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Circulating T-Cell Response to *Helicobacter pylori* Infection in Chronic Gastritis

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ABSTRACT

Background. *Helicobacter pylori* elicits a specific humoral and cellular immune response. There is increasing evidence that the type of T-cell response contributes to clinical outcome in *H. pylori* infection.
Materials and Methods. The host response to *H. pylori* infection in 34 subjects with chronic gastritis was examined in terms of T-cell proliferation and cytokine production in whole-blood cultures stimulated or unstimulated with *H. pylori* acid-glycine extract antigens (AGE).
Results. The proliferative response in whole-blood cultures was similar for both *H. pylori*–positive and –negative subjects stimulated with *H. pylori* AGE. While an increase in interferon-γ (IFN-γ) production was observed from both *H. pylori*–positive and –negative subjects with gastritis, significantly higher levels of IFN-γ were detected in the former when stimulated with *H. pylori* AGE. In contrast, interleukin 4 (IL-4) was undetectable regardless of antigen stimulation. However, if an in situ IL-4 antibody capture assay was used, antigen-independent production of IL-4 was detected, but there was no difference between *H. pylori*–positive and –negative subjects with gastritis. After eradication of *H. pylori*, antigen-induced production of IL-4 was increased, with no decrease in the levels of secretion of IFN-γ. IL-4 production was dependent on CD4+ T cells, as addition of anti-CD4 but not anti-CD8 mouse monoclonal antibody or matched IgG isotype to the whole-blood culture inhibited the production of IL-4.
Conclusion. The results suggest that a shift toward a balanced Th1-Th2 response due to an increase in antigen-induced IL-4 production from CD4+ T cells follows eradication. We suggest that the downregulation of mucosal inflammation consequent on reduction in antigen levels or removal of downregulation after eradication of *H. pylori* contributes to this shift in cytokine balance.

Persistent colonization of the human gastric mucosa by *Helicobacter pylori* leads to chronic gastritis and, on occasions, cancer of the stomach [1,2]. The inflammatory process in *H. pylori*–associated gastritis is mediated largely through the release of cytokines from cells within the gastric mucosa [3–5]. For example, increase in interferon-γ (IFN-γ) but not interleukin 4–secreting T cells in the gastric mucosa has been reported in *H. pylori*–associated chronic gastritis [6]. *H. pylori*–specific CD4+ T-cell clones generated from gastric biopsy tissues can be capable of T-cell help for B-cell antibody production [7,8], and they secrete interleukin-4 (IL-4) and IFN-γ in response to antigen stimulation [9].

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Many studies of the pathogenesis of *H. pylori*–associated gastritis have been based on the analysis of T-cell proliferation and cytokine production in peripheral blood lymphocytes as indicators of the inflammatory response in infected gastric mucosa [10,11]. However, it has been shown that changes in the T-cell response in peripheral blood do not reflect those in the mucosa [11,12]. For instance, in some studies, both *H. pylori*–positive and –negative subjects have similar levels of T-cell proliferative response [12,13], whereas in others it is lower in *H. pylori*–positive subjects [3,14]. In other studies, T-cell proliferation is increased irrespective of disease, antibody, or infection status with *H. pylori*–associated gastritis when peripheral blood lymphocytes were stimulated with whole killed *H. pylori* bacteria [15]. Production of IFN-γ by peripheral blood mononuclear cells (PBMNC) stimulated with *H. pylori* is lower in *H. pylori*–positive subjects than in *H. pylori*–negative sub-

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jects [14,16], whereas IL-4 is lower in the latter [11].

Collectively, these various studies suggest experimental variation as a cause for the lack of correlation between T-cell response and infection. To address these issues, an analysis of T-cell proliferation and cytokine production in cultures stimulated with *H. pylori* antigens was performed using whole-blood cultures from *H. pylori*–positive and –negative subjects with chronic gastritis. These immune parameters were also evaluated in cultures of whole blood stimulated with *H. pylori* antigens in subjects who received eradication antibiotic therapy. The results of these studies identify reproducible and specific activation of circulating T cells that secrete different patterns of IL-4 and IFN-γ depending on the colonization status of the gastric mucosa.

