Interferon gamma and interleukin 4 secreting cells in the gastric antrum in *Helicobacter pylori* positive and negative gastritis

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Abstract

Little is known of the function of the T cells in the inflammatory infiltrate in *Helicobacter pylori* associated gastritis. This study thus measured T cell in vivo activation by enumerating the frequency of interferon gamma (IFN gamma) and interleukin 4 (IL 4) secreting cells isolated from the gastric antral mucosa in patients with or without gastritis and in *H pylori* positive and negative gastritis. Fifty four samples were examined for cytokine secretion. Four antral biopsy specimens from each patient (n=51) were taken during diagnostic endoscopy. One was used to estimate histological gastritis and the presence of *H pylori*, and three of the samples were used to isolate T cells by enzymatic digestion. IFN gamma and IL 4 secreting cells were enumerated by ELISPOT. Thirty four samples had gastritis and 79% of those were *H pylori* positive. None of the samples from non-inflamed mucosa had *H pylori*. The numbers of IFN gamma secreting cells per 10^5 T cells were higher in gastritis than in normal mucosa (145 *v* 20 IFN gamma spots, $p<0.01$), and higher in *H pylori* negative than *H pylori* positive gastritis (371 *v* 110 IFN gamma spots, $p<0.05$). The frequencies of IL 4 secreting cells did not differ between gastritis and non-inflamed mucosa. In conclusion, there is an increase in IFN gamma secreting cells but not in IL 4 secreting cells in *H pylori* positive and negative gastritis. It is not known if this TH1 type reaction has a pathogenetic or protective role.

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Keywords: *Helicobacter pylori*, interferon gamma, interleukin 4, gastritis.

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Helicobacter pylori causes chronic inflammation, gastritis, which does not generally resolve until the bacterium is eradicated by antimicrobial treatment. Gastric hormone functions are aberrant in this infection, and peptic ulcers, atrophic gastritis, and stomach carcinoma and lymphoma may be longterm consequences of *H pylori* infection.[1]

The inflammatory infiltrate in *H pylori* associated gastritis consists mainly of mononuclear and polymorphonuclear leucocytes but may include eosinophilic leucocytes, and increased intraepithelial lymphocytes or intestinal metaplasia are sometimes seen.[2] Lymphoid follicles often develop. An increased secretion of the cytokines tumour necrosis factor alpha (TNFα), interleukin 6 and 8 has been reported in *H pylori* associated gastritis.[3 4]

There is increasing evidence that the type of T cell response controls tissue immunopathology. Originally based on animal experiments, CD4 positive T cells ('helper' cells) have been classified into two main classes: TH1 and TH2, based on differential cytokine responses,[5] for instance TH1 subtype secretes interleukin 2 (IL 2) and interferon gamma (IFN gamma) whereas IL 4 and IL 5 are TH2 products. The biological relevance of this distinction has been shown especially in experimental animal models for parasitic infections such as schistosomiasis and leishmaniasis where TH1 cells seem to give protection against the pathogen while TH2 cells exacerbate the disease.[6] The pattern of TH1 versus TH2 activation also explains immunopathology in human leprosy as IL 2 and IFN gamma are prominent in tuberculoid leprosy and IL 4, IL 5, IL 10 in lepromatous leprosy.[7]

H pylori has been found to stimulate peripheral blood lymphocytes to proliferate and secrete cytokines IFN gamma and TNFα and the stimulation occurred both in the sensitised (=antibody positive) and non-sensitised patients. Interestingly, the stimulation responses were lower in the patients with specific antibodies.[8 9] The results have been confirmed in other studies.[10–12]

We present a hypothesis that the inadequacy of the immune system to cure *H pylori* infection might be caused by an incorrect response elicited, namely an overreaction of TH2 activation. The finding of a low secretion of IFN gamma in the lymphocyte cultures with *H pylori* in the antibody positive patients fits into this hypothesis.[8] It is, however, important to get information on the actual local T cell activation process that occurs in the mucosa in gastritis as the in vitro stimulation responses do not always parallel those in vivo. We have extracted the cells from the biopsy specimens in the patients with and without *H pylori* gastritis and measured spontaneous cytokine secretion of the extracted gastric antral cells. By applying a sensitive ELISPOT (enzyme linked immuno spot) method for IFN gamma and IL 4 it was possible to detect cytokine secretion at a single cell level from a very small number of T cells in biopsy specimens. Choosing IFN gamma and IL 4 as our objects it was possible to test the hypothesis related to TH1 versus TH2 pattern of the cell mediated response in gastric inflammation.

