HELMICOBACTER PYLORI AND MOLECULAR MECHANISMS OF GASTRIC MUCOSA PROLIFERATION.

M. Limatelli, C. Menas, F. Russo, I. Giorgio, A. Di Leo

H. pylori (Hp) causes chronic gastritis, it is strongly associated with peptic ulcer disease, and recently it has also been associated with gastric carcinoma. Using a monoclonal anti-BF1, it has been shown that eradication of Hp reduces gastric cell proliferation (1). Besides, Hp eradication decreases ODC activity, the rate limiting enzyme in polyamine synthesis (2). Either polyamines, putreanine (Put), spermidine (Spd) and spermine (Spm) or Epidermal Growth Factor (EGF) are involved in regulating the cellular proliferation, both normal and neoplastic (3). Therefore, aim of this study was to assess the effect of Hp infection on cellular proliferation of gastric mucosa by evaluating polyamine levels in biopsy samples of the body and antrum and EGF levels in biopsy of the antrum of 11 Hp positive patients (5 M, 28-61 yrs and 6 F, 42-62 yrs) before and 8 weeks after eradicating therapy (DeNol tabs qid, Amoxicillin qid, Metronidazole 400 mg tid). After therapy Hp was eradicated in all patients. The presence of Hp was evaluated by CLO test and histology (Warthin Starry). Polyamine levels were analyzed by HPLC. EGF levels were evaluated by ELISA. The results were analyzed by Student's t test. Polyamines are expressed as nmol/mg prot. EGF levels are expressed as pg/mg prot.

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<tr>
<th>Group</th>
<th>EGF</th>
<th>Polyamines</th>
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<tbody>
<tr>
<td>Body</td>
<td>0.170±0.65</td>
<td>2.23±1.52</td>
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<tr>
<td>Before</td>
<td>* (p&lt;0.05), ns: not significant</td>
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<tr>
<td>After</td>
<td>0.27±0.14</td>
<td>0.16±0.06</td>
</tr>
<tr>
<td>p</td>
<td>0.002</td>
<td>0.06</td>
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Our findings show lower polyamine levels and higher EGF levels in gastric biopsies taken after therapy than in those taken before Hp eradication. This preliminary study provides new data to the assumption that Hp infection actively affects cellular proliferation in gastric mucosa.


Previous studies indicated that the prevalence of H. pylori infection among gastroenterologists is higher than expected and that performing endoscopies may be a risk factor. The goal of the present study was to confirm these data using a large sample of gastroenterologists as a national level. 1,296 gastroenterologists (2/3 of the total number of registered gastroenterologists in the country) participated in the study. A questionnaire concerning personal data and information of past and actual risk factors was filled out. The Hp status was determined on a mixture of saliva and transgingival exudate collected with OraSure® devices. The samples were sent by mail and tested by using the Roche multiliner well H. pylori kit. Cut-off value was established by testing 20 proven H. pylori negative subjects (mean + 3SD).

1,276 cases were available for analysis: 442 were considered H. pylori positive (34.6%). Fifty-four cases with OD close to the cut-off value are being confirmed and are not included in the analysis. The prevalence increased with age from 18.7% (30-34 yrs), 19.8% (35-39 yrs), 38.4% (40-44 yrs), 48.3% (45-49 yrs), 63.6% (50-54 yrs), 55.3% (55-59 yrs) and 50% (60 yrs and over). When stratified by age group, the prevalence of the infection increased significantly with the number of endoscopies performed for those aged 50-59 yrs it was borderline for those aged 40-49 yrs, but not significant for the others. These data show a high level of H. pylori infection in gastroenterologists 40 years and older and a dose-response effect with regards to endoscopy which favours this practice as a risk factor for H. pylori infection.

One of the major characteristics of type B gastritis is the induction of a local inflammation including the development and activation of the mucosal immune system. Nevertheless, the causative agent Helicobacter pylori (HP) is able to persist in the mucosa for years or even decades, possibly due to immune evasive mechanisms. Previous studies by our group have revealed that HP in vitro suppresses the proliferative response of human mononuclear cells to mitogen and antigen.

