

## Short reports

## Analysis of TH1 and TH2 cytokine production in low grade B cell gastric MALT-type lymphomas stimulated in vitro with *Helicobacter pylori*

A C Hauer, T M Finn, T T MacDonald, J Spencer, P G Isaacson

### Abstract

Previous studies have suggested that the dependence of low grade B cell gastric lymphoma on infection of the gastric mucosa with *Helicobacter pylori* results from help provided by *H pylori* specific tumour infiltrating T cells. ELISPOT analysis was used to characterise functional subpopulations of tumour infiltrating T cells. The production of the TH1 cytokine interferon  $\gamma$  and TH2 cytokines interleukin (IL)-4, IL-5, and IL-10 were measured in tumour cell suspensions from two cases of low grade B cell gastric lymphoma, one case of thyroid gland lymphoma, and one case of salivary gland lymphoma. Cells were assayed on day 0 and following 24 hours incubation either in culture medium or with a range of strains of *H pylori*. There was a dominant TH1-type (pro-inflammatory) response consistent with the TH1 response observed in *H pylori* gastritis.

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Low grade B cell gastric lymphoma of MALT-type is associated with infection of the gastric mucosa with *Helicobacter pylori*. The regression of most cases of gastric lymphoma following the eradication of *H pylori* suggests there is a causal relation between infection and lymphoma growth.<sup>1</sup> Previous studies have shown that tumour cells from low grade B cell gastric lymphoma of MALT-type proliferate when cultured in vitro with heat killed whole cell preparations of *H pylori*.<sup>2</sup> The in vitro response was strain specific and dependent on the presence of tumour infiltrating T cells, which are abundant in low grade MALT-type lymphomas. In addition, T cells freshly isolated and purified from these tumours proliferated in response to *H pylori* presented by autologous Epstein-Barr virus transformed B cells, whereas splenic T cells from the same patient did not.<sup>3</sup> The malignant B cells in all cases of gastric MALT-type lymphoma published to

date recognise autoantigens. Current data therefore suggest that the association between the tumour cells in gastric lymphoma and infection with *H pylori* is indirect, and that the link between the two is possibly tumour infiltrating *H pylori* specific T cells that promote tumour growth.

Most tumour infiltrating T cells in low grade B cell gastric lymphomas express CD4. CD4+ T cells can be subdivided functionally into TH1 and TH2 types according to the profile of cytokines produced: TH1 cells produce pro-inflammatory cytokines such as interferon  $\gamma$  (IFN- $\gamma$ ) and interleukin (IL)-2; TH2 cells produce cytokines involved in the regulation of the B cell response such as IL-4, IL-5, IL-6, and IL-10. Others have reported the production of TH1 cytokines in response to *H pylori* infection.<sup>4,5</sup> Given that in gastric lymphoma it is T cells that recognise *H pylori*, it is pertinent to characterise the cytokines they produce. The nature of the T cell help provided is not yet fully understood.

In this study we characterised the production of the TH1 cytokine IFN- $\gamma$ , and the TH2 cytokines IL-4, IL-5, and IL-10 in cell suspensions from two cases of gastric lymphoma before and after stimulation with *H pylori*.

### Materials and methods

#### CELLS AND TISSUES

Two surgically resected low grade gastric MALT-type lymphomas with associated *H pylori* infection were studied. The diagnosis of MALT-type lymphoma in each case was confirmed using immunohistochemistry and gene rearrangement analysis. Both cases have been studied previously.<sup>2,6</sup> Lymphoma from case 1 has been shown to proliferate in vitro in response to *H pylori*, responding optimally to NCTC 11637.<sup>2</sup> Lymphoma from case 2 was shown to proliferate spontaneously in vitro, which is unusual behaviour for lymphomas of this type.<sup>6</sup> The tumour cells in this case secrete rheumatoid factor. The response of this case to *H pylori* has not been previously investigated. Two further low grade MALT-type lymphomas (one salivary gland and one thyroid) were used as controls. Cells were teased from fresh tissues

Department of Paediatric Gastroenterology, St Bartholomew's and the Royal London School of Medicine and Dentistry, St Bartholomew's Hospital, London EC1A 7BE, UK

A C Hauer  
T T MacDonald

Department of Histopathology, UCL Medical School, University Street, London WC1E 6JJ, UK  
T M Finn  
P G Isaacson

Department of Histopathology, UMDS, St Thomas' Campus, Lambeth Palace Rd, London SE1 7EH, UK  
J Spencer

