BACTERIAL PATHOGENICITY

A low molecular weight factor is a significant mediator of non-opsonic neutrophil activation by *Helicobacter pylori*

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Helicobacter pylori is believed to trigger neutrophil activation through several factors, including the *H. pylori* neutrophil-activating protein (HpNAP). The aim of this study was to characterise the factors within *H. pylori* cell-free extracts that stimulate neutrophil activation. Neutrophil activation was found to be dose-dependent and exhibited considerable variation between different clinical isolates. Activity was attributable to more than one protein factor. A low mol. wt fraction of <3 kDa was found to contribute a large proportion of the neutrophil-stimulating activity within *H. pylori* cell-free extract. Additional activity was provided by a high mol. wt fraction, possibly representing HpNAP. An inhibition ELISA and neutralisation experiments failed to identify or exclude formyl methionyl leucyl phenylalanine as the active factor within the low mol. wt fraction. The importance of the putative, low mol. wt neutrophil-activating factor may have been overlooked by those studies that have used concentrated *H. pylori* extracts.

Introduction

The pathogenesis of peptic ulcer disease (PUD) is multifactorial and includes the dual damage caused by reactive oxygen intermediates (ROI) released by activated neutrophils and the action of the *Helicobacter pylori* vacuolating cytotoxin on the gastric epithelium. However, not all *H. pylori* isolates stimulate the production of ROI from neutrophils *in vitro* [1–4]. Similarly, only a subset of *H. pylori* isolates express vacuolating cytotoxin activity [5–7]. Together and independently, these characteristics have been found to be associated with the development of PUD [1–4, 8–11].

A previous study found that the ability to activate neutrophils and vacuolating cytotoxin activity are found more frequently in *H. pylori* isolates from patients with PUD than in patients without ulceration [12]. Through the action of the vacuolating cytotoxin, the gastric epithelium may become more permeable to the passage

of *H. pylori* neutrophil-activating factors, increasing the susceptibility of the lower tissue layers to the deleterious effects of ROI.

Previous studies have attempted to characterise the neutrophil-activating and chemo-attractive factors present in *H. pylori* extracts [13–18]. However, different research groups have reported factors with disparate mol. wts and activities. As a result, the size and nature of *H. pylori* activating and chemo-attractive factors remain unclear. Some authors have reported activating and chemo-attractive factors to be heat-labile [18, 19], in contrast to others who have found activating factors to be resistant to the effects of high temperature [14, 16]. Nonetheless, neutrophil-activating and attractant factors in *H. pylori* extracts have been concluded generally to be primarily proteinaceous, with additional possible activity originating from non-protein components including lipopolysaccharide (LPS) [18, 20], despite the fact that *H. pylori* LPS is thought to be of low biological activity [21].

Several proteins with neutrophil-activating and chemo-attractive properties have been identified in *H. pylori* extracts. The *H. pylori* urease protein may display chemo-attractive activity [15], as have uncharacterised 25–35-kDa and 10.5-kDa proteins [16, 17]. The same 25–35-kDa chemo-attractant protein described by

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Nielsen and Andersen (1992) was also shown to activate neutrophils [16]. However, the best described of all the *H. pylori* neutrophil-activating proteins is the *H. pylori* neutrophil-activating protein (HpNAP). In its native form, HpNAP is a 150-kDa homodecamer of 15-kDa subunits [22, 23].

Several groups have observed a low mol. wt activating and chemo-attractant factor in *H. pylori* sonicates and conditioned media [13, 14, 18]. However, conclusions regarding the exact size and nature of the low mol. wt *H. pylori* neutrophil-activating factor have varied. Mooney and associates attributed activating properties in *H. pylori* extracts to chemotactic formylated peptides and found activity to elute in the same fraction as commercially available formyl methionyl leucyl phenylalanine (fMLP) [13]. This fraction and that from several intestinal bacteria also appeared to be antigenically cross-reactive with fMLP [13, 24]. In contrast, activity was concluded by others to be distinct from bacterial formylated peptides [15, 18]. This study addressed these contradictory findings.