In the present study the susceptibilities of different mammalian cell lines including U 937, Jurkat, EBV-transformed B-cells, KATO 3, AGS, and HeLa (human origin) and P388D1, W311, and B132.3 (murine origin) were investigated for the antiproliferative activity of HP. The proliferation (3H-thymidine incorporation) of all cell lines was suppressed in the presence of HP after 48 hours and showed only slight differences. This result shows that this effect is neither limited to human cells nor to immunocompetent cells. Comparing the antiproliferative activity of 13 strains including an isogenic urease negative mutant of HP N6 and cytotoxic negative strains revealed no significant differences, so that urease and the vacuolizing cytotoxin could be excluded as the causative agents of the observed effect. In a kinetic experiment using U937 cells the antiproliferative effect was obvious in the first 16 hours of incubation and maximal between 24 and 48 hours. In addition, HP inhibition was found to be dose-dependent. Effects of the cells in the first 5 hours of incubation, compared to cycloheximide and diphtheria toxin. There was no evidence that the inhibitory effects were due to lytic or other lethal activities of HP. A preliminary physico-chemical characterization showed that the proliferation inhibiting factor was non-dialyzable, heat-labile (70°C, 30 min), sensitive to proteases and had an apparent native molecular weight of 100 ± 10 kDa. These results suggest the presence of a new antiproliferative factor with antiproliferative activity for immunocompetent and epithelial mammalian cells. It is reasonable to presume that this property may contribute to the pathogenesis of HP infected diseases.

INDUCTION OF GASTRIC EPITHELIAL INTERLEUKIN 8 EXPRESSION WITH NATURAL H. PYLORI INFECTION, DETECTED BY A NEW ENZYME LINKED OLIGO-NUCLEOTIDE CHEMILUMINESCENCE ASSAY (ELOCA). J. McLaughlin, R. Seth, D. Jenkins, A. Robins, J. C. Hawkey. Depts Gastroenterology, Histopathology & Immunology, University Hospital, Nottingham, UK.

INTRODUCTION: Interleukin (IL) 8 from epithelial cells could mediate the persistent neutrophilic gastritis of H. pylori infection. Although induction in cell lines has been shown, this has not hitherto been quantitated in natural infection. To achieve this, we developed a sensitive new method to quantitate RT-PCR products.

METHODS: Total RNA was extracted from 13 whole gastric mucosal biopsy samples and 32 samples of gastric epithelial cells (GEC) collagenase digested from single biopsy samples. After reverse transcription, PCR amplification was performed for actin and IL8 mRNA for 30 cycles. Products were blot transferred onto nylon membrane, hybridised to specific oligonucleotide probes labelled with alkaline phosphate and quantitated by ELOCA, using the chemiluminescent substitute lumigen® and scintillation counting the product. IL8 peptide was assayed by ELISA.

RESULTS: The assay of RT-PCR product was shown to give quantitative results over a 32,768 fold range of template and over 20-30 cycles. Reactivity was found with H. pylori, and no more IL8 peptide (pg/mg) and mRNA (ratio to actin mRNA x 10^9) in both whole biopsy and epithelial cells.

Whole Biopsy

<table>
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<th>IL8 Peptide (pg/mg)</th>
<th>IL8 mRNA (ratio to actin mRNA x 10^9)</th>
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<tbody>
<tr>
<td>H. pylori 0.7 (0.3-0.9)</td>
<td>25 (7-26)</td>
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<td>H. pylori 11 (4-49)</td>
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HspA is a groES homolog in H. pylori of 118 amino acids with a unique carboxy-terminus. Previously, using recombinant techniques, a fusion of HspA with the mouse heat shock protein 60 (Hsp60) of MaEL E. coli was used to assess the human serum antibodies to HspA. In preliminary studies, only 39% of infected persons were seroreactive. We now sought to further characterize the basis of the dichotomous response to HspA. Checkerboard titrations established that antigen concentration of 125 ng/well and serum dilution of 1:100 were optimal. Among 60 infected persons, none was seroreactive for HspA. Among 40 infected patients, only 50% were positive, similar to the previous result. Analysis of these results allowed us to establish thresholds with which to study 258 H. pylori-infected patients with defined clinical features. For infected asymptomatic (n=71), NUD (n=38), GU (n=25), and DU (n=41) patients, seropositivity rates were tightly clustered between 39.5% and 43.7%. Among 36 patients with atrophy, 27.5% were seropositive (p<0.05), but among 49 patients who had gastric cancer, 67.3% (p<0.001) were seropositive. Analysis of 20 infected persons each who were HspA-seropositive or seronegative showed that the proportion of patients with a positive test that was cagA positive (60% vs. 55%), or possessing particular naaA genotypes were not substantially different. However, persons who were seropositive for HspA had substantially higher serum IgG levels to whole cell sonicates (5.4±0.6 vs. 3.3±0.6; p<0.005). In total, these studies indicate that H. pylori-infected persons can be subdivided by whether or not they develop a serum IgG response to HspA. Our preliminary studies suggest that persons with gastric cancer or those who have heightened seroreactivity to pooled H. pylori antigens are more likely to be HspA seropositive.
Previous studies have shown an association with mucosal antibodies to CagA with active inflammation. We, therefore, chose 37 Hp+ cases with and without serum IgG titters to CagA (21 CagA negative, 16 CagA positive) to evaluate blindly the degree of mucosal inflammation in both the antrum and the gastric corpus. Jumbo forces mucosal biopsies were examined without knowledge of the diagnosis or CagA antibody status. Number of Hp, severity of polymorphonuclear cell, mononuclear cell infiltration, and an overall impression of the severity of inflammation were scored on a 6 point scale (0 to 5).