Correspondence to: Dr Spencer; email: j.spencer@umds.ac.uk

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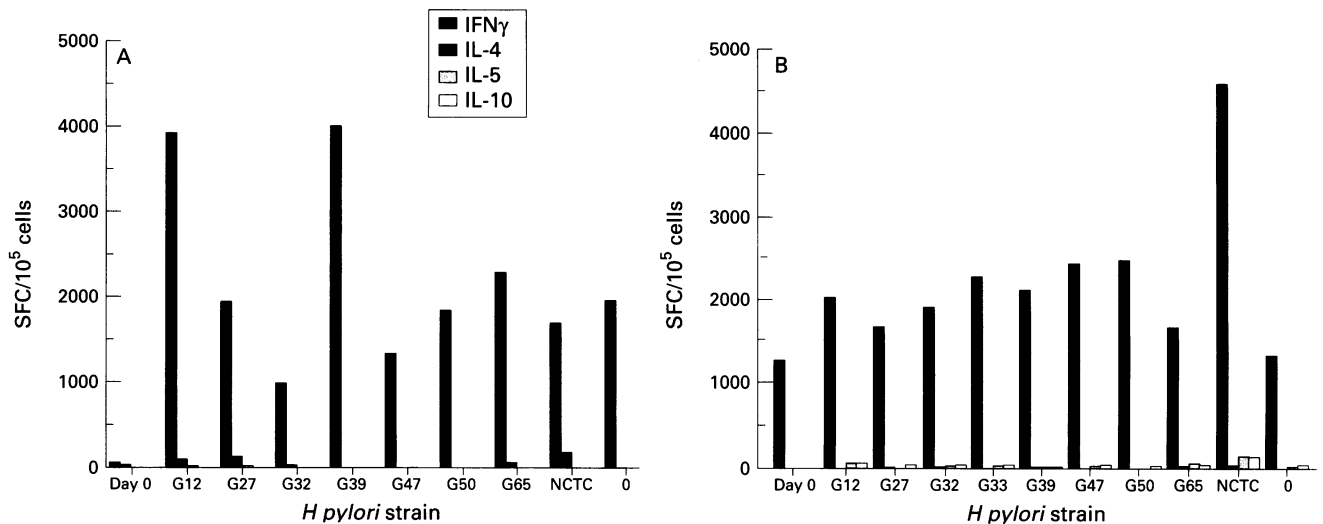


Figure 1 Production of T cell cytokines in cell suspensions prepared from two cases of gastric lymphoma. (A) case 1; (B) case 2. The number of IFN- $\gamma$ , IL-4, IL-5, and IL-10 spot forming cells/ $10^5$  cells was determined using ELISPOT assays on day 0, and following 24 hours incubation with whole heat killed strains of *H pylori*.

and stored in liquid nitrogen as viable cell suspensions until required.

#### BACTERIAL PREPARATIONS AND CULTURES

Heat killed whole cell preparations from *H pylori* strain NCTC 11637 (cytotoxin positive) and seven clinical isolates (including cytotoxin positive (G27, G32, G39, G65) and cytotoxin negative (G12, G47, G50) strains) were kindly provided by Dr J E Crabtree, department of clinical medicine, St James' Hospital, Leeds. Protein contents of the bacterial preparations were measured by a modified Lowry method.<sup>7</sup> In all experiments *H pylori* were added to cultures at a concentration of 10  $\mu$ g/ml, shown to be optimal in earlier experiments.

#### CYTOKINE ANALYSIS

Tumour cells ( $1.5 \times 10^5$  viable cells per well) were incubated in 200  $\mu$ l RPMI 1640 containing 10% fetal calf serum alone or with the addition of *H pylori*. B cell and T cell mitogens were used as positive controls to confirm the viability of cells studied; either 20 ng/ml phorbol 12-myristate 13-acetate or 50  $\mu$ g/ml phytohaemagglutinin. The number of cytokine producing cells was analysed after four hours in culture and after 24 hours' incubation with various strains of *H pylori*. The number of cells producing IFN- $\gamma$ , IL-4, IL-5, and IL-10 was measured using ELISPOT (enzyme linked immunospot) analysis as described in detail previously.<sup>8</sup> Briefly, this assay involved coating nitrocellulose-bottomed microtitre wells (Millipore Co, Bedford, Massachusetts, USA) with capture monoclonal antibody with specificity for the cytokine under investigation. After washing, cells were added to the wells and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for 20 hours. After extensive washing, the bound cytokine was identified using a biotin labelled cytokine specific detection monoclonal antibody followed by streptavidin alkaline phosphatase (Mabtech AB, Stockholm, Sweden) and BCIP/NBT substrate (Biorad Laboratories, Hercules, California, USA). The number of spots on the plate, which reflected the number of cells secreting cyto-