Materials and methods

Source and culture of H. pylori strains

The two *H. pylori* strains chosen for this study were selected from a bank of 46 isolates that were originally cultured from gastric biopsy specimens, collected at endoscopy from patients in several hospitals in North Queensland, Australia. The identification and culture of these isolates has been described previously [12].

Strain 96T/KAD was isolated from a patient with endoscopically observed peptic ulcer. This strain produced cytotoxin, but was *cagA*(-). Strain 96T/PRI was also obtained from a patient with PUD, was *cagA*(+), but did not produce cytotoxin. Strain 96T/KAD had previously been found to activate neutrophils, whereas strain 96T/PRI did not [12]. The measurement of vacuolating cytotoxin activity and the ability to activate neutrophils has been described previously [12]. The *cagA* gene was detected in isolates by the method described by Weel and colleagues [11].

Measurement of neutrophil activation

Two methods were used to measure neutrophil activation, a luminol-enhanced chemiluminescent (LEC) assay and a flow cytometric assay based on the oxidation of dihydrorhodamine-123 (DHR). The LEC assay provided a convenient method of monitoring neutrophil activation over a period of time, yielding measures of ROI release at regular intervals from the same samples. In contrast, the DHR assay provided one measure per sample, but was advantageous because of consistently low levels of background activation in negatively controlled samples.

LEC assay. Neutrophils were purified from whole blood collected from one adult volunteer by density gradient centrifugation. Two-ml volumes of whole blood were layered on to 2-ml volumes of Histopaque (Sigma) before centrifugation (1000 *g* for 15 min at 15°C). The neutrophil-erythrocyte plug was then obtained. Erythrocytes were lysed for 10 min in 10 ml of lysis buffer that had been pre-warmed to 37°C. Leucocytes were centrifuged (400 *g* for 5 min at room temperature), washed and resuspended in 1 ml of Hanks's Balanced Salts Solution (HBSS) + EDTA (Sigma). The resultant neutrophil population (1×10^6 /ml) was added to the chemiluminescent substrate 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol; Sigma) at 1×10^{-5} M in HBSS + EDTA to achieve a final suspension of 5×10^5 neutrophils/ml.

This preparation was dispensed as 180- μ l volumes into black 96-well plates (Fluoroplates, Flow Laboratories, Finland), to which 20- μ l test samples were added. Buffer only was added to other samples as negative controls and phorbol myristate acetate (PMA; Sigma) 40 ng and fMLP (Sigma) 1 μ g, were used as positive stimuli of ROI release. A Packard TopCount Luminometer set to single photon count (SPC) mode was used to measure the emission of light at 25°C for 90 min following the addition of samples.

DHR flow cytometric assay. Samples of whole blood (100- μ l) were lysed in 4 ml of pre-warmed (37°C) lysis buffer for 5 min to destroy erythrocytes. After centrifugation at 400 *g* for 3 min at room temperature, leucocytes were washed and resuspended in 400 μ l of suspension buffer. DHR-123 (Molecular Probes, Eugene, OR, USA) was added to a final concentration of 1×10^5 nM and incubated for 5 min at 37°C. Suspension buffer, PMA 40 ng, fMLP 1 μ g or *H. pylori* cell-free extract 100 μ g was added to triplicate tubes unless otherwise stated. Samples were incubated for a further 30 min at 37°C before immediate flow cytometric analysis with a Becton Dickinson FACScan Flow Cytometer.

Fractionation of active factors in the cell-free extracts of H. pylori by ammonium sulphate precipitation

Ammonium sulphate (Sigma) cuts of 0–40%, 40–60%, 60–80% and 80%+ were prepared from cell-free extracts of strain 96T/KAD. At each stage, the mixtures were centrifuged at 10 000 *g* for 10 min at 4°C. Pellets were resuspended in ddH₂O before passage through desalting HiTrap columns (Pharmacia Biotech, Uppsala, Sweden). The 80%+ fraction, which represented the constituents of the cell-free extract that could not be precipitated by an 80% ammonium sulphate cut, was similarly desalted. Samples of 100 μ g/20 μ l of each precipitated fraction were used to stimulate neutrophils. Neutrophil ROI release was measured by the LEC assay.