Material and Methods

Study Subjects

Thirty-four subjects with dyspepsia (16 male and 18 female; mean age, 53.6 yrs; range, 27–81) and with a histological diagnosis of chronic gastritis according to the Sydney System [17] were studied, none of whom had taken nonsteroidal antiinflammatory drugs or antibiotics during the previous 3 months. Fourteen subjects with chronic gastritis infected with *H. pylori* were given eradication treatment with clarithromycin, amoxycillin, and omeprazole for 1 week. Thirteen of 14 subjects cleared *H. pylori* infection validated by histopathological examination and CLO test (Delta West, Pty, Ltd, Perth, Australia) at 6 weeks after therapy. Multiple biopsy specimens were obtained during upper gastrointestinal endoscopy from the antrum and the body for assessment of infection by histology and rapid urease test (CLOtest). Peripheral blood samples were drawn from each subject for antibody, cytokine, and cell proliferation assays. The study was approved by the Hunter Area Human Ethics Committee, and informed consent was obtained from all patients.

Preparation of H. pylori *Antigens*

H. pylori antigens from the NCTC 11637 strain were prepared by acid-glycine extraction (AGE) according to the method described by Goodwin et al. [18]. Briefly, a 2-day culture of *H. pylori* NCTC 11637 was collected and washed twice by sterile phosphate-buffered saline solution (PBS). The bacteria were then resuspended in PBS and mixed with 0.2 M acid glycine (pH 2.2) for 20 minutes on ice. The mixture was centrifuged at 11,000 × *g* for 20 minutes at 4°C. The supernatant was retained and was dialyzed against distilled water for 24 hours at 4°C. The preparation was sterilized by using 0.2 μm sterile filter (Sartorius, Göttingen, Germany), and protein concentrations in the extract were measured by using a Bio-Rad Kit (Bio-Rad Laboratories, Australia). The preparation was then aliquoted and stored at −70°C.

Lymphocyte Proliferation in Whole-Blood Culture

Whole blood was cultured in the presence or absence of *H. pylori* AGE using a modified method described by Petrovsky et al. [19]. Briefly, 150 μl of heparinized whole blood was added in triplicate to the wells of 96-well flat-bottomed microtiter plates loaded with 150 μl of AIM-V medium containing *H. pylori* AGE antigen at final concentrations of 0, 1, 10, and 50 μg/ml. After incubation at 37°C for various times, the cultures were pulsed with tritiated thymidine for the final 6 hours before harvesting and counting in a scintillation counter (Packard, Downers' Grove, IL). Thymidine incorporation was expressed as mean counts per minute (cpm).

IL-4 Production in Whole-Blood Culture by Antibody Capture Enzyme-Linked Immunosorbent Assay

Heparinized venous blood was collected (as described). After washing and blocking of the plates with 3% bovine serum albumin in PBS-tween 20, 150 μl of blood was pipetted into 96-well flat-bottomed plates coated or uncoated with capture polyclonal mouse antibody to IL-4 (Endogen, Woburn, MA) and loaded with 150 μl of AIM-V serum-free medium and antigen at various concentrations or medium alone. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. Then standard preparations of recombinant IL-4 (Endogen) were loaded into wells for 90 minutes. The plates were then washed, and biotinylated mouse monoclonal anti-IL-4 antibody (Endogen) was added in 0.5 μg/ml. After incubation, the wells were washed, and streptavidin-conjugated horseradish peroxidase (Selinus, Australia) were applied at 1:400 dilutions. The plates were washed, and then tetramethyl benzidine (TMB, Sigma-Aldrich, Castle Hill, NSW, Australia) substrate was added to each well. The absorbance was read at 450 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad 450, Japan). The limit of sensitivity for IL-4 was 9.4 pg/ml. The amount of IL-4 in samples were determined using a Softmax program (Version 2.3 FPU, USA).

IFN-γ ELISA Assays

Wells of a 96-well flat-bottomed microtiter plate (Nunc, Roskilde, Denmark) were coated with mouse anti-IFN-γ monoclonal antibody at 2 μg/ml overnight at 4°C. After washing and blocking, plasma supernatants of whole blood culture and IFN-γ standards (Endogen) were added in duplicate and incubated for 90 minutes. The plates were washed, and biotinylated mouse monoclonal anti-IFN-γ antibody (Endogen) was added (0.25 μg/ml). After incubation, the wells were washed, and streptavidin-conjugated horseradish peroxidase (Selinus) was applied at a 1:2000 dilution. The plates were washed, and then tetramethyl benzidine (TMB, Sigma-Aldrich) substrate was added to each well. The absorbance was read at 450 nm in an ELISA plate reader (Bio-Rad 450, Japan). 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).

