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GASTRIC CARCINOMA: T-CELL RESPONSE AND VASCULARITY

Shift of the gastric T-cell response in gastric carcinoma

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Abstract

Background and Aims: The etiology and pathophysiology of stomach carcinoma is complex, and the mechanism whereby *H. pylori* directly or indirectly induces carcinoma remains unclear. In this study, interleukin (IL)-8, IL-4 and interferon (IFN)-γ were measured in the tissue culture supernatant of gastric organ cultures from subjects with chronic gastritis with or without *H. pylori* infection, and with or without gastric cancer and gastric dysplasia.

Results: Interleukin-8 levels were higher in cancer- and *H. pylori*-infected gastritis subjects than in *H. pylori*-negative subjects (12.95 ± 3.16, 10.48 ± 1.55 and 4.49 ± 1.28 ng/mL, respectively). Elevated levels of IFN-γ were detected in both *H. pylori*-infected and non-infected subjects with uncomplicated gastritis (72.23 ± 19.0 and 34.61 ± 5.30 pg/mL) and in non-infected dysplasia subjects (88 ± 20.5 pg/mL). Background levels of IL-4 (≤ 9.4 pg/mL) in uncomplicated gastritis subjects and relatively high levels of IL-4 in dysplasia subjects (25.8 ± 7.3 pg/mL) were detected. In contrast, trace amounts of IFN-γ (16.01 ± 0.35 pg/mL) and high levels of IL-4 (42.81 ± 8.49 pg/mL) in gastric biopsy culture supernatants were found in cancer subjects. Mucosal IL-4 levels (but not IL-8 levels) correlated with infection and mucosal anti-*H. pylori* immunoglobulin G antibody.

Conclusions: The significant differences between gastritis with and without cancer and dysplasia indicated a shift from a Th1 to a Th2 helper cell pattern of cytokine secretion. This study has identified a local mucosal defect in gastric cancer. The near absence of IFN-γ production from the mucosa at the margins of the tumor may be a critical factor in promoting growth of neoplastic cells.

Key words: cytokines, gastric carcinoma, gastric dysplasia, *Helicobacter pylori*.

INTRODUCTION

The strength of epidemiological data supporting an association between *Helicobacter pylori* (*H. pylori*) infection and gastric carcinoma, supported by morphological analysis of the gastritis-precancer-cancer sequence,[1,2] led to the bacterium being classified as a class 1 carcinogen by the International Agency for Research on Cancer.[3] The mechanism whereby *H. pylori* contributes to gastric carcinogenesis is, however, unclear. Genetic, bacterial and host factors may contribute, although genetic abnormalities have generally behaved independently of colonization characteristics.[4–6] The observation of epithelial damage characterized by enhanced proliferation and cytokine secretion without a parallel increase in apoptosis[7,8] suggests that characteristics of the host response to infection may contribute at an early stage of carcinogenesis.[6] Interest

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in clinical disease representing a particular outcome of the host–parasite relationship has focused attention on the mucosal immune response to *H. pylori*. Mucosal T lymphocytes are strongly polarized towards the expression of 'Th1 cytokines' (such as interferon (IFN)-γ) in subjects with peptic ulcer disease,[9–11] whereas a more balanced cytokine profile (the Th0 helper cell phenotype, with both Th1 and Th2 cytokines) has been described in subjects with uncomplicated gastritis.[12] These observations led to the view that pro-inflammatory Th1 cytokines promote mucosal damage, whereas a shift towards a Th2 response, with promotion of a mucosal antibody response, would favor protection, with a clearance of infection.[10] This study of the cytokine secretion profile within the gastric mucosa from subjects with gastric dysplasia and gastric carcinoma identifies a distortion of the cytokine profiles previously described in uncomplicated *H. pylori*-infected mucosa, which further supports the argument that host factors contribute to carcinogenesis and/or tumor growth.

