Neutrophil activation by *Helicobacter pylori*

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Abstract

Helicobacter pylori infection of the stomach is accompanied by a persistent polymorphonuclear leukocyte (PMNL) infiltrate of the mucosa. The aim of this work was to study the activation of human PMNL by substances produced by *H. pylori*. Filtered *H. pylori* conditioned media stimulated a significant PMNL oxidative burst ($p < 0.002$). This was equal to 26% of the maximal response stimulated by the PMNL chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP, $1 \mu\text{mol/l}$). The response to FMLP was prolonged by the combined presence of complement inactivated human anti-*H. pylori* plasma and conditioned medium ($p < 0.002$). High pressure liquid chromatography of an extract of conditioned medium showed a fraction that stimulated PMNL, eluted, and antigenically cross reacted with FMLP. Washed *H. pylori* cells, and those opsonised with complement inactivated human anti-*H. pylori* plasma, did not induce a significant oxidative burst. Opsonised *H. pylori*, however, prolonged the oxidative burst induced by FMLP ($p < 0.02$). In conclusion, *H. pylori* synthesises and secretes a substance, probably FMLP, that may account for the PMNL accumulation that accompanies *H. pylori* infections. Immune complexes composed of *H. pylori* antigen and specific antibody potentiate the PMNL oxidative burst. This combination of *H. pylori* derived products, and host PMNL and antibodies, may be involved in the mucosal damage observed in *H. pylori* associated gastritis.

Helicobacter pylori, which infects gastric epithelium, is closely associated with type B (or antral) gastritis and with peptic ulceration. Although the infection, if untreated, is chronic, it is characterised by high numbers of acute inflammatory polymorphonuclear leukocytes (PMNL) in the underlying superficial mucosa.¹⁻³ PMNL phagocytose and kill *H. pylori* in vitro.⁴⁻⁶ Despite this, and a strong secretory and systemic antibody response to *H. pylori*,⁷ the infection persists.

Host PMNL may be continuously attracted to the site of an infection by either damaged host tissue⁸ or by the infecting organism itself. In general, products of bacterial origin known to activate neutrophils include formyl-methionyl-leucyl-phenylalanine (FMLP) and related peptides,⁹ endotoxin,¹⁰ whole cells, and cell wall components. However, no specific neutrophil activating substances have previously been reported for *H. pylori*. This study describes the initiation and potentiation of the PMNL oxidative burst by substances produced by

cultured *H. pylori*, and the effects of specific human antisera.

Methods

H PYLORI

H. pylori isolates were obtained from consenting patients undergoing routine upper gastrointestinal endoscopy. *H. pylori* was identified in gastric biopsy samples by urease assay,¹¹ by Gram stain and culture of the macerated tissue, and by modified Giemsa staining of the fixed tissue.

Specimens were cultured at 35°C in a micro-aerobic atmosphere on Columbia sheep blood agar plates. Colonies resembling *H. pylori* were subcultured, and *H. pylori* was identified as urease, oxidase, and catalase positive Gram negative curved or spiral bacilli. For broth culture, *H. pylori* from blood agar plates were suspended in sterile normal saline, transferred into Brucella broth (Difco Laboratories, Detroit, USA) supplemented with 10% heat inactivated fetal calf serum, and incubated as above.

For PMNL oxidative burst experiments, 20 ml of *H. pylori* broth culture was centrifuged at 1400 g for 20 minutes and the supernatant was filtered (0.2 μm). This 'conditioned medium' was used within four hours. The pellet was washed once by recentrifugation in fresh broth medium and resuspended in medium to give an absorbance at 540 nm of 0.5 (approximately 15×10^7 colony forming units (cfu) per ml). For opsonisation experiments, the pellet was washed once in phosphate buffered saline (PBS) and resuspended in 10% complement inactivated (56°C for 30 minutes) anti-*H. pylori* positive or negative human plasma in PBS. This was incubated at 37°C for 30 minutes, washed twice in PBS, and resuspended as above in fresh broth medium.

DETECTION OF ANTIBODIES TO H PYLORI

Specific anti-*H. pylori* IgG in patient plasma was detected by indirect enzyme linked immunosorbent assay (ELISA). Microtitre plate wells (NUNC Immunoplate II), coated with *H. pylori* antigen,¹² each received 100 μl of 1:1000 diluted plasma sample. Bound IgG was detected with a 1:20 000 dilution of goat anti-human IgG/horse-radish peroxidase conjugate (Tago), colour was developed with o-phenylenediamine, and absorbances were read at 492 nm.

PREPARATION OF PMNL

PMNL were isolated with dextran and Ficoll-Hypaque¹³ from heparinised blood obtained from the above mentioned consenting patients.

