

ORIGINAL ARTICLE

Helicobacter pylori water soluble surface proteins prime human neutrophils for enhanced production of reactive oxygen species and stimulate chemokine production

T Shimoyama, S Fukuda, Q Liu, S Nakaji, Y Fukuda, K Sugawara

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Backgrounds/Aims: Chronic gastritis induced by *Helicobacter pylori* is characterised by considerable neutrophil infiltration into the gastric mucosa without mucosal invasion of bacteria. Bacteria have different characteristics with respect to their ability to stimulate human neutrophils to produce reactive oxygen species and chemokines. The aim of this study was to examine the effects of *H pylori* water extracts on the oxidative burst and chemokine production of human neutrophils.

Methods: *Helicobacter pylori* cells were extracted by harvesting into distilled water and centrifugation. Neutrophils were incubated with *H pylori* water extracts and the production of reactive oxygen species was measured using luminol dependent chemiluminescence (LmCL). In addition, the concentrations of chemokines (interleukin 8 (IL-8), macrophage inflammatory protein 1- α (MIP1- α), and MIP1- β) were measured by enzyme linked immunosorbent assay. Neutrophils were also stimulated by opsonised zymosan (OZ) after preincubation with *H pylori* water extracts.

Results: *Helicobacter pylori* water extracts alone induced only a weak oxidative burst but preincubation of neutrophils with water extracts dose dependently enhanced the LmCL response stimulated by OZ. *Helicobacter pylori* water extracts also stimulated neutrophil IL-8 production, although MIP-1 β production was only stimulated weakly, and MIP-1 α was not stimulated at all.

Conclusions: *Helicobacter pylori* products in water extracts may have a role in the activation and migration of neutrophils, which results in enhanced oxidative damage to gastric mucosa. These findings may explain the pathology of *H pylori* induced gastritis, in which there is little invasion of bacteria into the gastric mucosa.

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H*elicobacter pylori* infection induces chronic gastritis, which is characterised histologically by considerable neutrophil infiltration. Reactive oxygen species (ROS) produced by neutrophils are thought to play an important role in the oxidative damage to the gastric mucosa and the severe clinical outcome of *H pylori* induced chronic gastritis.^{1,2} *Helicobacter pylori* activates neutrophil oxidative metabolism^{3,4} and the surface proteins of *H pylori*, including *H pylori* neutrophil activating protein (HP-NAP), modulate ROS production in neutrophils.^{5,6} However, the stimulating and/or priming effects of the surface proteins of *H pylori* on the production of toxic oxidants by human neutrophils have not been fully investigated.

"Because *Helicobacter pylori* is non-invasive, bacterial surface proteins may play a role in the chronic active gastritis induced by this bacterium"

Human neutrophils can produce chemokines, which are a family of proinflammatory cytokines that have leucocyte chemotactic and activating properties.⁷ The chemokine superfamily has several subgroups including two major subgroups, the CXC and CC chemokines. The CXC chemokines primarily act on neutrophils, whereas the CC chemokines have functional effects on monocytes and lymphocytes. Infection with *H pylori* has been associated with increased concentrations of CXC and CC chemokines in the gastric mucosa.⁸ Previous studies showed that the surface proteins of *H pylori* stimulated the production of interleukin 8 (IL-8) and growth related oncogene (GRO) proteins, members of the CXC chemokines, by neutrophils.^{9,10} However, human neutrophils can also produce several CC chemokines in response to certain

stimuli,¹¹ such as macrophage inflammatory protein 1 α (MIP-1 α) and MIP-1 β , which are CC chemokines that are chemotactic to T helper type 1 lymphocytes.¹² Although human neutrophils produce MIP-1 α and MIP-1 β , the effects of *H pylori* on the production of MIP-1 α and MIP-1 β by human neutrophils have not been elucidated.

Because *H pylori* is non-invasive, bacterial surface proteins may play a role in the chronic active gastritis induced by this bacterium. Thus, it would be useful to understand the responses of neutrophils to stimulation by *H pylori* surface proteins. The aim of our study was to characterise the effects of *H pylori* water soluble surface proteins on the production of ROS and chemokines by human neutrophils.

