Local and systemic antibody responses in humans with *Helicobacter pylori* infection

Thomas G Blanchard PhD[1], John G Nedrud PhD[1], Steven J Czinn MD[2]

TG Blanchard, JG Nedrud, SJ Czinn. Local and systemic antibody responses in humans with *Helicobacter pylori* infection Can J Gastroenterol 1999;13(7):591-594. Immunization can prevent or cure an otherwise chronic helicobacter infection in several animal models despite the chronic nature of natural helicobacter infections. Differences in the antigenic specificity of the antibodies may contribute to the protection observed in these experimental animals. The goal of the present study was to compare the local and systemic antibody responses of humans with chronic *Helicobacter pylori* infection with those of an individual with spontaneous resolution of infection to find an immunological correlate of protection. Spontaneous resolution of infection was accompanied by a change in immunoblot profiles. Whereas a broad range of *H pylori* antigens was recognized in chronically infected patients (including the patient who ultimately cleared the infection spontaneously), resolution of infection in the absence of therapeutic agents resulted in the recognition of only several immunodominant antigens. The most dominant antigen was approximately 66 kDa in molecular mass. Immunoblot analysis demonstrated that these antibodies were specific for the structural subunits of the urease enzyme. These studies suggest that the success of antihelicobacter immunization may be due to the ability of vaccination to induce an immune response against antigens that are normally not immunodominant during the course of infection.

Key Words: Helicobacter pylori; *Urease enzyme*

Productions systémique et locale d'anticorps chez les humains infectés à *Helicobacter pylori*

RÉSUMÉ : L'immunisation peut prévenir ou guérir une infection à *Helicobacter* autrement chronique chez plusieurs modèles animaux malgré la nature chronique des infections naturelles à *Helicobacter*. Les différences dans la spécificité antigénique des anticorps pourraient contribuer à la protection qui a été observée dans ces modèles expérimentaux. L'objectif de la présente étude était de comparer les productions systémique et locale d'anticorps chez les humains atteints d'une infection chronique à *Helicobacter pylori* avec celles d'un sujet chez qui l'infection s'est résolue spontanément pour mettre en évidence un corrélat immunologique de protection. Une résolution spontanée de l'infection s'accompagnait d'un changement dans les profils d'immunotransfert. Alors qu'un large éventail d'antigènes de *H pylori* ont été décelés chez les patients infectés de façon chronique (y compris le patient chez qui l'infection s'est finalement résolue spontanément), la résolution de l'infection en l'absence d'agents thérapeutiques a permis d'identifier seulement quelques antigènes immunodominants. L'antigène le plus dominant avait environ 66kDa de masse moléculaire. L'analyse par immunotransfert a démontré que ces anticorps étaient spécifiques aux sous-unités de structure de l'uréase. Ces études laissent à penser que la réussite de l'immunisation contre *Helicobacter* peut être due au fait que la vaccination est capable d'induire une réponse immunitaire contre les antigènes qui normalement ne sont pas immunodominants au cours de l'infection.

The Gram-negative bacterium *Helicobacter pylori* is well established as an etiological agent of gastritis and peptic ulcers (1). Chronic infection with *H pylori* is also recognized as a risk factor for the development of gastric cancer (2). The prevalence of these diseases worldwide and the cost of treatment make *H pylori* a significant human pathogen.

Chronic *H pylori* infection is accompanied by both serum and local antibody responses (3-5). The mechanisms by which *H pylori* persist and colonize the stomach despite such a rigorous immune response are unknown. However, several laboratories have employed animal models of helicobacter infection to demonstrate that oral immunization not only can prevent the establishment of a chronic infection, but also can eradicate an established infection when given therapeutically (6-9). Thus, whereas the immune response that is induced by infection is generally not adequate to eradicate the organism, the immune response induced by immunization can both prevent and clear the *H pylori* infection.

Despite these observations, no correlates of protective immunity have been described. It is likely that immune re-

[1]*Institute of Pathology, and* [2]*Department of Pediatrics, Case Western Reserve University, Cleveland, Ohio, USA*

Correspondence and reprints: Dr Steven J Czinn, Department of Pediatrics, Rainbow Babies & Childrens Hospital, 11100 Euclid Avenue, Cleveland, Ohio 44106, USA. Telephone 216-844-1767, fax 216-368-1357, e-mail sjc3@po.cwru.edu

TABLE 1
Anti-*Helicobacter pylori* titres (log_{10}) from *H pylori*-positive patients and *H pylori*-negative patients