Methods

PATIENTS AND SAMPLES

Fifty four samples were collected from 51 dyspeptic patients (mean age 56, range 29–83). The patients came to a diagnostic gastroscopy at the Department of Gastroenterology, St Bartholomew's Hospital. The patients with immunosuppressive therapy or disease, anticoagulant treatment, antimicrobial treatment, HIV infection or hepatitis, serious heart or lung disease or earlier stomach resection were excluded, as well as those who during endoscopy were found to have candidiasis of the oesophagus or stomach. Forty nine of the patients had a single endoscopy while one patient with normal gastric mucosa had two endoscopies and specimens taken on both occasions. One patient had three endoscopies and triple therapy against *H pylori* before the third one. The first two samples showed active gastritis and were *H pylori* positive while the third sample did not have *H pylori* and gastritis had become inactive. Four biopsy specimens were taken with forceps from antrum, from normal or inflamed looking mucosa but not from erosion site. One specimen was used for histological examination and three for the extraction of mucosal cells.

HISTOLOGICAL ANALYSIS

One antral specimen from each patient was fixed in formalin and sent to the Department of Pathology, University of Oulu, Finland where haematoxylin and eosin and modified Giemsa stainings were done. A pathologist (TK) estimated the grade of gastritis (score 0–4) in which score 0 indicated normal (non-inflamed) mucosa, 1 superficial gastritis, 2 slight, 3 moderate, and 4 severe atrophic gastritis.[13] Score 0 was used to classify the samples into non-gastritic and scores 1–4 to gastritic samples. The gastritic samples were also classified into inactive (mononuclear cells present, polymorphonuclear cells absent) and active (both cells present). The presence of *H pylori* was estimated (positive versus negative). The histological analysis was done without knowing the symptoms of the patients, endoscopy findings or cytokine results.

EXTRACTION OF THE ANTRAL MUCOSAL CELLS

The three samples were first treated with collagenase (5 mg/ml, Type 1, Sigma) diluted in RPMI (Flow, Irvine, Scotland) medium containing heparin (5 U/ml), L-glutamine, mercaptoethanol, penicillin, streptomycin, amphotericin B, and gentamycin. The tubes were incubated at 37°C and vortexed every 30 minutes. Collagenase was used as the only enzyme to treat 14 samples and 40 specimens had additional deoxyribonuclease and thermolysin treatment. After four to six hours, collagenase was washed away and deoxyribonuclease (2 mg/ml, crude preparation, DN-25, Sigma Chemical, St Louis, MO) and thermolysin (1 mg/ml, T 7902, Sigma Chemical, St Louis, MO)[14] were added and the samples were incubated further for 30–60 minutes. Incubation was finished when the large tissue pieces were dissolved into small pieces. The overall enzyme treatment took four to seven hours. Thereafter the cells were washed three times with RPMI medium. The cells were suspended in a small volume (250–500 μl) of RPMI medium containing 10% fetal calf serum, and the total number of cells obtained from three specimens were counted. Two to three cytospin preparations were made, and kept frozen at −70°C until immunostaining.