As the data are ordered categories, the results were analyzed using nonparametric statistics. The only statistical significant difference was a greater severity of mononuclear infiltration in the antrum in CagA seropositive individuals. Among those with IgG antibodies to CagA, there was no suggestion of a relation between antibody titer and severity of the polymorphonuclear cell infiltration (Fig). Using blinded evaluation of large gastric mucosal biopsies we were unable to find a relation between serum IgG to CagA and active mucosal inflammation.

**CORRELATION BETWEEN INFLAMMATION OF Hp-INFEC TED GASTRIC MUCOSA AND INTERLEUKIN-10, GAMMA INTERFERON AND INTERLEUKIN-12. R. Karttunen, F.A.K. El-Zaatari, T.J. Karttunen, H.M.T. El-Zimaty, M.M. Yousif, D.Y. Graham. Department of Medicine, VAMC and Baylor College of Medicine, Houston, TX, USA.

**BACKGROUND:** We studied the expression of mRNA’s for IFN-γ, IL-10 and IL-12 in gastric mucosa. IFN-γ is an important factor in TH1 lymphocyte activation while IL-12 is a newly found cytokine prerequisite for IFN-γ secretion. IL-10 is speculated to be vital for immune down regulation.

**METHODS:** Antrum biopsies were taken from dyspeptic patients or volunteers for RNA extraction and RT-PCR. Cytokine amounts were estimated semiquantitatively according to band intensities after Southern hybridization. Histology was used for Hp detection and estimation of inflammatory cells.

**RESULTS:** The expression of cytokines were as follows:

<table>
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<tr>
<th>Cytokine</th>
<th>IFN-γ</th>
<th>IL-10</th>
<th>IL-12</th>
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<tbody>
<tr>
<td>HP-positive</td>
<td>6/9 (67%)</td>
<td>7/8 (78%)</td>
<td>4/9 (44%)</td>
</tr>
<tr>
<td>Hp-negative or gastritis</td>
<td>3/5 (59%)</td>
<td>2/5 (40%)</td>
<td>2/4 (50%)</td>
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IL-10 expression was more common among Hp-positive gastritis or ulcer vs. Hp-negative normal or treated gastritis. There was a positive correlation between mucosal mononuclear cell counts, estimated histologically, and IL-12 amounts (r=0.68, P=0.045) and a negative correlation between mucosal neutrophils and IL-10 (r = -0.73, P=0.03) among Hp-positive subjects.

**CONCLUSION:** There was an expression of mRNA for all three cytokines in many subjects with Hp-positive gastritis and a correlation between cytokines and mucosal inflammation.

**CIRCULATING IGA ANTIBODIES TO THE 60KDA HEAT SHOCK PROTEIN FAMILY ARE RAISED IN PATIENTS WITH HELICOBACTER PYLORI-RELATED GASTRIC ATROPHY. S.R.G. Barton, ILP Beales*, VB Varow, DS Rampton, J Calam*, London Hospital Medical College and *Hammersmith Hospital, London, U.K.

Heat shock proteins (HSP’s) are ubiquitous, highly immunogenic intracellular molecules induced in vitro by inflammatory mediators. It has been suggested that they may induce autoimmune phenomena in vivo. Helicobacter pylori (HP) is known to produce a 60Kda HSP and causes varying gastroduodenal pathologies in different patient groups although the exact mechanisms are not clear. We have now assayed sera of 147 patients with either gastritis (G), gastric atrophy (A), duodenal ulcer (DU) or gastric ulcer (GU), all with HP infection, as well as sera of HP-negative controls (N), for antibodies to the 60Kda HSP.

**Methods:** All sera were tested for IgA, IgG and IgM antibodies against the recombinant 65Kda protein of M.leprae (Dr Jo Colston, Mill Hill) using an established ELISA. Results are expressed as optical density ratios at 1:50 dilution using a standard control throughout.