Whole-Blood Culture with Mouse Anti-CD4 Antibody

To study the effect of anti-CD4 antibody on cytokine production, a separate experiment was conducted with five *H. pylori*–positive subjects with gastritis. Whole-blood cultures were set up in the presence of *H. pylori* AGE antigen at 10 μg/ml with or without mouse anti-CD4 antibody (mouse IgG1, Dako, Glostrup, Denmark) at 10 μg/ml. Anti-CD8 (mouse IgG1, Dako) and isotype mouse IgG1 (Dako) were used as control antibodies. After 24 hours' incubation, levels of IL-4 were measured (as mentioned).

Statistical Analysis

Data were expressed as mean plus or minus the standard error of the mean. Analysis of variance paired *t*-test and unpaired *t*-test were used to compare the data between the patient groups, using a StatView 4.5 program (Abacus Concepts, Berkeley, CA).

Results

Subjects were selected and classified as being *H. pylori*–positive or –negative after endoscopy, histology, and urease testing. Of 34 patients, 19 were positive and 15 were negative for *H. pylori* infection. Assays for antibody to *H. pylori* gave results consistent with this classification. Histological analysis of gastritis was based on the Sydney System [17].

Proliferative and Cytokine Responses to H. pylori *in Whole-Blood Culture from Chronic Gastritis Subjects*

To determine dose-response curves for T-cell proliferative and cytokine responses, whole-blood cultures from chronic gastritis subjects with or without *H. pylori* infection were studied. A dose-dependent increase in thymidine uptake was observed irrespective of infection status, with no significant difference between the *H. pylori*–positive and –negative groups (10 subjects per group; Fig. 1A). A similar pattern was observed with separated blood mononuclear cells (data not shown). In both *H. pylori*–positive and *H. pylori*–negative subjects (six per group), increase in IFN-γ production was detected in culture supernatant in response to antigen stimulation in a dose-related manner (see Fig. 1B). While significantly higher levels of IFN-γ

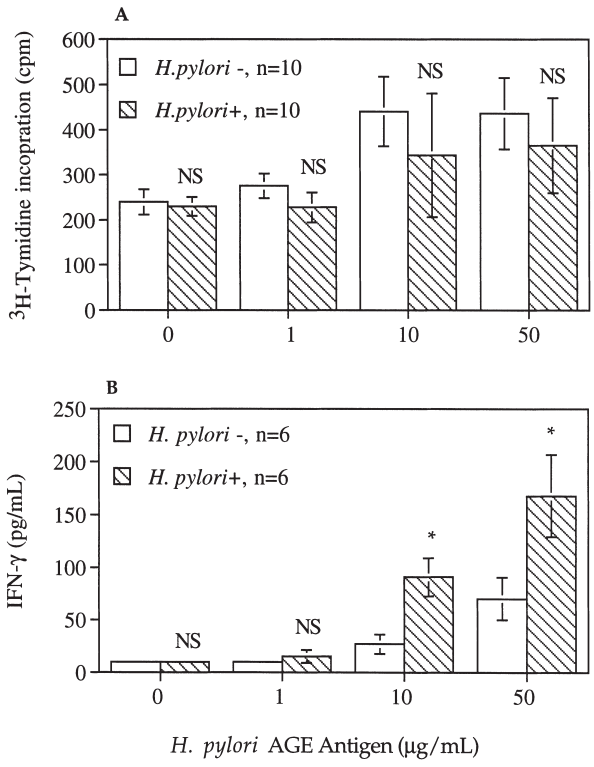

Figure 1 T-cell proliferation and cytokine production in whole-blood culture stimulated with *H. pylori*. Peripheral blood from chronic gastritis subjects with or without infection was diluted with equal volumes of AIM-V medium in the absence or presence of graded doses of *H. pylori* acid-glycine extract. After incubation for 3 days, thymidine incorporation was determined by pulsing the cultures for the final 6 hours before harvesting and counting (*A*). The culture supernatants were determined for interleukin-4 and interferon-γ by enzyme-linked immunosorbent assay (*B*). Results shown are the mean ± standard error of the mean. *$p < .05$ compared with values from *H. pylori*–negative subjects. NS = not significant.

were detected in supernatants of whole-blood cultures stimulated with *H. pylori* AGE at 10 and 50 μg/ml from *H. pylori*–positive subjects than from *H. pylori*–negative subjects, IL-4 was undetectable.

