Post-immunisation gastritis and *Helicobacter* infection in the mouse: a long term study

P Sutton, S J Danon, M Walker, L J Thompson, J Wilson, T Kosaka, A Lee

**Abstract**

**Background and aims**—*Helicobacter pylori* is a major cause of peptic ulcers and gastric cancer. Vaccine development is progressing but there is concern that immunisation may exacerbate *Helicobacter* induced gastritis: prophylactic immunisation followed by challenge with *H. felis* or *H. pylori* can induce a more severe gastritis in mice than seen with infection alone. The aim of this study was to investigate the relationship between immunity to *Helicobacter* infection and post-immunisation gastritis.

**Methods**—(1) C57BL/6 mice were prophylactically immunised before challenge with either *H. felis* or *H. pylori*. Histopathology and colonisation were assessed one month post-challenge. (2) C57BL/6 mice were prophylactically immunised against *H. felis* infection and gastritis assessed up to 18 months post-challenge.

**Results**—Prophylactic immunisation induced a reduction in bacterial colonisation following *H. felis* challenge which was associated with increased severity of active gastritis with neutrophil infiltration and atrophy. However, immunised mice challenged with *H. pylori* SS1 had little evidence of pathology. Long term follow up showed that post-immunisation gastritis was evident at three months. However, from six months onwards, although immunised/challenged mice still developed gastritis, there was no significant difference between inflammation in these mice and infected controls. Post-immunisation gastritis was not associated with the serum antibody response. Immunisation prevented the formation of secondary lymphoid aggregates in the gastric tissue.

**Conclusion**—The *H. felis* mouse model of post-immunisation gastritis is the most extreme example of this type of pathology. We have shown in this model that post-immunisation gastritis is a transient event which does not produce long term exacerbation of pathology.

*Helicobacter pylori* is a Gram negative bacterium which infects solely the gastric mucosa. It has a known association with peptic ulcers, gastric cancer, and mucosal associated lymphoid tissue (MALT) lymphoma. The current eradication regimen for infection is antimicrobial therapy and concurrent acid suppression. Unfortunately, the development of antibiotic resistant strains of *H. pylori* is already raising concerns about the long term use of these combination treatments. For this reason, much attention has focused on the production of an effective vaccine. Using mouse models of infection with *H. pylori* or the closely related *H. felis*, it has been shown that immunisation with whole cell sonicate or single antigens protects mice against later bacterial challenge or even when given therapeutically if administered with a mucosal adjuvant.

A consistent observation has been that prophylactic immunisation is often accompanied by an increase in the severity of gastritis in the challenged animals. This phenomenon has been termed post-immunisation gastritis and is an important issue which needs to be addressed if a human vaccine is to become a reality. It has mainly been seen following challenge with *H. felis* but has also been reported with *H. pylori*.

Post-immunisation gastritis has only been studied in short term experiments and its long term effects are unknown. Similarly, no published studies have followed immunised and challenged mice to assess the life long effect of vaccination on bacterial colonisation. This study reports on two immunisation studies in C57BL/6 mice. The first is a short term study which compares post-immunisation gastritis induced by challenge of immunised mice with *H. felis* and the Sydney strain of *H. pylori*. The second study examines the course of gastritis in immunised mice for up to 18 months after bacterial challenge with *H. felis*.

**Materials and methods**

**ANIMALS**

C57BL/6 age matched female mice were obtained from the Animal Resources Centre, Perth, Australia. All protocols involving animal

**Abbreviations used in this paper:** BHI, brain heart infusion; PBS, phosphate buffered saline; CT, cholera toxin; GAIS, gland active inflammatory score; BSA, bovine serum albumin; MALT, mucosal associated lymphoid tissue.
experimenation were approved by the Animal Care and Ethics Committee at the University of New South Wales (ACE 96/113).

EXPERIMENTAL PROCEDURE
The time frames used for these studies are shown in table 1.