METHODS

Subjects

Fifty-five patients with dyspepsia investigated by the use of endoscopy were studied. Eighteen subjects (mean 56.9 years, range 27–77 years, 12 males and six females) were confirmed as having gastric cancer by the use of histopathology. Twenty-three (mean 52.0 years, range 23–88 years, 10 males and 13 females) were diagnosed as having chronic gastritis by using histopathology. No patient took non-steroidal anti-inflammatory drugs at the time of endoscopy, nor had any taken antibiotics during the 3 months prior to the study. Fourteen subjects with gastric dysplasia (mean 61.6 years, range 40–83 years, seven males and seven females) had been in a surveillance program following the eradication of *H. pylori* for 24–48 months prior to the study. All but one of this latter group were *H. pylori* negative at the time of study. The present study was approved by the Hunter Area Human Ethics Committee and informed consent was obtained from all patients. Multiple biopsy specimens were obtained during upper gastrointestinal endoscopy from the antrum and the body of stomach for *in vitro* culture, histology, rapid urease test (CLO test; Delta West, Bentley, WA, Australia) and PCR. Biopsies from subjects with cancer were taken from the mucosa adjoining the carcinoma. Cancer patients attended the outpatient department of the First Teaching Hospital of Shanxi Medical University, People's Republic of China. All other subjects were studied within Australia. A heparinized venous blood sample was drawn from each patient for serologic testing. Sera were aliquoted and stored at −70°C until assayed.

In vitro culture

Biopsy specimens were collected at endoscopy and placed immediately in AIM-V medium (Life Technology, Melbourne, Vic., Australia). The tissues were weighed and then cultured at a ratio of 50 μL serum-free AIM-V medium per mg tissue (wet weight) for 24 h. The culture supernatants were collected and spun briefly (10 s, 5600 *g* at room temperature). Aliquots were stored at −70°C until assayed.

Histology

The presence and severity of gastritis was assessed by using formalin-fixed biopsy tissues stained with hematoxylin and eosin. Gastritis was assessed according to the criteria of The Sydney System[13] and active gastritis was graded according to the presence of intraepithelial neutrophils. Gastric carcinoma was classified as 'intestinal' ($n=13$) or 'diffuse' ($n=5$) type, according to Lauren's classification.[14] *Helicobacter pylori* was identified in tissue sections stained with Giemsa. Gastric dysplasia was diagnosed in the presence of dysplastic epithelial cells.

Antibody detection by ELISA

Helicobacter pylori antigens were isolated from the NCTC 11637 strain according to the method described by Goodwin *et al.*[15] The protein concentration in the extract was measured by using a Bio-Rad Kit (Bio-Rad Laboratories, Regents Park, NSW, Australia).

For antibody detection, wells of a 96-well flat-bottomed microtiter Polysorb plate (Nunc, Roskilde, Denmark) were coated with 7 μg/mL *H. pylori* antigen at 4 °C overnight. After washing and blocking the plates, serum samples at 1:1000 dilution for immunoglobulin (Ig)G detection and 1:50 dilution for IgA were then added to individual wells in triplicate. Culture supernatants were assayed at a 1:20 dilution for both isotype antibodies. After incubation, the wells were washed and horseradish peroxidase conjugated-sheep anti-IgG or IgA (Silenus, Boronia, Vic., Australia) at 1:2000 dilution was added to each well. Following a further incubation, the plates were washed and tetramethyl benzidine (TMB) substrate (Sigma, Castle Hill, NSW, Australia) was then added to each well. The reaction was stopped by using 1 mol/L H_2SO_4 and the absorbance was read at 450 nm in an ELISA plate reader (Bio-Rad, 450, Richmond, CA, USA). The results were expressed as an ELISA INDEX, which was the mean OD_{450} of a given serum divided by the mean OD_{450} of the calibrating sera. Positive and negative quality control sera were included in each run to control for intra- and interassay variation.

