Chemotactic activity of *Helicobacter pylori* sonicate for human polymorphonuclear leucocytes and monocytes

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Abstract

The immunopathology of *Helicobacter pylori* associated active chronic gastritis, which is characterised by predominance of polymorphonuclear leucocyte infiltration, is largely unknown. To evaluate the role of bacterial components as inflammatory mediators ultracentrifuged sonicated preparations were made of clinical isolates of *Helicobacter pylori*. The crude sonicates were shown to exhibit chemotactic activity for human polymorphonuclear leucocytes and blood monocytes in a concentration dependent fashion. The potency was comparable with previously described bacterial derived cytotoxins. The cytotoxin(s) was non-dialysable and completely destroyed by proteinase. Heat treatment did not decrease the chemotactic activity, but in sonicate subjected to 100°C for 15 minutes all activity disappeared after dialysis suggesting the breakdown of a larger protein to small fragments that are still biologically active. By ammonium sulphate precipitation at increasing concentrations the cytotoxin(s) was selectively found in 10% ammonium sulphate saturation, and by further molecular gel separation the chemotactic activity was found in the molecular size range from 25 to 35 kDa. The demonstration of a polymorphonuclear leucocyte and monocyte cytotoxin from *Helicobacter pylori* sonicate may help in understanding the mucosal immune response in gastric inflammatory diseases.

The close association between gastric inflammatory disease and the Gram negative bacterium *Helicobacter pylori* (formerly *Campylobacter pylori*)¹ is now well established.²⁻⁴ *H. pylori* has a particular tropism for the gastric epithelium, because it specifically colonises the surface mucous layer of the antral mucosa and in cases of gastric metaplasia also the duodenal mucosa⁵ and it may even penetrate the epithelium and its basement membrane.⁶ There appears now to be little doubt that the bacterium is causally related to gastric inflammatory diseases and this is supported by epidemiological data⁷ as well as the presence of gastritis after ingestion of viable organisms.⁸

Patients with *H. pylori* gastritis develop a local and systemic humoral response with IgG and IgA antibodies, but despite this immune response the disease persists.^{9,10} On histological examination *H. pylori* is localised in areas of inflammation dominated by infiltration with polymorphonuclear leucocytes, but also

macrophages and T-lymphocytes are found in increased numbers. *H. pylori* is only occasionally cultured from gastric specimens without active chronic inflammation.^{11,12} After pharmacological elimination of *H. pylori* a marked improvement in histology have been reported,^{13,14} which contrasts the persistence of active chronic gastritis with polymorphonuclear leucocytes in patients given placebo.

The mechanisms involved in regulating the inflammatory response to *H. pylori* infection of the gastric mucosa is unknown. The close histological association of *H. pylori* and polymorphonuclear leucocytes suggests, however, recruitment by inflammatory mediators. Binding of specific IgG to the bacterium increases complement dependent phagocytosis by polymorphonuclear leucocytes,^{15,16} and incubation of *H. pylori* in human serum generates the chemotactic complement split products C3a and C5a.¹⁷ The role of bacterial factors as inflammatory mediators have not been studied. We report here the identification and partial characterisation of a bacterial protein from *H. pylori* sonicate with chemoattractant activity for human polymorphonuclear leucocytes and monocytes.

Methods

BACTERIAL STRAINS

Bacteria recovered from gastric biopsy specimens were identified as *H. pylori* based on colony morphology after growth under microaerophilic conditions at 37°C for three to six days, Gram's stain, and the production of urease, catalase, and oxidase. The test strain of *H. pylori* used for most of the studies was a clinical isolate (CH-20249) from the antral gastric biopsy of a 53 year old man with duodenal ulcer. Confirmatory studies were carried out with two additional clinical isolates (GE-40001 and GE-40003) obtained from the antral part of the stomach from two children with abdominal pain and normal endoscopic findings. Bacteria used in these studies was subcultured from five to 15 times subsequent to primary isolation before large scale sonicate preparations were performed.

PREPARATION OF *HELICOBACTER PYLORI* SONICATE

Pure cultures of *H. pylori* were grown on chocolate agar plates under microaerophilic conditions. After 24 to 48 hours of incubation at 37°C, the non-contaminated plates were harvested in sterile distilled water. Bacteria were washed twice in distilled water and disintegrated

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by sonication three times for 45 seconds at 20 000 Hz, using a Rapidis 350, 19-mm probe with a 9.5-mm tip, and centrifuged at 48 000×g for one hour at 4°C. In some experiments the bacteria were sonicated five times for 45 seconds, and in others the crude sonic extract was tested before centrifugation. The supernatant was filtered through a 0.22 µm Millipore filter. Sterility of the sonicate was controlled on 5% blood agar plates incubated microaerophilic and aerobic at 37°C. The antigen concentration was measured by refractometry using human immunoglobulin as a standard. The sonicate was stored in small aliquots at -20°C.

