Helicobacter pylori increases proliferation of gastric epithelial cells

X G Fan, D Kelleher, X J Fan, H X Xia, P W N Keeling

Abstract

The direct and indirect effects of *Helicobacter pylori* on cell kinetics of gastric epithelial cell line AGS were investigated by flow cytometric analysis of Ki-67 positive cells and by MTT assay. Flow cytometric analysis of Ki-67 positivity permits detection of cells that are in S-phase, whereas the MTT assay is a colometric measure of the number of viable cells. In the absence of added stimulants, 23·06 (4·88)% mean (SD) of AGS cells were Ki-67 positive. When cells were preincubated in the presence of *H pylori*, there was a significant increase in Ki-67 positivity (66·20 (7·89)%, p<0·001). This increase was not seen in cells cultured in the presence of *Campylobacter jejuni* (24·63 (8·11)% or *Escherichia coli* (21·66 (9·78)%). Pre-incubation of AGS cells with supernatants from both *H pylori* and mitogen activated peripheral blood lymphocytes also increased the per cent of cells that were Ki-67 positive (72·93 (8·68) and 69·96 (12·35)%; p,0·001) respectively. Similar results were also found in MTT assay. These data show that both *H pylori* directly and the immune/inflammatory response to *H pylori* indirectly can influence the rate of epithelial cell proliferation, suggesting this bacterium may be an initiating step in gastric carcinogenesis and an important co-carcinogenic factor in *H pylori* positive subjects.

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Keywords: *Helicobacter pylori*, cytokines, epithelial cell line, proliferation.

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Helicobacter pylori has been implicated in the pathogenesis of gastric carcinoma as well as active chronic gastritis and peptic ulcer disease.[1–3] Recently, it has been reported that *H pylori* infection, as a risk factor for gastric cancer, is associated with proliferation of gastric epithelial cells in *H pylori* positive hosts[4 5] and eradication of *H pylori* significantly reduces gastric epithelial cell proliferation.[4 6] Increased proliferation of epithelial cells is an important biomarker for increased risk of gastrointestinal adenocarcinoma.[7] Demonstration of an association between *H pylori* and gastric cancer would be of great importance, because it would indicate the possibility that eradication of the organism might prevent the disease in infected patients. Little is known, however, about the mechanism of the effect of *H pylori* on epithelial cell kinetics and as to whether this is a direct or indirect effect. A study of the effect of *H pylori* on gastric epithelial cell kinetics may provide clues to *H pylori* associated gastric carcinogenesis.

Monoclonal antibody Ki-67 reacts predominantly with a nuclear antigen expressed in S-phase of the cell cycle and is hence a marker for proliferating cells.[8 9] Ki-67 has frequently been used to assess the number of cells in S-phase and hence inferentially the number of proliferating cells in tissue sections. These techniques may be modified for flow cytometry. Flow cytometry permits the objective quantification of the percentage of positive cells.[8] Proliferative activity as determined by this antibody has been shown to be reliable in the assessment of the growth fraction in a wide variety of normal and malignant human tissues.[10] A colorimetric assay of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is a reliable colorimetric assay for evaluating cell proliferation.[11] To better assess the role of *H pylori* in gastric epithelial cell proliferation, direct and indirect effects of *H pylori* on cell kinetics of gastric epithelial cell line AGS were investigated using flow cytometric analysis of Ki-67 positive cells and colorimetric MTT assay in our study. The aim of this study, therefore, was to find out if *H pylori* directly or cytokines produced by lymphocytes exposed to *H pylori* could influence the rate of proliferation of gastric epithelial cells.

Methods

Bacterium preparations

H pylori preparation was made by sonicating a mixture of *H pylori* bacterial cultures obtained from six patients (four duodenal ulcer and two gastritis alone) on ice using six x 15 seconds, 100 watt pulses, with 30 seconds cooling intervals followed by irradiation at 40 Gy to sterilise the preparation. It was then washed with phosphate buffered saline (PBS) and stored at −20°C.[12] *Campylobacter jejuni* and *Escherichia coli* (NCTC 8007) preparations were made by the same procedures described above.

Cytokine rich supernatants

Cytokine rich supernatants were prepared by co-culturing peripheral blood lymphocytes from healthy volunteers negative for anti-*H pylori* antibody at a concentration of 2 x 10^6/ml for 18 hours with *H pylori* (15 μg/ml) or phytohaemagglutinin (10 μg/ml) plus phorbol myristate acetate (10 ng/ml) respectively in carbon dioxide independent medium (Gibco)

containing 100 U/ml penicillin, 10 μg/ml streptomycin, 20 mM L-glutamine, and 10% fetal calf serum. After 18 hours stimulation, lymphocytes were washed three times and incubated for a further 48 hours in the medium. Medium at the end of this period was collected and used as cytokine rich supernatants.

