Gastric T lymphocyte responses to *Helicobacter pylori* in patients with *H pylori* colonisation

X J Fan, A Chua, C N Shahi, J McDevitt, P W N Keeling, D Kelleher

Abstract

*Helicobacter pylori* has been identified as a dominant factor in the pathogenesis of duodenal ulcer. The aim of this study was to examine peripheral blood and gastric lymphocyte proliferation and cytokine production in patients with *H pylori* colonisation. Sixty five dyspeptic patients attending for endoscopy were studied; 35 of these were *H pylori* positive and 30 *H pylori* negative as assessed by culture, histology, and rapid urease test. *H pylori* antigen was capable of stimulating peripheral blood lymphocyte proliferative responses even in *H pylori* negative patients. Peripheral blood lymphocyte proliferative responses to *H pylori* (but not to purified protein derivative or phythaemagglutinin) were significantly lower in *H pylori* positive than *H pylori* negative patients. Similarly, antigen specific proliferative responses and interferon production by gastric lamina propria lymphocytes were also depressed in *H pylori* positive patients compared with *H pylori* negative patients. CD8 and CD22 positive lamina propria lymphocytes were increased in *H pylori* positive patients. These data show that antigen specific responses to *H pylori* are significantly lower in *H pylori* positive patients and could indicate activation of antigen specific suppression.

(Gut 1994; 35: 1379-1384)

*Helicobacter pylori* (H pylori, previously known as *Campylobacter pylori*) is a spiral organism that colonises the human gastric epithelium. It is strongly associated with chronic type B gastritis and peptic ulcer disease. Eradication of the bacterium is associated with healing of gastric and duodenal ulcer and longterm remission suggesting that this is a dominant factor in the pathogenesis of peptic ulcer.

Methods

Sixty five dyspeptic patients attending for upper gastrointestinal endoscopy for dyspepsia were studied (35 females, 30 males, age range: 17-78 years; mean age: 49-6), all of whom had antral biopsies performed. None of the patients studied had received non-steroidal anti-inflammatory drugs, bismuth compounds or antibiotics recently. Patients with evidence of malignant disease or immunosuppression were excluded. Multiple biopsy specimens were obtained during upper gastrointestinal endoscopy from adjacent sites of the gastric antrum for *H pylori* culture, histological examination, and rapid urease test (CLO test). *H pylori* was identified histologically by a Giemsa stain. *H pylori* were grown on 7% lysed horse blood agar under microaerophilic conditions at 37°C for three days. Patients were designated as *H pylori* positive on the basis of CLO testing. Preliminary analysis on 37 patients showed that CLO testing had a 94% sensitivity for *H pylori* positivity with respect to culture and a 100% specificity. Table I summarises the patient characteristics.

ANTIGEN AND MITOGEN PREPARATION

*H pylori* antigen was prepared from a mixture of *H pylori* cultures obtained from six patients. Bacteria cells were harvested in phosphate buffered saline and then washed (×3) in phosphate buffered saline by centrifugation (12 000×g) for 15 minutes at 10°C. The cells were resuspended in phosphate buffered saline (1.2 vol/vol). This suspension was then sonicated on ice using 6×15 second 100 Watt pulses, with 30 second cooling intervals in between, using a DAWE Soniprobe 7532A.

**Tables**

1. Colonisation of the stomach with *H pylori* is accompanied in the acute stage by an increased number of neutrophils in the lamina propria.
2. In the chronic stage, an increase in the number of lymphocytes, plasma cells, and eosinophils in the lamina propria is evident. The infection is also associated with both a local and systemic specific antibody immune response. The contribution, however, of T cell responses to host defences to *H pylori* is little understood. It has previously been reported that peripheral blood lymphocyte responses to *H pylori* are reduced in *H pylori* positive patients. It has been suggested that reduction in peripheral blood lymphocyte responses might simply reflect a selective accumulation of antigen specific T cells in mucosal sites. In this study, we have developed a system to examine antigen specific T cell responses to *H pylori* in the gastric lamina propria lymphocytes. Gastric lymphocytes were isolated by a modification of techniques developed for the isolation of small bowel lymphocytes from intestinal biopsy specimens. Antigen specific responses could be analysed if an exogenous source of antigen presenting cells and cytokine was provided in the form of irradiated autologous peripheral blood mononuclear cells and low dose interleukin 2, respectively.