ENUMERATION OF IFN GAMMA AND IL 4 SECRETING CELLS

The mucosal and blood cells were incubated for 20 hours in 100 μl volumes in a humidified 5% carbon dioxide atmosphere at 37°C in nitrocellulose bottomed microtitre wells (Millipore, Bedford, MA), which had been coated with a capture antibody. For IFN gamma measurement, two identical wells (about 5×10^4 mucosal cells/100 μl) were cultured without any stimulant (spontaneous cultures) and for some samples, additional two wells with staphylococcal enterotoxin B (SEB) (final concentration 5 μg/ml, Sigma Chemical, St Louis, MO) as a control stimulant. Mononuclear cells from the peripheral blood samples of healthy volunteers (n=12) were purified using Ficoll density gradient centrifugation and 10^5 mononuclear cells/well were cultured both with and without SEB stimulant simultaneously with mucosal cell cultures. For IL 4 secretion, mucosal cells were cultured in double wells, without any stimulant, to measure spontaneous secretion.

An earlier described ELISPOT method[15] was modified for the mucosal cells as they contained mucus and cell debris. It was found necessary to enhance washing at all phases and not incubate more than $5–7\times10^4$ mucosal cells per well. This made it possible to get rid of artefactual background staining and so distinguish the true spots. Non-conjugated monoclonal antibodies were used as capture and biotin labelled monoclonals to detect secreted cytokine. IFN gamma antibodies were purchased from Chromogenix AB, Mölndal, Sweden, and IL 4 antibodies from Mabtech AB, Stockholm, Sweden. The wells were first coated with a capture antibody (0·7–1 μg/well), diluted in phosphate buffered saline and incubated for three to 24 hours at room temperature. The unbound antibody was washed away with phosphate buffered saline. The cells were removed after 20 hour incubation by washing the wells eight times with phosphate buffered saline containing 0·05% TWEEN 20 (200 μl/well). Biotin labelled antibody (0·15–0·3 μg/well) was added and incubated for two to three hours at room temperature. After six washes with phosphate buffered saline+TWEEN, streptavidin alkaline phosphatase, in 1:1000 dilution (Mabtech AB, Stockholm, Sweden or Bio-Rad Laboratories, Hercules, CA, USA), was added and incubated for one to two hours

Numbers of antral mucosal cells in biopsy specimens (three pieces) in normal and gastritis mucosa. Median and range

	Normal mucosa (n=20)	*Gastritis (n=34)*	*p Value*
Total cells	382 500 (37 500–680 000)	626 000 (36 000–3 600 000)	0·016
T cells	42 220 (2625–126 000)	94 750 (5400–1 044 000)	0·006

at 37°C. The wells were washed six times with buffer containing TRIS (pH 7·6) and 100 µl aliquots of BCIP/NBT substrate solution (Bio-Rad Laboratories, Hercules, CA, USA) was added. The plate was incubated at 37°C until the appearance of dark blue spots (20–40 min). The enzyme reaction was stopped by washing with tap water. The number of spots/well were enumerated using a dissection microscope, and a mean of two wells was taken as a result.

ENUMERATION OF T CELLS AMONG THE EXTRACTED ANTRAL MUCOSAL CELLS

T lymphocyte percentages were estimated on cytospin preparations stained with monoclonal anti- CD3 antibody UCHT-1 and an APAAP method. In short, slides were fixed with acetone after which UCHT-1 (a cell line supernatant) was added for 60 minutes at room temperature. Rabbit antimouse immunoglobulin (Sigma Chemical, St Louis, MO) in dilution of 1:50 was used as the secondary antibody and, after removing it by washing, an APAAP complex (calf alkaline phosphatase coupled with mouse antialkaline phosphatase, Sigma Chemical, St Louis, MO) in dilution of 1:50 was added. The last two steps were repeated to enhance staining. After washing, BCIP/NBT substrate (Bio-Rad Laboratories, Hercules, CA, USA) was added. After a final wash with water, the cells were counterstained with Mayer's haemalum. In control slides where primary antibody was replaced by a TRIS containing buffer, less than 1% of cells stained positive. T cell percentages were used to calculate the absolute numbers of T cells per well and for three biopsy specimens from the known total number of mucosal cells. The numbers of cytokine secreting cells (=spots) were expressed in two ways: per 10^5 T cells (the frequency of secreting cells) and for three biopsy specimens (total numbers of secreting cells).