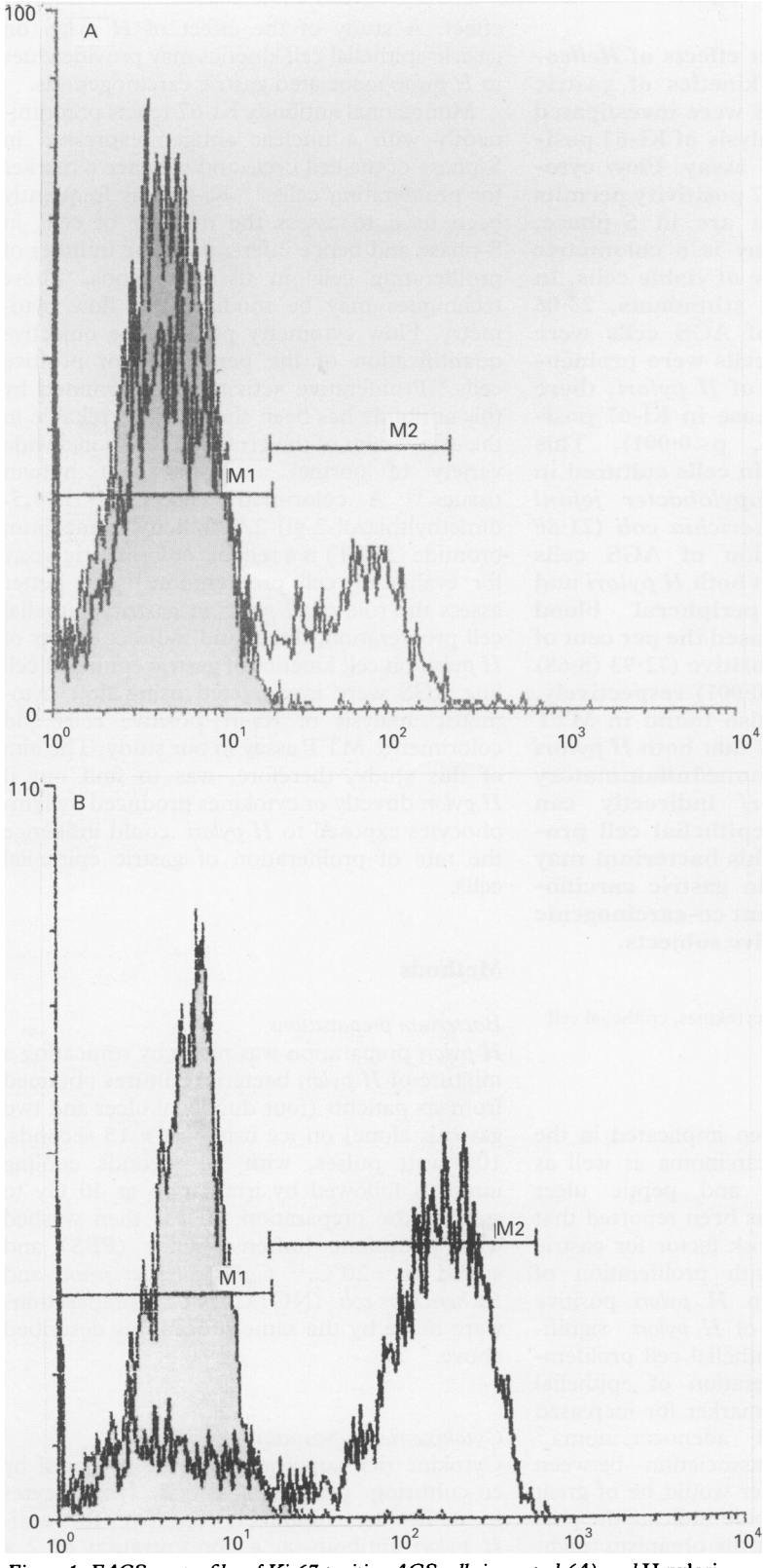

Figure 1: FACScan profiles of Ki-67 positive AGS cells in control (A) and H pylori *groups (B). The shaded histograms represent background and open histograms represent the profiles of Ki-67 positive cells. The marker 2 (M2) outlines the positive cell population and shows that there is a significant increase in Ki-67 positive cells in* H pylori *stimulated group (B) than that in control (A).*

Epithelial cell culture

A gastric epithelial cell line (ATCC), AGS, derived from a human gastric adenocarcinoma was used in the study.[13] AGS cells were cultured as monolayer in Ham F12 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM L-glutamine, and 10% fetal calf serum in flasks at 37°C in a humidified atmosphere of 5% carbon dioxide in air.