	H pylori-positive (n=37)	*H pylori*-negative (n=16)
Serum IgA	3.09±0.49*	2.23±0.73
Serum IgG	4.32±0.68*	3.26±0.58
Gastric juice IgA	0.59±0.73*	0.13±0.29
Gastric juice IgG	0.19±0.39*	0.02±0.07
Saliva IgA	1.84±0.63	1.87±0.68
Saliva IgG	1.22±0.61*	0.71±0.69

**Difference is significant by Student's two-tailed* t *test. Ig Immunoglobulin*

sponses induced by immunization are either quantitatively or qualitatively different from those induced by infection. Human immunization studies parallel to those in mice and ferrets cannot be performed because a safe, efficacious *H pylori* vaccine for use in humans has not yet been developed. We obtained serum samples from a patient who spontaneously cleared *H pylori* infection without therapeutic intervention. The goal of the present study was to compare quantitatively and qualitatively the humoral immune response to *H pylori* of infected patients with the antibody response of the patient who spontaneously resolved the infection. Defining these differences should help elucidate the mechanisms by which helicobacter immunity is achieved and thereby define markers for protective immunity.

PATIENTS AND METHODS

Patients: The study group consisted of 53 (26 male and 27 female) patients with dyspepsia or peptic ulcer disease who were referred for gastrointestinal endoscopy. The study was approved by The Investigational Review Board of University Hospitals of Cleveland, Ohio.

Bacteria: The *H pylori* test strain (HpP12) used for the production of outer membrane proteins (OMPs) was isolated from a pediatric patient and identified as *H pylori* based on colony and bacterial morphology, Gram stain, and the production of urease, catalase and oxidase. The urease-negative strain of *H pylori* used as a source of antigen for immunoblots was created by insertional inactivation of the urease genes and was a generous gift from OraVax Inc (Cambridge, Massachusetts). Helicobacter strains were grown on Columbia agar (Difco, Detroit, Michigan) containing 7% horse blood under microaerobic conditions (5% oxygen and 10% carbon dioxide) at 37°C for 96 h. Bacteria were stored at –70°C in 0.1 M phosphate-buffered saline (pH 7.2) (PBS) with 25% glycerol and 25% heat-inactivated fetal calf serum.

Helicobacter lysates: Sonicates of *H pylori* were prepared as previously described for *Helicobacter felis* (10). Confluent plates of *H pylori* were harvested in 1 mL sterile PBS and transferred to 200 mL brain heart infusion medium (Difco Laboratories, Franklin Lakes, New Jersey) supplemented with 10% fetal bovine serum and 0.25% yeast extract in a 500 mL flask. Flasks were placed in bell jars with CampyPak Plus envelopes (Becton Dickinson, Sparks, Maryland) to achieve a microaerobic environment. Jars were placed on an orbital platform shaker at 37°C and incubated overnight while shaking at 125 revolutions/min. Bacteria were harvested by centrifugation at 4000 *g* for 20 mins. Cell pellets were resuspended in 2 mL PBS each. The cells were lysed while on ice by three 30 s bursts of power using a probe sonicator (Sonics and Materials Inc, Newtown, Connecticut) set at 5% duty cycle and a power setting of 5. Whole cells were removed by centrifugation at 4000 *g* for 20 mins, and the lysate was filtered through a 0.22 µM pore filter. The concentration of protein present in the supernatants was determined by the method of Lowry et al (11).

***H pylori* OMPs:** OMPs were isolated from *H pylori* as previously described for *H felis* (12). *H pylori* was harvested from 200 confluent plates by using 1 mL of 50 mM Tris (pH 7.8) and 1 mM EDTA per plate. One milligram each of DNase and RNase (Sigma Chemical Co, St Louis, Missouri) was then added, and iced bacteria were sonicated for four 30 s intervals. Unbroken cells were removed by centrifugation at 10,000 *g* for 30 mins at 4°C. OMP were recovered by centrifugation of the supernatant at 135,000 *g* for 1 h at 4°C. The pellet was resuspended in 2 mL of 2% *N*-lauroyl sarcosine (Sigma Chemical Co) and allowed to sit at room temperature for 20 mins. Proteins were recovered by centrifugation at 106,000 *g* for 1 h at 4°C. The pellet was washed three times by resuspension in 1% *N*-lauroyl sarcosine followed by centrifugation at 106,000 *g* for 1 h at 4°C. 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 (11).

Recombinant *H pylori* urease: Recombinant *H pylori* urease used for immunoblots was a generous gift from OraVax Inc.