MATERIALS AND METHODS

Preparation of neutrophil suspension

Neutrophils were isolated from six healthy volunteers using Histopaque density gradient separation (Sigma, St Louis, Michigan, USA). Briefly, peripheral blood samples were diluted twofold in Hank's balanced salt solution (HBSS) and decanted on to an equal volume of Histopaque 1077 and 1119. After centrifugation at 500 $\times g$ for 30 minutes at 4°C, the neutrophil fraction, located at the 1077–1119 interface, was harvested and washed with HBSS. This procedure yields a neutrophil population that is 96–99% viable (using trypan

Abbreviations: GRO, growth related oncogene; HBSS, Hank's balanced salt solution; HP-NAP, *Helicobacter pylori* neutrophil activating protein; IL, interleukin; LmCL, luminol dependent chemiluminescence; MIP, macrophage inflammatory protein; MPO, myeloperoxidase; OZ, opsonised zymosan; ROS, reactive oxygen species

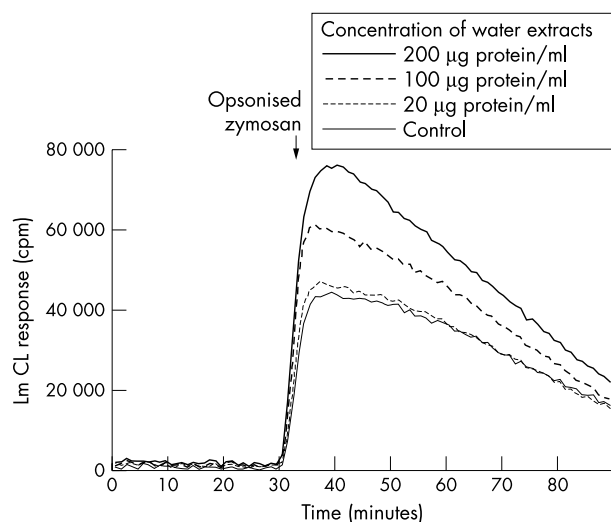


Figure 1 Luminol dependent chemiluminescence (LmCL) response pattern stimulated by *Helicobacter pylori* water extracts and *H pylori* cells. Stimulation of the production of neutrophil reactive oxygen species by *H pylori* cells depended on the concentration of the preincubation *H pylori* water extracts.

blue exclusion) and > 98% pure. Neutrophils were suspended in HBSS to the required concentration (2.0×10^3 / μ l) and the suspension was used within 20 minutes.

Water extracts and bacterial suspension

Two clinically isolated *H pylori* strains were used (both were cagA positive with a vacA s1/m1 genotype). *Helicobacter pylori* strains were extracted by harvesting into 1.0 ml/plate distilled water. The bacterial suspension was kept at room temperature for 20 minutes, and after centrifugation at $17\,000 \times g$ for 15 minutes the supernatant was stored at -70°C . Before use, the extracts were centrifuged at $39\,000 \times g$ for 20 minutes, and the supernatant was passed through a $0.2 \mu\text{m}$ filter. Filtered water extracts were suspended in HBSS to the required concentrations.

Luminol dependent chemiluminescence

Luminol dependent chemiluminescence (LmCL) was measured in a 96 well black flat bottom plastic microplate (Greiner Japan, Tokyo, Japan). Luminol was prepared by dissolving 5-amino-2,3-di-hydro-1,4-phthalazinedione (Sigma) in 0.9% NaCl at pH 7.4. A $50 \mu\text{l}$ aliquot of neutrophil suspension and the same volume of luminol solution (final concentration, 0.1mM) were added to each well of the microplate. After the addition of $100 \mu\text{l}$ of HBSS, with or without *H pylori* water extracts, the plate was immediately transferred to the dark box of a Lumi Box H-1000 (Microtec, Funabashi, Japan) and the LmCL response was recorded simultaneously and automatically with the CCD camera by means of photon counting, agitating the plate constantly.¹³ After measuring for 30 minutes at 37°C , $50 \mu\text{l}$ of opsonised zymosan (OZ; Sigma; final concentration, 1.0 mg/ml) was added to the wells and the measurement of the LmCL response was continued. The measurement interval was 60 seconds, the run time was one hour, and the temperature inside the chamber was 37°C . The highest value on the LmCL response curve (fig 1) was expressed as the peak height and used for the assessment.