Cytokine assay of tissue culture supernatants

Interleukin-8 concentrations in culture supernatant were measured by using ELISA kits (Biotrak, Amersham, UK). The sensitivity of this assay was

31.3 pg/mL. Interleukin-4 and IFN-γ concentrations in culture supernatant were measured by using paired antibodies (Endogen, Woburn, MA, USA). The sensitivity was 9.4 pg/mL for IL-4 and 15.6 pg/mL for IFN-γ. Briefly, the plates were coated with 2 μg/mL of mouse antihuman IL-4 or IFN-γ in sodium bicarbonate buffer (pH 9.6) at 4 °C overnight. After washing and blocking the plates with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)-tween20, 100 μL tissue culture supernatant samples were then added to individual wells in duplicate. After a 90 min incubation, the wells were washed and biotinylated mouse antihuman IL-4 or IFN-γ antibodies were added (at a concentration of 0.5 μg/mL for IL-4 and 0.25 μg/mL for IFN-γ). Plates were washed after the 90 min incubation, and horseradish peroxidase-conjugated streptavidin (Silenus) was added to each well (at 1:400 dilution for IL-4 and 1:2000 dilution for IFN-γ). Following a further incubation, the plates were washed and then TMB substrate (Sigma) was added to each well. The reaction was stopped by using 1 mol/L H_2SO_4 and the absorbance was read at 450 nm in an ELISA plate reader. Recombinant IL-4 and IFN-γ were used as standards for each plate. The amount of IL-4 and IFN-γ was calculated by using SOFTMAX software (Version 2.3, FPU, Cupertino, CA, USA).

Polymerase chain reaction amplification for *Helicobacter pylori* detection and the *cagA* strain

Biopsy tissues were ground by the use of a sterile disposable pestle, and total DNA was extracted by using a DNA purification Kit (Promega, Madison, WI, USA). DNA was then dissolved in 100 μL DNA rehydration solution. Sense primer 5′-ATAATGCTAAATTAGA CAACTTGAGCGA-3′ and the antisense primer 5′-TTAGAATAATCAACAAACATCACGCCAT-3′ (298 bp) for *cagA*, and sense primer 5′-TGGCGTGTC TATTGACAGCGAGC-3′ and antisense primer 5′-CCTGCTGGGCATACTTCACCATG-3′ (298 bp) for a 26 kDa protein encoding gene were synthesized (Brestec, Adelaide, SA, Australia) according to published sequences of *H. pylori*.[16,17] For the PCR assay, the template DNA was added to 50 μL of a reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L each deoxynucleotide (Promega) and 1 μmol/L of each oligonucleotide primer. The Taq DNA polymerase (1 U; Promega) was added and the reaction mixture was overlaid with 50 μL of mineral oil to prevent evaporation. Both known positive and negative controls were included for each assay. The reaction was initiated with a thermal cycler (Hybaid, Ltd., Twickenham, UK). The amplification cycle consisted of an initial denaturation of target DNA at 94°C for 5 min followed by a denaturation at 94°C for 1 min; annealing at 60°C for *cagA* and 55°C for 26 kDa for 1 min and an extension at 72°C for 1 min. The final cycle included extension for 5 min at 72°C to ensure the full extension of the products. After 38 cycles of amplification, the samples were analyzed using ethidium bromide-agarose gel electrophoresis and the PCR products were visualized under a UV transilluminator (Carson, CA, USA).

Statistical analysis

Data are expressed as mean ± SEM. Differences between the means were analyzed with a two-tailed Mann–Whitney *U*-test. The non-parametric Spearman's rank correlation was used to test for a correlation between cytokine production and *H. pylori* infection and mucosal IgG antibody measurements. *P* values of < 0.05 were considered significant. All calculations were performed by using a statistical software program (GRAPHPAD PRISM Version 2; GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Helicobacter pylori infection in patients with gastric cancer and gastritis

Two groups (*H. pylori* positive and negative) were defined according to the Campylobacter-like organism (CLO) urease test and histology (Table 1). Of the 18 subjects with adenocarcinoma of the stomach, 13 were of the intestinal type and five were of the diffuse type. There were 11 subjects with a lesion in the antrum, and seven with a corpus lesion. Nine subjects (seven intestinal-type and two diffuse-type) tested positive for *H.*

Table 1 Patient characteristics

	Gastric cancer		Chronic gastritis		Dysplasia*
	Helicobacter pylori status				
	Positive	Negative	Positive	Negative	
No.	9	9	12	11	14
Age (range)	52.2 (27–61)	61.7 (40–77)	58.6 (31–88)	44.9 (23–73)	61.6 (40–83)
Sex (M/F)	6/3	6/3	5/7	5/6	7/7
26 kDa gene	9	0	12	0	1
cag A gene	7	0	2	0	0