STATISTICAL ANALYSIS

The Mann-Whitney test was used to estimate significances of the differences and non-parametric test to estimate correlation between the parameters.

This study was approved by the local ethics committee of the Hackney and District Health authority.

Results

ENDOSCOPICAL, HISTOLOGICAL, AND BACTERIAL FINDINGS

Histological analysis showed gastritis in 34 samples (32 patients, mean age 58, range 29–83). These samples had the following endoscopical findings: normal mucosa (15 samples), gastric ulcer (three samples), DU (three samples), duodenal or prepyloric erosion (six samples), stenosis or deformed pylorus (six samples), atrophic gastritis (one sample), and oesophagitis (one sample). Twenty samples had histologically non-inflamed antral mucosa (19 patients, mean age 53, range 30–77). Endoscopy was normal except for oesophagitis in four samples. A stain for *H pylori* gave positive results in 27 of 34 samples with gastritis (79%) and in none of the cases with normal mucosa. Inactive gastritis (=increased number of mononuclear cells but no polymorphonuclear cells) was found in 12 samples while 22 had active gastritis. Six of seven samples with *H pylori* negative gastritis showed inactive type of gastritis.

NUMBERS OF EXTRACTED ANTRAL CELLS AND T LYMPHOCYTES

Total cell counts include all cell types, that is, epithelial, mononuclear, and polymorphonuclear cells. T cell counts were obtained using the total cell counts and T cell percentages. More cells and T cells were extracted in gastritis samples compared with normal mucosa (Table).

FREQUENCIES OF IFN GAMMA SECRETING CELLS

ELISPOT for IFN gamma was performed on 41 samples (11 normal, 30 gastritis). The gastritis samples had a higher frequency of IFN gamma secreting cells than non-inflamed samples (145 *v* 20 spots/10^5 T cells, $p<0·01$) (Fig 1). The highest frequency was found in the patients with *H pylori* negative gastritis, which differed from that of *H pylori* positive gastritis (371 *v* 110 spots, $p<0·05$) (columns 3 and 4, Fig 1).

The frequencies of IFN gamma secreting cells tended to be higher in inactive versus active gastritis (199 *v* 110 spots, $p=0·15$).

The frequencies of mucosal IFN gamma secreting cells were increased in cultures with SEB versus spontaneous cultures (229 *v* 110 spots, $p<0·01$; n=31, both normal and gastritis samples included). SEB also stimulated peripheral blood mononuclear cells, obtained from the volunteers of the laboratory staff, to secrete more IFN gamma than they spontaneously did (101 *v* 12 spots, $p<0·01$).

TOTAL NUMBERS OF IFN GAMMA SECRETING CELLS

The total numbers of IFN gamma secreting cells in biopsy specimens were higher in all types of gastritis than the numbers in the normal mucosa ($p<0·01$) but there were no differences between the different forms of gastritis (Fig 2).

When the gastritis samples were divided into those with duodenal findings (DU, erosion, pyloric stenosis or deformed pylorus or healed ulcer at the region, n=12) and those without (n=18), the figures for IFN gamma secreting cells (frequencies and total numbers) did not