Chemokine ELISA

The microplates containing $50 \mu\text{l}$ of neutrophil suspension and $150 \mu\text{l}$ of *H pylori* water extracts at various concentrations in each well were incubated at 37°C . After incubation for 12 hours, cell free supernatants were collected by centrifugation and IL-8, MIP-1 α , and MIP-1 β were measured by enzyme

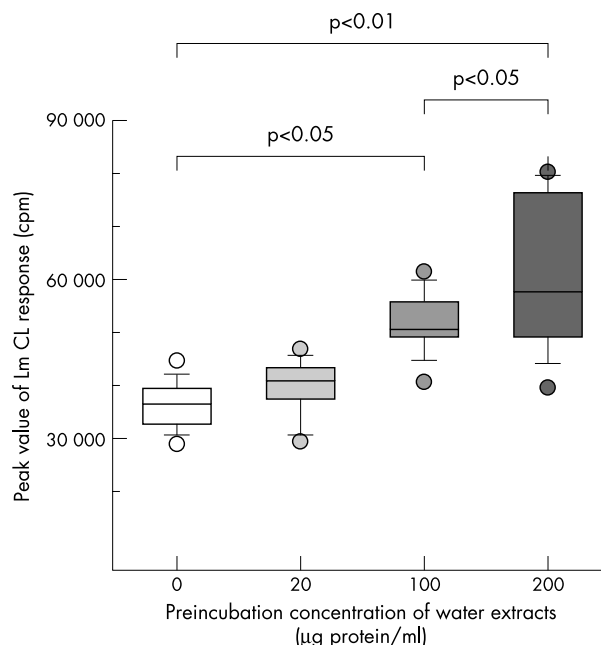


Figure 2 The peak value of the luminol dependent chemiluminescence (LmCL) response of neutrophils stimulated by *Helicobacter pylori* after 30 minutes of preincubation with *H pylori* water extracts. The highest LmCL response significantly correlated with the concentration of the water extracts ($r = 0.770$; $p < 0.001$). Data are the average of the results of two different experiments run in at least duplicate, and the bars represent SD.

linked immunosorbent assay (BioSource, Camarillo, California, USA), according to the manufacturer's instructions. The minimum detectable concentrations were 5.0, 5.0, and 2.0 pg/ml for IL-8, MIP-1 α , and MIP-1 β , respectively.

Statistical analysis

One way analysis of variance was used to test for significance. Pair comparisons based on the Bonferroni's standard were used to compare the effects of different concentrations of water extracts. The correlation coefficient (r) was determined to assess the dose dependent effect. A p value of less than 0.05 was considered significant.

RESULTS

Luminol dependent chemiluminescence response

Figure 1 shows a typical LmCL response pattern after preincubation with *H pylori* water extracts. *Helicobacter pylori* water extracts induced only a weak neutrophil oxidative burst (0–30 minutes) and no difference was seen in the highest value of the LmCL response between the various concentrations. In contrast, strong LmCL responses were seen when OZ was added after incubation with water extracts. The LmCL response stimulated by OZ was stronger when the cells had been preincubated with higher concentrations of water extracts (fig 2). The increase in the LmCL response correlated significantly with the concentration of the water extracts used for preincubation ($r = 0.770$; $p < 0.001$).