IL-4 and IFN-γ Production in Whole-Blood Culture Stimulated with H. pylori

As IL-4 was undetectable in the culture supernatant, its production was examined using a capture IL-4 assay in whole-blood cultures stimulated or unstimulated with *H. pylori* AGE in 14 *H. pylori*–positive and 15 *H. pylori*–negative subjects with chronic gastritis as determined by histological analysis using the Sydney System. After incubation for 24 hours, significant levels of IL-4 were detected in both unstimulated and stimulated cultures of whole blood from subjects with chronic gastritis irrespective of their infection status (Fig. 2). While a capture assay in whole-blood culture was essential for IL-4 detection, it was not necessary for IFN-γ, which was readily detectable. Increase in IFN-γ production was detected in an antigen dose-dependent manner in cultures from both *H. pylori*–positive and *H. pylori*–negative subjects, but significantly higher levels were detected in the former, indicating that an antigen-dependent increase in the production of IFN-γ is associated with *H. pylori* infection. By contrast, similar amounts of IL-4 were produced irrespective of antigen presence or concentration.

IL-4 and IFN-γ Production in Whole-Blood Culture After Eradication of H. pylori

To determine whether IL-4 production from circulating T cells is linked to *H. pylori* infection in the gastric mucosa, Whole-blood cultures from 13 *H. pylori*–infected subjects were stimulated with *H. pylori* before and 6 weeks after completion of eradication therapy. As shown in Figure 3, an antigen-dependent increase in IL-4 production was observed in whole-blood cultures stimulated with *H. pylori* after eradication therapy ($p < .05$). By contrast, there was no significant difference in the production of IFN-γ before or after eradication.

Effect of Anti-CD4 Antibody on IL-4 Production

To identify the source of IL-4, antigen-induced production of IL-4 in the presence or absence of monoclonal mouse anti-CD4 antibody in whole-blood cultures from five chronic gastritis subjects with *H. pylori* infection was examined. As shown in Figure 4, increase in IL-4 production was dependent on antigen stimulation of CD4+ T cells in whole blood, since the addition of anti-CD4 mouse monoclonal antibody, but not matched IgG isotype or anti-CD8+ mouse monoclonal antibody to whole-blood cultures, resulted in the inhibition of IL-4 production.

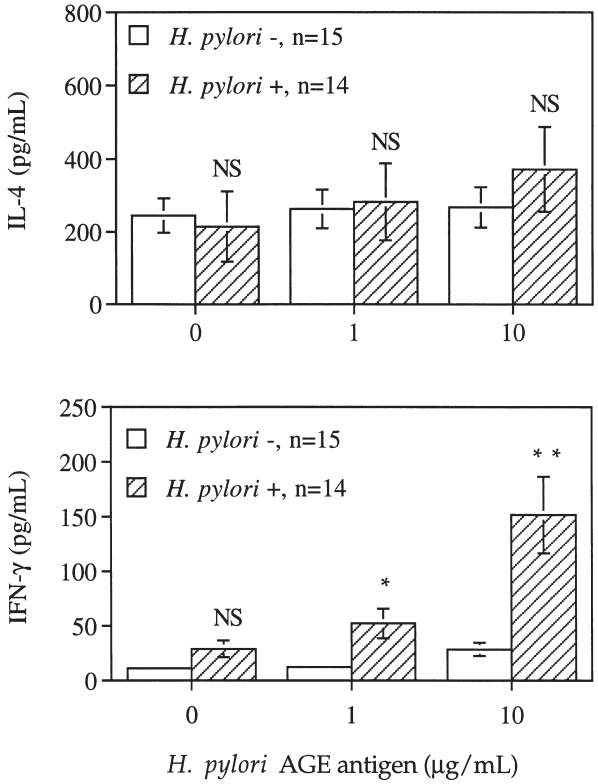

Figure 2 Interleukin 4 and interferon-γ production in response to *H. pylori* acid-glycine extract stimulation in whole-blood cultures. Peripheral blood obtained from chronic gastritis subjects with infection (n = 14) or without infection (n = 15) was added to equal volumes of AIM-V medium containing 0, 1, or 10 μg/ml of *H. pylori* acid-glycine extract. After 24 hours' incubation, the plasma supernatants were collected and assayed for interferon-γ and enzyme-linked immunosorbent assay and interleukin 4 was measured by solid-phase enzyme-linked immunosorbent assay capture. Results shown are the mean ± standard error of the mean. *$p < .05$; **$p < .01$ compared with values from *H. pylori*–negative subjects. NS = not significant.