Diagnosis of *H pylori* infection: Patients were considered infected with *H pylori* if gastric biopsies were positive for urease activity, if *H pylori* could be cultured from gastric biopsies or if *H pylori* was visualized directly in histological sections of gastric biopsies.

Sample collection for antibody measurement: Serum, saliva and gastric juice were obtained from fasting patients at the time of endoscopy. Gastric juice was neutralized with an equal volume of 0.67 M Tris-HCl (pH 7.4). Insoluble contents of saliva and gastric juice were removed by centrifugation at 10,000 *g* for 10 mins. All samples were stored at –70°C until assayed.

RESULTS

Infection with *H pylori* results in a rise in both local and systemic antibody responses. Antihelicobacter-specific immunoglobulin (Ig) G and IgA responses were determined for *H pylori*-positive patients and compared with those of patients presenting with dyspepsia or peptic ulcer without documented *H pylori* infection. Serum samples from *H pylori*-positive patients had antihelicobacter IgG and IgA titres significantly higher than those of noninfected controls (Table 1). Similar results were observed when the IgG and IgA levels in gastric washes were compared. As expected, IgA titres were higher than IgG titres at the gastric mucosa. Titres for salivary IgA were comparable between the two groups,

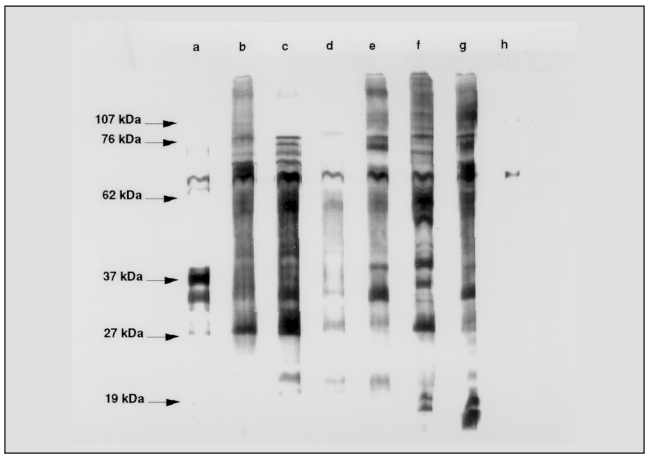

Figure 1) *Comparison of the antigenic specificities of serum immunoglobulin A obtained from patients presenting with chronic* Helicobacter pylori *infections (lanes a to g) and a patient who spontaneously cleared* H pylori *infection without therapeutic intervention (lane h). Whole cell sonicate of* H pylori *was used as antigen for immunoblotting. Approximate molecular masses are presented to the left of lane a*

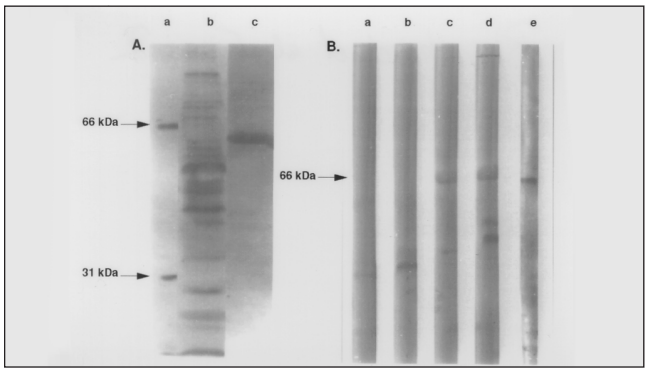

Figure 2) A *Antigenic specificity of serum immunoglobulin A from a patient before (lane b) and after (lane c) spontaneous clearing of the* Helicobacter pylori *infection. Lane a contains molecular weight markers.* **B** *Binding activity of serum used in A, lane c against whole cell lysate (lane a) or outer membrane proteins (lane b) from a urease-negative* H pylori *strain, against whole cell lysate (lane c) and outer membrane proteins (lane d) from wild-type* H pylori *and against purified recombinant* H pylori *urease*

while salivary antihelicobacter IgG was significantly higher in infected patients than in uninfected controls. These results confirm those of previous studies on which serological tests for *H pylori* are based.

Unique antigenic specificity of serum IgA from a patient who spontaneously cleared *H pylori* infection: Serum samples were obtained from an *H pylori*-infected 10-year-old boy with a duodenal bulb ulcer and chronic gastric inflammation before and after spontaneous clearance of the *H pylori* infection. If it is assumed that the subject's immune response was responsible for the clearance of infection, comparison of the antibody specificity of the patient with that of infected individuals might yield important information as to important vaccine candidate molecules. Previous studies have determined that, unlike serum IgG, serum IgA gives an immunoblot profile similar to that of gastric IgA in both mice and humans. Therefore, although gastric juice was not available from this patient for analysis, the serum IgA response in this patient was compared with that of seven *H pylori*-positive patients. Antibodies in the patient's serum collected postclearance recognized a single immunodominant protein of approximately 66 kDa (Figure 1, lane h). Although this protein was recognized by serum samples obtained from other patients, it was only one of a broad spectrum of immunodominant antigens in those patients (lanes a to g).