Chemokine production

The production of IL-8 by neutrophils was stimulated by *H pylori* water extracts (fig 3). When the *H pylori* water extracts contained $200 \mu\text{g}$ protein/ml, the mean concentration of IL-8 was 34.5 pg/ml , which was significantly higher than that seen when the concentrations of the *H pylori* water extracts were 0 and $20 \mu\text{g}$ protein/ml ($p < 0.01$). However, there was no significant correlation between the concentration of *H pylori* water extracts and amount of IL-8 produced.

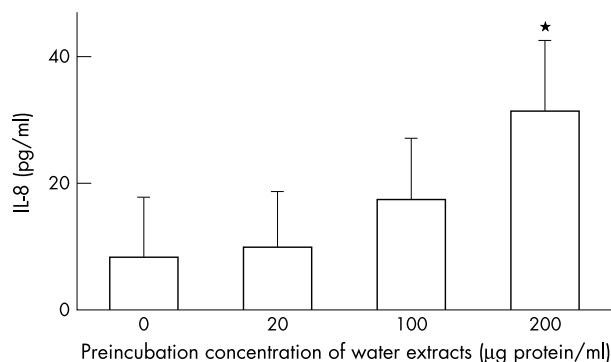


Figure 3 Interleukin 8 (IL-8) production by neutrophils stimulated with *Helicobacter pylori* or zymosan. Data are presented as mean (SE). *The IL-8 concentration is significantly higher compared with neutrophils not stimulated by *H pylori* water extracts ($p < 0.01$). Data are the average of the results of two different experiments run in at least duplicate, and the bars represent SD.

In contrast, the effects of the *H pylori* water extracts on neutrophil production of CC chemokines were much weaker than that seen for the production of IL-8. MIP-1 β was detectable (mean concentration, 12.3 pg/ml) in the neutrophils of three subjects when the concentration of the water extracts was 200 µg protein/ml, but it could not be detected when lower concentrations of protein were used. MIP-1 α was not detected at all.

DISCUSSION

Infection with *H pylori* has been associated with peptic ulcer diseases and the development of gastric cancer. Increased oxidative DNA damage has been implicated in the carcinogenic process,¹⁴ and oxidative DNA damage is clearly seen in the gastroduodenal mucosa of patients with peptic ulcer diseases.^{15, 16} Neutrophils are a major source of oxygen derived free radicals and *H pylori* induced gastritis is characterised histologically by chronic infiltration of neutrophils. *Helicobacter pylori* stimulated ROS production by neutrophils could be relevant in gastric mucosal damage. However, the histopathology of *H pylori* induced gastritis shows that this bacterium is non-invasive, so that it is hard to see how substances produced by *H pylori* could modulate the neutrophil oxidative burst. Several studies have examined the mechanisms involved in the production of ROS stimulated by *H pylori*. An early study demonstrated that water extracts of *H pylori* increased the expression of CD11b, which plays an important role in neutrophil phagocytic activity.⁵ Recently, the activation of neutrophil NADPH oxidase by HP-NAP was also demonstrated.⁶ In addition, the neutrophil priming effects of several substances, such as smoke, have been investigated. Primed neutrophils are capable of producing large amount of ROS more rapidly than non-primed neutrophils. Increased ROS production by peripheral neutrophils has been shown in smokers and this is considered to explain, at least in part, the delay in wound healing often seen in cigarette smokers.¹⁷ In our present study, water extracts of *H pylori* induced only a weak LmCL response, whereas neutrophils incubated with water extracts before stimulation by OZ showed increased LmCL responses. These observations suggest that *H pylori* water extracts may have priming effects on human neutrophil ROS production. In the gastric mucosa, neutrophils usually produce ROS when they ingest foreign bodies. Therefore, to stimulate neutrophils, we used OZ, which is a phagocytic particle that binds to receptors on the neutrophil cell surface. Overall, our present results suggest that neutrophils that have infiltrated the gastric mucosa after *H pylori* infection have an enhanced capacity to produce ROS, even though the bacteria exist within the lumen.