* One patient in this group was *H. pylori* positive confirmed by CLO urease test and Giemsa stain.

pylori infection. Seven of these were positive for the *cagA* gene. Fifteen of the 18 subjects with carcinoma had nests of tumor cells included in a biopsy of the mucosa adjoining the tumor, although the predominant histology was that of chronic gastritis. In the gastritis group, 12 subjects tested positive for *H. pylori*. Two of these tested positive for the *cagA* gene. None of those subjects that were negative for *H. pylori* in either group of subjects on standard diagnostic criteria were positive for the 26 kDa gene. The 14 subjects with gastric dysplasia had been infected with *H. pylori* 24–48 months prior to the present study and all had been given antibiotic therapy. This group was in a long-term follow-up program because dysplasia was judged to be premalignant. Endoscopic and histological examination at the time of the present study confirmed chronic gastritis with dysplasia, but without evidence of cancer. All patients except one were negative for *H. pylori* in urease testing and histological examination.

Serum antibody IgG and IgA antibody to *Helicobacter pylori*

Serum specific IgG antibody levels were significantly higher in *H. pylori*-positive subjects than in -negative subjects with both cancer and gastritis, respectively ($P<0.05$ and $P<0.01$). Immunoglobulin G antibody levels were higher in gastritis subjects than in cancer subjects, but this difference was not statistically significant ($P=0.11$). Similar results were obtained for serum IgA antibody, with higher levels in *H. pylori*-positive subjects than in -negative subjects ($P<0.05$ in both gastritis and cancer subjects). Serum IgG and IgA levels in subjects with dysplasia were similar to those in gastritis patients without infection of *H. pylori* (Fig. 1).

Production of IgG and IgA antibody to *Helicobacter pylori* in organ culture

To determine local *H. pylori* antibody production, gastric biopsy tissues were cultured in AIM-V medium for 24 h, after which time the supernatants were assayed for IgG and IgA antibodies. As shown in Fig. 2, both IgG and IgA antibodies were higher in *H. pylori*-positive subjects with gastritis ($P<0.001$ for IgG and IgA) or cancer ($P<0.01$ for IgG; $P<0.05$ for IgA) than in *H. pylori*-negative subjects. Although higher IgA levels were observed in gastritis subjects than in cancer subjects, this difference did not reach statistical signifi-

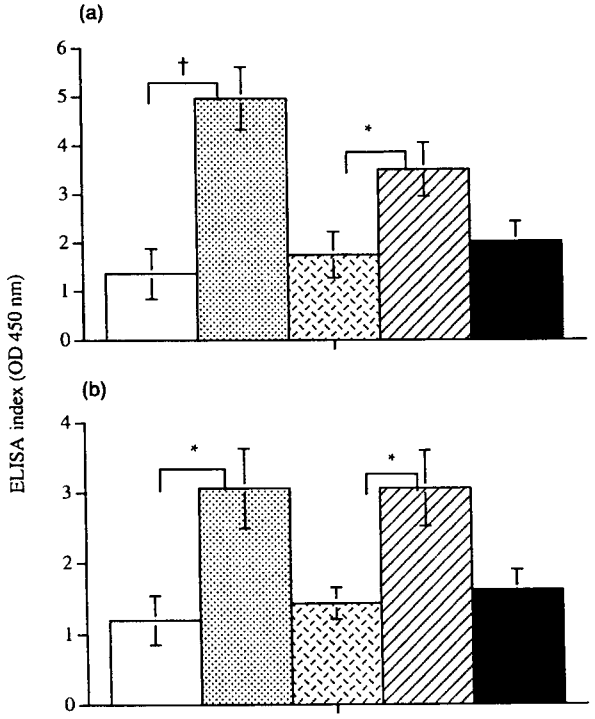

Figure 1 Serum *Helicobacter pylori*-specific (a) IgG and (b) IgA antibody in subjects with (⊡) chronic gastritis ($n=12$), (▨) gastric cancer ($n=9$) and (■) dysplasia ($n=14$). Results were compared with *H. pylori*-negative subjects with (□) gastritis ($n=11$) and (▨) with cancer ($n=9$). OD, optical density. Results shown are presented as mean ± SEM. *$P<0.05$; †$P<0.01$.