Discussion

Our study in subjects with chronic gastritis demonstrated that the production of IL-4 and IFN-γ by circulating antigen-reactive CD4+ T cells in whole-blood assays is associated with the gastric mucosal infection with *H. pylori*. No difference in the proliferative response to an antigen extract was detected between *H. pylori*–positive and *H. pylori*–negative subjects with chronic gastritis. Higher levels of IFN-γ production, however, were detected in *H. pylori*–positive subjects with gastritis,

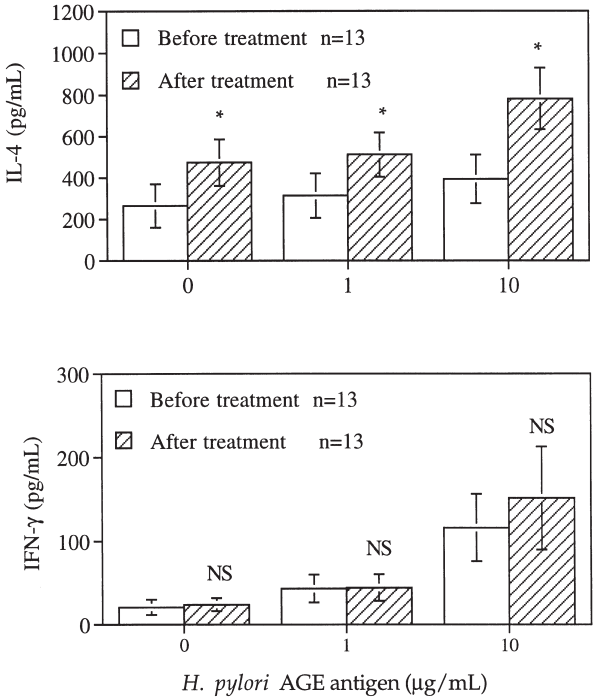

Figure 3 Effects of eradication therapy on interleukin 4 and interferon-γ production in whole-blood cultures stimulated with *H. pylori* acid-glycine extract. Peripheral blood obtained from 13 chronic gastritis subjects with *H. pylori* infection before and 6 weeks after eradication therapy was stimulated with or without *H. pylori* acid-glycine extract in culture with AIM-V medium for 24 hours, after which interferon-γ production was measured in plasma supernatant by enzyme-linked immunosorbent assay and interleukin 4 production was measured by solid-phase enzyme-linked immunosorbent assay capture. Results shown are the mean ± standard error of the mean. *$p < .05$ compared with values from subjects before eradication therapy. NS = not significant.

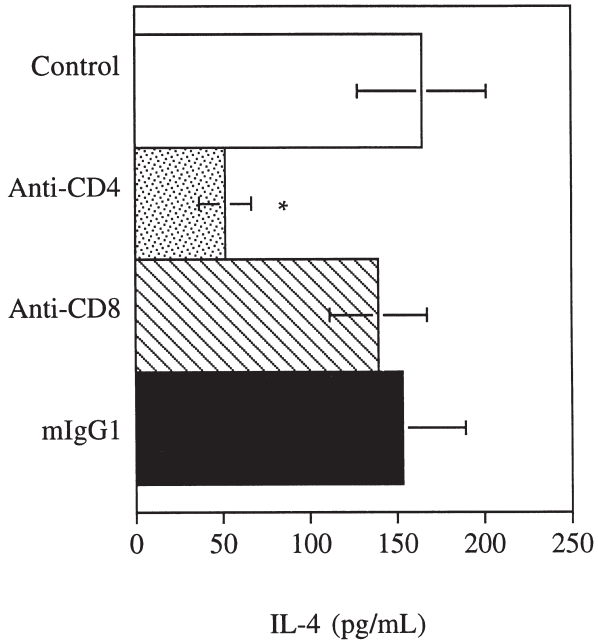

Figure 4 Inhibition of interleukin 4 production by anti-CD4 antibody in whole-blood cultures stimulated with *H. pylori* acid-glycine. Peripheral blood obtained from 5 chronic gastritis subjects with *H. pylori* infection was stimulated with equal volumes of AIM-V medium containing *H. pylori* acid-glycine extract (10 μg/ml) with or without 10 μg/ml of mouse monoclonal anti-CD4, anti-CD8, or mouse IgG1. After 24 hours, interleukin 4 production was determined by solid-phase enzyme-linked immunosorbent capture assay. Results shown are the mean ± standard error of the mean. *$p < .05$ compared with values obtained from control cultures.

whereas IL-4 secretion was neither specific for *H. pylori* infection nor dependent on antigen stimulation. After eradication of *H. pylori*, a selective and significant increase in IL-4 secretion was detected.