Fractionation of the activating factors within H. pylori cell-free extract by gel filtration column chromatography

H. pylori cell-free extract (20 mg at 5 mg/ml) was treated with 1 mM phenylmethylsulphonyl fluoride (PMSF) and concentrated four-fold with UltraFree Biomax-5000 MW cut-off filters (Millipore, Bedford, MA, USA). The supernate (>3 kDa) and filtrate (α fraction, <3 kDa) were collected. Proteins in the supernate were separated on the basis of size by gel filtration with a Sephadex G-100 (Pharmacia Biotech), 300 $\times$ 16 mm column (1 ml sample; 0.6 ml/min in PBS + EDTA, pH 7.2; 5.5 ml fractions) and a GradiFracTM Chromatography System (Pharmacia Biotech). The column had previously been calibrated with blue dextran (2000 kDa), catalase from bovine liver (BC, 232 kDa), bovine albumin fraction V (BSA, Sigma; 67 kDa), cytochrome *c* from horse heart (12 kDa) and phenol red (300 Da). All mol. wt standards were obtained from Sigma.

After separation, column fractions were concentrated by 60% ammonium sulphate precipitation, resuspended in suspension buffer and desalted with HiTrap columns (Pharmacia Biotech). The resultant fractions were assayed for neutrophil-stimulating activity in the LEC assay after adjustment to a protein concentration of 100 μ g/20 μ l.

Characterisation of the low mol. wt α fraction with anti-fMLP mouse serum

To investigate the nature of active components within the α fraction, an antiserum was prepared against commercial fMLP for use in ELISA and neutralisation experiments.

Conjugation of fMLP to protein carriers. Commercially available fMLP (Sigma) was conjugated *via* the C terminus to BSA or keyhole limpet haemocyanin (KLH; Sigma) by the carbodiimide reaction. Ten mg of 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC; Sigma) were dissolved in 20 ml of ddH₂O. Then 5 mg of fMLP, reconstituted in DMSO (Sigma), were added and the reaction was allowed to proceed for 5 min at room temperature. After the addition of 7.5 mg of the protein carrier, the reaction was incubated at 4°C overnight. Dialysis with a 10 000 mol.wt cut-off membrane (Millipore) was performed overnight against ddH₂O before drying down to one-tenth of volume overnight in a fume cupboard.

Antiserum production. Half of the fMLP-BSA conjugate preparation was emulsified in an equal volume of Freund's complete adjuvant (FCA; Sigma) before subcutaneous injection as 200- μ l volumes into five 6-week-old male BALB/c mice. Then, 14 days later, boosting injections of 200 μ l of the same preparation emulsified in Freund's incomplete adjuvant (FIA;

Sigma) were given intraperitoneally. After 10 days, animals were killed with CO₂ and the total blood volume was obtained immediately, following dissection of the carotid artery. Serum was separated, heat-inactivated (56°C for 30 min) and stored in divided volumes at -70°C. All five animals had generated similar antibody titres when tested against a reciprocal fMLP-KLH conjugate (mouse 1, 500; 2, 500; 3, 500; 4, 400; 5, 500).

Mice were housed at the Small Animal Experimental Facility at James Cook University and were provided with water and dried, pelleted food *ad libitum*. Ethical approval for antiserum production was obtained from the James Cook University Experimental Ethics Review Committee.

