Opsonic requirements of *Helicobacter pylori*

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Summary. The opsonic requirements of *Helicobacter pylori* were investigated in a series of experiments with human polymorphonuclear leucocytes (PMNL). Pre-incubation of *H. pylori* with pooled normal human serum (NHS) in concentrations of 5–20% significantly increased the uptake of radiolabelled bacteria by PMNL. Treatment of the bacteria with NHS 30% caused the release of radiolabel and this effect was abolished by heating serum to 56°C, suggesting that *H. pylori* is serum-sensitive and that complement is involved. Opsonisation of *H. pylori* with NHS concentrations of 10–30% significantly increased PMNL chemiluminescence. Removal of specific antibody had no effect. Removal of either the classical or alternative complement pathways produced no significant change in PMNL chemiluminescence, indicating that either pathway is sufficient for opsonisation on its own. The results confirm that complement is the most efficient opsonin for *H. pylori*.

Introduction

Helicobacter pylori was first isolated in 1983.¹ It is a small gram-negative organism that inhabits the mucous layer of the stomach.² The organism is fragile and slow growing *in vitro*, tolerating exposure to air only poorly. By contrast, it is more robust *in vivo* and colonisation of the gastric mucosa appears to be life-long. Eradication of the organism is difficult to achieve, even with appropriate antibiotics.

H. pylori is closely associated with type B gastritis, particularly when this is “active” with large numbers of polymorphonuclear neutrophil leucocytes (PMNL) present within the mucosa.³ The infection persists despite the profusion of PMNL, some of which appear to be ingesting bacteria,⁴ and the presence of specific systemic antibody of all classes.⁵ Mucosal IgG and IgA, apparently directed at the bacteria,⁶ are also easily demonstrated. Whole *H. pylori* and extracted lipopolysaccharide (LPS) have been shown to induce HLA-DR and interleukin-2 receptors on human monocytes *in vitro*. They also stimulate the production of inflammatory cytokines such as interleukin 1 and TNF- α , and the active oxygen intermediate, superoxide anion.⁷ These observations indicate that the monocyte-macrophage limb of the cellular immune system is likely to be active in gastritis.

Although the gastric mucosa responds actively to *H. pylori* infection, both *via* the humoral immune system and by the influx of inflammatory cells, the organism manages to evade the host response. We have investi-

gated the opsonic requirements of *H. pylori* to try and determine why the immune response is ineffective in eradicating the organism from the gastric mucosa.

Materials and methods

Culture of *H. pylori*

Isolation of bacterial strains. Three strains of *H. pylori*, originally isolated from patients with gastritis, were used in the experiments. In the opsonisation experiments there were no differences between these three strains and the results shown represent a compilation of data obtained with the strains. Bacteria were isolated from antral biopsy specimens and transported to the laboratory within 3 h, by a method modified from Goodwin *et al.*⁸ Specimens were macerated with a PTFE (Teflon)-tipped piston (McQuilken, Glasgow), in a small amount of saline 0.9% in a 5-ml glass tube. The resulting suspension was then transferred to Columbia Agar with horse blood 7%, Heated Blood Agar (L), and Skirrow's Campylobacter Selective Medium containing vancomycin 10 mg, trimethoprim 5 mg, polymyxin B 2500 IU (all from Oxoid). All isolates grew on all three media. Incubation was in sealed culture jars (Don Whitley Scientific Ltd, Shipley, W Yorks) with catalysts and “Campypak” gas generators (BBL, Cockeysville, MD, USA) to provide humid micro-aerophilic conditions. When incubated at 37°C, *H. pylori* appeared as small translucent colonies c. 1 mm in diameter at 48–72 h. Identification of *H. pylori* was confirmed by its characteristic appearance on Gram's stain and positive results in tests for oxidase and urease pro-

duction. All strains were maintained by twice-weekly subculture on to Columbia blood agar and Skirrow's agar.

Growth of bacteria in broth cultures. Surface bacterial growth on a Columbia blood agar plate was harvested with a sterile swab and suspended in 5 ml of sterile saline 0.9% to form a concentrated suspension. A 1-ml inoculum was added to Brain Heart Infusion Broth (BHIB, Oxoid) supplemented with fetal calf serum (Flow Laboratories, Irvine) 8%, and vancomycin (AddaTab, Mast Laboratories, Bootle) 6.7 mg/L. The cultures were grown as shallow layers, 2–3 ml deep, in petri dishes (Bibby Sterilin, Stone) with the side walls perforated just below the lid, to promote gas exchange during incubation.