<table>
<thead>
<tr>
<th>Results: Mean (SEM)</th>
<th>HP</th>
<th>No.</th>
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<tr>
<td>Normal</td>
<td>-</td>
<td>45</td>
</tr>
<tr>
<td>Atrophy</td>
<td>+</td>
<td>43</td>
</tr>
<tr>
<td>Gastritis</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>DU</td>
<td>+</td>
<td>15</td>
</tr>
<tr>
<td>GU</td>
<td>+</td>
<td>4</td>
</tr>
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*p<0.0005 from normal  *p<0.05 from gastritis (Mann-Whitney U)

**Conclusions:** Elevated IgA antibody levels to the 60Kda HSP in patients with HP-related gastric atrophy compared with HP-negative normal patients may reflect (a) a response to increased release of HSP from damaged gastric epithelial cells, and (b) a generation of antibody cross-reacting with HP antigens. Elevated IgA antibody levels in atrophy patients compared to gastritis patients may (1) indicate more HSP production and (2) contribute to gastric atrophy through an adverse effect on epithelial cell protection by HSP’s.

**MOLECULAR CHARACTERISATION OF IMMUNOGLOBULINS IN GASTRIC ASPARITES OF HELICOBACTER PYLORI INFECTED PATIENTS. AT Prach*, PDP McBride*, BW Senior*, FE Murray*, MA Kerr*, Departments of Clinical Pharmacology1, Pathology2 and Medical Microbiology3, University of Dundee, Ninewells Hospital and Medical School, Dundee, DDI S3Y, Scotland, UK.

The effects of Helicobacter pylori (Hp) infection on gastric immunoglobulin metabolism are poorly characterised. The aim of this study was to investigate the immunoglobulins present in gastric aspirates of patients with proven Hp infection.

**Methods and Results:** Immunoblotting of SDS-PAGE gels of aspirates using affinity-purified anti-IgA α-chain antiserum showed IgA was present mainly in the polymeric secretory IgA form (Mr ~450Kda) but was also present in the monomeric (180Kda) form. In some aspirates IgA was cleaved to lower molecular weight forms. The amount of IgA detected by immunoblotting correlated with the level of IgA anti-Hp antibodies measured by ELISA. IgG detected in the same aspirates by the use of anti-IgG chain specific antiserum was always monomeric although again lower molecular weight fragments were present in some aspirates. Interestingly, the aspirates containing cleaved IgA were not the same as those containing cleaved IgA. Again the levels of IgG detected by blotting correlated with levels of IgG anti-Hp assayed by ELISA. Analysis of the gastric aspirates by staining of SDS PAGE gels by Coomassie Blue or by gold-staining of blots of the aspirates failed to show any trace of albumin.

**Conclusion:** The results suggest that IgG in the aspirates could not have been due to bleeding and must therefore have been secreted by an, as yet, uncharacterised process. The presence of cleaved fragments of IgA and IgG in some aspirates could explain differences in the inflammatory response to Hp in different individuals.
DETECTION OF LOCALLY PRODUCED IGA- AND IGG-ANTIBODIES AGAINST HELICOBACTER PYLORI IN HUMAN GASTRIC MUCOSA BY ELISPOT METHOD

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Aim: Helicobacter pylori (H.p.)-infected individuals develop high IgG-antibody titers against H.p. in serum. IgA and IgG against H.p. have also been detected in gastric juice. The aim of the study was to define whether these specifically against H.p. and antigen directed immunoglobulins are locally produced by plasma cells within the gastric mucosa.

Methods: An enzyme-linked immunospot assay (ELISPOT) was developed, coded with water soluble proteins (WSP) from the H.p. reference strain NCTC 11637. Lympohocytes were separated by percoll gradient from single cell suspensions obtained from gastric biopsies of patients with gastritis, ulcer and of non-infected persons. H.p.-status was defined by serological (ELISA) and histopathological findings (Warthin-Starry-staining). The Sydney-system was used to classify chronicity and activity of gastritis.

Results: Immunoglobulin-producing cells were found in 63 % of H.p.-positive patients. All of them showed IgA-class immunospots and in 60 % in addition IgG-class immunospots. The detection of immunospots correlated significantly with the diagnosis gastritis or ulcer and with the extent of activity and chronicity. In some cases showing no gastritis or ulcer but high serum IgG-titers against H.p. were found. All H.p.-negative individuals were negative in the ELISPOT. Conclusions: H.p. induces an antigen-specific local humoral immune response which correlates well with the grade of inflammation and the degree of H.p.-colonization. The specificity of the ELISPOT assay is high. It may be used as a method to detect antigen-specific local immunoglobulin production against selected H.p.-antigens.


