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Impaired Host Immunity Contributes to *Helicobacter pylori* Eradication Failure

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OBJECTIVES: Effective eradication of *Helicobacter pylori* (*H. pylori*) infection has often proved more difficult than expected. Antimicrobial resistance incompletely explains eradication failure. This study tests the hypothesis that an impaired immune response may contribute to failed eradication after standard antibiotic therapy.

METHODS: Parameters of host immunity were assessed as blood T lymphocyte production of interferon-γ (IFN-γ) and interleukin-4 (IL-4) being surrogate markers of mucosal Th1 and Th2 responses, respectively. The validity of using circulating T cell cytokines as surrogate markers of mucosal immunity was established (unstimulated lymphocyte IL-4 level correlation $r^2 = 0.549$, $p < 0.001$; antigen-stimulated lymphocyte correlation $r^2 = 0.62$, $p < 0.001$).

RESULTS: A total of 52 dyspeptic patients and 11 patients with previous *H. pylori* eradication failure were recruited into the study. There was no significant difference in secretion of IFN-γ from peripheral blood T cells, in either unstimulated or antigen-stimulated cultures, between clinical groups. There was, however, a significant reduction in secretion of IL-4 from blood T cells in subjects failing to eradicate *H. pylori* compared with those who successfully eradicated the infection in both unstimulated and stimulated cultures. A significant difference in IL-4 secretion was also detected in antigen-stimulated cultures compared with that in *H. pylori*-positive subjects ($p < 0.05$). Low levels of IL-4 secretion were detected irrespective of the number of courses of antibiotic therapy. Lower levels of IgG anti–*H. pylori* antibody were detected in both serum and saliva of subjects with persistent *H. pylori* infection after use of antibiotics compared with untreated *H. pylori*-positive subjects (difference not statistically significant).

CONCLUSIONS: These results support the hypothesis that impaired mucosal immunity, particularly involving the secretion of IL-4, may contribute to *H. pylori* eradication failure. Measurement of whole blood secretion of IL-4 may predict which patients are more likely to fail standard antibiotic therapy. (Am J Gastroenterol 2002;97:3032–3037. © 2002 by Am. Coll. of Gastroenterology)

INTRODUCTION

Helicobacter pylori (*H. pylori*) is arguably the most common chronic infection in man, with clinical manifestations varying from asymptomatic infection to mucosal ulceration to gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphomas (1, 2). Particular patterns of host response to mucosal colonization determine variation in clinical disease (3). Although *H. pylori* is sensitive to many antibiotics, eradication has proved relatively difficult, requiring at least three antimicrobial agents used simultaneously to achieve cure. With these regimens in selected patient trials, eradication rates in excess of 90% are claimed; but in general clinical practice, a more realistic figure is 60–70% (4–7). Subjects with nonulcer dyspepsia have a higher eradication failure rate than those with classic peptic ulcer disease (8, 9). Failure of eradication cannot entirely be explained by noncompliance and resistance to antibiotics (10). Furthermore, oral immunization with *H. pylori* was shown to clear *H. pylori* infection in mice (11). Such observations point to the contributory role of host immunity in antibiotic-induced eradication of infection. The corollary is that subjects failing to eradicate *H. pylori* with antibiotics may have a deficient host response. Current theory suggests that a Th1 response in the gastric mucosa favors immunity (12, 13), although successful eradication is associated with a switch toward a more balanced Th0 cytokine pattern caused by an increase in secretion of interleukin-4 (IL-4) from *H. pylori*–stimulated T lymphocytes (14). This study tested the hypotheses that an impaired mucosal immune response may contribute to eradication failure in *H. pylori* infection, and that whole blood culture T lymphocytes are reliable surrogate markers for a mucosal response.

MATERIALS AND METHODS

Study Subjects

A total of 52 subjects referred for investigation of dyspepsia and 11 subjects with persistent *H. pylori* infection after one or more courses of antibiotics were recruited for this study. Subjects with dyspepsia had not taken any antibiotics for at least 3 months before the study began. The study was

approved by the Human Research Ethics Committee of the Centre for Digestive Diseases, Sydney, Australia. Informed consent was obtained from each patient. Multiple biopsy specimens were obtained from the antrum and body of the stomach for tissue culture, histology, and urease test (CLO test, Delta West, WA, Australia). Blood samples were incubated at 37°C within 2 h of collection. Serum was stored at −70°C for *H. pylori*-specific antibody.

Saliva Sample Collection

Saliva samples, collected before endoscopy, were centrifuged at 1000 × *g* for 10 min at 4°C. Aliquots were stored at −70°C.

