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

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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 MIP-1β) 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-1β production was only stimulated weakly, and MIP-1α 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.

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 × 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 blue exclusion).

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
Neutrophil activation by *H. pylori* water extracts

**, results and discussion**

**Chemokine ELISA**

The microwells containing 50 μl of neutrophil suspension and 150 μl 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-1α, and MIP-1β were measured by enzyme 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-1α, and MIP-1β, 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.

**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 μg 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 μg 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.
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-1α was not 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-1β 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, and oxidative DNA damage is clearly seen in the gastroduodenal mucosa of patients with peptic ulcer diseases. 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. Recently, the activation of neutrophil NADPH oxidase by HP-NAP was also demonstrated. 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. In our present study, water extracts of *H. 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. 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 chemotactant protein 1 (MCP-1) and RANTES. 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-1α and MIP-1β. 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. *Helicobacter pylori* water extracts have been shown to have chemotactic effects on neutrophils, and the results suggested that water extracts might also stimulate granulocyte infiltration to produce CC chemokines. Therefore, in *H. pylori* water extracts infected with *H. pylori*.

**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 1α (MIP-1α) and MIP-1β.
- 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.
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-1α and MIP-1β. 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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