Chemotactic signals are necessary for local accumulation of granulocytes as a response to acute bacterial infection. Therefore, we investigated the chemotactic factors interleukin-8 (IL8) and ENA78 in H. pylori-associated gastritis. Furthermore, we analysed whether inflammatory mediators such as interleukin-18 (IL18) and tumor necrosis factor α (TNFα) are transcribed in gastritis tissues. The purpose of our study was to obtain further information on the immune-mediated pathogenesis of H. pylori-associated gastritis.

Methods: Antral biopsies were obtained from H. pylori-associated gastritis and non-gastritis patients. Total RNA was extracted from homogenized biopsies by means of a silica-based membrane technique. In order to monitor small quantities of mRNA transcripts out of total RNA we used the RT-PCR technique. PCR was performed on synthesized cDNA using specific primers for IL8, ENA78, IL18, TNFα, ICAM-1 and the housekeeping gene superoxide dismutase (Cu, Zn-SOD).

Results: The comparison of amplified CuZn-SOD assured an equal concentration of cDNA used for PCR. mRNA transcript for proinflammatory cytokines IL18 and TNFα as well as for the adhesion molecule ICAM-1 was found only in tissues of gastritis patients but not in our negative control individuals. The amount of mRNA transcript of IL18, TNFα and ICAM-1 was correlated with the degree of gastritis. The grade and activity of gastritis were evaluated by histopathology and Warthin-Starry stain. However, we did not find mRNA transcript for the neutrophil-activation peptide ENA78.

Conclusions: H. pylori infection upregulates IL18 and TNFα gene transcript. These proinflammatory cytokines again stimulate the transcription of ICAM-1. This implicates the importance of the inflammatory response as a major factor in the pathogenesis of H. pylori. Interestingly enough, the epithelial cell-specific chemokine ENA78 could not be found in H. pylori-associated gastritis.


Infection of human gastric mucosa with H. pylori results in a local immune response leading to an increase in mucosal production of IgG and IgA antibodies. Beyond this, a characteristic feature of inflammation is the marked infiltration with neutrophils. These cells produce and release defensins as a further defence against bacterial infection. The aim of our study was to confirm the above defence mechanisms against H. pylori and to investigate further inflammatory mechanisms such as the expression of the inducible nitric oxide synthase (iNOS).

Methods: Antral biopsies were taken endoscopically from H. pylori-associated gastritis and non-gastritis patients. Defensins were analysed immunohistochemically and RNA was extracted from homogenized biopsies by means of a silica-based membrane technique. PCR was performed on synthesized cDNA using specific primers for iNOS and the housekeeping gene superoxide dismutase (Cu, Zn-SOD).

Results: An equal concentration of cDNA was assured by the amplification of CuZn-SOD. iNOS mRNA amplification was only obtained in gastritis patients but not in negative control individuals. The amount of mRNA coding for the enzyme iNOS was strictly correlated with the degree of gastritis. Furthermore, we could show a local increase of defensins in submucosal gastric tissues.

Conclusions: NO, a cytotoxic substance, is produced by the enzyme NOS. Therefore, the increased amount of mRNA for iNOS in tissues of active gastritis may reveal a further local defence mechanism against H. pylori infection. Furthermore, it is assumed that NO and other oxygen radicals produced by chronic inflammatory processes may initiate or enhance carcinogenesis in humans like gastric cancer due to chronic H. pylori infection.

HELCOBACTER PYLORI AND G CELL BINDING

Chris Holcombe, Andrew Willls, Deborah Sunderland

Departments of Surgery & Microbiology, University of Liverpool

The cause of H. pylori hypergastrinemia remains unknown. Pathogenic E.coli cause a rise in cytosolic free calcium on binding. A similar process may occur if H. pylori bind to G cells. This rise in cytosolic free calcium may then cause gastrin secretion. It is important to know if H. pylori binds specifically to G cells.

Methods

Human endoscopic antral biopsies were microdissected, digested with collagenase and enriched by centrifuge through a Histopaque density gradient. Using this method G cells made up 8% of the final cell population (range 6-10%). The G cells were mixed with a dense suspension of H. pylori at 37 degrees for one hour. The cells were heat fixed and stained using a monoclonal antibody to gastrin and a fluorescent monoclonal antibody to H. pylori.

Results

In biopsies from 5 different patients a total of 242 G cells were studied, of these 123 (50%) had H. pylori bound to them. Usually there were several H. pylori bound to each G cell.

Conclusion

H. pylori do bind to isolated G cells. Further work is needed to establish if this binding causes an increase in cytosolic free calcium and subsequent secretion of gastrin.