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TABLE I Characteristics of the patients studied for peripheral blood mononuclear cells and lamina propria lymphocytes response. Patients were designated as H pylori positive on the basis of CLO testing. Preliminary analysis of 37 patients showed that CLO testing has a 94% sensitivity for H pylori positivity with respect to culture and a 100% specificity. Patient characteristics are summarised in the Table.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HP+tve on the CLO test</th>
<th>HP+tve on the CLO test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoscopic gastritis</td>
<td>30/33 (91) including 2 cases of peptic ulcer</td>
<td>5/32 (16)</td>
</tr>
<tr>
<td>Histological gastritis</td>
<td>33/33 (100)</td>
<td>4/32 (13)</td>
</tr>
<tr>
<td>HP seen on staining</td>
<td>31/33 (94)</td>
<td>0/32 (0)</td>
</tr>
</tbody>
</table>

HP = H pylori.

The H pylori protein concentration in the preparation was measured by the method of Bradford using bovine serum albumin as the standard. The sonicated preparation of H pylori was irradiated at 40 Gy and stored at −20°C. Purified protein derivative (DK-2300 Copenhagen, Denmark) and phythaemagglutinin (Sigma Chemical, St Louis, MO, USA) were used at a concentration of 100 U/ml and 10 µg/ml, respectively. Escherichia coli (E coli) antigen was prepared by inactivating the bacteria by irradiation and sonication as described above and used at a protein concentration of 2 mg/ml. The optimum concentration of H pylori for use in the lymphocyte proliferation studies was determined in preliminary experiments.

PERIPHERAL BLOOD LYMPHOCYTE PROLIFERATION STUDIES

Peripheral blood samples were obtained by venepuncture for isolation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells were separated by Ficoll-hypaque density gradient centrifugation at 250×g for 30 minutes at 4°C (Lymphoprep Nycomed Pharma AS, Oslo, Norway), then washed (×3) and resuspended in RPMI 1640 (Gibco, Life Technologies Ltd, Paisley, Scotland) medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum. To assess antigen specific lymphocyte proliferation, 1×10⁶ peripheral blood mononuclear cells were cultured in a total volume of 200 µl for five days with varying amounts of H pylori, purified protein derivative, phythaemagglutinin, E coli or without stimulant (spontaneous cultures). ³H-thymidine incorporation (cpm) was determined using a Packard Tri-Carb scintillation counter. Lymphocyte proliferation assays were expressed as ³H-thymidine incorporation (cpm).

GASTRIC MUCOSAL LYMPHOCYTE CULTURE

Lamina propria lymphocytes were separated by modification of techniques developed for the isolation of lymphocytes from duodenal mucosal biopsy specimens. Five gastric mucosa specimens were placed in Hank’s balanced salt solution without calcium and magnesium (CMF HBSS Gibco, +5% fetal calf serum) containing 1 mM dithiothreitol (Sigma) and 1 mM ethylenediamine tetra-acetic acid (Analar, BDH Chemicals Ltd, Poole, England) in a 20 ml V bottomed container. The specimens were then agitation for one hour at 37°C (Matburn blood cell suspension mixer), to remove the epithelial layer and washed with RPMI 1640. The specimens were treated with collagenase, type I (120 U/ml, Sigma) for three hours at 37°C with agitation. The cellular supernatant was then washed three times and the number and viability (>85%) of the isolated lamina propria lymphocytes determined using acridine orange/ethidium bromide. Viability of lamina propria lymphocytes was consistently greater than 85%. Lymphocytes were cultured (4×10⁶/ml) with H pylori (300 µg/ml), phythaemagglutinin (5 µg/ml), and anti-CD3 (OKT3, 1:50) for three days (at 37°C in 5% carbon dioxide) with irradiated (250 Gy) autologous peripheral blood mononuclear cells (2×10⁶/ml) in RPMI 1640 medium containing interleukin 2 (20 U/ml) in 24 well bottomed microplates. ³H-thymidine was added for the last 24 hours of culture. All samples were measured in triplicate. The cultured cells were processed as described earlier.