TREATMENT OF H PYLORI SONICATE

To further characterise the active component(s) of *H. pylori* sonicate the following were performed. (i) Heat treatment for 15 and 60 minutes at 56°C, 70°C, or 100°C by immersion a sample of sonicate in a water bath; (ii) dialysis of untreated as well as heat treated sonicate overnight against phosphate buffered saline with a cut off at 6-8 kDa; (iii) ammonium sulphate precipitation with increasing concentrations: 10, 15, 25, 40, 50, 75, 85, and 95%; (iv) molecular gel chromatography on a Sephadex G-50 column; (vi) treatment with 2 mg/ml pronase (Sigma) for one hour with heat destroyed pronase (100°C for 10 minutes) as a control.

ISOLATION OF POLYMORPHONUCLEAR LEUCOCYTES AND MONOCYTES

Peripheral venous blood of healthy donors was separated on Dextran sedimentation followed by density gradient centrifugation on metrizoate polysucrose (Lymphoprep®, Nyegaard, Oslo, Norway) as described by Böyum.¹⁸ Mononuclear cells were washed twice in Eagle's minimal essential medium (MEM; Difco) and adjusted to 1×10⁶ monocytes per ml in MEM with 2% human serum albumin. The percentage of monocytes on cytocentrifuge preparations was in the range 18%-24% as assessed by morphology on Wright's stain and cytochemical identification of non-specific esterase.¹⁹ Polymorphonuclear leucocytes were washed twice in Gey's solution, remaining erythrocytes removed by hypotonic lysis, and cells adjusted to 2×10⁷ per ml in Gey's solution with 1% human serum albumin. The purity of polymorphonuclear leucocytes was always >95%.

CHEMOTAXIS

The chemotactic response was determined in modified, reversible Boyden chambers as previously described.^{20, 21} Polymorphonuclear leucocytes chemotaxis was performed in triplicate by placing 0.5 ml of the cell suspension in the upper compartment of the Boyden chamber separated from the lower compartment by a nitrocellulose filter with a pore size of 3 µm (Sartorius, Germany). The lower compartment was filled with 0.5 ml of the appropriate dilutions of *H. pylori* sonicate or a known cytotoxin as indicated below. In each experiment the spontaneous migration towards medium was assessed

Figure 1: Migration of human polymorphonuclear leucocytes and monocytes towards various concentrations of a *Helicobacter pylori* sonicate. Results represent the mean (SEM) of three experiments performed in triplicate with polymorphonuclear leucocytes and in duplicate with monocytes. Results are expressed as number of cells per high power field in chambers with sonicate minus number of cells per high power field in chambers with medium.

as a negative control. The chambers were incubated at 37°C for 150 minutes, after which the filters were fixed in ethanol, stained with haematoxylin and mounted on slides. The cells which had migrated completely through the filter were counted by direct microscopy in five fields on each filter, and the results expressed as number of polymorphonuclear leucocytes per field.

Monocyte chemotaxis was assessed in duplicate and with polycarbonate filters with a pore size of 5 µm (Nuclepore, Pleasanton, Ca, USA). The chambers were incubated in 90 minutes at 37°C and the monocytes that had migrated completely through the filter were counted in 10 random fields in each filter. The results are expressed as the number of monocytes per field.

CYTOTAXINS

The following agents were used: (i) N-f-methionyl-leucyl-phenylalanine 10⁻⁸ M (fMLP; Sigma Chemical Co, St Louis, Mo, USA), (ii) 5% zymosan-activated serum (ZAS) prepared as described,²⁰ (iii) *Pseudomonas aeruginosa* sonicate prepared from a patient isolate,²² and (iv) 1% casein (Merck, Germany). All reagents were diluted in Gey's solution or Eagles minimal essential medium at their optimal chemotactic concentration and adjusted to pH 7.2 for polymorphonuclear leucocyte assays and pH 7.0 for monocyte assays.

TABLE I Comparison of human polymorphonuclear leucocyte and monocyte chemotaxis towards *H. pylori* sonicate and four other cytotoxins: 1% casein, 5% zymosan-activated serum (ZAS), N-f-methionyl-leucyl-phenylalanine (fMLP), and *Pseudomonas aeruginosa* sonicate 750 µg/ml

Cytotoxin	PMNs	Monocytes
<i>H. pylori</i> 4000 µg/ml	112 (8)	15 (4)
<i>H. pylori</i> 2000 µg/ml	36 (3)	12 (3)
<i>P. aeruginosa</i> 750 µg/ml	165 (14)	21 (4)
ZAS 5%	ND	23 (3)
Casein 1%	ND	20 (2)
fMLP 10 ⁻⁸ M	181 (21)	16 (3)

Results represent the number of cells per high power field, expressed as the mean (SEM) of three experiments. ND=Not done.

