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Neutrophil degranulation by *Helicobacter pylori* proteins

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Abstract

Mucosal biopsy specimens from patients with *Helicobacter pylori* infection in gastric antrum contain an increased amount of myeloperoxidase. This study was performed to elucidate the interaction of *H pylori* sonicate protein(s) and neutrophils concerning myeloperoxidase release. Neutrophil degranulation with myeloperoxidase release was examined in a direct stimulating assay. Priming activity of *H pylori* was examined after preincubating neutrophils in sonicate, either crude or modified by heat treatment, pronase inactivation and dialysis, and stimulating with *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or serum opzonised zymosan (OZ). It was found that *H pylori* sonicate protein(s) stimulates neutrophil degranulation with myeloperoxidase release in a concentration dependent way. The activity was distinct from fMLP and capable of priming the subsequent fMLP and OZ response. Experiments with the modified bacterial sonicate suggest the activity is caused by a protein, but the findings show that non-protein molecules, for example, lipopolysaccarides were also part of the *H pylori* sonicate priming activity. The increased mucosal myeloperoxidase in *H pylori* associated disease can be a direct consequence of bacteria derived stimulation of inflammatory neutrophils.

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Helicobacter pylori is known to cause gastritis in the human stomach,[1 2] to be an important factor in the development of peptic ulcer,[1 3] and possibly also gastric cancer.[4] Histological examination of the infected gastric mucosa shows abundant accumulation of neutrophils. Despite this and high concentrations of antibodies against *H pylori*,[5] the infection persists. Neutrophils release inflammatory mediators into the surrounding milieu on contact with a variety of stimuli.[6] The pathogenesis of the tissue damage in chronic gastritis and duodenal ulcer is unknown but it is reasonable to believe that degranulation of proteolytic enzymes from neutrophils may contribute to the tissue damage as described for other chronic infections and for inflammatory disorders.[7 8] The intracytoplasmic neutrophil granules contain a wide assortment of lysosomal enzymes including myeloperoxidase in the azurophilic granules. Leakage or secretion of myeloperoxidase to the outside of the cell can occur either during phagocytosis or after exposure of neutrophils to an antibody coated surface or a soluble stimulus. Myeloperoxidase is a crucial component in oxygen dependent microbial killing. Micro-organisms ingested by neutrophils initiate a process by which H_2O_2 formed by the respiratory burst reacts in the phagosome with myeloperoxidase released by degranulation to form a potent oxidant. The components of the myeloperoxidase system can be released to the outside of the cell, where they can attack extracellular targets.[9]

We have previously shown that *H pylori* induces phagocyte chemotaxis and oxidative burst response and that this is caused by a molecule that is different from *N*-formylated-oligopeptides (fMLP).[10–12] In this study we have measured myeloperoxidase release from neutrophils stimulated with fMLP, serum opzonised zymosan (OZ), and *H pylori* sonicate protein(s). We found that *H pylori* sonicate protein(s) did stimulate neutrophils to degranulation of the azurophilic granules caused by a protein distinct from fMLP.

Methods

ISOLATION OF NEUTROPHILS

Peripheral blood from healthy donors was drawn into citrated polypropylene tubes. Neutrophils were separated by dextran sedimentation followed by metrizoate/polysucrose gradient centrifugation (Lymphoprep, Nyegaard, Oslo, Norway). The remaining erythrocytes were removed by hypotonic lysis. Neutrophils were resuspended in Gey's balanced salt solution. The purity of neutrophils was >98%.