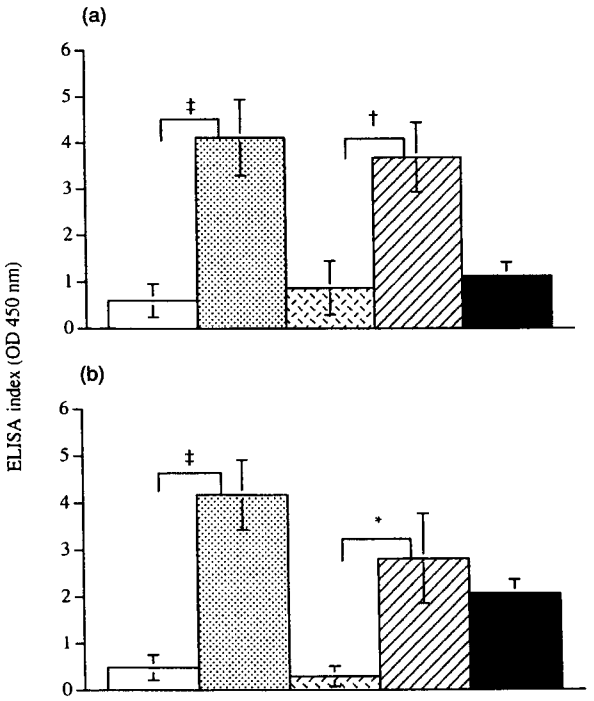

Figure 2 Mucosal *Helicobacter pylori*-specific (a) IgG and (b) IgA antibody in subjects with (⊡) chronic gastritis ($n=12$), (▨) gastric cancer ($n=9$) and (■) dysplasia ($n=14$). Results were compared with *H. pylori*-negative subjects with (□) gastritis ($n=11$) and (▨) with cancer ($n=9$). OD, optical density. Results shown are presented as mean ± SEM. *$P<0.05$; †$P<0.01$; ‡$P<0.001$.

cance. Mucosal IgA anti *H. pylori* antibody (but not mucosal IgG) levels remained at relatively high levels in most of the dysplasia group.

IL-4, IL-8 and IFN-γ production in organ cultures

Interleukin-4 production by gastric mucosal tissue was significantly higher in 18 cancer subjects compared with subjects with gastritis (irrespective of infection status) ($P<0.05$). None of the uncomplicated gastritis subjects had a level of IL-4 detected above the sensitivity of the assay (9.4 pg/mL), whereas only three of 18 cancer subjects had undetectable levels. Interleukin-4 levels in the dysplasia group were intermediate between the gastritis and cancer group; five of 14 had undetectable levels. In contrast, significantly higher levels of IFN-γ were detected in both *H. pylori*-positive ($P<0.01$) and -negative gastritis subjects ($P<0.01$) and dysplasia subjects ($P<0.01$) compared with cancer subjects (Fig. 3). While IFN-γ production was higher in *H. pylori*-positive subjects than in -negative subjects with gastritis, this difference was not statistically significant. Interleukin-8 levels were higher in both cancer and gastritis subjects who were positive for *H. pylori*. Levels of IL-8 in dysplasia subjects were similar to those in chronic gastritis subjects without *H. pylori*. There was a positive correlation between IL-4 ($r=0.615$, $P<0.05$) and mucosal *H. pylori* antibodies in cancer subjects. The IL-4 levels also correlated with *H. pylori* infection status ($P<0.05$). Interleukin-8 levels did not correlate with *H. pylori* antibody status in the gastric cancer group.