TABLE II Effect of heat treatment of *H. pylori* sonicate on chemotactic activity against human polymorphonuclear leucocytes and monocytes. All experiments performed with protein concentration 1000 µg/ml

Treatment	PMNs [n]	Monocytes [n]
Untreated	63 (15) [5]	12.2 (3.7) [11]
56°C 15'	55 (22) [3]	15.6 (3.4) [3]
70°C 15'	60 (13) [3]	16.1 (2.5) [5]
100°C 15'	71 (17) [5]	10.6 (3.0) [11]
100°C 60'	Not done	10.8 (3.1) [4]
100°C 15' + dialysis	3 (1) [3]	0.9 (0.2) [3]

Results represent the number of cells per high power field, expressed as the mean (SEM) migrating towards sonicate after subtracting spontaneous migration towards medium.

Results

The crude sonicate preparation of *Helicobacter pylori* exhibited a concentration dependent chemotactic activity towards human polymorphonuclear leucocytes and blood monocytes (Fig 1). Below 100 µg/ml no activity with polymorphonuclear leucocytes was observed, whereas monocytes migrated towards sonicate concentrations at 1 µg/ml, although with weak activity. Peak activity with monocytes was observed at concentrations of 1000–8000 µg/ml and with polymorphonuclear leucocytes 8000 µg/ml gave a peak chemotactic response of the same magnitude as with optimal fMLP concentration (10^{-8} M). The chemotactic activity was similar in preparations before and after high speed centrifugation of sonicate (data not shown), and the activity was not different in preparations sonicated three or five times for 45 seconds (data not shown). The chemotactic response elicited by *H. pylori* sonicate was compared with the optimal response to previously described cytotoxins (Table I). The response to 4000 µg/ml was 62% for polymorphonuclear leucocytes and 93% for monocytes when compared with the response to fMPL. In additional control experiments, sonicate preparations of two unrelated clinical isolates gave comparable chemotactic activity (data not shown).

Further experiments were undertaken to partially characterise the chemoattractant nature of the crude sonicate. Dialysis overnight with a cut off at 6–8 kDa did not reduce the chemotactic activity for polymorphonuclear leucocytes or monocytes (data not shown). Preincubation of the sonicate at 1000 µg/ml with proteinase completely destroyed activity (0 cells/field *v* 18 (3) cells/field in untreated sonicate), whereas sonicate incubated with preboiled proteinase had a slightly lower activity towards monocytes (15 (3) cells/field). Proteinase 2 mg/ml itself had no chemotactic activity.

Heat treatment of *Helicobacter pylori* sonicate did not reduce the chemotactic activity and treatment for 15 minutes at 56°C or 70°C even increased the activity, but without statistical significance (Table II). The activity against monocytes after treatment for 15 minutes at 100°C was 87% of control (*n* = 11; not statistically significant). Dialysis of the boiled sonicate completely removed the chemotactic activity for both polymorphonuclear leucocytes and monocytes, however, reflecting low molecular weight property of this activity.

Characterisation of the non-dialysable pro-

teinous cytotoxin(s) of the crude sonicate was attempted by graded ammonium sulphate precipitations and molecular gel filtration. With increasing concentrations of ammonium sulphate most of the chemotactic activity for polymorphonuclear leucocytes was found in 10% precipitate, whereas activity for monocytes also was observed in 15% and 25% fractions (Table III). The chemotactic responsiveness of the phagocytes was assessed at suboptimal concentrations of bacterial protein thus allowing detection of the concentration of activity in single fractions – that is, in 10% ammonium sulphate. Dose response experiments of 10% ammonium sulphate precipitated sonicate revealed chemotactic activity for monocytes at 200, 100, 50, 10, and 1 µg/ml, whereas the activity for polymorphonuclear leucocytes was undetectable below 200 µg/ml. Molecular size chromatography of the 10% ammonium sulphate precipitation gave maximal chemotactic activity for monocytes in the fractions 10–15 corresponding to molecular weights between 25 and 35 kDa as assessed by immunoblotting (Fig 2), although the activity was also seen outside this size range, but at a much lower level.

Discussion

The selective localisation of *Helicobacter pylori* to the gastric epithelium in patients with active chronic gastritis suggests an aetiological role of this microorganism in maintaining a chronic infection in the mucosal lesions. The cellular immunopathology of this interaction is largely unknown, but histopathological examinations of the mucosa show a high correlation of *H. pylori* with polymorphonuclear leucocyte and macrophage infiltration.^{11,12} The present study provides evidence that *H. pylori* contains chemotactic activity for human phagocytes, and this suggests an important role for this bacterial product(s) in attracting polymorphonuclear leucocytes to the site of infection. In addition, bacterial activation of complement factors may generate the cytotoxic split product C5a,¹⁷ but it is unclear if the gastric mucosal surface contains sufficient amounts of complement proteins to give this mechanism *in vivo* significance.

