Helicobacter pylori secretes a chemotactic factor for monocytes and neutrophils

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Abstract
Helicobacter pylori is associated with an inflammatory reaction in the stomach and duodenum, yet the mechanism of this inflammatory infiltrate is unknown. The ability of Helicobacter pylori to secrete a factor that attracts leucocytes is investigated. Helicobacter pylori conditioned supernatant attracted neutrophils and monocytes with 50–100% of the activity of control chemotactic factor, 10−8M formyl-methionine-leucine-phenylalanine. Strains derived from individuals with ulcer or non-ulcer associated H pylori infections displayed similar chemotactic activity. Preliminary characterisation shows that the factor has a molecular weight of less than 3000, is heat stable, is acid resistant, and can be diluted at least 10-fold. Checkerboard analysis confirmed that the activity was chemotactic rather than chemokinetic. This chemotactic activity could play a role in the pathogenesis of Helicobacter pylori gastritis.

(Helicobacter pylori is strongly associated with superficial antral gastritis. There is growing evidence that Helicobacter pylori is the causal agent of this inflammation. Bacteria are rarely seen in the setting of histologically normal antral mucous. Microscopic examination of infected gastric tissue shows H pylori on the gastric luminal side of the mucosa, in and beneath the mucus layer, and with an inflammatory infiltrate just below an intact gastric mucosa. There is a strong correlation between the severity of the inflammation and the number of bacteria seen. Gastritis resolves after eradication of the bacterial infection. Based on these observations we sought to determine if Helicobacter pylori secretes a factor that attracts leucocytes. Many bacteria have been shown to secrete such factors, often characterised as low molecular weight, amino-formylated peptides. The prototype is formyl-methionine-leucine-phenylalanine or f-mort-leu-phe, a tripeptide isolated from E coli. Because H pylori is associated with an inflammatory infiltrate, we investigated the possibility that H pylori secretes a chemotactic factor for monocytes and neutrophils.

Methods
MATERIAL
Helicobacter pylori was grown under microaerophilic conditions at 37°C in brucella broth with 1% pig serum for 36 to 72 hours. The bacteria conformed to standard phenotypic criteria. When log phase growth was achieved, the bacteria were harvested, washed twice with phosphate buffered saline, and resuspended in phosphate buffered saline at a density of 107 colony forming units per millilitre. The bacteria were incubated in the phosphate buffered saline for four hours at 37°C, then harvested, and the supernatant passed through a 0.2 μm filter to remove bacterial debris. This supernatant was then tested for chemotactic activity.

Human leucocytes were obtained from heparinised peripheral blood by Ficoll-Hypaque density gradient separation (Histopaque, Sigma, St Louis, Missouri, USA) to obtain mononuclear cells, followed by dextran sedimentation to obtain neutrophils. The cells were washed and resuspended at a concentration of 106 monocytes or neutrophils/ml in Hanks balanced salt solution containing 0.15% bovine serum albumin.

Chemotaxis was determined in multiwell chambers (Neuro Probe, Inc, Cabin John, MD, USA) using a 5 μm pore size filter (Nucleopore Corp, Pleasanton, CA) that separated the upper well, containing the leucocytes, from the lower well which contained the putative chemotactic factors. Formyl-methionine-leucine-phenylalanine (FMLP), a well characterised chemotactic peptide for monocytes and neutrophils, was used at 10−8 M as the positive control, and negative control using phosphate buffered saline were run simultaneously. All experiments were performed in triplicate. After one hour of incubation at 30°C, the filters were removed, fixed in methanol, and stained with giemsa. The number of cells that had migrated to the lower aspect of the filter, toward the putative chemotactic factor were counted microscopically to determine the average number of cells per high power field (cells/hpf). The ratio of the activity of the H pylori supernatant compared to the activity of FMLP is used to determine the % of FMLP activity.

Checkerboard analysis was carried out to distinguish chemotactic from chemokinetic activity. Serial dilutions of the test factor were placed in the lower well of the chamber, and similar concentrations of the factor were used to dilute the leucocytes used in the upper well of the chamber. Directional movement of the cells in response to a concentration gradient indicates chemotaxis, whereas increased locomotion of the cells induced by the factor unrelated to the concentration gradient indicates chemokinesis. Results are expressed as the per cent of the undiluted activity.

Results
The chemotactic activity of Helicobacter pylori supernatants is summarised in Figure 1. The supernatants showed chemotactic activity for...
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neutrophils, attracting about 50% of the cell activity seen with FMLP (Fig 1A). Monocytes were attracted to *H pylori* supernatants with approximately 90% of the activity of FMLP (Fig 1B).

Figure 2 shows the chemotactic activity of *Helicobacter pylori* strains isolated from patients with active ulcer disease, compared with strains isolated from patients without ulcer disease. All strains tested produced neutrophil and monocyte chemotactic activity. The non-ulcer derived strains produced similar chemotactic activity as the ulcer derived *H pylori* strains.

The time course of chemotactic factor production is shown in Figure 3. Chemotactic activity was evident by one hour after washing and resuspending the organisms at 10^8 bacteria/ml in phosphate buffered saline and remained active for at least 18 hours of incubation. Colony counts were performed on the bacteria after incubation, and there was no change in the number of bacteria from nought to four hours, when chemotactic activity became apparent. At 18 hours, more than half of the bacteria had died. These data suggest that chemotactic activity becomes apparent before there is any change in the number of viable colony forming units and remains detectable after the bacteria begin to die. Similar time course results were obtained for neutrophils (data not shown).