The cultures were incubated at 37°C for 72 h under micro-aerophilic conditions. The purity of each culture was checked by Gram's stain and urease activity. Bacteria were harvested by centrifugation at 2500 *g* for 10 min, and washed three times in saline 0.9%.

Preparation of radiolabelled bacteria. Bacteria were grown in supplemented BHIB, containing ³H adenine 10 µl/10 ml of broth. Uptake of radiolabel was satisfactory after incubation for 72 h. The bacteria were harvested by centrifugation at 750 *g* for 15 min, washed three times with phosphate-buffered saline (PBS), pH 7, and suspended in saline 0.9% to give an OD₆₁₀ of 0.13, which had been demonstrated previously to be equivalent to a viable count of 1 × 10⁷ cfu/ml.

Opsonisation of bacteria

A single pool of normal human serum (NHS) was derived from the blood of c. 20 healthy laboratory workers, and stored in small quantities at –70°C until use. NHS was assayed for anti-*H. pylori* surface antigen and anti-urease specific antibody by ELISA^{9,10} at the Public Health Laboratory, Preston. *H. pylori* antibody titres varied between 2000 and 4000, and urease antibody titres between 2000 and 8000.

NHS was diluted in Hanks's Balanced Salts Solution 10% supplemented with gelatin 0.1% (GHBSS) to obtain various serum concentrations. Complement activity was destroyed by heating NHS to 56°C for 30 min and the alternative pathway activity was destroyed by heating NHS to 50°C for 30 min.¹¹ Components of the classical pathway were blocked by treating NHS with ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetra-acetic acid (final concentration 10 mM) in the presence of equimolar MgCl₂ (EGTA-MgCl₂).¹²

H. pylori-specific antibody was removed by triple absorption against a pool of three strains of *H. pylori* at 0°C for 15 min. Titres of *H. pylori* antibody fell by between four- and eight-fold and anti-urease antibody titres by between eight- and 32-fold. Specific antibody and complement were removed by sequential heating and absorption. In some experiments, serum absorbed with *H. pylori* was further treated by triple absorption

(0°C for 15 min) with *Staphylococcus aureus* (Cowan 1 strain) as a source of protein A. Protein A binds to the Fc region, inactivating immunoglobulin irrespective of antigenic specificity.¹³

Suspensions of *H. pylori* in saline 0.9% were adjusted to either an OD₆₁₀ of 0.13 (viable count 1 × 10⁷ cfu) for the radiolabelled bacterial experiments, or OD₆₁₀ of 0.3 (viable count 2 × 10⁷ cfu) for the assessment of PMNL chemiluminescence. Equal quantities of bacterial suspension and NHS diluted in GHBSS were mixed and incubated at 37°C for 15 min in a rotary incubator. The bacteria were sedimented by centrifugation at 2500 *g* for 15 min and resuspended in their original volume in GHBSS. Opsonised bacteria were used immediately.

Preparation of PMNL

PMNL were freshly prepared for each experiment, by a method based on that of Böyum.¹⁴ Sixty ml of heparinised blood was taken from a series of healthy volunteers and mixed with 20 ml of dextran 150 in saline 0.9% for 30 min to sediment erythrocytes. Ten-ml volumes of leucocyte-rich supernate were decanted into conical centrifuge tubes containing 3–5 ml of Leucocyte Separation Medium (Flow Laboratories) and centrifuged at 750 *g* for 30 min. The PMNL-rich layer was then resuspended in NH₄Cl 0.87% to lyse any remaining red blood cells. PMNL were washed in GHBSS and resuspended to a concentration of 2 × 10⁷ PMNL/ml. In all experiments PMNL were used within 3 h of separation.

Measurement of phagocytosis by uptake of radiolabelled bacteria

The method was based on that previously described by Verhoef *et al.*¹⁵ in which each experimental limb consisted of a set of four reaction mixtures. Two of the four contained 0.1 ml of opsonised bacteria in GHBSS and an equal volume of PMNL. The other two contained 0.1 ml of bacteria with an equal volume of GHBSS but no PMNL. These acted as controls, allowing an estimate to be made of any non-specific binding of bacteria to the walls of the reaction vessels. Each experimental limb was performed in duplicate. After incubation at 37°C for 15 min, one vial containing PMNL (washed cells) and one containing GHBSS alone (control) were centrifuged at 750 *g* for 5 min to pellet the PMNL; unphagocytosed bacteria remained in suspension and were discarded with the supernate. Pellets were washed three times in ice-cold PBS to remove extracellular radiolabel, and then scintillation fluid (Canberra Packard, Caversham) was added to lyse all the remaining cells. The remaining two vials had scintillant added immediately after incubation to produce an estimate of the total radiolabel present (total count).