HELMICOBACTER: ANTIGEN PREPARATION AND BACTERIAL CHALLENGE
H felis CS1 (ATCC 49179) was grown on Campylobacter selective agar consisting of 5% (v/v) sterile horse blood in blood agar base No 2 (Oxoid Ltd, Basingstoke, UK) and Skirrow’s supplement (10 µg/ml vancomycin (Sigma Chemical Co., St Louis, Missouri, USA), 5 µg/ml trimethoprim lactate (Sigma), 2500 IU/l polymyxin B (Sigma), and 5 µg/ml amphotericin B (ER Squibb & Sons, Princeton, New Jersey, USA)). Plates were incubated in an anaerobic jar with a microaerophilic gas generating kit (code No BR 56; Oxoid) for two days at 37°C. H pylori strain SS1 was grown for two days at 37°C with 10% CO2 and 95% humidity in brain heart infusion (BHI) broth culture (Oxoid) with 5% (v/v) horse serum and Skirrow’s supplement.

For antigen preparation, cells were harvested in phosphate buffered saline (PBS) and sonicated with a Branson Sonifier fitted with a microtip (Branson Ultrasonics Corporation, Danbury, Connecticut, USA). The protein content of the sonicate was determined using the Bio-Rad DC protein assay (Bio-Rad, Danbury, Connecticut, USA). The protein content of the sonicate was determined using the Bio-Rad DC protein assay (Bio-Rad, Danbury, Connecticut, USA). The protein content of the sonicate was determined using the Bio-Rad DC protein assay (Bio-Rad, Danbury, Connecticut, USA).

IMMUNISATION
Mice were immunised with four weekly orogastric doses of 1 mg of either H felis or H pylori whole cell sonicate plus 10 µg of cholera toxin (CT) as adjuvant (Sigma) in PBS. Age and sex matched control animals were dosed with PBS alone.

SAMPLING
At termination of the experiments, mice were anaesthetised by intraperitoneal injection of 50 mg/kg of ketamine and xylazine (Parnell Laboratories, NSW, Australia). Blood was collected from the aortic arch and sera stored at -20°C. Mice were sacrificed by cervical dislocation and the stomachs removed. Half of each stomach was fixed in 10% formal buffered saline and processed for histopathology. For H pylori infected mice, half of the stomachs were collected into a preweighed bijou containing 2 ml of BHI broth for quantitative culture.

ASSessment of colonisation and gastric pathology
H felis does not form colonies in culture and therefore colonisation was assessed histologically. Sections of stomach (4 µm) were cut and stained by the modified Steiner silver method. The degree of colonisation in H felis infected mice was assessed by semiquantitative analysis of bacteria. The most dense region of bacterial colonisation was noted and graded from 0 to 4 where 0=no bacteria, 1=1–2 bacteria/crypt, 2=3–10 bacteria/crypt, 3=11–20 bacteria/crypt, and 4>=20 bacteria/crypt.

For assessment of colonisation by H pylori, half stomachs were weighed and homogenised with an Ultra Turrax homogeniser (John Morris Scientific Ltd). One in 10 serial dilutions were prepared in BHI broth and 200 µl aliquots spread over GSSA selective agar plates—a blood agar base with 5% horse blood supplemented with vancomycin (10 mg/ml), polymyxin B (0.33 mg/ml), bacitracin (20 mg/ml), nalidixic acid (1.07 mg/ml), and amphotericin B (5 mg/ml). After five days of incubation under humidified microaerophilic conditions, colonies were counted. Colony forming units per gram of stomach tissue were calculated by multiplying the number of colonies counted by the dilution factor and dividing by the weight of the stomach tissue.

For assessment of gastric histopathology, blinded sections stained with haematoxylin and cosin were examined by light microscopy. Antral and body mucosa were graded separately. Active inflammation was assessed by the presence of neutrophils and chronic inflammation by the presence of lymphocytes. The scoring system was graded as: 1=mild multifocal; 2=mild widespread or moderate multifocal; 3=mild widespread and moderate multifocal or severe multifocal; 4=moderate widespread;

Table 1 Time frames used for the studies

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunisation (week)</th>
<th>Bacterial challenge (week)</th>
<th>Collection (time (months) post-challenge)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td>1 2 3 4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Study 2</td>
<td>1 2 3 4</td>
<td>8</td>
<td>3, 6, 12, 18</td>
</tr>
</tbody>
</table>