Inhibition ELISA for the detection of fMLP in clinical isolates of H. pylori. Volumes (100 μ l) of a 1 in 8 dilution of fMLP-KLH conjugate in PBS (final concentration 5 μ g/100 μ l) were used to coat 96-well plates (Nunc, Nunc, Denmark) at 4°C overnight. Anti-fMLP-BSA polyclonal antiserum, diluted 1 in 100 in PBS-T-G, was combined with variable concentrations of competitive antigen (10 μ g, 1 μ g, 100 ng, 50 ng of fMLP or α fraction) and added to plates in a total volume of 100 μ l before incubation for 1 h at 37°C. After washing (3 $\times$ 10 min in PBS-T), 100 μ l of a 1 in 2000 dilution of goat anti-mouse horseradish peroxidase (HRP) conjugate (BioRad Laboratories, Hercules, CA, USA) were added and plates were incubated for a further 1 h at 37°C before washing and colour development with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; Sigma) solution. Absorbance at 405 nm was measured with an LP400 SANOFI plate reader (Pasteur Diagnostics, Marnes-la-Coquette, France). Dilutions of coating antigen, primary antiserum and secondary antibody conjugate had been optimised previously by a checkerboard titration. Therefore, the amount of fMLP present in the test sample was directly related to the degree of inhibition of primary antibody binding to the fMLP-KLH conjugate and was measured by a decrease in colour development.

Neutralisation of neutrophil-stimulating activity in the α fraction. Twenty- μ l samples of the α fraction and 1 μ g/20 μ l and 100 ng/20 μ l quantities of commercially available fMLP were incubated in triplicate with equal volumes of a 1 in 10 dilution of the anti-fMLP-BSA antiserum described above. Following incubation for 30 min at 37°C, these samples were assayed for neutrophil-stimulating activity by the DHR assay.

Statistical analysis

Statistical analyses were performed with one-way ANOVA models and the SPSS Statistical Program.

Results

Effect of H. pylori cell-free extract concentration, temperature and protease activity on neutrophil activation

To examine the effect of concentration upon the activation of neutrophils by *H. pylori* extract, stimulation experiments were performed with two strains of *H. pylori* over a range of concentrations of the cell-free extract. A neutrophil-activating strain (96T/KAD) and a non-activating strain (96T/PRI) were chosen from a group of isolates characterised previously [12]. The cell-free extracts of these strains were added in triplicate to chemiluminescent suspensions in 20- μ l volumes containing 100 μ g, 50 μ g, 10 μ g or 1 μ g of total protein. The effect of concentration on the activation of neutrophils by *H. pylori* cell-free extract was also measured by the DHR assay. Neutrophils

were stimulated by the addition of 100 μ g, 50 μ g or 10 μ g of *H. pylori* cell-free extract (strain 96T/KAD) or buffer only.

Neutrophil activation by cell-free extracts of *H. pylori* was found to be concentration-dependent. Strain 96T/KAD stimulated H_2O_2 release in response to 100, 50 and 10 μ g of extract in a dose-dependent manner (Fig. 1). In comparison, strain 96T/PRI only caused activation after the addition of 100 μ g of cell-free extract (Fig. 1); 100 μ g of strain 96T/PRI extract stimulated neutrophils to a similar degree to 10 μ g of strain 96T/KAD extract.

When the effect of cell-free extract concentration on neutrophil activation was examined by the DHR assay, stimulation indices increased with increasing concentrations of *H. pylori* cell-free extract ($F_{(1,3)} = 81.3802$, $p = 0.029$) (Fig. 2).

Fig. 1. Neutrophil activation by cell-free extracts of *H. pylori*: ■—■, strain 96T/KAD 100 μ g; ▲—▲, strain 96T/KAD 50 μ g; ●—●, strain 96T/KAD 10 μ g; ×—×, strain 96T/PRI 100 μ g; ◆—◆, negative control.

Fig. 2. Concentration-dependent neutrophil activation by *H. pylori* 96T/KAD extract in the DHR assay. Bar = SD.