Biopsy Culture

Gastric biopsy tissues were weighed and cultured at a ratio of 50 μl serum-free AIM-V medium (Life Technology, Sydney, Australia) milligram of tissue (wet weight) for 24 h. The culture supernatants were collected and centrifuged. Aliquots were stored at −70°C until assay.

H. pylori *Antigen Preparation*

H. pylori antigens from the NCTC 11637 strain were prepared by acid-glycine extraction (AGE) according to the method described by Goodwin *et al.* (15). *H. pylori* AGE was used for cell culture and specific antibody measurement.

ELISA Capture Assay for Secreted IL-4 in Whole Blood Culture

Il-4 levels in whole blood culture were measured following the assay Secril-4 Alert (VRI Biomedical, Perth, Australia). Principles of this assay, which measures IL-4 secreted from blood CD4 T-cells, have been described (14).

IFN-γ ELISA Assay

Wells of a 96-well, flat-bottomed microtiter plate (Nunc, Roskilde, Denmark) were coated with mouse antihuman IFN-γ monoclonal antibody (Endogen, Woburn, MA) at 2 μg/ml overnight at 4°C. After washing and blocking, supernatants from whole blood culture or IFN-γ standards (Endogen) were added in duplicate, and incubated for 90 min. The plates were washed and biotinylated mouse monoclonal antihuman IFN-γ antibody (Endogen) was added (0.25 μg/ml). After 90 min of incubation, the wells were washed and streptavidin-conjugated horseradish peroxidase (Selinus, Melbourne, Australia) was applied at a 1:2000 dilution. The plates were washed and tetramethyl benzidine chromagen (Sigma-Aldrich, St. Louis, MO) was added to each well. The absorbance was read at 450 nm in an ELISA plate reader (Bio-Rad 450, Richmond, CA). The limit of sensitivity for IFN-γ was 9.4 pg/ml. The amount of IFN-γ in samples was determined using a Softmax program (version 2.3 FPU, Cupertino, CA).

Detection of H. pylori *antibody*

Wells of a 96-well, flat-bottomed microtiter plate were coated with *H. pylori* AGE at 5 μg/ml at 4°C overnight. After washing and blocking, serum samples at 1:3000 dilution and saliva sample at 1:4 dilution were added to wells in triplicate. Horseradish peroxidase conjugated sheep anti-IgG (Selinus) was applied at 1:2000 dilution. Tetramethyl benzidine substrate (Sigma-Aldrich) was used for color development. The absorbance was read at 450 nm in an ELISA plate reader (Bio-Rad, 450). The results were expressed as ELISA units against a reference standard of pooled positive sera. Intra- and interassay variation was less than 10%.

Sensitivity and Specificity of Serum H. pylori *Antibody IgG*

Serum *H. pylori* antibody IgG was measured from 112 *H. pylori*-negative and 52 *H. pylori*-positive subjects. The *H. pylori* infection status of those subjects were confirmed by CLO test and histopathological examination. A cut-off value of serum *H. pylori* IgG was established from 112 *H. pylori* negative subjects (data not shown). Sensitivity and specificity of the ELISA test was 90.38% and 97.32%, respectively.

Statistical Analysis

Data were expressed as mean ± SE. Correlation *Z* test was used to test for a correlation between mucosal and blood cytokine production. Differences in means among patient groups were analyzed by analysis of variance (ANOVA). All statistical analyses were performed using StatView 4.5 software program (Abacus Concepts, Berkeley, CA). Differences were considered significant when p was <0.05.

RESULTS

Subjects were divided into four groups according to *H. pylori* infection status and results of antibiotic treatment. There were 23 *H. pylori*-negative subjects, 20 *H. pylori*-positive subjects, nine subjects with successful *H. pylori* eradication (confirmed by histology or carbon-14. [^{14}C] breath test at 6–8 wk after eradication therapy), and 11 subjects with *H. pylori* eradication failure after antibiotic therapy. Details of diagnosis, antibiotic sensitivity, and therapeutic regimens in subjects with eradication failure are shown in Table 1.

Comparison of Blood and Mucosal IL-4 Response

To determine whether there was a correlation between blood and mucosal cytokine responses to *H. pylori* infection, levels of IL-4 production in whole blood cultures stimulated or not stimulated with *H. pylori* antigens were compared with levels in gastric mucosa cultures (Fig. 1; data from antigen stimulated cultures not shown). The results from *H. pylori*-positive (n = 6) and negative (n = 11) subjects, as well as subjects with failed eradication (n = 8) showed that IL-4 production in whole blood cultures (stimulated or not stimulated) correlated with that in gastric mucosa ($r^2 = 0.549$, $p < 0.001$).