Table 2 Corpal gastritis in immunised and challenged C57BL/6 mice. Post-immunisation gastritis in short term immunised/challenged mice. Bacterial colonisation and gastric pathology were assessed in control, infected, or immunised/challenged mice one month after challenge with Helicobacter felis or H pylori

<table>
<thead>
<tr>
<th>Group</th>
<th>Antrum Neutrophils</th>
<th>CI</th>
<th>Body Neutrophils</th>
<th>CI</th>
<th>Lymphoid aggregates/follicles</th>
<th>GAlS</th>
<th>Atrophy</th>
<th>Submucosal inflammation</th>
<th>Colonisation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H felis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control infected</td>
<td>2 (1–3)</td>
<td>2 (1–3)</td>
<td>3 (0–5)</td>
<td>2 (1–4)</td>
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<td>1 (0–3)</td>
<td>1 (0–4)</td>
<td>3.5 (2–4)</td>
</tr>
<tr>
<td>Immunised/challenged</td>
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<td>4.5 (4–5)*</td>
<td>2 (1–3)</td>
<td>0 (0)</td>
<td>2.5 (0–11)</td>
<td>2 (0–3)*</td>
<td>1.5 (0–3)</td>
<td>0 (0–3)*</td>
</tr>
<tr>
<td><strong>H pylori</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control infected</td>
<td>1 (0–2)</td>
<td>0 (0–1)</td>
<td>0 (0–2)</td>
<td>1 (0–1)</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>10.1 (6.6)</td>
</tr>
<tr>
<td>Immunised/challenged</td>
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<td>1 (0–2)*</td>
<td>1.5 (0–2)*</td>
<td>1 (0–2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1.1 (1.3)‡</td>
</tr>
</tbody>
</table>

CI, chronic inflammation; GAI S, gland active inflammatory score; CFUs, colony forming units, ×106/g stomach tissue. Scores given are median (range), except colony counts for H pylori which are mean (SD).

*Significantly greater than the corresponding value in the infected control group (p<0.05; Mann-Whitney); †significantly lower than the corresponding value in the infected control group (p<0.05; Mann-Whitney); ‡significantly lower than the corresponding value in the infected control group (p<0.01; one way ANOVA).
MEASUREMENT OF ANTI-HELCOBACTER SERUM ANTIBODIES

Serum anti- Helicobacter antibody levels were measured by standard enzyme linked immunosorbent assays. In brief, duplicate wells of Maxisorp microtitre plates (Nunc, Denmark) were coated with 50 µl of H. felis or H. pylori lysate (100 µg/ml) in carbonate buffer, pH 9.6. After washing with PBS-0.5% (v/v) Tween 20, the wells were blocked for two hours with PBS containing 1% (w/v) bovine serum albumin (PBS-BSA) and then incubated overnight at 4°C with 100 µl aliquots of sera, diluted 1 in 20 in PBS-BSA. Bound antibody was detected by incubation at room temperature with rat-antimouse IgG, or IgA conjugated with alkaline phosphatase (Pharmingen, San Diego, California, USA), used at a dilution of 1/2000 and 1/250, respectively. Enzyme activity was determined by incubation with Sigma 104 phosphatase substrate tablets dissolved in diethanolamine buffer and absorbance measured at a wavelength of 405 nm.

STATISTICS

Comparisons of H. felis colonisation, gastric pathology scores, and serum antibody levels between infected and immunised mice were assessed by non-parametric Mann-Whitney analysis. Colonisation by H. pylori colony forming assay was assessed by the parametric ANOVA one way means test. Changes in H. felis colonisation, gastric pathology scores, and serum antibody levels over time were assessed by non-parametric Kruskal-Wallis analysis.

Results

POST-IMMUNISATION GASTRITIS IN IMMUNISED MICE CHALLENGED WITH EITHER H. FELIS OR H. PYLORI

Groups of 10 C57BL/6 mice were either prophylactically immunised with H. felis lysate and CT before challenge with H. felis or with H. pylori lysate and CT before challenge with H. pylori. Control mice were dosed with PBS before infecting with the respective Helicobacter. Colonisation by H. pylori was assessed at one month post-challenge by colony count assay. H. felis does not readily form colonies and so colonisation was assessed histologically. In both cases, immunisation significantly decreased bacterial colonisation in all immunised mice compared with infected controls (table 2).