Several bacterial species have been reported to exhibit chemotactic activity for human phagocytes, but only few have been characterised.^{23–25} The cytotoxin(s) identified from our *H. pylori*

TABLE III Chemotactic activity of non-dialysable *H. pylori* sonicate after precipitation with ammonium sulphate (AS) of different concentrations. All experiments performed with protein concentration 200 µg/ml

Treatment	PMNs	Monocytes
Untreated (AS)	2 (1)	9 (2)
10%	90 (16)	15 (3)
15%	6 (2)	15 (3)
25%	5 (2)	8 (2)
40%	3 (1)	5 (2)
50%	3 (1)	5 (1)
75%	7 (2)	3 (1)
85%	1 (1)	5 (2)
95%	9 (1)	5 (2)

The results are expressed as mean (SEM) number of cells per high power field of three experiments performed in triplicate (PMNs) or duplicate (monocytes).

Figure 2: Gel filtration of dialysed and 10% ammonium sulphate precipitated *Helicobacter pylori* sonicate on a Sephadex G-50 column. The figure shows chemoattractant activity towards monocytes tested in duplicate.

sonicates differs from previously reported bacterial cytotoxins in several ways. In contrast with the low molecular weight factors derived from *Escherichia coli*²³ and *Staphylococcus aureus*,²⁵ the present study has identified the activity from *Helicobacter pylori* to be non-dialysable and in the molecular range of 25 to 35 kDa. We conclude that N-formyl oligopeptides are not the active cytotoxins in *H. pylori* preparations. The potency of the sonicate cytotoxin(s) was comparable with other bacterial derived factors, and blood monocytes were attracted at even lower concentrations than reported for *Legionella pneumophila* and *Pseudomonas aeruginosa*.²⁶ It is at present unknown, if the cytotoxin(s) identified by us is similar to the recently reported *H. pylori* surface protein(s) with chemotactic activity for human monocytes, as the preliminary report by Mai *et al.*²⁷ did not characterise the chemical nature of the activity.

As the active component(s) was partially purified by ammonium sulphate precipitation and molecular gel filtration the relative activity increased reflecting selective removal of non-cytotactic proteins. Eluted fractions were assayed at a minimum of two dilutions in order to avoid the possibility of depressed chemotactic responses that may occur at high concentrations of attractant. The chemotactic response of polymorphonuclear leucocytes and blood monocytes was uniformly observed with sonicates of three different clinical isolates of *H. pylori*, but further studies are needed to confirm if the production of a cytotoxin for human phagocytes is a general feature of this bacterial species.

As the activity was completely destroyed by proteinase incubation the chemical nature of the active component(s) probably is a polypeptide. It was, however, not abolished by heat treatment as might be expected of a protein. Further experiments of boiled sonicate revealed that all activity disappeared after dialysis suggesting the breakdown of a larger protein to smaller fragments retaining biological activity. The finding of increased cytotoxic activity after heat treatment at 56°C and 70°C may be explained by changes of protein conformation so that active sites are

exposed to a higher degree than in the native form.

The chemotactic responsiveness was established in polymorphonuclear leucocytes and blood monocytes probably reflecting a common reactivity of myeloid derived cells to the 25 to 35 kDa cytotoxin(s) in 10% ammonium sulphate precipitation preparations, although monocytes appears to respond weakly to additional molecules in other fractions. Preliminary experiments suggest that the influence of *H. pylori* sonicate on oxidative burst responsiveness involves both phagocytic cell types as well (Nielsen and Andersen, unpublished results). No information on the nature of the cellular interaction with the cytotoxin at the receptor level is yet available.

The immune response to *H. pylori* infection of the gastric mucosa includes a pronounced generation of IgG and IgA.^{7,10} In cases of chronic gastritis and relapsing ulcers it is clear that immunoglobulins are insufficient to eradicate the infection despite of the high antibody titres against *H. pylori*. Therefore, other immunological mechanisms are required to control the infection, of which the killing capacity of polymorphonuclear leucocytes and mononuclear phagocytes could be important. Killing of *H. pylori* by human phagocytes, however, requires a high proportion of phagocytes per bacteria (Andersen, Blom, and Nielsen; manuscript submitted), which makes this function ineffective in vivo as the bacteria generally exceed polymorphonuclear leucocytes in areas of active chronic gastritis. Release of reactive oxygen radicals or proteolytic enzymes from stimulated polymorphonuclear leucocytes may contribute to the chronicity and tissue damage characteristic of gastric inflammatory diseases, and the continued accumulation of phagocytes at the inflammatory site caused by the production of a cytotoxin by *H. pylori* could be an important pathogenic mechanism in this situation.

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