TABLE II Proliferative response of peripheral blood mononuclear cells to H pylori. Results are expressed as ³H-thymidine incorporation (cpm) in peripheral blood mononuclear cells cultured for five days

<table>
<thead>
<tr>
<th>³H-thymidine incorporation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H pylori (+)</td>
</tr>
<tr>
<td>(n=21)</td>
</tr>
<tr>
<td>Phythaemagglutinin (10 µg/ml)</td>
</tr>
<tr>
<td>Purified protein derivative (100 U/ml)</td>
</tr>
<tr>
<td>H pylori (5 µg/ml)</td>
</tr>
<tr>
<td>E coli (2 µg/ml)</td>
</tr>
<tr>
<td>Spontaneous RPMI</td>
</tr>
</tbody>
</table>

*Proliferative responses were significantly lower (p<0.02 in the H pylori positive group compared with H pylori negative patients. There were no significant differences between the two groups after stimulation with phythaemagglutinin, purified protein derivative, E coli, and RPMI control. Data shown as mean (SEM).
viability (>85%) of the isolated cells after CD22 depletion was determined.

INDUCTION AND ASSAY OF INTERFERON \( \gamma \)
Peripheral blood mononuclear cells (1x10^6/ml) and lamina propria lymphocytes (4x10^5/ml) were cultured with inactivated \( H \) pylori (3 \( \mu \)g/ml, 300 \( \mu \)g/ml), purified protein derivative (100 \( \mu \)g/ml), phythaemagglutinin (10 \( \mu \)g/ml, 5 \( \mu \)g/ml), OKT3 (1:50), and RPMI 1640 only in RPMI 1640 for either five days or three days at 37°C in 5% carbon dioxide. The culture supernatants were collected and stored at -70°C.

Interferon \( \gamma \) was measured by enzyme linked immunosorbent assay (ELISA) using anti-interferon \( \gamma \) antibodies kindly donated by Dr Kingston Mills, National Institute for Biological Standards, UK. Purified mouse anti-interferon monoclonal Ab was coated (50 \( \mu \)l/well) onto 96 well round bottomed ELISA plates and incubated for two hours at 37°C. The wells were then blocked overnight (4°C) with 150 \( \mu \)l phosphate buffered saline containing bovine serum albumin (0.5%, wt/vol).

The plates were washed four times with phosphate buffered saline-TWEEN 20 (200 \( \mu \)l/well). Supernatants were added (50 \( \mu \)l/well) in triplicate. Tripleticate serial dilutions of interferon \( \gamma \) (100 IU to 1 IU/ml) were added to standard wells. The plates were incubated for one hour at 37°C and then washed four times with phosphate buffered saline-TWEEN 20.

Rabbit polyclonal anti-interferon \( \gamma \) was added (50 \( \mu \)l/well) in bovine serum albumin-phosphate buffered saline and incubated for one hour at 37°C. After washing the plates, biotinylated antirabbit IgG (50 \( \mu \)l/well) was added in bovine serum albumin-phosphate buffered saline and incubated for one hour at 37°C. Strepavidin biotinylated horseradish peroxidase conjugate (50 \( \mu \)l/well) in bovine serum albumin-phosphate buffered saline was added and incubated for 30 minutes at 37°C, washed four times with bovine serum albumin-phosphate buffered saline followed by two washes with 0.1 M citrate phosphate buffer (pH 5.0).

The substrate, o-phenylenediamine (1 mg/ml in citrate phosphate buffer) was then added (50 \( \mu \)l/well) and the reaction was developed in the dark at room temperature. The reaction was stopped after 10 to 15 minutes by addition of 1 M \( \text{H}_2\text{SO}_4 \) (50 \( \mu \)l/well). The plates were read using an ELISA reader at 450 nm. A standard curve was constructed and the amount of interferon \( \gamma \) present in the supernatant was determined with reference to this standard curve. The assay sensitivity was 2 IU/ml.

