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

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 (MIP-1-α), and MIP-1-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.

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
Neutrophil activation by *H pylori* water extracts

**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 *Helicobacter pylori* water extracts on neutrophil production of CC chemokines were much weaker than those seen for the production of IL-8. MIP-1β 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-1α was not detected at all.

**DISCUSSION**

Infection with *Helicobacter 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 *Helicobacter 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 *Helicobacter pylori* induced gastritis shows that this bacterium is non-invasive, so that it is hard to see how substances produced by *Helicobacter pylori* could modulate the neutrophil oxidative burst. Several studies have examined the mechanisms involved in the production of ROS stimulated by *Helicobacter pylori*. An early study demonstrated that water extracts of *Helicobacter 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 amounts 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 *Helicobacter 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 *Helicobacter 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 *Helicobacter pylori* infection have an enhanced capacity to produce ROS, even though the bacteria exist within the lumen.

The ROS detected by LmCL are mainly hypochlorites (HOCl/OCl−), which are generated by myeloperoxidase (MPO) activity caused by degranulation. In a recent study, the expression of MPO was found to be upregulated by water extracts of *Helicobacter pylori*. Therefore, at least in part, the increased LmCL responses seen in neutrophils incubated with *Helicobacter pylori* water extracts can be explained by the upregulation of MPO activity. A recent study showed that HP-NAP stimulated the production of HOCl by neutrophils, but the response was slower than that seen with other stimulants. 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 HOCl or superoxide. In particular, hypochlorous acid can react with ammonia, generated by *Helicobacter pylori* urease activity, to produce a highly toxic molecule, monochloramine. Thus, the LmCL response would be a better method to examine *Helicobacter pylori* associated oxidative stress than intracellular HOCl 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 stimuli. In our previous study, *Helicobacter pylori* cells stimulated the production of IL-8 by human neutrophils but did not stimulate the production of two CC chemokines, macrophage chemotactic protein 1 and RANTES. Therefore, in our present study, we examined the effects of *Helicobacter 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 *Helicobacter pylori* water extracts in a dose dependent manner. These results are in accordance with previous studies, which showed upregulation of the expression of CX3C chemokines (IL-8, GROα proteins) by neutrophils in response to *Helicobacter 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 migrated neutrophils to produce CX3C chemokines. In contrast, *Helicobacter pylori* water extracts had weak or no stimulatory effects on the production of CC chemokines, MIP-1α and MIP-1β. Such chemokine production by neutrophils may play a role in the pathology of *Helicobacter pylori* associated gastritis, which is characterised by chronic neutrophil infiltration. CX3C chemokines are primarily chemotactic to neutrophils and the expression of IL-8 and GROα correlates significantly with the degree of neutrophil infiltration in gastric mucosa infected with *Helicobacter pylori*. Therefore, in *Helicobacter 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-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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