Table 1. Clinical Characterization of Subjects With Failed Antibiotic Therapy—Including *Helicobacter pylori* Antibiotic Susceptibilities

Patient No.	Age (yr)	Diagnosis	Eradication Therapy Used	Times Failed	Symptom Duration (mo)	Antibiotic Susceptibility	
						Metronidazole	Clarithromycin
1	40	NUD	ABM	1	24		
						R	R
2	58	NUD	ACM + PPI	2			
			AC + PPI	1	12	R	R
3	55	Esophagitis/NUD	BMT	1	>36	–	–
			AC + PPI	1			
4	47	NUD	AC + PPI	2	20	–	–
5	37	NUD	M	1			
			AC + PPI	3	5	R	S
6	45	NUD	AC + PPI	3	28	–	–
7	27	NUD	AC + PPI	3			
			CMT + PPI	1	6	S	R
8	33	NUD, duodenal ulcer disease	BMT	2	>36	S	R
9	26	NUD	AC + PPI	2	10	S	S
10	47	NUD	AC + PPI	3	>36	R	R
11	73	Esophagitis, NUD, duodenal ulcer disease	AC + PPI	3	>36	S	R

A = amoxicillin; B = bismuth; C = clarithromycin; M = metronidazole; NUD = nonulcer dyspepsia; PPI = proton-pump inhibitor; R = resistant; S = sensitive; T = tetracycline; – = susceptibility testing not performed;

IL-4 and IFN-γ Production in Whole Blood Culture

Significantly lower levels of IL-4 were detected in whole blood stimulated or not stimulated with *H. pylori* AGE from subjects with eradication failure compared with subjects in whom *H. pylori* was successfully eradicated ($p < 0.05$, 0 and 1.0 μg/ml *H. pylori* AGE; $p < 0.01$, 10 μg/ml *H. pylori* AGE) or in subjects with untreated infection ($p < 0.05$, 10 μg/ml *H. pylori* AGE) (Fig. 2). IL-4 levels were similar in noninfected and infected subjects and were not significantly different when compared to those in subjects with successful eradication (although there was a trend toward increased levels after eradication). There was no difference in the levels of IFN-γ between the different groups, although lower levels were detected in subjects with successful *H. pylori* (Fig. 3). Low levels of IL-4 secretion were seen in most subjects with ongoing infection with *H. pylori* irrespective of the number of courses of therapy (Table 2).

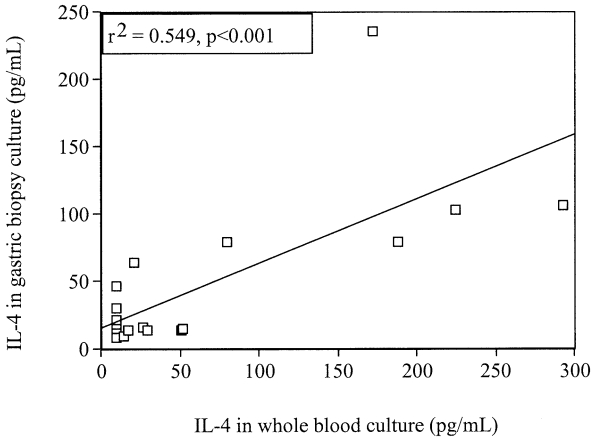

Figure 1. Correlation between IL-4 production in whole blood and gastric tissue cultures. Whole blood cultures or gastric antrum biopsy cultures were incubated for 24 h at 37°C, after which the levels of IL-4 were measured by ELISA capture assay. Results shown are a correlation between mucosal and whole blood IL-4 ($p < 0.001$).