LAMINA PROPRIA LYMPHOCYTE PHENOTYPIC ANALYSIS
Lamina propria lymphocytes were isolated as described above. The number and viability of the isolated cells were determined using acridine orange/ethidium bromide. One\( \times 10^5 \) cells were washed with phosphate buffered saline containing bovine serum albumin (1%, wt/vol) and sodium azide (0.02%, wt/vol). Monoclonal antibodies including Leu 4 (CD3), Leu 3a (CD4), Leu 2a (CD8), Leu M3 (CD14), Leu 14 (CD22), and Leu 19 (CD56), control phycoerythrin and fluorescein isothiocyanate were added (5 \( \mu l \)) to the lamina propria lymphocytes, respectively. Lymphocyte subsets were analysed by a combination of direct and indirect immunofluorescence. Data were acquired on a Beckton Dickinson FACSCAN and analysed using Lysis 3 software. Lamina propria lymphocyte phenotypic analysis was expressed as a percentage of the positive cells. All the monoclonal antibodies were obtained from Becton Dickinson Immunocytometry Systems, San Jose, CA, USA.

STATISTICAL ANALYSIS
Data are expressed as mean (SEM). Differences between the results obtained with \( H \) pylori

### Table III

<table>
<thead>
<tr>
<th>Phenotypic Analysis</th>
<th>( H ) pylori (+)</th>
<th>( H ) pylori (-)</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phythaemagglutinin (5 ( \mu )g/ml)</td>
<td>7171 (1613)</td>
<td>7755 (156)</td>
<td>0.52</td>
</tr>
<tr>
<td>ORT3 (1:50)</td>
<td>6104 (493)</td>
<td>6313 (821)</td>
<td>0.25</td>
</tr>
<tr>
<td>( H ) pylori (300 ( \mu )g/ml)</td>
<td>1400 (448)*</td>
<td>3013 (418)*</td>
<td>0.015</td>
</tr>
<tr>
<td>Spontaneous RPMI</td>
<td>562 (192)</td>
<td>650 (281)</td>
<td>0.22</td>
</tr>
<tr>
<td>Irradiated feeder cells alone</td>
<td>435 (110)</td>
<td>502 (150)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Results are expressed as \( ^3 \)H-thymidine incorporation (cpm) in lamina propria lymphocytes (4x10^5/ml) cultured for 72 hours with autologous irradiated (250 Gy) peripheral blood mononuclear cells (2x10^6/ml) containing interleukin 2 (2 U/ml). *Lamina propria lymphocyte response to \( H \) pylori in culture was significantly lower \((p<0.05)\) in \( H \) pylori positive subjects compared with \( H \) pylori negative subjects. Data shown as mean (SEM).
Results

PROLIFERATIVE RESPONSES OF PERIPHERAL BLOOD MONONUCLEAR CELLS IN RESPONSE TO H PYLORI ANTIGEN

Lymphocyte proliferative responses were performed using H pylori antigen (3 μg/ml), a dose found to give maximal peripheral blood mononuclear cells proliferation results in preliminary experiments. Lymphocyte proliferative responses were detected in both H pylori positive and negative subjects. Proliferative responses were significantly lower in the H pylori positive subjects (p<0.02). No significant differences were seen between the H pylori positive and negative groups as analysed using the two tailed Mann-Whitney U test.

PROLIFERATIVE RESPONSES OF GASTRIC MUCOSA LAMINA PROPRIA LYMPHOCYTES IN RESPONSE TO H PYLORI ANTIGEN

A high dose of H pylori (300 μg/ml) resulted in optimal stimulation of lamina propria lymphocytes and this amount was used in subsequent experiments (Fig 1). This may reflect a lower efficiency of antigen processing by the irradiated antigen presenting cells used in these assays. In addition low amounts of interleukin 2 were required to induce proliferation in this system. Exogenous interleukin 2 was added at the lowest concentration that permitted measurement of proliferation. No proliferation was seen in the absence of irradiated peripheral blood feeder cells or exogenous interleukin 2. Also no proliferation was seen in the irradiated peripheral blood feeder cells alone in the presence of interleukin 2 and H pylori (Table III). Proliferation of gastric lamina propria lymphocytes was found to be maximal at three days in preliminary studies. The proliferative responses to H pylori (300 μg/ml) were significantly lower in the H pylori positive patients compared with negative patients (p<0.02). There was no significant difference, however, between the two groups in their responses to phythaemagglutinin and OKT3 (Table III).