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The isolated PMNL ($5\text{--}15 \times 10^6$ cells/ml), comprising $\geq 95\%$ neutrophils (Wright's Giemsa stain), were usually incubated at 37°C for 30 minutes with heat treated anti-*H. pylori* positive or negative plasma, and then diluted to 2×10^6 cells/ml with PBS containing 1.3 mmol/l CaCl_2 , 0.5 mmol/l MgCl_2 , and 5.6 mmol/l glucose. PMNL viability, assessed by eosin-Y exclusion,¹⁴ was normally higher than 95%.

PMNL OXIDATIVE BURST ASSAY

The oxidative burst response of 10^6 PMNL to 0.2 ml of putative stimulant was measured in a final volume of 1.0 ml . This was done by monitoring the rate of reduction of ferricytochrome C by superoxide ion,¹⁵ in the presence of $5\text{ }\mu\text{g/ml}$ cytochalasin B. Mean initial rates were calculated from absorbances read 0.5 to 1.5 minutes after the addition of test substance (Fig 1). To correct for between donor variations in PMNL responsiveness, these rates were expressed as a percentage of the initial rate stimulated by FMLP (final concentration $1\text{ }\mu\text{mol/l}$), which was added four to five minutes after each test substance (Fig 1). The response to FMLP is normally rapidly attenuated,¹⁶ so that the rate of superoxide production three to four minutes after addition of FMLP (secondary rate) is typically 10% of the initial rate. Some test substances were found to block this attenuation, so secondary rates after FMLP stimulation were also recorded (Fig 1).

HPLC

Some 40 ml of five day *H. pylori* broth culture containing 1.6×10^7 cfu/ml and an equal volume of control medium were separately centrifuged at 3000 g for 20 minutes and filtered ($0.2\text{ }\mu\text{m}$). Each filtrate was then acidified with trifluoroacetic acid to pH 2.5 and loaded onto a Sep-pak TM C18-cartridge (Waters Associates, Milford, Mass, USA). This was washed, eluted, and subjected to reverse phase HPLC analysis.¹⁷ Fifty sequential 2 ml fractions were collected and lyophilised. They were reconstituted in $500\text{ }\mu\text{l}$ of PBS before bioassay and radioimmunoassay.

BIOASSAY OF HPLC FRACTIONS

Duplicate $100\text{ }\mu\text{l}$ aliquots of HPLC fractions were used to challenge PMNL (0.5×10^6) in aliquots of heparinised whole blood from human volunteers which had been pre-incubated for five minutes with cytochalasin B at 37°C . After incubation at 37°C for 10 minutes the cells were pelleted and the supernatant assayed for unsaturated vitamin B_{12} binding capacity (UBBC),¹⁸ which, after subtraction of values in blanks, represents release of vitamin B_{12} binding protein from the specific granules of PMNL in response to the challenge.

The maximum possible UBBC was determined by performing the assay on supernatants from cells lysed in 0.5% Triton X-100, and results from challenged samples were expressed as percentages of the maximum. From a standard curve relating per cent maximal UBBC ν log molar concentration of synthetic FMLP, bio-

Figure 1: Measurement of oxidative burst. Reduction of ferricytochrome C by superoxide produced by 10^6 polymorphonuclear leukocytes is monitored at 550 nm . The initial rate of superoxide production was measured for 1 minute, beginning 30 seconds after the addition of test substance (= conditioned medium or *Helicobacter pylori* cells, solid line). This rate was expressed as a percentage of the initial rate stimulated by $1\text{ }\mu\text{mol/l}$ *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) which was added to the same cuvette 4–5 minutes after the test substance. The secondary rate (7.5–8.5 minutes) was also recorded. Broken line – controls (test substances = unconditioned medium).

activity in each HPLC fraction was converted to FMLP equivalents.

RADIOIMMUNOASSAY OF HPLC FRACTIONS

The radioimmunoassay for FMLP was performed as previously described by one of the authors.¹⁹ The antiserum used here was, however, from a different rabbit. It showed similar specificity to that previously reported, recognising only FMLP and closely related sequences (F-nle-leu-phe, F-met-leu-tyr), and showing minimal cross-reactivity with other formylated di- and tri-peptides and none with non-formylated peptides.

Results

ELISA RESULTS

Plasma containing a high titre of specific anti-*H. pylori* IgG was obtained from six patients. *H. pylori* was successfully cultured from biopsy specimens of three of these patients. The urease, Gram stain, and histology tests were positive for *H. pylori* for five of them.

The ranking order of ELISA absorbances for the six positive plasmas was dependent to a degree on the antigen preparation employed, but the absorbances remained consistently higher than for negative plasma samples (Fig 2). These results, and those of others,⁷ suggest that antibodies from any positive patient would recognise antigen from any *H. pylori* isolate. The data presented below are for *H. pylori* and plasma from one patient (= patient 1, antigen preparation F in Fig 2). Comparable results were found for other isolates.