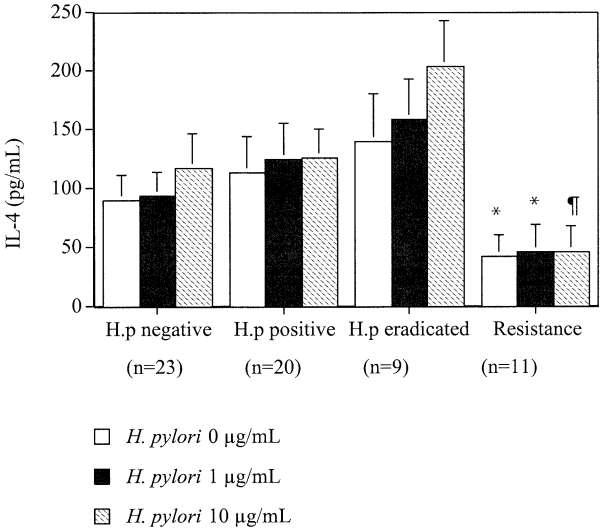

Figure 2. Levels of IL-4 in whole blood culture stimulated with *H. pylori* AGE antigen. Peripheral blood obtained from subjects with or without *H. pylori* infection or with eradication failure was added to an equal volume of AIM-V culture medium containing graded concentrations of *H. pylori* AGE antigen as indicated. After 24 h of culture, levels of IL-4 were measured by ELISA capture assay. Results shown are the mean ± SEM. *$p < 0.05$ compared with *H. pylori*–eradicated subjects; ¶$p < 0.01$ and $p < 0.05$ compared with values from subjects with *H. pylori* eradicated and *H. pylori* positive, respectively.

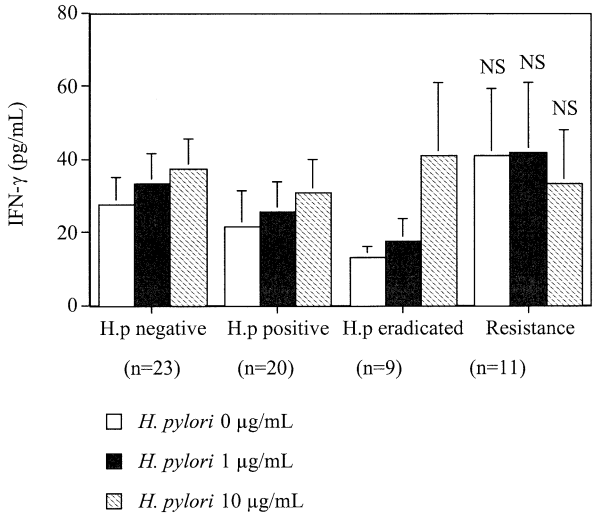

Figure 3. IFN-γ production in response to *H. pylori* acid-glycine extract stimulation in whole blood. Peripheral blood was collected from individual subjects and cultured in the presence of graded concentration of *H. pylori* AGE antigen for 24 h. Culture supernatants were collected and assayed for IFN-γ by ELISA. Results shown are mean ± SEM. NS = not significant.

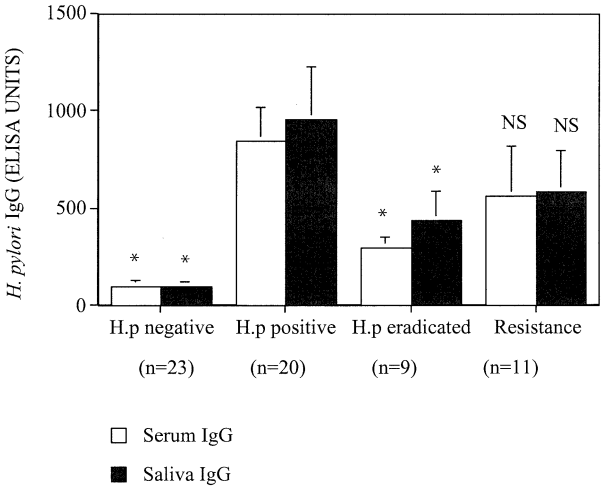

Figure 4. Levels of specific *H. pylori* IgG antibody in serum and saliva. Serum and saliva samples were collected from individual subjects. Levels of specific *H. pylori* IgG were measured by ELISA. Results shown are mean ± SEM. *$p < 0.05$ compared with mean from *H. pylori*-positive group. NS = not significant.

*Anti–*H. pylori *IgG Levels in Serum and Saliva*

Both serum and saliva IgG antibody levels were significantly lower in noninfected subjects ($p < 0.05$) and in subjects at 6–8 wk after eradication therapy ($p < 0.05$) than in subjects who were positive for *H. pylori*. For both saliva and serum antibody, a trend toward lower levels of antibody in those failing to eradicate infection was seen, but this did not reach statistical significance (Fig. 4).

DISCUSSION

This study demonstrated that subjects who have failed to respond to antibiotic eradication therapy for *H. pylori* have lower levels of IL-4 production in whole blood cultures when compared with patients with untreated *H. pylori* infection, and those with successful eradication of bacteria after antibiotic treatment. In contrast, there was no significant difference in levels of IFN-γ between the various groups. Serum and saliva anti–*H. pylori* antibody levels were lower in subjects failing to eradicate but this did not reach statistical significance.