The radioactivity of each sample was measured in an "Ultrabeta" scintillation counter (LKB, Milton

Fig. 1. Ingestion of radiolabelled *H. pylori* by PMNL after opsonisation with increasing concentrations of NHS (mean with 95% confidence intervals).

Fig. 2. The release of radiolabel from *H. pylori* exposed to increasing concentrations of heated (56°C for 30 min) (●—●) and unheated (○—○) NHS.

Keynes) set to record β emissions. In this way it was possible to calculate the phagocytic uptake according to the formula:

$$\text{Uptake (\%)} = \frac{\frac{\text{Washed cells (counts/s)}}{\text{Total radiolabel (counts/s)}}}{\frac{\text{Control (counts/s)}}{\text{Total radiolabel (counts/s)}}} \times 100$$

Demonstration of serum sensitivity

One ml of radiolabelled *H. pylori* in saline 0.9% was added to 1-ml volumes of NHS diluted in GHBSS at initial concentrations of 5, 10, 20 and 30% and incubated at 37°C in a rotary incubator for 15 min. To assay total radioactivity, 0.1-ml amounts from each suspension were transferred to polythene scintillation vials containing 3 ml of hydroxyluminol scintillant and counted as previously described. The remainder of the serum-bacteria suspension was then centrifuged at 2500 *g* for 15 min and a 0.1-ml volume was mixed with 3 ml of scintillant; the radioactivity measured represented the loss of radiolabel from lysed bacteria. The remaining supernate was then discarded, the bacterial pellet was resuspended in 1 ml of GHBSS, and a 0.1-ml sample was taken to determine the quantity of radiolabel remaining in whole bacteria. The supernate and bacterial counts of radioactivity were expressed as a percentage of the total radioactivity present. The amounts of radiolabel released into the supernate and retained in the bacterial pellet were inversely related. For convenience the results were expressed as the percentage of radiolabel present in the supernate.

Measurement of chemiluminescence

Opsonised bacteria (0.2 ml; 2×10^7 cfu) were mixed with 50 μ l of 10^{-5} M luminol in polystyrene tubes (Sarstedt) and a baseline chemiluminescent measurement was made in an automated luminometer (Pico-lite, Canberra Packard) with the temperature set at 37°C. PMNL (0.1 ml; 2×10^7) were added *via* an

Fig. 3. PMNL chemiluminescence after exposure to *H. pylori* pre-opsonised with increasing concentrations of NHS (mean with 95% confidence intervals).

automatic injection port and further readings were taken at 5-min intervals for 60 min. Results were analysed on an IBM personal computer and expressed as peak counts/s/cell.

Samples of GHBSS and luminol without bacteria were run as controls and showed no significant activity. Typically, PMNL chemiluminescence increased rapidly after exposure to opsonised *H. pylori*, reaching a peak value before falling towards baseline over the course of 1 h. The response curve was analysed by determining the maximum value for each experimental limb and expressing this as the peak count/s/cell.

Statistical analysis

Analysis of variance¹⁶ was used to determine the variance between repetitions, and between the different serum fractions. The residual variance for the experimental set was used to calculate the SE of the mean PMNL activity for each serum fraction; 95% confidence intervals could then be calculated for the difference between each serum concentration and baseline activity.

Data concerning the release of radiolabel during opsonisation were derived from paired experiments with heated and unheated serum and compared by Wilcoxon's Rank sum test.

Calculations were performed with the Statgraphics (Statistical Graphics Corporation, USA) statistical analysis package, and Lotus 123 spreadsheet (Lotus Development Corporation, USA).