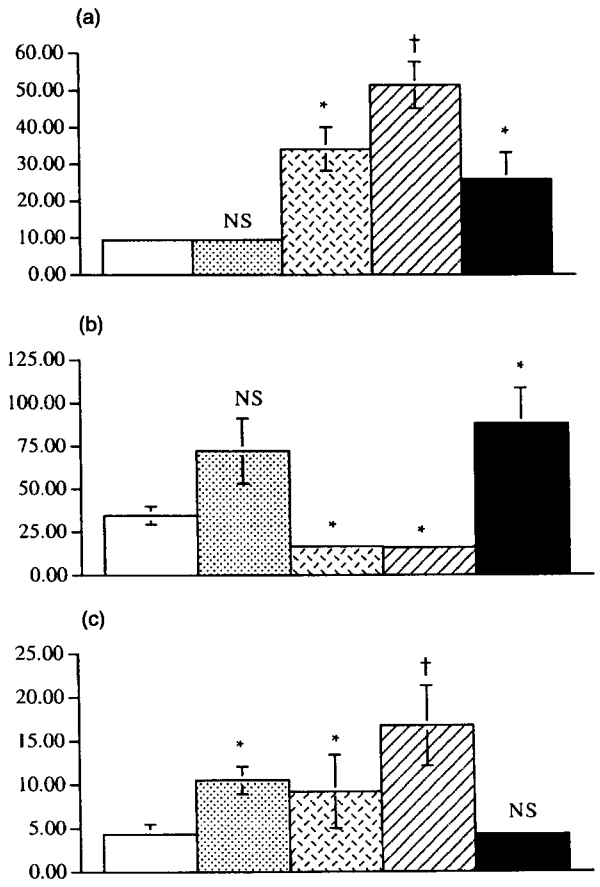

Figure 3 Mucosal (a) IL-4 (pg/mL), (b) IFN-γ (pg/mL) and (c) IL-8 production (ng/mL) in subjects with (⊡) *Helicobacter pylori* positive-chronic gastritis ($n=12$), (◱) *H. pylori* negative-gastric cancer ($n=9$) (▨) *H. pylori* positive-gastric cancer ($n=9$) and (■) dysplasia ($n=14$). Results were compared with (□) *H. pylori*-negative subjects with chronic gastritis. The results are represented as mean ± SEM. NS, not significant. *$P<0.05$; †$P<0.01$.

DISCUSSION

The present study identified a significant shift in the pattern of cytokines secreted from the gastric mucosa of subjects with carcinoma of the stomach, when compared to that found in uncomplicated chronic gastritis subjects. Antibody secretion from the mucosa, however, was similar in both groups. Subjects with dysplasia secreted both IL-4 and IFN-γ in mucosal cultures.

T cells have been identified as a significant source of IFN-γ or IL-4 as measured by using ELISA-spot and RT-PCR assay in subjects with chronic gastritis.[18,19] Although mucosal biopsies adjoining the tumor in the present study contained numerous CD3-positive cells (data not shown), nests of tumor cells were present in the majority of biopsies, suggesting that tumor cells may be an alternative source of IL-4. This is consistent with a report that tumor cells can express mRNA for IL-4.[20] However, no IL-4 or IFN-γ could be detected in the supernatant of a gastric carcinoma cell line (data not shown), whereas relatively high levels of IL-4 were present in the supernatants of gastric mucosa organ cultures from the three subjects with no detected carcinoma cells. A powerful argument for a T-lymphocyte origin of IL-4 is the secretion of IL-4 into the culture media containing mucosal biopsies from subjects with (premalignant) gastric dysplasia. The detection of IL-4 secretion in the supernatants of cultured mucosa from subjects with gastric dysplasia makes ethnic or geographic factors an unlikely basis for the profound difference noted in cytokine balance in the present study. The level of IL-4 in the cancer subjects was in part linked to colonization with *H. pylori*, although significant secretion in *H. pylori*-negative subjects suggests alternative control mechanisms.[21-23]

Similar levels of IL-4 were found in those subjects with gastric dysplastic (post *H. pylolri* eradication) and those with gastric cancer, but without current colonization with *H. pylori*. A similar close correlation to that between IgG antibody and *H. pylori* seen here and in the literature[24,25] was not noted between IL-4 secretion and *H. pylori* in either the cancer or the dysplastic groups. A positive correlation in the cancer group was found between mucosal IL-4 and local IgG antibody secretion, but not with serum antibody levels. This may

reflect B-lymphocyte 'help' mediated by IL-4 (and related cytokines) within the mucosa.