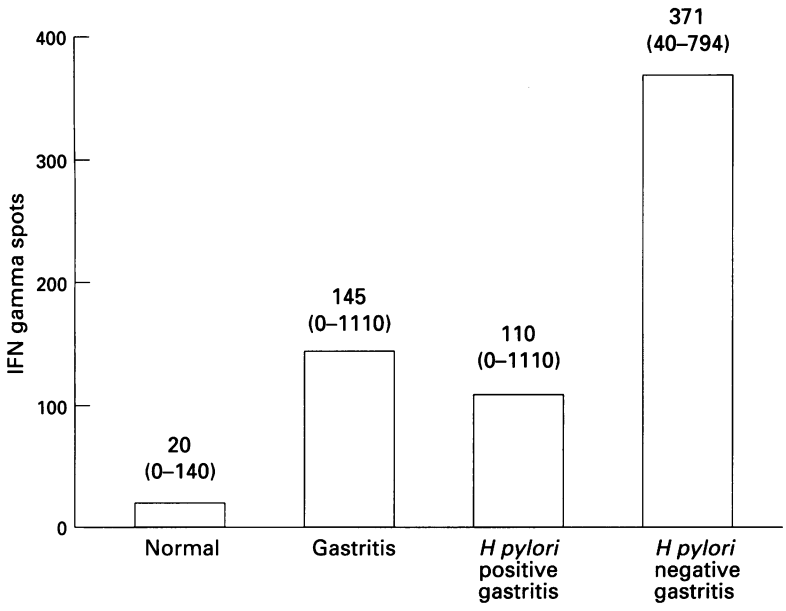

Figure 1: The frequencies of IFN gamma secreting cells/10^5 T cells isolated from gastric antral biopsy specimens. Samples are from patients with normal mucosa (n=11), gastritis (n=30), H pylori *positive gastritis (n=23) or* H pylori *negative gastritis (n=7). (Gastritis* v *normal, p<0·01;* H pylori *positive gastritis* v *normal, p<0·05;* H pylori *negative gastritis* v *normal, p<0·01.) Median and range.*

differ between the two groups (data not shown).

IL 4 SECRETING CELLS

ELISPOT for IL 4 was performed on 26 samples (15 normal, 11 gastritis). The gastritis samples had a slightly higher frequency of IL 4 secreting cells than the normal samples but the difference was not significant (90 *v* 40 spots/10^5 T cells, p=0·68). Neither was there any difference between the total numbers of IL 4 secreting cells in gastritis versus non-inflamed samples (32 *v*12 spots, p=0·25).

Discussion

We have successfully measured IFN gamma and IL 4 secreting cells in the normal and inflamed gastric antral mucosa. The numbers of IFN gamma secreting cells were increased in gastritis with or without *H pylori* while the numbers of IL 4 secreting cells remained the same as in the non-inflamed mucosa thus pointing to a TH1 pattern of activation in gastritis. We used the collagenase extraction method that has been applied earlier to intestinal cells and their cytokine measurements.[16] The digestion of antral biopsy specimens took more time than that of intestinal samples. The extraction did leave lymphocytes functionally intact, as IFN gamma secretion was boosted in vitro by SEB.

ELISPOT method was used to detect cytokine secreting cells as other methods (ELISA, immunocytochemistry) may not give as reliable results. There seems to be technical problems when immunocytochemistry is applied to cytokines secreted by T cells, and ELISA would also need to work at its lower detection level as we are measuring spontaneous IFN gamma and IL 4 secretion.

The frequency of IFN gamma secreting cells was increased in gastritis, but the type of secreting cell remains unclear. We suggest that T cells were the secreting cells, as they are the main producers of IFN gamma, but cannot exclude the possibility that some of the cells were natural killer cells, which can also secrete IFN gamma.[17] There are no reports on the frequency of natural killer cells in the gastritic mucosa.

IFN gamma is induced in vivo by antigenic or superantigenic stimulation and has multiple effects. It induces HLA class II molecule expression on epithelial and endothelial cells.[17] It decreases epithelial barrier function, but is not toxic to epithelial cells.[18 19] These two effects are especially important with regard to gastric mucosa. An enhanced HLA-DR expression on epithelial cells occurs in *H pylori* associated gastritis[20 21] so this is supposingly caused by local IFN gamma.

Quiding *et al*[14] have reported an average number of 10 IFN gamma spots/10^5 extracted mucosal mononuclear cells from normal duodenum, which is comparable with our finding in normal antral mucosa. The higher frequency of spontaneous IFN gamma secreting cells in the gastric mucosa than in the blood (145 spots in gastritis; 20 spots in normal mucosa *v* 12 spots in blood cells) shows that mucosal lymphocytes tend to be activated in vivo. Only a few samples from the blood lymphocytes were measured for spontaneous IL 4 response and the scores were <5/10^5 cells. It seems from our data that T cells in the stomach mucosa, also with regard to IL 4 (40 spots in normal mucosa), are in a higher state of activation than the blood cells, even if there is no inflammation. This high figure for mucosal cells also shows that extraction was not harmful for the capacity of the cells to secrete IL 4.