Figure 2: Detection of specific anti-*H. pylori* IgG. ELISA absorbances for plasma samples from patients (numbered 1–23) are shown v antigen prepared from 11 different *H. pylori* isolates (A–K). The six positive patients are shown in the upper band, and the lower band shows five randomly selected *H. pylori* negative patients (by urease, Gram stain, culture).

EFFECT OF *H. PYLORI*-CONDITIONED MEDIUM ON PMNL OXIDATIVE BURST

Filtered *H. pylori* culture supernatants induced a variable but statistically significant oxidative burst from human PMNL (Table I). In the presence of heat treated anti-*H. pylori* plasma, conditioned media also prolonged the response to 1 $\mu\text{mol/l}$ FMLP by increasing the secondary oxidative burst rate 2.5 fold (Table I). The initial rate, in response to 1 $\mu\text{mol/l}$ FMLP, was unaffected by anti-*H. pylori* plasma (data not shown).

Compared with PBS, unconditioned medium had no effect on PMNL. It did not stimulate PMNL oxidative burst activity, it had no effect on that induced subsequently by FMLP, and it did not cause leakage of intracellular lactate dehydrogenase (data not shown).²⁰

EFFECT OF *H. PYLORI* CELLS ON PMNL OXIDATIVE BURST

Washed *H. pylori* cells, whether opsonised with anti-*H. pylori* antibody or not, induced a small, highly variable, and consequently statistically insignificant oxidative burst. Opsonized *H. pylori* cells did, however, increase the secondary rate of

TABLE II Effect of antibody on *Helicobacter pylori* cell induced polymorphonuclear leukocyte (PMNL) oxidative burst*

	Initial rate	Secondary rate
Negative serum† (mean (SD) (no))	2.0 (4.0) (7)	13.4 (4.7) (7)
Positive serum† (mean (SD) (no))	9.2 (9.9) (18)	31.2 (15.9) (18)
p†	>0.10	<0.02

*The mean initial rates of the oxidative burst stimulated by *H. pylori* cells are expressed as a percentage of the initial rate induced by 1 $\mu\text{mol/l}$ N-formyl-methionyl-leucyl-phenylalanine (FMLP) (which was added to each cuvette 4–5 minutes after the test substance, Fig 1). The mean secondary rates of the FMLP induced oxidative burst are also shown, expressed as a percentage of the initial rate. PMNL in diluted, anti-*H. pylori* antibody negative, heat treated, plasma.

†The effects of *H. pylori* cells preincubated in heat treated human serum which was either *H. pylori* antibody negative or positive, were compared using the Mann-Whitney U test.

the FMLP induced oxidative burst by a factor of 2.3 (Table II).

FMLP LIKE ACTIVITY

Figure 3 shows the results of UBBC bioassay (upper panel) and anti-FMLP immunoassay (lower panel) of HPLC fractions from processed *H. pylori* conditioned and unconditioned media. In conditioned medium the major peaks of bioactivity and immunoactivity were found in fraction 25. In terms of FMLP equivalents, both assays gave values of 640–650 nmol/l, representing concentrations of 8 nmol/l in the original culture supernatant. Bioassay and immunoassay of the extract before HPLC fractionation each gave 15 nmol/l in FMLP equivalents. The nature of the immunoreactive and bioactive factor in fraction 25 was not determined further. Synthetic FMLP, however, also elutes in this fraction (see Discussion).

Fractions from unconditioned medium showed minimal amounts of immunoreactivity (<30 nmol/l in the fractions). A number of fractions showed low values of UBBC bioactivity, and in fraction 13 a prominent peak was observed. This apparently UBBC bioactive material from unconditioned medium was not present in conditioned medium and was not immunoreactive. Also, unconditioned medium did not stimulate the PMNL oxidative burst (Table I). It presumably represents a constituent of the medium which is metabolised by bacteria during growth.

Discussion

H. pylori clearly produced substances in vitro which stimulate PMNL function. It secreted substance(s) which induced the oxidative burst. Also, UBBC bioassay of HPLC fractions from processed *H. pylori* culture supernatant indicated that a factor eluting in fraction 25 was the major stimulus for vitamin B₁₂ binding protein secretion by PMNL. The radioimmunoassay, using an antiserum raised against FMLP, confirmed that the bioactive material was most likely to be an N-formyl-met oligopeptide. Authentic FMLP elutes in fraction 25. One of the authors (VC) has processed *Escherichia coli* supernatant, found similar bioactivity and immunoactivity in

TABLE I Effect of filtered *Helicobacter pylori* culture supernatant on polymorphonuclear leukocyte (PMNL) oxidative burst*

