Helicobacter 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. Helicobacter pylori activates neutrophil oxidative metabolism and the surface proteins of H pylori, including H pylori neutrophil activating protein (HP-NAP), modulate ROS production in the surface proteins of H pylori. The aim of our study was to characterise the effects of human neutrophils 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. 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. 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. However, human neutrophils can also produce several CC chemokines in response to certain stimuli, such as macrophage inflammatory protein 1α (MIP-1α) and MIP-1β, which are CC chemokines that are chemotactic to T helper type 1 lymphocytes. Although human neutrophils produce MIP-1α and MIP-1β, the effects of H pylori on the production of MIP-1α and MIP-1β 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 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) and > 98% pure. Neutrophils were suspended in HBSS to the required concentration (2.0 × 10^6/µl) 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* water extracts 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 × g for 15 minutes the supernatant was stored at −70°C. Before use, the extracts were centrifuged at 39,000 × g for 20 minutes, and the supernatant was passed through a 0.2 µm 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 µl aliquot of luminophor solution (final concentration, 0.1 mM) were added to each well of the microplate. After the addition of 200 µl 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.

The peak value of the luminol dependent chemiluminescence (LmCL) response of neutrophils stimulated by *Helicobacter pylori* water extracts 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.

**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 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.
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 H. pylori. 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. 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. 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 luminal 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 chemotactic protein 1 (MCP-1) 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, GROα) 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 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-1α and MIP-1β. 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 GROα correlates significantly with the degree of neutrophil infiltration in gastric mucosa infected with H. pylori. Therefore, in H. pylori
Neutrophil activation by *H. pylori* water extracts

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

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