Take home messages

- *Helicobacter pylori* water soluble products can induce human neutrophils to produce interleukin 8, although they have little effect on the CC chemokines, macrophage inflammatory protein 1 α (MIP-1 α) and MIP-1 β
- Stimulated neutrophils may play a role in the persistent neutrophil infiltration seen in the gastric mucosa infected with *H pylori*
- *Helicobacter pylori* water extracts prime human neutrophils for enhanced production of reactive oxygen species
- Thus, these products may have an important role in *H pylori* induced gastritis, which is characterised by neutrophil infiltration and increased oxidative damage, without the invasion of bacteria

The ROS detected by LmCL are mainly hypochlorites (HOCl/OCl⁻), which are generated by myeloperoxidase (MPO) activity caused by degranulation.¹⁸ In a recent study, the expression of MPO was found to be upregulated by water extracts of *H pylori*.¹⁰ Therefore, at least in part, the increased LmCL responses seen in neutrophils incubated with *H pylori* water extracts can be explained by the upregulation of MPO activity. A recent study showed that HP-NAP stimulated the production of H₂O₂ by neutrophils, but the response was slower than that seen with other stimulants.⁶ We also measured the LmCL response in the presence of water extracts before the addition of OZ but the measurement period was only 30 minutes. Longer incubation periods might show an increase in the LmCL response. However, MPO generated ROS are capable of causing more oxidative damage than H₂O₂ or superoxide. In particular, hypochlorous acid can react with ammonia, generated by *H pylori* urease activity, to produce a highly toxic molecule, monochloramine.¹⁹ Thus, the LmCL response would be a better method to examine *H pylori* associated oxidative stress than intracellular H₂O₂ production.

“Water extracts of *Helicobacter pylori* induced only a weak luminol dependent chemiluminescence response, whereas neutrophils incubated with water extracts before stimulation by opsonised zymosan showed increased responses”

Human neutrophils can also produce several chemokines and the amounts and/or types of chemokines produced are differentially regulated by the species of pathogens or stimulants.^{5, 6} In our previous study, *H pylori* cells stimulated the production of IL-8 by human neutrophils but did not stimulate the production of two CC chemokines, macrophage chemoattractant protein 1 and RANTES.²⁰ Therefore, in our present study, we examined the effects of *H pylori* water soluble extracts on the neutrophil production of IL-8 and different CC chemokines, MIP-1 α and MIP-1 β . The production of IL-8 by neutrophils was stimulated by *H pylori* water extracts in a dose dependent manner. These results are in accordance with previous studies, which showed upregulation of the expression of CXC chemokines (IL-8, GRO proteins) by neutrophils in response to *H pylori* water extracts.^{9, 10} *Helicobacter pylori* water extracts have been shown to have chemotactic effects on neutrophils,²¹ and the results suggested that water extracts might also stimulate migrated neutrophils to produce CXC chemokines. In contrast, *H pylori* water extracts had weak or no stimulatory effects on the production of CC chemokines, MIP-1 α and MIP-1 β . Such chemokine production by neutrophils may play a role in the pathology of *H pylori* associated gastritis, which is characterised by chronic neutrophil infiltration. CXC chemokines are primarily chemotactic to neutrophils and mRNA expression of IL-8 and GRO α correlates significantly with the degree of neutrophil infiltration in gastric mucosa infected with *H pylori*.⁸ Therefore, in *H pylori*

induced gastritis, the neutrophil itself seems to contribute to further neutrophil migration into the gastric mucosa. These mechanisms may play a role in maintaining chronic neutrophil infiltration without the invasion of bacteria into the mucosa.

In conclusion, *H pylori* water extracts are capable of inducing human neutrophils to produce IL-8, but had few effects on the CC chemokines, MIP-1 α and MIP-1 β . Stimulated neutrophils may play a role in the persistent neutrophil infiltration seen in the gastric mucosa infected with *H pylori*. *Helicobacter pylori* water extracts also prime human neutrophils for enhanced ROS production. These effects of *H pylori* water extracts may participate in *H pylori* induced gastritis, which is characterised by neutrophil infiltration and increased oxidative damage without the invasion of bacteria.

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