B CELL ELIMINATION

After B cell elimination from lamina propria lymphocytes, similar proliferative results were obtained. T lymphocyte responses to H pylori (300 μg/ml) were statistically lower mean (SEM) (2356 (342) v 4425 (626) cpm, p<0.02) in H pylori positive patients compared with the H pylori negative patients. There was no significant difference, however, between the two groups in their responses to phythaemagglutinin (7021 (1023) v 8456 (844), p=0.325) and OKT3 (7285 (783) v 8651 (744), p=0.251) (Fig 2).

INTERFERON Γ PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS AND LAMINA PROPRIA LYMPHOCYTES IN CULTURE

Interferon γ secretion by peripheral blood mononuclear cells in response to H pylori antigen was significantly lower in H pylori positive patients compared with H pylori negative controls (p<0.02). Neither spontaneous production of the interferon γ or interferon γ production in response to phythaemagglutinin (10 μg/ml) stimulation were significantly different in the two groups (Fig 3A). Interferon γ secretion by lamina propria lymphocytes in response to H pylori antigen was significantly lower in patients with H pylori infection (p<0.05). Neither spontaneous production of interferon γ or interferon γ production in
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TABLE IV Characterisation of lamina propria lymphocyte population

<table>
<thead>
<tr>
<th>Percentage of positive cells</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD22</th>
<th>CD14</th>
<th>CD56</th>
</tr>
</thead>
<tbody>
<tr>
<td>HP +ve (n=8)</td>
<td>50(4-9)</td>
<td>19.2(1-6)</td>
<td>28(3-1)*</td>
<td>21(5-2)*</td>
<td>7.7(1-2)</td>
<td>&lt;2</td>
</tr>
<tr>
<td>HP -ve (n=8)</td>
<td>55(5-1)</td>
<td>21.3(3-6)</td>
<td>21(2-1)*</td>
<td>8.4(1-5)*</td>
<td>6.4(1-4)</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

Results are expressed as a percentage of positive cells in lamina propria lymphocyte suspensions. All cells were quantitated by flow cytometry. *Note that there was a significant (p<0.05) increase in CD8+ and CD22+ cells in lamina propria lymphocyte populations from H pylori positive subjects, but no significant difference in the number of CD3+, CD4+, CD14+, and CD56+ cells from either H pylori positive or negative groups. Data shown as mean (SEM).

response to phthaemagglutinin (5 μg/ml) and OKT3 (1:50) stimulation were significantly different in the two groups (Fig 3B).

CHARACTERISATION OF LAMINA PROPIA LYMPHOCYTE POPULATIONS

The percentage of CD3, CD4 (helper), CD8 (suppressor), CD22, CD14, and CD56 positive cells in lamina propria lymphocyte populations from H pylori positive and negative patients was assessed by flow cytometry. There was a significant (p<0.05) increase in CD8+ and CD22+ cells present in lamina propria lymphocytes subpopulations in H pylori positive subjects, but no significant difference in the number of CD3+, CD4+, CD14+, and CD56+ cells in H pylori positive and negative groups (Table IV).

Discussion
In this study we have shown that an inactivated H pylori preparation is capable of stimulating T cell activation as reflected in the secretion of the T cell cytokine interferon-γ, as well as T cell proliferation. This activation is seen not only in subjects with previous contact with H pylori and established serological immunity but also in subjects who are negative for H pylori colonisation. Furthermore, our data suggest a significantly attenuated response to H pylori in H pylori positive patients. It is conceivable that the observed lymphocyte response reported in H pylori negative patients resulted from cross-reactivity with certain ubiquitous bacterial antigens. Lymphocyte proliferative responses to E coli, purified protein derivative or phthaemagglutinin were not different, however, when compared between the two groups.