Helicobacter pylori was found within the mucosa of half the subjects with gastric tumors, although four of nine subjects considered as negative by the use of histology and urease testing had antibodies detected in serum. This presumably reflects previous infection. Given the high incidence of *H. pylori* infection in China[26] and the epidemiologic link between carcinoma and infection,[5] it is probable that the long-standing colonization had cleared because of changes in the physical milieu of the gastric mucosa associated with neoplasia, and/or the evolution of more effective host protection mechanisms.

Unanswered are questions related to the mechanism of the switch to a Th2 profile and the effect this cytokine balance may have on carcinogenesis and tumor growth. Current studies involve subjects with established gastric carcinoma, making it difficult to assess whether the skew towards a Th2 cytokine profile is a cause or effect of tumor growth (or indeed irrelevant to the presence of a tumor). The study of subjects with premalignant changes of dysplasia showing mucosal secretion of IL-4 suggests the Th1 to Th2 switch predates clinical tumor growth. The retention of IFN-γ secretion in subjects with dysplasia differed from the cancer group, where essentially no IFN-γ secretion was detected. Time-course studies in subjects with gastric dysplasia should better define the relationship between *H. pylori* infection, the mucosal immune response, factors that modulate the host response, and the development of gastric carcinoma. The continued secretion of IL-4 from the gastric mucosa in subjects with dysplasia and carcinoma negative for *H. pylori* suggests continued T-cell activation through an alternative antigen, the development of autocrine mechanisms (which are a recognized feature of T cells), or the presence of paracrine stimulation from neoplastic or dysplastic epithelial cells. Data from subjects with chronic gastritis confirmed the previous demonstration that IL-8 secretion from gastric mucosal epithelial cells is increased in the presence of *H. pylori*, specifically *cagA*-positive strains.[27] In subjects with gastric cancer, however, IL-8 secretion was not dependent on infection. A possible source of the IL-8 is the dysplastic or neoplastic epithelial cell, although this suggestion is not supported by the lower level of IL-8 secretion found in cultures from subjects with dysplasia. Eradication of *H. pylori* reduces secretion of IL-8 in gastric mucosa;[28] differences in the level of epithelial cell dysfunction may explain the differ-ences in IL-8 secretion. The mechanisms involved in producing an uncontrolled switch in cytokine production by genetic, environmental or host factors are unclear. Prospective time-course studies in the murine and human models should clarify these relationships.

What is the relationship between the change in cytokine profile and the development and progression of neoplasia involving gastric epithelial cells? Interleukin-4 has a direct antitumor effect and thus may control the growth of an established tumor.[29] Eosinophils secrete IL-4, and clinical studies correlate survival from gastric carcinoma with the degree of tissue eosinophilia.[23] Perhaps of more importance is the absence of IFN-γ from the supernatants of gastric mucosa organ cultures in those subjects with carcinoma, but not in subjects with gastric dysplasia. Interferon-γ has an antitumor effect.[30,31] This cytokine also controls the rate of apoptosis in the normal gastric mucosa.[32] The apoptosis rate in the epithelium of *H. pylori*-infected mucosa is increased, downregulating the accumulation of dysplastic cells.[32] Chronically infected mucosa with increased epithelial cell proliferation, not associated with an increase in apoptosis, may result from a switch in cytokine balance involving reduced IFN-γ production. Reduced secretion of IFN-γ in blood mononuclear cells has been described in other malignant conditions, such as cancer of the breast, cervix, ovary and colon,[33,34] suggesting a generic relationship between carcinoma and the downregulation of IFN-γ production by T cells and natural killer cells, although direct studies on tissue T cells are lacking. The present study identifies a local mucosal defect in gastric carcinoma. The retention of mucosal production of IFN-γ in subjects with gastric dysplasia may be a critical factor in preventing progression to cancer. Furthermore, a speculation could be made that in patients with pre-gastric cancer, progression of mucosal changes to gastric cancer may in the future be predictable on the basis of altered mucosal secretion.

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