kine, was counted using a dissecting microscope ( $\times 25$  magnification). The capture and detection monoclonal antibodies were purchased from Chromogenix AB, Molndal, Sweden (IFN- $\gamma$ ), Mabtech AB (IL-4), and Pharmingen, San Diego, California, USA (IL-5 and IL-10).

For case 1, stimulation with all strains of *H pylori* was carried out once. The duplicate experiment used strains NCTC 11637 and G39, which were selected as stimulating and non-stimulating strains of *H pylori* based on earlier studies. All experiments using case 2 were duplicated. Data shown is the average of three replicates within an experiment.

#### ESTIMATION OF T CELL CONTENT

Samples of cells from the suspensions taken before and after culture were used to prepare cytocentrifuge preparations. These were then acetone fixed and stained with an indirect immunoperoxidase technique using monoclonal antibodies to CD3, CD4, and CD8 (Dako Ltd, High Wycombe, Bucks, UK). The percentage of CD3+, CD4+, and CD8+ cells was determined to deduce the percentage of cytokine producing T cells.

#### Results

Cytokine producing cells, predominantly producing IFN- $\gamma$  were observed in both cases of gastric MALT-type lymphomas in the cell suspensions following four hours in culture with no external stimulus (fig 1). The number of IFN- $\gamma$  producing cells was generally lower in case 1 than in case 2. After 24 hours incubation with *H pylori*, an increase in the number of IFN- $\gamma$  producing cells was observed for both cases in response to some strains (fig 1). Both cases of gastric MALT-type lymphoma responded optimally to strain NCTC 11637. The sizes of the ELISPOTs also increased in response to the stimulating strains of *H pylori*, suggesting that the amount of IFN- $\gamma$  produced also increased (data not shown). The cell suspensions in both cases of gastric MALT-type lymphoma contained approximately 10%

T cells before and after culture with a CD4:CD8 ratio of approximately 5:1. By deduction, approximately 1% of freshly isolated T cells from case 1 and 18% of T cells from case 2 produced IFN- $\gamma$ . This figure increased to approximately 20% of T cells in case 1 and between 60% and 75% of T cells in case 2 following the addition of *H pylori* NCTC 11637.

The number of IFN- $\gamma$  spot forming cells in cases of extragastric MALT lymphomas was less than 80/10<sup>7</sup> cells with or without stimulation with *H pylori*. However, the cells were viable as they proliferated in response to the positive control mitogens (data not shown).

Synthesis of IL-4, IL-5, and IL-10 was induced by *H pylori* in some experiments, but the number of spot forming cells was small compared to the IFN- $\gamma$  response (fig 1).

### Discussion

The dominant cytokine produced in cases of low grade B cell gastric lymphoma was IFN- $\gamma$  regardless of stimulation with *H pylori*, indicating the dominance of a TH1-type response. This is also observed in *H pylori* gastritis.<sup>4</sup> However, in both cases of gastric MALT lymphoma studied, the number of IFN- $\gamma$  producing cells following stimulation with *H pylori* was remarkably high. The number of T cells producing IFN- $\gamma$  was at least an order of magnitude higher than that observed in either normal stomach or *H pylori* gastritis, using the same methodology.<sup>4</sup> This suggests that either the immunological stimulus for cytokine production is much higher in gastric MALT lymphoma than in *H pylori* gastritis, or there is imbalance in the regulation of cytokine production in these tumours.

The population of cells producing cytokines IL-4, IL-5, and IL-10, the functions of which include provision of help for B cell proliferation and differentiation, is relatively small in these experiments. However, it is possible that the activity of these cytokines is potent, and that

their secretion into the microenvironment by relatively few cells may be functionally significant.

IFN- $\gamma$  provides co-stimulatory signals for B cell proliferation and differentiation, and could potentially enhance tumour progression.<sup>9</sup> In addition, IFN- $\gamma$  increases the expression of class II antigens. As the presentation of *H pylori* to T cells is class II restricted,<sup>10</sup> this could enhance the immune response to *H pylori* and could be a significant factor in the pathogenesis of the tumour.

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