We have shown in subjects with successful eradication that the cytokine profile shifted, with an increase in antigen-induced IL-4 production by circulating T cells, without change in the production of IFN-γ (14). Although the mechanism of this shift is uncertain, the increase in IL-4 may reflect a reduction in Th1 cytokines caused by a reduction in the bacterial load (16). The trend toward an increase in IL-4 in subjects who successfully eradicated infection in this study is consistent with our previous findings; the failure to reach significance in the current study probably reflects comparison on this occasion of different subject groups (as opposed to a longitudinal study of a homogeneous group). Thus, a primary mucosal defect might be a failure to switch to a Th0 (or balanced) cytokine response, which reflects optimal mucosal resistance (17). The sustained and similar levels of IFN-γ would be consistent with this view. Increased amount for mRNA of IL-4 was also reported in stomachs of mice with successful eradication of *H. pylori* infection compared with those that failed to eradicate after oral vaccination (18). These results are consistent with the view that a mucosal cytokine switch from Th1 to Th2 is linked to host protection, whereas a defective switch mechanism contributes to eradication failure (19, 20). Furthermore, low IL-4 levels were not transient, as they persisted irrespective of the number of antibiotic courses taken. The levels of IL-4 secreted in whole blood cultures correlated

Table 2. IL-4 and *Helicobacter pylori* Antibody IgG in Subjects With Failure Eradication

Times Failed	No. of Subjects	IL-4 Levels (pg/ml)*			*H. pylori* Antibody IgG	
		H. pylori Antigen (0 μg/ml)	*H. pylori* Antigen (1 μg/ml)	*H. pylori* Antigen (10 μg/ml)	Serum (ELISA Unit)*	Saliva (ELISA Unit)*
One	1	20.76	28.21	44.20	214	116.3
Two	4	40.49 ± 29.36	54.07 ± 43.14	65.22 ± 45.86	224 ± 101.58	1000.2 ± 866.5
Three	4	45.16 ± 36.16	53.34 ± 44.34	55.63 ± 44.19	410.95 ± 167.29	418.9 ± 151.96
Four	2	18.82 ± 9.82	22.56 ± 13.58	12.60 ± 3.6	1453.6 ± 1244.4	523.7 ± 235.3

* ± SEM.

with those from gastric mucosal biopsies in both spontaneous and stimulated cultures. The levels of IFN-γ in whole blood were similar in all groups, though there was a trend towards lower levels in those subjects successfully eradicating infection, suggesting a diminished response to a reduced antigen load. The sustained levels of IFN-γ in those failing to eradicate suggest IFN-γ alone has limited protection capabilities.

Although short of statistical significance, IgG anti–*H. pylori* antibody levels in both serum and saliva (a mucosal secretion) in subjects failing to eradicate infection were similar to those in subjects who had successfully eradicated infection and were lower than in untreated subjects. This, despite a continued antigen load through persistent infection in those with failed eradication therapy, suggests that defective specific immunity may also be a marker identifying subjects who are less likely to eradicate infection with standard antibiotic therapy. Further study is needed to ascertain whether low levels of specific antibody characterize a resistance-prone group, and whether these levels are of value in determining management strategy. These will be important studies, as the current database predominantly relates to spontaneous cytokine secretion, with little evidence of antigen-specific immune response. That may pertain to insensitive assay or to inappropriate endpoint measurement. One data point suggesting defective specific T cell immunity, however, is the significant difference between IL-4 secretion levels in untreated infected patients and those failing to respond to antibiotic therapy when blood T cells are stimulated with high antigen concentration. A link between impaired Th2 immunity and reduction in antibody levels is consistent with the view that Th2 cytokines are particularly relevant to humoral immunity (13, 21).

Clinical implications of these findings relate to the observation that some patients who lack an appropriate mucosal immune response may not effectively eradicate *H. pylori* with routine combination antibiotic therapy. Low IL-4 levels detected before commencement of therapy could identify subjects at risk for failing routine antibiotic therapy, but larger prospective studies are needed to provide sufficient data to validate this hypothesis. Subjects with low IL-4 should be carefully assessed before therapy to ensure that additional factors such as bacterial sensitivity are properly addressed. Although considerable data support the importance of antibiotic resistance to failed eradication, bacteria-related factors alone cannot explain the observed levels of failed eradication (10). These subjects could then be offered therapeutic regimens of antibiotic combinations and duration known to give maximal chance of eradication guided by sensitivity assessment, and could be counseled regarding the importance of compliance. The corollary is that higher levels of spontaneous IL-4 production would predict that less aggressive and shorter duration regimens will lead to successful eradication.

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