PREPARATION OF *H PYLORI* SONICATE

Clinical isolates of *H pylori* were prepared as previously described.[10 12] Briefly, pure cultures of *H pylori* (strain CH-20249) were cultured under microaerophil conditions on chocolate agar plates and harvested after 48 hours of incubation at 37°C into a phosphate buffered saline and disintegrated by sonication three times for 45 seconds at 20 000 Hz, on ice, using a Rapidis 350, 19 mm probe with a 9·5 mm tip, and centrifuged at 14 000 *g* for 30 minutes at 4°C. The supernatant protein concentration was measured by refractometry using human immunoglobulin as a standard. The sonicate was stored in small aliquots at −20°C. Two fresh clinical isolates were cultured and sonicated as described. In other experiments water extracted surface proteins

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were prepared by resuspending *H pylori* 0·2 mg/ml in phosphate buffered saline, pH 7·4, vortex mixed for 15 seconds, and centrifuged at 5000 *g* for 10 minutes. The supernatant was filtered through a 0·22 μm Millipore filter. The supernatant was stored in small aliquots at −20°C.

REAGENTS

fMLP (a synthetic peptide and a major neutrophil activator, which is a product of bacterial origin from, for example, *Escherichia coli* and *Streptococcus sanguis*), cytochalasin B (a fungal metabolite, which facilitates secretion of neutrophil enzymes from granules), zymosan A (synthetic particles, when opzonised with serum represent the cell wall component particularly involved in the complement related stimulation of phagocytes), and 3,3'-dimethoxybenzidine (DMB, o-dianisidin) (the drug, which reacts with myeloperoxidase giving a colour change) were obtained from Sigma Chemical Co, St Louis, MO, USA. NaN_3 was obtained from E Merck, Darmstadt, Germany.

DEGRANULATION OF NEUTROPHILS

Neutrophils (2×10^7/ml) were pretreated in cytochalasin B (2·5 μg/ml) 10 minutes at 37°C, washed once, and resuspended in Gey's balanced salt solution with 0·25% human serum albumin. Neutrophils were mixed with Gey's balanced salt solution, fMLP (1×10^{-5} M, final concentration) or *H pylori* sonicate at a concentration of 100 or 1000 μg/ml, for 30 minutes at 37°C. The neutrophils were washed once and stimulated with either Gey's balanced salt solution, Triton X-100 (2%), fMLP (1×10^{-5} M, final concentration), *H pylori* sonicate at 100 or 2000 μg/ml for 15 minutes at 25°C or OZ (20 mg/ml) 45 minutes at 25°C. All incubations were made in triplicate. After preincubation and stimulation the tubes were centrifuged for five minutes at 1500 *g*. One hundred μl supernatant was added to 800 μl of phosphate buffer, pH 6·4 and 100 μl of an equal mixture of 3·9 mM DMB (1·25 mg/ml in distilled water) and 15 mM H_2O_2 (0·17 ml stock 30% H_2O_2 freshly diluted in 100 ml of distilled water). The tubes were gently mixed and incubated for exactly 10 minutes at 25°C. Further colour development was halted by addition of 50 μl of 1% NaN_3 to all tubes. The absorbency of the supernatants were measured at 450 nm in a double beam spectrophotometer (Shimadzu UV-190, Kyoto, Japan). Degranulation activity was calculated as:

$$\frac{\text{absorbance (stimulus)} - \text{absorbance (medium)}}{\text{absorbance (Triton-X 100)}}.$$

TREATMENT OF *H PYLORI* SONICATE

To characterise the active component(s) of *H pylori* sonicate further, the following modifications were performed: (a) heat treatment for 15 and 60 minutes at 56°C, 70°C, or 100°C by immersing a sample of sonicate in a water bath; (b) dialysis of untreated as well as heat treated sonicate overnight against phosphate buffered saline with a cut off at 6–8 kDa; (c) treatment with 2 mg/ml pronase for one hour and with heat destroyed pronase (100°C for 10 minutes) as a control.