Results

Uptake of radiolabelled *H. pylori* by PMNL

Fig. 1 shows the effect of various concentrations of NHS on the mean uptake of *H. pylori* by human

PMNL. Some phagocytosis occurred with unopsonised bacteria but only to a limited extent. Significantly enhanced uptake of radiolabel occurred with concentrations of NHS up to 20%, but above this concentration uptake fell. A possible explanation for this finding is that the organism is serum-sensitive. This hypothesis was tested by incubating three strains of *H. pylori* with increasing concentrations of NHS, and measuring the amount of radiolabel released (fig. 2). When *H. pylori* was incubated with GHBSS alone, c. 15% of the radiolabel was recovered from the supernate. In the presence of NHS this percentage rose progressively, and at a serum concentration of 30% most of the radiolabel was present in the supernate (fig. 2). In contrast, heated NHS (56°C for 30 min) had little effect on the release of radiolabel.

H. pylori-induced PMNL chemiluminescence

Unopsonised *H. pylori* stimulated PMNL chemiluminescence, but peak levels were increased with opsonised bacteria (fig. 3). *H. pylori* opsonised with NHS 10% induced significantly higher levels of PMNL chemiluminescence than unopsonised bacteria. At serum concentrations > 10% there was no further increase in PMNL chemiluminescence. Although the response curve flattened there was no fall-off at higher concentrations of serum as occurred in the experiments with radiolabelled bacteria.

Determination of serum components necessary for opsonisation

The removal of complement significantly reduced PMNL chemiluminescence, but absorption of antibody had no significant effect (table I). Removal of both antibody and complement equated with removal of complement alone.

In a further series of experiments, inactivation of the

Table I. The effect of the selective removal of antibody and complement from NHS on opsonisation of *H. pylori* as measured by PMNL chemiluminescence

Serum fraction	Number of observations	Mean peak counts/s/cell	Difference in mean from baseline	SE difference in mean*	95 % confidence intervals
Whole serum (baseline)	6	0.68	...	...	...
Complement depleted	6	0.25	0.43	0.12	0.17-0.69
Antibody depleted	6	0.65	0.03	0.12	-0.23-0.29
Antibody and complement depleted	6	0.23	0.44	0.12	0.18-0.71

*df = 15.

Table II. The effect of selective removal of antibody and complement pathways on opsonisation of *H. pylori* as measured by PMNL chemiluminescence

Serum fraction	Number of observations	Mean peak counts/s/cell	Difference in mean from baseline	SE difference in mean*	95 % confidence intervals
Whole serum (baseline)	8	0.47	...	...	...
Alternate pathway depleted	8	0.47	0	0.05	-0.1-0.1
Classical pathway depleted	8	0.37	0.09	0.05	0-0.19
Total complement depleted	8	0.3	0.17	0.05	0.07-0.27
Specific antibody depleted	8	0.39	0.08	0.05	-0.02-0.17
Total antibody depleted	7	0.4	0.06	0.05	-0.04-0.16
Antibody and complement depleted	7	0.29	0.18	0.05	0.08-0.28

*df = 40.

alternative complement pathway had no effect on opsonisation and, although depletion of the classical pathway reduced chemiluminescence, differences were not significant (table II). As previously observed, the removal of both the alternative and the classical pathways significantly reduced PMNL chemiluminescence, but absorption of *H. pylori*-specific antibody had no effect. Further treatment of absorbed serum with staphylococcal protein A was also ineffective in reducing opsonisation.

Discussion

Various methods have been used to study the interaction between *H. pylori* and PMNL *in vitro*, including chemiluminescence, immunofluorescence¹⁷

and bactericidal assays,¹⁸ and have been described previously. In the present study we used PMNL chemiluminescence and a radiolabelled bacteria assay and, in agreement with others,^{17,18} found human serum to be opsonic for *H. pylori*. In common with some other gram-negative bacteria, *H. pylori* appears to be serum-sensitive; we found exposure of *H. pylori* to serum resulted in the release of radiolabel into the supernate. A lytic, or part-lytic, process would seem to be the most likely explanation, although we have not demonstrated the destruction of *H. pylori* microscopically. The virtual abolition of the effect by heating NHS to 56°C indicates that complement is responsible, and suggests that *H. pylori* may be sensitive to non-specific humoral defences *in vivo*.