Test substance	Initial rate		Secondary rate	
	Negative serum	Positive serum	Negative serum	Positive serum
Unconditioned medium (mean (SD) (no))	1.2 (2.7) (18)	1.6 (2.9) (13)	11.2 (13.0) (18)	10.2 (11.4) (13)
<i>H. pylori</i> conditioned medium (mean (SD) (no))	26.3 (26.1) (19)	25.2 (21.2) (16)	15.0 (10.2) (19)	25.5 (9.0) (16)
p†	<0.002	<0.002	>0.1	<0.002

*The mean initial rates of the oxidative burst stimulated by test substances are expressed as a percentage of the initial rate induced by 1 $\mu\text{mol/l}$ N-formyl-methionyl-leucyl-phenylalanine (FMLP) (which was added to each cuvette 4–5 minutes after the test substance, Fig 1). The mean secondary rates of the FMLP induced oxidative burst are also shown, expressed as a percentage of the initial rate. *H. pylori* from up to 16 different donors, in diluted anti-*H. pylori* antibody negative or positive, heat treated plasma.

†The effects of conditioned and unconditioned media (200 μl) were compared, within each column, using the Mann-Whitney U test.

Figure 3: Polymorphonuclear leukocyte unsaturated vitamin B₁₂ binding capacity (UBBC) bioactivity (top) and anti-N-formyl-methionyl leucyl-phenylalanine (FMLP) immunoactivity (bottom) of high performance liquid chromatography fractions of an extract of *H. pylori* conditioned medium (x) and of unconditioned medium (●). Results are expressed as molar equivalents of FMLP. Note the scale change in both panels.

fraction 25, and shown this fraction to contain FMLP by amino acid sequencing.²¹ It is, therefore, likely that the active material in fraction 25 derived from *H. pylori* is also FMLP. Since both UBBC bioassay and immunoassay gave identical values in terms of FMLP equivalents, it is unlikely that other bioactive factors were present in the fraction.

The calculated FMLP equivalent concentration in the original culture supernatant was approximately 10^{-8} mol/l, which is the lower limit for which a measurable oxidative burst can be detected.²² This may account for the variability that was observed in oxidative burst responsiveness to conditioned media.

FMLP is the best characterised PMNL chemotactic N-formylated peptide. These compounds are produced in vitro by *E. coli*,⁹ *Streptococcus sanguis*²³ and other bacteria.¹⁷ In tissues infected by these organisms, FMLP and related peptides are believed to be at least partly responsible for the resulting accumulation of inflammatory cells. These peptides are also produced by commensal flora in the colon.¹⁷ Endogenous luminal FMLP does not normally result in a PMNL inflammatory response in the bowel because the normal colon is relatively impermeable to FMLP, and the small intestine has high activities of carboxypeptidase C which

inactivates FMLP.²⁴ Introduction of higher concentrations of FMLP does, however, activate neutrophils in ileal mucosa²⁵ and attract neutrophils to the colon.²⁶ It seems likely that *H. pylori* secretes FMLP in infected patients, but neither this nor the passage of FMLP across the gastric epithelium have been shown.

H. pylori also secreted into the medium a factor that, only in the presence of anti-*H. pylori* antibodies, prolonged the FMLP stimulated oxidative burst. The dependence on anti-*H. pylori* antibodies implies that the factor is soluble *H. pylori* antigen.

Direct stimulation of the oxidative burst by *H. pylori* cells was not sufficiently consistent to reach significance, even after opsonisation with heat treated anti-*H. pylori* plasma (Table II). Others have also found such a weak response by PMNL to *H. pylori* opsonised with specific antibody.⁵ The secondary rate of the oxidative burst stimulated by 1 µmol/l FMLP was enhanced by *H. pylori* cells, but only if these were opsonised with anti-*H. pylori* plasma (Table II). The in vivo relevance of this in vitro observation is not known. *H. pylori* is normally found on gastric epithelium, where it is predominantly coated with secretory IgA.²⁷ Although the role of IgA in opsonisation and complement activation is not yet clear,^{28,29} *H. pylori* is capable of directly activating the classic complement pathway.⁶ The exudation of complement components and IgG into mucosal sites in gastritis and the presence of soluble antigen raises questions regarding the outcome of *H. pylori* infection and associated tissue damage.

It is not known if the gastric epithelial damage observed in type B gastritis is caused by *H. pylori* itself or by its accompanying PMNL mucosal infiltrate. *H. pylori* may secrete potentially damaging substances such as ammonia,^{30,31} a high molecular weight cytotoxin,³² and a mucinolytic protease.³³ PMNL can also damage host tissue as is believed to be the case in a number of chronic inflammatory diseases. Degradative enzymes and reactive oxygen metabolites may be released by PMNL activated by FMLP, in conjunction with immune complexes and activated complement. These secretory products, no longer confined to phagolysosomes within the PMNL, could deleteriously alter epithelial function²⁵ and the mucus layer.³⁴ This may result in a functional loss of the gastric mucosal barrier and render the mucosa more susceptible to peptic ulceration.

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