TABLE I *Myeloperoxidase release from human neutrophils stimulated with* H pylori *sonicate proteins, serum OZ, and fMLP*

H pylori *sonicate (μg/ml)*					*OZ*	*fMLP*
10	*100*	*500*	*1000*	*2000*	*2%*	*10^{-5}M*
0·5	5 (4)	0·5 (0·5)	5 (4)	9 (5)	30 (7)	9 (4)

Values are expressed as mean (SEM) of absorbance at 450 nm, corrected for the absorbance when stimulated with medium, in percentage of absorbance, when the neutrophils are treated with Triton-X 100 (total amount of myeloperoxidase). Number of experiments two to 12, except for *H pylori* 10 μg/ml where only one experiment was performed.

STATISTICAL ANALYSIS

The Mann-Whitney U test was used to analyse cross stimulation experiments. Wilcoxon's rank sum test was used to analyse the influence of modification of bacterial sonicate.

Results

Neutrophil degranulation was seen after direct stimulation with *H pylori* sonicate proteins in a concentration dependent way. Two 'degranulation' optima were obtained; (a) *H pylori* 100–250 μg/ml and (b) 2000 μg/ml (Table I). The potency was comparable with that of fMLP, but the response was less pronounced than in neutrophils stimulated with OZ.

Priming of the neutrophil responsiveness was seen in a cross stimulation assay. We found that neutrophils when incubated in *H pylori* sonicate had an increased ($p<0·05$) subsequent responsiveness to fMLP and OZ. Preincubation in fMLP also primed responsiveness to OZ (Table II).

The cross reaction experiments showed, that the degranulation after stimulation with *H pylori* sonicate protein(s) could not be blocked by preincubation of the neutrophils in fMLP, but the responsiveness to fMLP itself was blocked (Table II). When neutrophils were preincubated in high concentrations of *H pylori* sonicate further response to the sonicate was blocked. Preincubation of neutrophils in low concentrations of *H pylori* sonicate did

TABLE II *Myeloperoxidase release from human neutrophils cross stimulated with fMLP and* H pylori *sonicate protein(s)*

Preincubation	H pylori *(μg/ml)*			*fMLP*
Stimulation	*100*	*1000*	*Medium*	*10^{-5}M*
H pylori	10 (5)	3 (2)	8 (4)	6 (2)*
OZ 2%	48 (27)	38 (17)*	28 (15)	39 (23)
fMLP	6 (3)	9 (3)*	4 (2)	0·1 (0·1)

Values are expressed as mean (SEM) of absorbance at 450 nm, corrected for the absorbance when stimulated with medium, in percentage of absorbance, when the neutrophils are treated with Triton-X 100 (total amount of myeloperoxidase). Number of experiments=4. *H pylori* stimulation concentration 2000 (μg/ml). *$p<0·05$ compared with the responsiveness of medium.

TABLE III *Priming of human neutrophil degranulation, to fMLP, by modified* H pylori *sonicate*

H pylori sonicate		40 (8)
Heat treated	56°C (15 min)	44 (15)
	70°C (15 min)	12 (3)*
	100°C (15 min)	6 (4)*
Dialysed	*H pylori* sonicate	17 (4)
	H pylori sonicate 100°C (15 min)	14 (3)*
Pronase treated	*H pylori* sonicate	12 (6)
(100°C (10 min) pronase)	*H pylori* sonicate	40 (8)

Values are expressed as mean (SEM) of absorbance at 450 nm, corrected for the absorbance when stimulated with medium, in percentage of absorbance, when the neutrophils are treated with Triton-X 100 (total amount of myeloperoxidase). Number of experiments=4. Concentrations: *H pylori* 1000 μg/ml, fMLP 10^{-5}M. *$p<0.05$ compared with *H pylori* sonicate activity.

not block the subsequent degranulation when stimulated with *H pylori* sonicate (Table II).

Experiments with modified *H pylori* sonicate (heat, dialysis, and pronase treatment) indicated that the phagocyte stimulating factor in *H pylori* is a protein.