The precise reactions which generate chemilum-

inescence, and the interaction with luminol are not completely established. The stimulus appears to be perturbation of the cellular membrane, and this probably equates with phagocytosis under most circumstances. However, other factors such as endotoxin,¹⁹ exotoxins,²⁰ and certain chemicals, e.g., f.metleu.phe,²¹ are known to be capable of stimulating chemiluminescence, so the response is not specific. Chemiluminescence indicates activation of the PMNL myeloperoxidase system and reflects the functional state of the phagocytic cell, but is not a direct measurement of phagocytosis. Therefore, it is possible that the greater chemiluminescence seen with opsonised *H. pylori* could have arisen from improved immune adherence rather than increased phagocytosis. In contrast, the uptake of radiolabelled bacteria is a more direct measurement of phagocytosis and is less sensitive to external chemical factors; bacteria must be either firmly adherent to, or already ingested by the phagocyte, to avoid being removed during the washing phase of the assay.

Our data indicate that complement is a more efficient opsonin than specific antibody, particularly in relation to chemiluminescence, and confirm a previous report.¹⁷ It is possible that the absorption process did not completely remove all anti-*H. pylori* antibody from NHS, and that sufficient remained to allow opsonisation. Absorption decreased the anti-surface antigen antibody and anti-urease antibody concentrations by between eight- and 32-fold; nevertheless, some antibody was detectable by ELISA. The absorption was performed with *H. pylori* strains used in the opsonisation experiments, and should have removed the most specific anti-*Helicobacter* antibody from NHS; any residual antibody would be expected to be of lower affinity and be a less efficient opsonin. The finding that PMNL chemiluminescence was unaffected by removal of specific antibody indicates either that specific antibody is an inefficient opsonin, or that small quantities of poorly functional antibody are sufficient to maintain opsonisation. Staphylococcal protein A binds to the Fc region of the immunoglobulin molecule and would be expected to reduce the concentration of functional antibody still further. No effect on the opsonic capacity of NHS could be demonstrated, implying that immunoglobulin has little role.

Das *et al.* used serum from a patient with agammaglobulinaemia as a source of antibody-free serum¹⁷ and reached similar conclusions regarding the poor opsonic efficacy of antibody. They also found complement to be a more efficient opsonin, but concluded that the alternative pathway predominated. Our data indicate that opsonisation can still occur after inactivation of the alternative pathway and that chemiluminescence is only reduced following the simul-

taneous removal of both pathways. This suggests that either the classical or the alternative pathways are sufficient to provide opsonisation for PMNL.

In contrast, Pruul *et al.*¹⁸ suggested that specific antibody plays an essential role in the opsonisation of *H. pylori* but their experimental approach differed; a bacterial killing assay was used and both immune and pooled human serum were employed for opsonisation. Immune serum 1% had little bactericidal effect on its own but augmented the bactericidal activity of normal, non-immune serum, and improved bacterial killing by PMNL. Interestingly, this occurred only in the presence of an intact complement system. Absorption of anti-*H. pylori* antibody reduced bacterial killing but this was restored by the addition of immune serum 1%.

There is general agreement that complement is an important opsonin for *H. pylori*. Antibody may have a role in bacterial killing,¹⁸ but its effects are increased in the presence of complement. The inefficiency of specific antibody may be related to non-specific binding of immunoglobulin by *H. pylori*, impeding access of specific antibody to the bacterial surface.²²

The distribution of complement within the gastric mucosa²³ may be of importance in host defence to *H. pylori*, but few data are available. C3b is present in the gastric mucous layer of patients with *H. pylori* infection, but its activity under conditions of low pH is not clear. There is some evidence that antibody does not reach the lower depths of the gastric glands²⁴ and this might also apply to complement.

The performance of PMNL may be influenced by physical factors unrelated to the immune system—PMNL may not function as efficiently in conditions of low pH. It is known that peritoneal dialysate fluids of low pH reduce PMNL chemiluminescence²⁵ and impair their ability to mount a respiratory burst.²⁶ The gastric lumen is likely to be equally inhospitable. The spiral shape of *H. pylori* improves motility in viscid environments such as the gastric mucous layer,²⁷ but PMNL may find their progress impeded by mucus. *H. pylori* produces a variety of exo-products including catalase, urease²⁸ and a vacuolating cytotoxin.²⁹ Catalase has been shown to reduce the bactericidal capacity of PMNL by interfering with the generation of H₂O₂ by the myeloperoxidase system, and ammonia, produced by urease, may combine with hypochlorous acid, and create intermediates toxic to PMNL.³⁰

The reasons why the immune system fails to eradicate *H. pylori*, despite the sensitivity *in vitro* to complement and PMNL killing, are complex and are likely to arise from a combination of factors.

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