Gastritis was independently assessed by two histopathologists (SD and MW). Mice infected with H. felis showed mild active and chronic gastritis, mild inflammation of the submucosa, and mild functional atrophy (table 2). However, immunised mice challenged with H. felis demonstrated post-immunisation gastritis, evidenced by a significant increase in active gastritis in both the antrum and body and the development of atrophy. Representative photomicrographs of the observed pathologies...
induced by *H. felis* infection or post-immunisation gastritis following *H. felis* challenge are shown in fig 1B and 1C, respectively.

In contrast with mice infected with *H. felis*, those infected with *H. pylori* developed mild chronic gastritis with no evidence of atrophy, lymphoid follicle/aggregates, active inflammation, or submucosal inflammation. This was also true for the immunised mice challenged with *H. pylori*. There was a small but significant increase in chronic gastritis in the antrum and both active and chronic gastritis in the gastric body of immunised *H. pylori* challenged mice but this still only represented a very mild degree of inflammation. Thus there was little evidence of post-immunisation gastritis in immunised mice challenged with *H. pylori*.

Analysis by ELISA revealed no correlation between the development of *Helicobacter* specific serum antibodies and the development of post-immunisation gastritis. Anti-*Helicobacter* antibodies in immunised mice challenged with *H. felis*—that is, those which exhibited post-immunisation gastritis in the antrum and both active and chronic gastritis in the gastric body of immunised *H. pylori* challenged mice but this still only represented a very mild degree of inflammation. Thus there was little evidence of post-immunisation gastritis in immunised mice challenged with *H. pylori*.

**Table 3 Assessment of post-immunisation gastritis and Helicobacter felis colonisation.** Long term assessment of post-immunisation gastritis and *H. felis* colonisation. Bacterial colonisation and gastric pathology were assessed in control, infected, or immunised/challenged mice, 3, 6, 12, and 18 months post-challenge with *H. felis*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time point (months)</th>
<th>Antrum</th>
<th>Body</th>
<th>Lymphoid aggregates/follicles</th>
<th>Gastritis (GAIS)</th>
<th>Atrophy</th>
<th>Submucosal inflammation</th>
<th>Colonisation grade</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Neutrophils</td>
<td>CI</td>
<td>Neutrophils</td>
<td>CI</td>
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<td>0 (0–1)</td>
<td>0 (0–1)</td>
</tr>
<tr>
<td>Non-infected</td>
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<td>0 (0–1)</td>
<td>0 (0–1)</td>
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<td>0 (0–1)</td>
<td>0 (0–1)</td>
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</tr>
<tr>
<td>Infected</td>
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<td>2 (1–2)</td>
<td>1.5 (0–3)</td>
<td>2 (1–4)</td>
<td>3.5 (0–8)</td>
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<tr>
<td></td>
<td>6</td>
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<td>2 (1–3)</td>
<td>3 (1–4)</td>
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<td>1 (0–2)</td>
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<tr>
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<td>3 (1–4)</td>
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<td>18</td>
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<td>2 (1–10)</td>
<td>8 (0–24)</td>
<td>2 (0–3)</td>
</tr>
<tr>
<td>Immunised/challenged</td>
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<td>5 (2–5)</td>
<td>3 (2–4)</td>
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<td>1 (0–3)</td>
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<tr>
<td></td>
<td>18</td>
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<td>2 (1–3)</td>
<td>4 (1–5)</td>
<td>0 (0–5)</td>
<td>2 (0–16)</td>
<td>2 (0–3)</td>
</tr>
</tbody>
</table>

CI, chronic inflammation; GAIS, gland active inflammatory score.

All scores are median (range).

*Significantly greater than the corresponding value in the infected control group (p<0.0125; Mann-Whitney); †significantly lower than the corresponding value in the infected control group (p<0.0125; Mann-Whitney).
mice which was maintained for the length of the study.