Many studies have reported that peripheral blood T-cell proliferation responses to *H. pylori* antigen do not correlate with infection, antibody, or the pattern of disease despite the isolation of antigen-specific T-cell clones with distinct cytokine secretion patterns from the gastric mucosa of *H. pylori*–infected subjects [12,14]. The proliferative response to *H. pylori*–soluble antigens in infected subjects has been reported as either similar or less than that found in seronegative subjects [12]. The results described in our study using whole-blood culture is consistent with these observations. The failure to show a difference in proliferation was not considered to be due to suppression by bacterial factors, as the blastogenic response measured was similar when stimulated by formalin-killed whole bacteria and was unrelated to the pathology or the serological status [10,16]. These observations, however, may reflect direct inhibition by components in the *H. pylori* AGE antigen preparation. Indeed, sonicates or acid extracts of *H. pylori* have been reported to suppress mitogen-stimulated cultures of normal PBMNC [10,14], and low mitogenic responses are described in *H. pylori*–positive and –negative lymphocyte cultures. Similar levels of IL-2 secretion have been reported in both populations [16,20]. Secretion of an antigen-dependent suppressive factor stimulated by one or more antigens in the *H. pylori* AGE antigen extract is an alternative explanation of these findings [19].

A lack of correlation between infection and cytokine production after antigen stimulation has been reported in *H. pylori* infection [21]. In one study, PBMNC from infected subjects secreted less IFN-γ in the presence of *H. pylori* than did nega-

tive subjects [12]. However, in our study, higher levels of IFN-γ were found in whole-blood culture with IL-4 undetectable in culture supernatants. This dominant IFN-γ response has been reported for mucosal cell preparations [6,22] and supports the view that the host response is a polarized Th1 response [9,23]. However, many T-cell clones derived from the mucosa of subjects infected with *H. pylori* secrete IL-4 [8,9], and some studies of PBMNC identify IL-4 production from both *H. pylori*–positive and –negative subjects [11]. PBMNC failed to secrete detectable IL-4 unless an antibody capture assay developed for use with whole-blood cultures was used (data not shown). It is concluded that in subjects with uncomplicated chronic gastritis, the dominant T-cell response to *H. pylori*, both at the level of gastric mucosa [9] and in circulating cells, is a Th1 response. The instability of IL-4 in culture without a capture assay is responsible for the variations in levels found in culture; stabilization of IL-4 in the capture assay enables detection of levels that reflect in vivo conditions [24,25]. Levels of IL-4 were higher in *H. pylori*–positive subjects than in *H. pylori*–negative control subjects but were independent of added antigen.

The balance of the cytokine profile shifted after eradication of *H. pylori*. This change was characterized by a selective increase in antigen-induced IL-4. A reduction in the production of the proinflammatory cytokines IL-1, IL-8, and tumor necrosis factor–alpha in the gastric mucosa after eradication of *H. pylori* has also been described [3,26], reflecting downregulation of mucosal inflammation when antigen is removed. In our study, IL-4 production was inhibited by anti-CD4 monoclonal antibody, indicating that T helper cells are the source of IL-4, with antigen presentation likely involving major histocompatibility complex class II expression on antigen-presenting cells. The increase in IL-4 production may in part be due to a reduction in mucosal Th1 cytokines after clearance of *H. pylori* [3,26] and thus may contribute to the downregulation of inflammation. This balanced Th1-Th2 (or Th0) cytokine response may also reflect more effective protection; studies are in place to examine this hypothesis directly by study of failed eradication therapy.

These studies document patterns of cytokine production in circulating T cells in uncomplicated chronic gastritis caused by *H. pylori* infection and the impact on these patterns after eradication of *H. pylori* infection. Ongoing studies are directed at direct correlation of results from whole-blood cultures with mucosal cytokine production. Successful correlation will prove of considerable value in monitoring the cellular response to mucosal infection, in studying the natural history of the host-parasite relationship and the development of optimal parameters of protection, and in the study of complicated gastritis. Subsequent study of vaccine candidate antigens in humans would be facilitated by a whole-blood assay.

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