immune response in resistance to *Helicobacter* infection.

Urease has been implicated as an important virulence factor in *Helicobacter* pathogenesis (10, 17, 30). It is highly conserved among *Helicobacter* species and functions to convert urea to ammonia, which has been suggested to protect it from the harmful acids of the gastric environment (17). Urease is loosely associated with the bacterial membrane and represents over 5% of the soluble bacterial protein (12). The importance of this enzyme in *Helicobacter* colonization has been underscored by experiments with chemically induced urease-negative mutants of *H. pylori* which fail to colonize barrier-born pigs (6). The present study supports the idea that urease is an important antigen in *Helicobacter* immunity.

Urease-specific sIgA is produced in both humans and mice infected with *Helicobacter* species. While our results may seem contrary to the observation that *Helicobacter* infections persist in the presence of such antibodies, our previous results (3) and those of others (1, 5, 14) have demonstrated that preexisting *Helicobacter*-specific antibodies can prevent colonization despite their inability to eradicate an established infection. Recently it has been demonstrated that protective immunity against *H. felis* can be achieved in mice by active oral immunization with the urease proteins (9, 19). Our results support these findings, indicating that urease may serve as a possible vaccine candidate and strengthen the notion that protection is antibody mediated.

We have also provided evidence that our infection neutralizing MAbs IgA 71 and IgG 40 are specific for the large subunit of the urease enzyme. Although IgA 71 precipitates both subunits of purified recombinant *H. pylori* urease, it precipitates only the heavy subunit of urease when reacted with *H. felis* OMPs. This is most likely due to a difference in protein preparation. The purified *H. pylori* urease apoenzyme was purified from bacterial supernatants by column chromatography under conditions which would not disrupt subunit association, and therefore both the large and small subunits would be precipitated simultaneously. The *H. felis* OMPs, however, were prepared by repeated washing in *n*-lauroyl sarkosine (see Materials and Methods), and therefore the subunits were dissociated prior to immunoprecipitation. Precipitation of the *H. pylori* urease with IgA 71 is consistent with published sequence analysis of *H. pylori* and *H. felis* urease proteins demonstrating a high degree of conservation (8). However, the specificity of IgG 40 for *H. felis* but not *H. pylori* urease demonstrates that there are species-specific epitopes on the enzyme. Interestingly, Ferrero et al. (9) have reported an increased efficacy of the urease B subunit relative to the A subunit by immunizing mice with various recombinant urease fusion proteins.

Although both antibodies bind to the *ureB* protein, IgA 71, which precipitates *H. pylori* urease, fails to inhibit the enzymatic activity of purified *H. pylori* urease. We postulate that antibody effectiveness is mediated in one of two ways. First, the urease proteins may possess some previously undescribed function, either enzymatic or receptor binding, which is inhibited by the MAbs. Second, the antibody may inhibit colonization by agglutinating the bacteria and promoting clearance by mucus flow. Microscopic examination of *H. felis* incubated with our two OMP-specific MAbs reveals that both are capable of agglutinating the bacteria at the concentrations used in the passive protection studies. It should be noted that several of our other *H. felis*-specific MAbs also agglutinate the bacteria in vitro yet fail to inhibit colonization (unpublished data). It is unlikely that the nature of the ascites fluid plays a role in the protection, as ascites fluids containing irrelevant MAbs fail to prevent colonization.

The ability of IgG 40 to prevent colonization of *H. felis* is interesting in light of the fact that IgG is not present in significant quantities at the mucosa and is more prone to the acid environment of the stomach than IgA. Several explanations may be made for its effectiveness in the present study. First, agglutination is taking place in vitro in a "protein-friendly" environment. The nature of the agglutinated complex might protect the antibodies from the stomach acid when introduced to the mouse. Second, the volume (400 μl) of ascites and bacteria given to the mouse by gastric intubation may be sufficient to change the stomach environment long enough to allow the IgG antibodies to prevent colonization. Presumably the bacteria are displaced by the peristaltic action of the gastric mucosa as immune complexes. Third, the gastric environment of the mouse may be much less hostile than that of the human. In fact the mouse stomach is routinely colonized by other bacterial species. We have also performed experiments in which MAbs are delivered to the stomachs of naive mice prior to challenge with *H. felis*. These mice are not protected from colonization (data not shown), a result most consistent with agglutination being of major importance in passive protection of mice. However, as our experimental system may not have used ideal IgG concentrations, the second two explanations cannot be ruled out. In conclusion, this study provides evidence that the humoral response can be sufficient to prevent colonization and that urease can serve as a protective antigen.

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