These results from peripheral blood cultures are in agreement with those previously described.16,17 In addition to the results obtained in peripheral blood lymphocyte populations, we have now been successful in examining the proliferative responses of gastric mucosal lymphocytes isolated from endoscopic biopsy specimens. This has permitted direct analysis of antigen specific responses at the site of mucosal inflammation. Our studies have shown an increase in local T cells adjacent to gastric epithelial cells in response to H pylori infection.25 In the development of this assay we have found that irradiated antigen presenting cells and exogenous interleukin 2 were necessary for the stimulation of proliferation. No proliferation was seen in the absence of exogenous interleukin 2 or in the presence of interleukin 2 U/ml alone (data not shown). No proliferation was seen in the irradiated peripheral blood feeder cells alone in the presence of interleukin 2 and H pylori. Gastric mucosal lymphocytes were stimulated by inactivated H pylori preparation in both H pylori positive and H pylori negative patients. Lymphocyte proliferative responses to H pylori positive patients was, however, significantly less intense when compared with H pylori negative patients. We excluded the possibility that this was a non-specific B cell response to lipopolysaccharide as we have performed these studies in lymphocyte preparations depleted of B cells by magnetic bead isolation. These results show that lamina propria T lymphocytes exhibit reduced proliferation in response to H pylori in H pylori positive patients. In addition, interferon γ production was also reduced in H pylori positive subjects. Interferon γ production was significantly higher in lamina propria lymphocytes than in peripheral blood mononuclear cells. This may reflect a differing clonal distribution of lymphocytes producing this cytokine at these sites. Data in intestinal T cell clones suggest that the majority of such T cells produce interferon γ. Similarly, intestinal lamina propria lymphocytes seem to be capable of expressing interleukin 2 receptor without proliferating, a finding that suggests that little interleukin 2 is available for stimulation.26

There are a number of potential mechanisms for this attenuated response to H pylori. Firstly, H pylori might produce an inhibitory factor or toxin in vivo, which blocks lymphocyte proliferation. Lymphocyte proliferation in response to the T cell mitogen phthaemagglutinin and anti-CD3 is unaltered, however, by H pylori infection suggesting that a non-specific toxin is unlikely. A second mechanism could be antigen specific suppression mediated by CD8+ suppressor cells. In this regard it is notable that we have shown an increase in the proportion of CD8+ cells in the lamina propria of H pylori positive patients. It is as yet unclear whether these cells have cytotoxic potential or whether they are functioning as suppressor cells. Further studies will be directed to finding out if antigen specific CD8+ suppressor cells within the lamina propria may be implicated in attenuated responses to H pylori. Such cells have been shown in the cutaneous lesion of lepromatous leprosy27 and are thought to play a part in the nature of the tissue response to M leprae.

Many studies have shown the structure and antigenicity of H pylori whole cell, outer membrane, acid extractable surface protein, and proteinase K treated whole cell lysate preparations from H pylori strains. Antibody reactivity is quite diverse and recognises a wide range of proteins.28 It is possible that H pylori positive patients recognise different epitopes than H pylori negative patients. It has been reported that the presence of antibodies reacting with a 120 kDa protein is associated with duodenal ulcer and gastric cancer and it seems that this antigen is associated with strains of increased cytotoxicity.29,30 It is...
possible that exposure to a potent immunogen from a low virulence strain may confer T cell reactivity and protection from infection.

In assessing these data, it is important to consider the question of previous contact with *H pylori*. *H pylori* is an extremely prevalent organism with a high rate of recurrence in the year after eradication. Furthermore, infection may be intrafamilial or sporadic within families suggesting a wide environmental exposure. As yet there is little evidence for elimination of the bacteria in non-susceptible patients. The finding of lower responses to *H pylori*, which we and others have described, however, suggest the possibility that poor T cell responses may be associated with an inability to clear the organism.

DK is a Wellcome Senior Fellow in clinical science.

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