To determine the effect of temperature and protease activity on *H. pylori* extract activity, triplicate extracts of strain 96T/KAD were incubated with or without the protease inhibitor PMSF at 4°, 24°, 37° and 65°C for 30 min. PMSF was dissolved in acetone at 1 M (0.1742 g/ml) concentration and dispersed into the cell-free extract as a 1 in 1000 dilution before incubation. After incubation, 100 µg/20 µl of sample was added to the neutrophil suspensions and activation was measured by the LEC and DHR assays.

The factors in *H. pylori* cell-free extract that stimulate neutrophil activation were found to be sensitive to heat and protease. A significant decrease in activity was observed after incubation of the strain 96T/KAD cell-free extract at 65°C for 30 min, in comparison with the same extract incubated at physiological temperature ($F_{(1,2)} = 2763.7226$, $p = 0.0004$). Pre-treatment with PMSF partially prevented this loss ($F_{(1,1)} = 598.2411$, $p = 0.0260$), indicating that the observed reduction in activity following incubation at 65°C was partially due to the action of proteases on the active factors within the cell-free extract (Fig. 3). In the DHR assay, a stimulation index of 9.058 SD 0.528 in response to 100 µg of cell-free extract of strain 96T/KAD was decreased to 3.323 SD 1.087 following incubation at 65°C for 30 min ($F_{(1,3)} = 45.3201$, $p = 0.0067$).

Activity within fractionated *H. pylori* cell-free extracts

Neutrophil-stimulating activity was found in many fractions of the *H. pylori* cell-free extract after separation on a Sephadex G-100 column. However, the column fraction that contained most activity was that expected to contain the HpNAP homodecamer (10^3 – 10^2 kDa). A large proportion of the activity was

also found in the low mol. wt (<3 kDa) α fraction (Fig. 4).

When column fractions were analysed by SDS-PAGE and were stained with Coomassie Brilliant Blue and silver, multiple protein bands were observed in each fraction, with the exception of the α fraction (Fig. 5). Neutrophil-stimulating activity in *H. pylori* cell-free extracts was distributed evenly between the 0–40% and 40–60% ammonium sulphate precipitated fractions. Minimal activity remained in the 60–80% and 80+% fractions. Attempts were made to investigate the dose-response to the α fraction. When the α fraction was diluted 50% in buffer, <15% of activity remained detectable. Further dilution produced negligible activity. Efforts to concentrate the activity within the α fraction were unsuccessful.

An inhibition ELISA was found to be capable of measuring synthetic fMLP concentrations of between 10 µg and 100 ng/50 µl. No measurable inhibition of antibody binding in response to cell-free extract of *H. pylori* was observed ($F_{(1,3)} = 0.454$, $p = 0.8449$). No significant neutralisation of activity was found when the α fraction was pre-incubated with anti-fMLP-BSA mouse antiserum for 30 min ($F_{(1,4)} = 0.0000$, $p = 0.9971$). Also, similar incubation of various concentrations of fMLP with the antiserum did not result in a decrease in neutrophil-stimulating activity. For example, there was no significant difference in the activity of 1 µg of fMLP and the same fMLP concentration pre-incubated with antiserum ($F_{(1,3)} = 0.1281$, $p = 0.7441$).

Discussion

From the results of this study, it would appear that the majority of activity in *H. pylori* extracts is attributable

Fig. 3. Heat and protease sensitivity of *H. pylori* neutrophil-stimulating activity: ■—■, incubation at 37°C; ×—×, incubation at 65°C; ▲—▲, incubation at 65°C in the presence of PMSF; ◆—◆, negative control.

Fig. 4. Fractionation of *H. pylori* 96T/KAD neutrophil-activating factors by gel filtration. Bar graph represents protein elution, line graph represents the fractionation of activity, as measured by the DHR assay. Bar = SD.