Serum IgA from a patient who spontaneously cleared *H pylori* is urease-specific: Immunoblot profiles using sera collected before and after spontaneous resolution of *H pylori* infection revealed marked differences in antigenic specificity (Figure 2A). While preclearance serum bound uniformly to a variety of OMPs (Figure 2A, lane b), postclearance serum recognized only one immunodominant protein of approximately 66 kDa (Figure 2A, lane c). Antibodies present in postclearance serum were urease specific (Figure 2B). Use of the postclearance serum against bacterial lysates and OMP from urease-deficient *H pylori* resulted in the absence of binding at 66 kDa (Figure 2B, lanes a,b). However, when the same serum was reacted with lysates and OMP from wild-type *H pylori* (Figure 2B, lanes c,d, respectively), a 66 kDa antigen was recognized. The use of purified recombinant urease (Figure 2B, lane e) confirmed that the serum from this patient specifically recognized urease.

DISCUSSION

The mechanisms by which *H pylori* is able to colonize the gastric mucosa persistently in the presence of an active local humoral antibody response remain unclear. Initial prophylactic immunization studies in animal models demonstrate that orogastric immunization can protect against challenge with infectious helicobacter (6,7). This finding suggests that developing an immune response before challenge is necessary to mediate protection. However, animals successfully treated with antibiotics to eradicate helicobacters are not resistant to reinfection, despite the immune response generated in response to the primary infection (7,13,14). In addition, several studies performed in both the mouse and ferret models of helicobacter infection demonstrate that therapeutic immunization of infected animals results in the eradication of the bacteria from the gastric mucosa (8,9,15). Thus, it seems that the immune response generated by immunization must be quantitatively and qualitatively different from the response generated during infection (16).

Early studies of helicobacter immunology addressed issues such as local antibody binding to organisms in vivo (5), local and serum antibody titres as correlates of inflammation and infection (17), and antigenic specificities of serum IgA and IgG (reviewed in 18). Immunization studies have examined salivary, intestinal and serum levels of IgG and IgA in both infected and immunized mice (6,7,19-24). While protective immunity in mice can be induced in the absence of antibody (25), such studies do not rule out the possibility that antibodies play a role in protective immunity when they are pres-

ent. To investigate this possibility further, we recently made a direct comparison of the magnitude and specificity of the gastric antibody response in immune mice versus infected animals (26). These studies indicated that immunization results in the generation of local and systemic antibodies with specificities that are distinct from those observed in infected mice. The present study extends that report by using human serum and gastric secretions.

Our previous study demonstrated that prophylactic immunization of mice does not result in higher levels of helicobacter-specific gastric IgA than chronic infection; that local IgA generated in response to oral immunization is qualitatively different in antigenic specificity from that of IgA induced by natural infection; and that the antigenic specificity of the serum IgA response accurately reflects the specificity of local IgA generated in response to infection in both mice and humans (26). Such observations allowed us to investigate the local immune response of infected patients for whom gastric washes were not available. Consistent with findings in the mouse study, clearance of *H pylori* infection in a human was accompanied by a change in immunoblot profiles against helicobacter antigens. Thus, the specificity of the humoral immune response appears to be an important element in the protective response induced by immunization. These studies also support a role for immunization as both a preventive (6,7) and therapeutic (8,9) intervention for *H pylori* infection.

The immunoblotting results also suggest that the induction of a strong antiurease antibody response may be an important component of this altered specificity. In most infected individuals, urease is not typically an immunodominant antigen. However, in a patient who spontaneously cleared *H pylori* infection, the large subunit of urease became immunodominant. Indeed, only one other antigen was recognized by this patient's serum by immunoblot techniques even though the same patient had a broad spectrum of antigenic specificity before clearance of the organism. These results are consistent with animal immunization studies showing that urease can be used as a vaccine antigen against helicobacter infections (27-30).

ACKNOWLEDGEMENTS: The authors are grateful to Howard Carr for technical assistance and OraVax, Inc for supplying purified recombinant urease. This work was supported by grants DK-46461 and AI-36359 from The National Institutes of Health.

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