Ki-67 labelling

AGS cells removed from flasks by trypsin treatment were seeded into 8 well culture plates at a concentration of 10^5 cells per well. When the cells were grown to subconfluency, the cells were incubated for a further 24 hours with medium alone, *H pylori* (15 μg/ml), and cytokine rich supernatants (0·5 ml/ml) from both *H pylori* and mitogen activated lymphocytes respectively. As bacterial control, AGS was also co-cultured with *C jejuni* and *E coli* respectively with the same procedures and concentrations as *H pylori*. After co-culture with bacteria or supernatants, the cells were removed from plates by trypsin treatment and resuspended in fresh medium, and labelled using monoclonal antibody Ki-67 (Dakopatts, Denmark) in a series of steps.[8] Some 200 μl lysis-DNA-staining solution (calcium and magnesium free Hanks's balanced salt solution, Nonidet P-40 (0·5% v/v), RNase 0·2 mg/ml, EDTA 0·5 mM, pH 7·2) were added to the appropriate cell suspension tubes and shaken for 15 minutes in ice. Ki-67 antibody (diluted in 1:25 in PBS with 1% bovine serum albumin) was added and incubated for 15 minutes in ice, then 100 μl of fluorescein-conjugated rabbit antimouse (Fab')2 fragments was added and incubated for a further 15 minutes in ice. An irrelevant antibody (anti-murine IE) was used as control. The cells were analysed by flow cytometry immediately.

MTT assay

Briefly, 100 μl containing 1 x 10^5 cells/ml trypsinised AGS cells were seeded into each well of the 96 well plates and incubated at 37°C for 24 hours. The cells were then incubated with medium alone or *H pylori* or cytokine rich supernatants in duplicate at the same concentration as Ki-67 experiment. After a further 48 hours in culture, 10 μl of MTT (6 mg/ml, Sigma) was added to each well and incubated again for four hours at 37°C. MTT was then removed and 200 μl of dimethylsulphoxide added to each well. The absorbance at 570 nm was determined using an ELISA reader.[11]

Flow cytometric analysis

AGS cells stained with Ki-67 were analysed using the Becton Dickinson FACScan and Lysis II software. The percentage of positive cells for K—67 was estimated from the gate accumulated histograms.

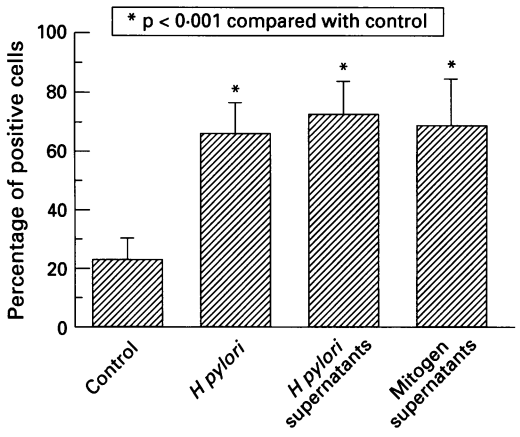

Figure 2: Comparison of Ki-67 positive cells between the control and H pylori *or cytokine rich supernatant stimulated groups. The data are presented as percentage of Ki-67 positive cells. Note that there is a significantly increased proliferative rate of epithelial cell line AGS in* H pylori *and cytokine rich supernatant stimulated groups.* H pylori *supernatants or mitogen supernatants: supernatants from* H pylori *or mitogen activated lymphocytes respectively. n=6 determinants for each group.*

Statistical analysis

The significance of the difference between control group and each experimental group was evaluated using Mann-Whitney U test and data were expressed as a mean (SD). Results were expressed as percentage of Ki-67 positive cells in flow cytometry and as optical density (OD) value in MTT assay.

Results

Flow cytometry

Figure 1 shows FACScan profiles of a representative experiment. As can be seen in Fig 1 non-specific background staining of AGS cells was low (shaded histogram), the marker 2 (M2) shows that there is a significant increase in Ki-67 positive cells in *H pylori* stimulated group (B) than that in control (A). In the absence of added bacteria or cytokine rich supernatants, 23·06 (4·88)% of AGS cells were Ki-67 positive. When cells were pre-incubated in the presence of *H pylori*, there was a significant increase in Ki-67 positivity (66·20 (7·89)%) (Fig 1B and Fig 2). Pre-incubation