Statistical analysis of changes in gastritis in infected mice over time revealed that there was no significant increase in gastritis from three to 18 months except for a progressive development of submucosal inflammation (Kruskal-Wallis; p<0.015). In contrast, immunised/challenged mice actually exhibited a significant decrease over time in both neutrophil infiltration into the body and in the number of GAIS (Kruskal-Wallis; p<0.02).

Discussion

A potential complication in developing a human vaccine against *H pylori* is the observation in mice that prophylactic immunisation against *Helicobacter* infection with subsequent bacterial challenge can lead to an exacerbated pathological response. The pathology associated with this post-immunisation gastritis can sometimes be severe and in the human would clearly be unacceptable as, if maintained, could potentially increase the chance of developing *Helicobacter* associated conditions such as peptic ulcer disease and gastric cancer. Here, we have described two experiments where this phenomenon was studied.

In the first short term study, post-immunisation gastritis was assessed following prophylactic immunisation of C57BL/6 mice and challenge with either *H felis* or *H pylori*. Challenge with *H felis* induced a significant increase in pathology in immunised mice, including the development of what we have termed functional atrophy. In contrast, immunised mice challenged with *H pylori* did not show gastric pathology, apart from a mild, degree of chronic gastritis in the antrum and mild active body gastritis. This gastritis, particularly the antral lymphocyte population, most likely constitutes the effective immune response induced by immunisation, as the antrum is the major site of bacterial colonisation. It is known that CD4+ T lymphocytes are essential for effective immunity against *H pylori* infection and thus migration of these cells to the site of infection is to be expected.

It has been proposed that post-immunisation gastritis is driven by residual bacteria which the immune response fails to clear. Thus the presence of these bacteria maintain the inflammatory response, explaining why the immunised mice did not have lower gastritis scores than the infected controls. If these mice are cleared of infection by antimicrobial therapy then gastritis abates. Despite realising the need for bacterial antigen to drive the observed post-immunisation gastritis, the mechanisms involved are not understood. However, our data suggest that the humoral response does not play an important role due to the inverse relationship between serum anti-*Helicobacter* antibody levels and development of post-immunisation gastritis. Immunised mice challenged with *H felis* which developed post-immunisation gastritis presented no change in their serum antibody response against *Helicobacter* antigen compared with infected controls. In contrast, immunised mice challenged with *H pylori* developed a large humoral response but without significant gastric pathology. Thus it appears that post-immunisation is a cell mediated phenomenon, although the effects of locally produced antibodies cannot be discounted.

It is worth noting that strain SS1 of *H pylori* is relatively avirulent in the mouse, as shown by the low gastritis scores in the infected mice. Thus it is possible that the absence of post-immunisation gastritis in these *H pylori* challenged mice may be at least partly due to the poor virulence of these bacteria. Against this argument however is the study of Goto and colleagues. In contrast to our findings, Goto et al, using basically the same model of C57BL/6 mice and *H pylori* SS1, reported that post-immunisation gastritis is associated with protection against *H pylori* challenge in mice. So why have two similar studies produced such different conclusions? We believe the explanation lies in the time at which the mice were challenged. Goto et al challenged mice one week after the final vaccination when the immunisation induced immune response was maximal. At this time, the immunised mouse would possess large numbers of highly activated *Helicobacter* specific lymphocytes. On challenge however the activated lymphocytes would migrate rapidly into the site of infection and induce the observed strong inflammatory response. In our experiment, mice were not challenged until four weeks after the final immunisation when the immune response induced by immunisation would have significantly reduced. Thus there would have been far fewer activated *Helicobacter* specific lymphoid cells in our mice to induce the rapid gastritis observed in post-immunisation gastritis. It is important to note however that the vast majority of infections with *H pylori* occur during childhood. For a prophylactic vaccine, it would clearly be necessary to immunise young children. Given the large numbers of individuals this would entail, a very low proportion of those immunised may well be infected shortly following vaccination and those individuals would possibly develop an accelerated inflammatory response. We propose that this phenomenon is part of the immune response induced by the vaccination to combat infection, and as suggested by this study, involves a transient increase in gastritis. Progression to the pathologies induced by *H pylori* infection, such as peptic ulcers and gastric cancer, occur due to a chronic gastritis normally requiring many years. If a truly effective human vaccine is developed that produces sterilising immunity, there should be no significant health risk as the acute increase in gastritis should resolve to a complete absence of gastritis, provided the final vaccine is truly effective.