The frequency of IFN gamma secreting cells per 10^5 T cells was highest in the gastritis samples that were negative for *H pylori*, even though there might actually be *H pylori* in one or two cases that were missed by our detection

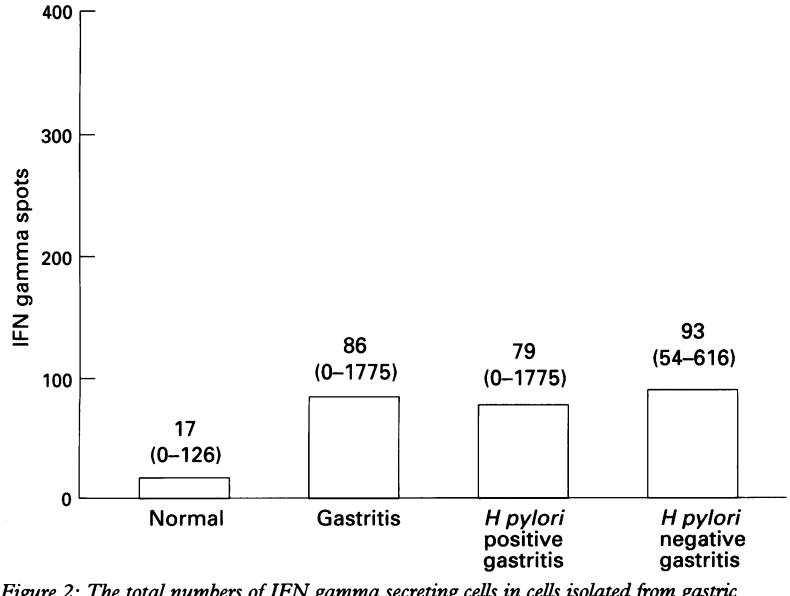

Figure 2: The total numbers of IFN gamma secreting cells in cells isolated from gastric antral specimens (three pieces). Samples are the same as in Fig 1. (Gastritis v *normal;* H pylori *positive gastritis* v *normal;* H pylori *negative gastritis* v *normal, p<0·01.) Median and range.*

method, which was based solely on histological examination. On the other hand, six of seven staining negative samples had inactive gastritis, which finding correlates with the absence of *H pylori* in them. The proportionally higher number of IFN gamma secreting cells in these negative samples might be related to the inactive nature of gastritis. On the other hand, the total numbers of secreting cells (in three biopsy specimens), while being generally higher in gastritis than in normal mucosa, were similar in different forms of gastritis (*H pylori* positive *v* negative, active *v* inactive gastritis). The design of our study made it possible to estimate cytokine secretion with regard to the frequency of secreting cells and also with regard to the total numbers of secreting cells per certain mucosal volume (three biopsy specimens), which reflects the local accumulation of T cells.

Our finding of an enhanced 'spontaneous' IFN gamma secretion in the antrum is seemingly in contradiction with the results obtained when blood or mucosal lymphocytes are stimulated with *H pylori* antigen and IFN gamma secretion is measured, which response is decreased in the patients with *H pylori*.[8 11 12] We think that the results can be explained on the basis that if the antigen specific lymphocytes are activated in vivo, they may not respond to further stimulation in vitro. Therefore there is a need to study the immune system both by in vivo and in vitro approaches.

In conclusion therefore, we have shown increased IFN gamma secreting cells in the stomach mucosa in gastritis. Unexpectedly, the highest frequencies were seen in *H pylori* negative gastritis, thus it is difficult to see what precise part *H pylori* has on the cytokine response. In view of the proinflammatory properties of IFN gamma, this cytokine may contribute to the local inflammation in gastritis. On the other hand, the presence of IFN gamma in mucosa may have beneficial effects, and our results do not support the hypothesis that the inability of the immune system to resist *H pylori* is caused by an overstrong TH2 activity.

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