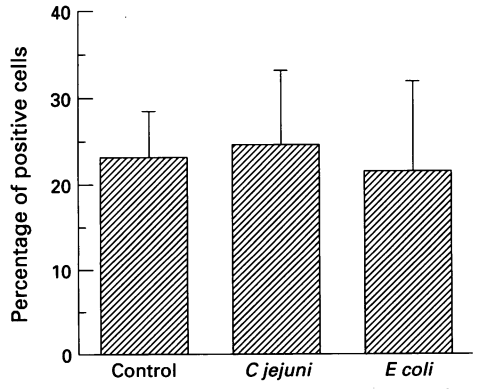

Figure 3: Comparison of Ki-67 positive cells between the control and C jejuni *or* E coli *stimulated groups. The results are presented as percentage of Ki-67 positive cells. Percentage of Ki-67 positive cells does not show significant difference between the control and* C jejuni *or* E coli *stimulated groups. n=6 determinants for each group.*

Effects of the different stimulants on cell proliferation of AGS (MTT assay results)

Stimulants	*OD values*	*p Values**
Control	0·486 (0·058)	
H pylori	0·632 (0·121)	<0·05
H pylori supernatants	0·624 (0·124)	<0·05
Mitogen supernatants	0·616 (0·111)	<0·05

*Compared with control. *H pylori* supernatants or mitogen supernatants: supernatants from *H pylori* or mitogen activated peripheral blood lymphocytes. *n*=6 determinants for each group. Data shown as mean (SD).

of AGS cells with supernatants from both *H pylori* and mitogen activated peripheral blood lymphocytes also increased the per cent of the cells that were Ki-67 positive (72·93 (8·68) and 69·86 (12·36)%) respectively (Fig 2). This increase was not seen, however, in the cells cultured in the presence of *C jejuni* (24·63 (8·11)%) or *E coli* (21·66 (9·78)%) (Fig 3).

MTT assay

Similar results were also seen on AGS cell proliferation using the MTT assay. The OD value reflects increasing cell numbers using this assay. A significant increased OD value was found in *H pylori* or cytokine rich supernatant stimulated group when compared with control, indicating an increase in cell numbers (Table).

Discussion

Gastric carcinoma develops through a sequence of events from normal mucosa to gastric carcinoma. H pylori infection has been thought, based on the epidemiological data and clinical investigations, to be closely associated with this sequence.[3 14] Increased proliferation of gastric epithelial cells has been seen in patients with *H pylori* gastritis.[4 6 15] These studies have used measurement of bromodeoxyuridine incorporation into cell nucleus on tissue sections.[6 15] Bromodeoxyuridine incorporation also represents the number of cells in S-phase and hence inferentially the proliferating rate of gastric epithelial cells. These data suggest the *H pylori* either directly or indirectly may influence the rate of proliferation of gastric epithelium. However, the direct experimental evidence on *H pylori* stimulated proliferation of epithelial cells is lacking. Our results showed that both *H pylori* itself and cytokine rich supernatants derived from peripheral blood lymphocytes can significantly increase the proliferative rate of the gastric epithelial cell line AGS. This change in cell proliferation provides suggestive evidence that this bacterium may be an initiating step in increased epithelial cell proliferation and hence potentially gastric carcinogenesis.

A number of studies have shown that *H pylori* can stimulate peripheral blood lymphocytes to produce various cytokines, including tumour necrosis factor and interferon γ,[16–18] both of which may influence epithelial cell proliferation.[19–21] Several reports have also shown that T cell mediated response may induce epithelial cell proliferation.[22 23] Factors

produced by activated T cells including granulocyte-macrophage colony stimulating factor have also been shown to affect the proliferative rate of a colonic epithelial cell line.[24] Hence, it is not surprising that T cell derived cytokines may influence epithelial cell proliferation. Our data suggest, however, that antigen specific lymphocyte activation may induce factors that affect gastric epithelial cell proliferation. High rates of local cytokine production have been shown in *H pylori* infection.[18 25] Hence, it is reasonable to speculate that this may result in increased gastric epithelial proliferation.

There is already preliminary evidence that *H pylori* itself may directly influence epithelial cell proliferation. Bode and his colleagues showed that *H pylori* stimulates proliferation of Morris hepatoma cells,[26] but a direct effect of *H pylori* on proliferation of an epithelial cell line has not previously been reported. This effect seems to be a specific response to *H pylori* because no effect of *C jejuni* or *E coli* on cell proliferation was found simultaneously. The precise mechanisms for this are unclear at present. Intriguingly, a novel cell proliferation associated gene (pag) has been shown to share approximately 50% identity with a 26-KDa antigen of *H pylori*.[27] Further studies will be required to determine which protein or factor of *H pylori* may be responsible for the proliferation of epithelial cell line AGS.

We can preliminarily conclude from this study that both *H pylori* directly and *H pylori* induced cytokines indirectly can stimulate proliferation of gastric epithelial cell line AGS. This may be of importance in clarifying the role of *H pylori* in gastric carcinogenesis. The use of this flow cytometric methodology could provide a tool for further more detailed analysis of the role of *H pylori* in epithelial cell proliferation.

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