Despite the lack of pathology in the immunised mice challenged with *H pylori*, the possibility of later development of post-immunisation gastritis cannot be ruled out by this study. However, we propose that this is unlikely as post-immunisation gastritis appears to be an acceleration of the normal long term inflammatory response observed following
Helicobacter infection of non-immunised animals. This view is supported by our second experiment which followed the long term consequences of challenging immunised mice with \(H\) felis. \(H\) felis was used as it produces much greater gastritis in mice than does \(H\) pylori and was thus considered the best model.

It is important to distinguish between gastritis and post-immunisation gastritis. Post-immunisation gastritis is exacerbation of gastritis induced by immunisation whereas gastritis is obviously the standard inflammatory response induced, in this case, by infection. In this study, post-immunisation gastritis was assessed between three and 18 months post-challenge. Post-immunisation gastritis was present at three months, as shown by active and chronic inflammation with atrophy in immunised/challenged mice (table 3). However, from six months onwards, by the same parameters this inflammatory response in the immunised and challenged group had significantly reduced compared with the infected group. This supports the view that post-immunisation gastritis—that is, exacerbation of gastritis induced by immunisation—is a transitory phenomenon which disappears over time. It is important to note that these mice still develop gastritis to the same level as control mice.

Immunisation produced a long term reduction in bacterial burden which lasted for at least a year, half the life of the mouse. There was apparently no significant effect after 18 months, but this may, at least partly, be due to the increased atrophy in the control infected mice reducing the colonisation score in these mice. The increased pathology makes the histological grading of colonisation more difficult, and could also possibly exert an environmental impact on the Helicobacter. However, it also needs to be noted that there was a slight increase in colonisation of the immunised mice. This suggests that a booster vaccination could be required for very long term protection, although the data required to conclude this are not currently available. Such boosters are a common feature of many currently available vaccines such as those against polio, tetanus, and hepatitis B. In the context of a \(H\) pylori vaccine, this may not be important given that most infections occur during childhood.

Also of note from this study is the fact that immunisation significantly reduced the formation of lymphoid aggregates/follicles in the gastric mucosa. This observation suggests that immunity may be redirected away from the formation of the MALT response. Infection with \(H\) pylori can lead to the development of B cell MALT lymphomas. These Helicobacter induced MALT lymphomas typically develop in the marginal zone of lymphoid follicles which are present in gastric tissue as a result of chronic infection. Thus the absence of a lymphoid follicular response may prevent development of MALT lymphomas.

In summary, we have presented evidence which suggests that prophylactic immunisation against \(H\) pylori infection can induce an effective immune response without the development of significant pathology. Most importantly, we have also shown that when an immunisation induced exacerbation of pathology did occur, the effects were transient and additionally prevented the formation of lymphoid follicles. Safety issues are paramount in the development of any human vaccine, and for \(H\) pylori this is no exception. The observation of post-immunisation gastritis seen in some animal models needs to be addressed. The \(H\) felis mouse model of post-immunisation gastritis is the most extreme example of this type of pathology. We have shown in this model that post-immunisation gastritis is a transient event with no long term exacerbation of pathology. This should provide reassurance regarding the safety of a final human vaccine against \(H\) pylori infection in humans. However, it is important to note that even though vaccination did not induce any long term increase in gastritis, neither did it produce any reduction in gastric inflammation, despite a significant reduction in bacterial burden. This is due to the unfortunate inability of current vaccination regimens to induce complete protection against Helicobacter infection. Antigens produced by the residual bacteria are still sufficient to drive an equivalent inflammatory response. The solution is to improve the vaccine so that sterilising immunity can be achieved and when this occurs, the gastritis should disappear once and for all.

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6 Czinn SJ, Gai A, Niedrud JG. Protection of germ-free mice from infection by Helicobacter felis after active oral or passive IgA immunisation. Vaccine 1993;11:637–42.
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