Fig. 5. Silver stain of fractions from Sephadex-G100 column. Lane 1, neat *H. pylori* extract; 2, blank; 3–9, fractions 1–7; 10, α fraction.

to protein factors. LPS may contribute to some background activation but is unlikely to be of significance due to low biological activity [21]. Neutrophil activation in response to *H. pylori* extracts was found to be dose-dependent. The peak ROI release in response to high concentrations was found to occur earlier than at lower concentrations. Nielsen and Andersen also found activation to be dose-dependent [2, 16].

Neutrophil activation in response to *H. pylori* cell-free extracts was not immediately apparent and maximum ROI release typically took up to 30 min to occur when the LEC assay was used. Activation by fMLP was similarly delayed, unlike PMA activation, which is thought to bypass cell-surface receptors [25]. The delay in response to *H. pylori* cell-free extract is suggestive of activation *via* a receptor-mediated process.

The results described in this study were found to be

consistent in both the LEC and DHR flow cytometric assays. While a non-activating strain of *H. pylori* (96T/PRI) was found to stimulate ROI release only at high concentrations, the extract from a highly activating strain was active even at low concentrations. These results may reflect different concentrations of active factors within the extracts of the strains in question or, alternatively, differences in the specific activities of those factors.

The fractionation of active factors by gel filtration revealed that many constituents of the *H. pylori* cell-free extract were able to activate neutrophils if added in sufficient concentrations. Significant levels of activity in *H. pylori* cell-free extract were found to be attributable to factors of 10^3 – 10^2 kDa, possibly representing the homodecamer of HpNAP. Multimers of the 15-kDa subunit may contribute to the low levels of activity found at other mol. wts. Evans *et al.* reported possible additional HpNAP cross-reactive bands by Western blotting [22]. In contrast to other studies, there

was no evidence of strong activity associated with the 61-kDa urease subunit [15] or unknown 25–35-kDa [16] and 10.5-kDa [17] proteins. Differences in the preparation of extracts may account for these findings.

Most activity was found to be attributable to a factor found within the low mol. wt α fraction. This factor, with a mol. wt of <3 kDa, may represent fMLP or some other small peptide. The activity within similar fractions of *H. pylori* has been attributed previously to formylated peptides [13, 14]. Other studies have used concentrated protein extracts for activation [15]. These techniques may have biased characterisation towards factors of higher mol. wt and the importance of small elements may have been discounted as a result.

Characterisation of a neutrophil-activating factor within the α fraction was made more difficult by its low mol. wt. Small peptides do not bind to membranes or plastic surfaces and quickly diffuse into electrophoresis gels [26]. Some peptides cannot be stained by either Coomassie Brilliant Blue or silver due to the absence of crucial amino acid residues and are present at such low levels within bacterial extracts [27] that concentration is required before electrophoresis can be performed. Ammonium sulphate precipitation was not successful in concentrating activity within extracts; significant activity was lost when this process was used. This may be due to the failure of ammonium sulphate to precipitate the active factor due to low mol. wt. Furthermore, when the α fraction was diluted more than twice, this process was found to result in a loss of activity and may indicate the sensitivity of the active factor once isolated from other extract proteins.

The possibility that the active factor within the low mol. wt α fraction was due to formylated peptides was investigated in some detail. As has been described previously in studies of formylated peptides, large volumes are required to provide sufficient material for purification and subsequent analysis [28, 29]. Other studies have used antibodies for immunoassays and neutralisation experiments [20, 24]. However, these approaches, including HPLC and sequencing (results not shown), all ultimately proved to be unsuccessful when applied to the α fraction. As a result, it was not possible to include or exclude a formylated peptide as the active factor within *H. pylori* extracts.

Lastly, the loss of activity in fractions following purification may be influenced by the separation of active factors from other proteins. Neutrophil activation and vacuolating cytotoxin activity have been demonstrated not only to be associated with PUD, but also with each other [1, 3, 12]. Although the vacuolating cytotoxin is not thought to be a factor that activates neutrophils, it may contribute to activity by some action on the neutrophil cell membrane [12]. The separation of the vacuolating cytotoxin from neutrophil-activating factors may contribute to an overall loss of activity in purified fractions.

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