After heat treatment the responsiveness of *H pylori* sonicate was considerably reduced with increasing temperature ($p<0.02$). There was no difference when the sonicate was treated for a longer period (one hour) (data not shown). Dialysis of *H pylori* sonicate with a cut off at 8 kDa reduced the degranulation activity by about 50% suggesting a small molecule to be, in part, responsible for the observed activity (Table III). When *H pylori* sonicate was pronase treated, the subsequent neutrophil degranulation seen was less than degranulation after dialyse treated *H pylori* sonicate, but it could not be reduced as much as when heat treated. *H pylori* sonicate treated with heat inactivated pronase had similar activity as untreated sonicate.

To see whether priming of the neutrophil responsiveness to fMLP was a common feature among *H pylori* strains, sonicate from two further clinical strains was tested. Comparable activity was obtained (data not shown). Experiments with supernatant from vortex mixed *H pylori* primed the subsequent responsiveness to fMLP equally to that obtained when neutrophils were preincubated with sonicated bacteria (data not shown).

With increasing incubation time in *H pylori* sonicate 1000 μg/ml up to 90 minutes a linear increase in 'priming' was seen upon fMLP stimulation. With extended preincubation time beyond 120 minutes a lower myeloperoxidase release could be detected, probably resulting from spontaneous degranulation during the preincubation.

Discussion

The pronounced association of *H pylori* and inflammatory cells in the gastric mucosa of patients with chronic gastritis supports the conclusion that reactive oxygen metabolites and degranulation products released from the inflammatory cells stimulated with *H pylori* is part of the pathogenesis of gastritis and duodenal ulcer disease. *H pylori* infection in the gastric antrum is associated with increased luminol and lucigenin amplified chemiluminescense in duodenal mucosal biopsy specimens, suggesting reactive oxygen metabolites to have a role in the pathogenesis of duodenitis and duodenal ulcer disease.[13] Primarily luminol enhanced chemiluminescense is increased reflecting the presence of myeloperoxidase in the mucosal tissue. This study was performed to elucidate the interaction of *H pylori* sonicate protein(s) and neutrophils concerning myeloperoxidase release.

This study, concerning cross reactions (Table II), supports previous data[10–12] suggesting that the interacting properties of *H pylori* with the neutrophil membrane is in part due to protein(s) distinct from fMLP. This is in contrast with the results of Mooney *et al* who found that *H pylori* synthesises and secretes a fMLP like substance to the culture media.[14] The substance was separated by high pressure liquid chromatography and cross reacted antigenically with fMLP. Culture of *H pylori* on solid media may be distinct from liquid medium, which may explain the difference. The results were confirmed by similar reaction of sonicate from two other clinical *H pylori* isolates, but it cannot be excluded that strain differences exist. This might also explain the difference between our results and those reported by Mooney *et al*.[14]

Nevertheless Mooney *et al* have also found that the bacteria itself or after it had been opzonised did not induce a significant oxidative burst response, but prolonged the subsequent fMLP response – that is, 'priming'.

The activity for stimulation of neutrophil degranulation had several characteristics in common with the previously identified chemotactic and oxidative burst stimulatory property of *H pylori* sonicate.[10–12] We found that preincubation in *H pylori* sonicate primed the subsequent degranulation of neutrophils stimulated with either fMLP or OZ and in cross stimulation experiments no desensitation was obtained between *H pylori* sonicate protein(s) and fMLP, but the neutrophils were deactivated to further reaction with the homologous stimulus (desensitation). The activity was destroyed by heat treatment and appreciably but not completely reduced by dialysis or pronase treatment. The results with the modified *H pylori* sonicate suggest that the myeloperoxidase releasing activity is in part caused by a protein, but the fact that the degranulation activity is completely destroyed by heat treatment is in contrast with previously reported data from our group,[10 11] where monocyte stimulatory potency was increased after heat treatment of *H pylori* sonicate, though priming activity to fMLP in a lucigenin enhanced chemiluminescense system was reduced after heat treatment.[11] The finding that priming activity was not removed, only reduced, after dialysis and pronase treatment reflects the presence of non-protein molecules capable of neutrophil priming, probably lipopolysaccharide. Experiments with supernatant from vortex shaked *H pylori* showed that the priming activity of *H pylori* was due to a surface structure, but priming experiments with whole bacteria have to be performed to elucidate this.