A checkerboard analysis was carried out to determine if the leucocyte migration was the result of chemotaxis with directed movement of leucocytes toward a concentration gradient, or to chemokinesis with an increase in non-directed movement. The Table shows that most of the movement of the leucocytes was chemotactic rather than chemokinetic.

Preliminary characterisation of the *Helicobacter pylori* chemotactic factor has shown that the activity of the *H pylori* supernatant is unaffected by boiling for 30 minutes, freezing overnight, lowering the pHi of the supernatant from 6.7 to 2.0 for 30 minutes, or raising the pHi from 6.7 to 10.0 for 30 minutes. Molecular sizing studies using Amicon ultrafiltration membranes (Amicon, Danvers, Mass, USA) showed that the chemotactic activity passed through membranes with pore sizes which excluded molecules of 10 000 and 3000 molecular weight suggesting that the factor has a molecular weight of less than 3000 (Fig 4).

**Discussion**

Our results indicate that *Helicobacter pylori* secretes a chemotactic factor for monocytes and neutrophils.

**Checkerboard analysis of the H pylori supernatants for neutrophils (top), and monocytes (bottom).** Various dilutions of the *H pylori* supernatants were placed in the lower well of the chamber as well as in the leucocyte-containing upper well of the chamber. Data are expressed as the per cent of migratory activity compared to the results when the undiluted *H pylori* supernatant (100%) was used in the lower well and the cells in the upper well were free of any *H pylori* supernatant (0%).

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**Figure 1:** Chemotactic activity of *H pylori* supernatants (■, phosphate buffered saline (negative control) ■, and 10^-7 M FMLP (positive control) ■), for neutrophil (1A), and monocytes (1B). Data are expressed as cells/high powered field (SDF) (n=26 for neutrophils, n=29 for monocytes). PBS=phosphate buffered saline, FMLP=formyl-methionine-leucine-phenyalanine.
secretes a chemoattractant factor for monocytes and neutrophils. *H. pylori* strains isolated from ulcer and non-ulcer patients produced chemotactic activity. Preliminary characterisation studies show that this factor is heat stable, acid and alkali stable, and has a molecular weight less than 3000.

This chemotactic factor may play an important role in the pathogenesis of the inflammatory reaction associated with *Helicobacter pylori* in the stomach and duodenum. As inflammation is present where there does not appear to be any break in mucosal integrity, and the bacteria are separated from the inflammatory infiltrate by an intact mucosa, a small acid stable hydrophobic peptide could permeate through an intact gastric barrier and recruit the leucocytes. Once the white cells are present, their products could induce the inflammatory damage, and possibly local disruption of cellular function. Further studies are needed to characterise and purify this chemotactic factor.

Many aerobic and anaerobic bacteria have been shown to secrete chemotactic factors for monocytes and neutrophils. Evidence for multiple bacterial peptides being chemotactic for neutrophils was reported by Ward and colleagues in 1968. Shiffman et al reported a size range of 150–1500 daltons for peptides from *E. coli* and suggested that they were formyl substituted peptides. Chadwick et al showed that culture supernatants of different bacteria were resistant to digestion by aminopeptidase, but destroyed by carboxypeptidase, suggesting they consist of peptides with amino terminal blocking groups. Knowledge of the precise structures of the natural bacterial chemotactic peptides has been defined for FMLP in *E. coli* cultures, and to several other formyl peptides in other bacteria. *Staphylococcus aureus* has been shown to secrete as many as nine different chemotactic peptides, all 3–5 amino acids in length. The most active is f-met-leu-phe-lysine, which binds to the same receptor of the white cell as FMLP. These formyl methionine peptides probably represent amino terminal signal peptides from newly synthesised bacterial proteins (the initiator peptide is always formylated in prokaryotic protein synthesis) which are cleaved by prokaryotic signal peptides in bacterial cell walls. Leucocytes have receptors for such peptides. In addition to stimulating chemotaxis, these peptides can also induce other leucocyte functions, such as lysosomal enzyme release, adhesion, aggregation, and superoxide production.

The gastric barrier against peptides in vivo includes mucosal carboxypeptidase. Whether chemotactic peptides can permeate an intact gastric mucosa is not known, however, luminal perfusion of the terminal ileum of rats with FMLP (10–10 M) produces signs of acute inflammation. Formyl-methionine-leucine-phenylalanine has also been shown to elicit neutrophil chemotaxis across epithelial monolayers in vitro with resultant disruption of epithelial barrier function, and increased blood flow and vascular permeability in rat small intestine.

Further studies are underway to characterise and purify this chemotactic factor in order to help determine its role in the inflammatory response associated with *Helicobacter pylori* gastritis. The role the chemotactic factor may be playing in the development of *H. pylori* related peptic ulcer disease is unclear. Our preliminary data indicating no difference in the production of the chemotactic activity between the ulcer derived and non-ulcer derived strains suggest that the chemotactic factor is unlikely to have a direct ulcerogenic effect, although secondary effects related to the release of inflammatory mediators may be involved. A more likely role for this chemotactic activity is in the production of the *H. pylori* related gastritis with the recruitment of the inflammatory cells into the stomach wall. How the variations in the inflammatory responses seen on biopsy in epidemiological studies of *Helicobacter pylori* gastritis, and the variations in the clinical findings, relate to differences in the type or concentration of the chemotactic factor, and how these relate to mucosal or other host factors is unknown at this time.

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