H pylori is capable of interacting in vitro with the phagocyte membrane and to stimulate

degranulation. This resembles the pathogenesis of other chronic infections[7] and inflammatory diseases.[8]

In conclusion, the previously reported increase in mucosal luminol reactive molecules (myeloperoxidase) in *H pylori* associated disease is probably a direct consequence of bacteria derived stimulation of neutrophils for degranulation and release of myeloperoxidase. Our results suggest that degranulation of phagocytes stimulated by *H pylori* protein(s), can together with release of toxic oxygen radicals,[11] be part of the mucosa tissue damage associated with *H pylori* infection.

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1 Blaser MJ. Helicobacter pylori and the pathogenesis of gastroduodenal inflammation. *J Infect Dis* 1990; **161:** 626–33.
2 Morris A, Nicholson G. Campylobacter pylori: human ingestion studies. In: Rathbone BJ, Heatley RV, eds. *Campylobacter pylori and gastroduodenal disease.* Oxford: Blackwell Scientific, 1989: 85–189.
3 Wyatt JI. Campylobacter pylori, duodenitis and duodenal ulceration. In: Rathbone BJ, Heatley RV, eds. *Campylobacter pylori and gastroduodenal disease.* Oxford: Blackwell Scientific, 1989: 117–24.
4 Talley NJ, Zinsmeister AR, Weaver A, DiMagno EP, Carpenter HA, Perez-Perez GI, *et al.* Gastric adenocarcinoma and Helicobacter pylori infection. *J Natl Cancer Inst* 1991; **83:** 1734–9.
5 Andersen LP. The antibody response to Helicobacter pylori infection, and the value of serologic tests to detect H pylori and for post-treatment monitoring. In: Goodwin CS, Worsley BW, eds. *Biology and clinical practice.* London: CRC Press, 1993: 285–305.
6 Webster RO, Henson PM. Rapid micromeasurement of neutrophil exocytosis. *Inflammation* 1978; **2:** 129–35.
7 Høiby N, Koch C. Pseudomonas aeruginosa infection in cystic fibrosis and its management. *Thorax* 1990; **45:** 881–4.
8 Wendling D, Didier JM, Vuitton DA. The phagocyte oxidative metabolism function in ankylosing spondylitis. *Rheumatology* 1991; **11:** 187–9.
9 Klebanoff SJ. Phagocytic cells: products of oxygen metabolism. In: Gallin JI, Goldstein IM, Snyderman R, eds. *Inflammation.* New York: Raven Press, 1988: 391–445.
10 Nielsen H, Andersen LP. Chemotactic activity of Helicobacter pylori sonicate for human polymorphonuclear leukocytes and monocytes. *Gut* 1992; **33:** 738–42.
11 Nielsen H, Andersen LP. Activation of human phagocyte oxidative metabolism by Helicobacter pylori. *Gastroenterology* 1992; **103:** 1747–53.
12 Nørgaard A, Nielsen H, Andersen LP. Activation of human phagocytes by Helicobacter pylori. A novel interaction with neutrophils and monocytes distinct from that of N-formylated oligopeptides. *Zeutralblatt für Bakteriologie* 1993; **280:** 86–92.
13 Davies GR, Simmonds NJ, Stevens TRJ, Grandison A, Blake DR, Rampton DS. Mucosal reactive oxygen metabolite production in duodenal ulcer disease. *Gut* 1992; **33:** 1467–72.
14 Mooney C, Keenan J, Munster D, Wilson I, Allardyce R, Bagshaw P, *et al.* Neutrophil activation by Helicobacter pylori. *Gut* 1991; **32:** 853–7.