Opsonophagocytosis of *Campylobacter pylori*

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Summary. The opsonic activity of human serum from various sources against *Campylobacter pylori* was compared. All sera, whether from control subjects with no symptoms of gastritis or peptic ulceration, or from symptomatic patients from whom *C. pylori* had or had not been isolated, opsonised *C. pylori* equally well. Opsonisation depended on the alternative pathway of complement activation but not on antibody. These findings suggest that antibody plays no role in protection against *C. pylori* and that the presence of antibody in patients' sera is mainly of diagnostic value.

Introduction

Campylobacter pylori, a spiral micro-aerophilic organism, was first detected in cultures of gastric antral mucosal biopsy specimens by Marshall in 1983. Since then many studies have demonstrated a strong correlation between the presence of *C. pylori* and histologically proven gastritis and peptic ulcer (McNulty and Watson, 1984; Price *et al.*, 1985). The aetiological significance of this organism in these diseases remains doubtful. *C. pylori* has been shown to provoke both a systemic and a local immune response (Jones *et al.*, 1984; Kalder *et al.*, 1985; Rathbone *et al.*, 1986; Wyatt *et al.*, 1986). The demonstration of *C. pylori*-specific IgG in serum and IgA in gastric juice have been taken to provide further evidence of a pathogenic role for the organism in gastro-duodenal disease. The aim of our study was to investigate whether the serum antibodies induced by *C. pylori* have opsonic activity for the organism and, accordingly whether they are likely to be protective.

Materials and methods

Patients and sera

One serum sample obtained from each of 35 patients undergoing investigation for upper abdominal symptoms was tested separately in all the experiments; the test sera were not pooled. *C. pylori* was isolated from mucosal biopsy specimens taken from the gastric antrum, duodenum, and oesophagus of 20 patients. Single serum

samples from 15 healthy individuals were taken and pooled to act as a control. Serum samples were heated at 56°C for 30 min to destroy complement. The classical complement activation pathway was inhibited selectively by treating with a solution containing 100 mM MgCl₂ and 100 mM ethylene glycol tetraacetate (MgEGTA; Sigma). Serum was also obtained from a patient with agammaglobulinaemia and used as a source of antibody-free complement. Serum devoid of antibodies to *C. pylori* was produced by absorption of serum three times at 4°C for 45 min with 10¹⁰ washed and packed *C. pylori* cells.

Preparation of micro-organisms

C. pylori was grown on 7% horse-blood agar incubated at 37°C in a micro-aerophilic atmosphere provided by CampyPak H₂-CO₂ generator envelopes (BBL Microbiology Systems) in GasPak jars (BBL) with no catalyst. A suspension of the organism was made in saline 0.9% w/v, centrifuged at 3000 *g* for 5 min and washed twice in saline. The organisms were then resuspended in Hanks's Balanced Salts Solution without phenol red (HBSS; Gibco) to give an optical density at 540 nm (OD₅₄₀) of 0.5, corresponding to a concentration of 1 × 10⁹ cfu/ml.

Preparation of human peripheral blood leucocytes

The same healthy human volunteer provided blood for preparation of leucocytes as and when required. Blood was taken by venepuncture and then heparinised with preservative-free heparin (10 u/ml). The erythrocytes were sedimented by gravity for 45 min at room temperature in dextran 6% w/v in saline 0.9% w/v. The leucocyte-rich buffy layer was centrifuged at 350 *g* for 5 min and the supernate discarded. The residual red cells were lysed by treatment with NH₄Cl 0.83% w/v in Tris-HCl buffer at pH 7.65 for 10 min. The cells were washed twice and a total and differential white cell count obtained. The cells were then resuspended in HBSS supplemented with fetal

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calf serum (Tissue Culture Services, Slough, Berks) 1% v/v to give a concentration of 1.5×10^6 polymorphonuclear leucocytes (PMN)/ml, accounting for 70% of the total number of leucocytes present in the final suspension.

Opsonisation

Each of the serum samples from the 20 *C. pylori* culture-positive and 15 culture-negative patients and the pooled normal human serum was duplicated and entered separately into the experiment.

In 2-ml plastic vials, 0.5 ml of bacterial suspension was added to 0.4 ml of HBSS and 0.1 ml of serum or other source of opsonin, to give a final opsonin concentration of 10% v/v. The mixture was incubated for 15 min at 37°C, centrifuged at 3000g for 5 min and washed twice in phosphate buffered saline, pH 7.2 (PBS). The opsonised particles were then resuspended in 0.5 ml of HBSS and kept at 4°C until ready to be used.

Opsonophagocytosis was studied by three methods that were essentially complementary to each other: luminol-dependent chemiluminescence; microscopical phagocytic assay; and indirect immunofluorescence. The first two are functional assays of opsonophagocytosis, measuring the uptake of bacteria by phagocytic cells. The IF test demonstrated the binding of opsonins to the surface of *C. pylori*.

Light emission in luminol-dependent chemiluminescence results from myeloperoxidase-dependent oxidation of luminol (Stevens *et al.*, 1978). The source of myeloperoxidase is the azurophilic granules of PMN, eosinophils and monocytes. The number of PMN present in a peripheral blood leucocyte preparation far exceeds that of eosinophils and monocytes. Accordingly, the phagocytic activity of PMN accounts for most of the light emission in luminol-dependent chemiluminescence.

Measurement of chemiluminescence

A stock solution of luminol was made by dissolving 1.77 mg of luminol (Sigma) in 1 ml of dimethylsulphoxide (DMSO; Sigma) to give a concentration of 10^{-2} M. This was diluted further in HBSS to the required concentration before use. Suspensions of opsonised bacteria (0.2 ml) were added to 0.5 ml of leucocyte suspension and 0.9 ml of 10^{-5} M luminol. The mixture was placed in the reaction chamber of a 1250 luminometer (LKB Co) for 15 min and the light generated was recorded in mV.

Microscopical phagocytic assay

Opsonised bacteria were diluted 100-fold in HBSS, and 0.2 ml of this suspension was incubated with an equal volume of leucocyte suspension at 37°C for 15 min, the tube being placed on a roller to ensure thorough mixing. Smears were prepared in a Cytospin centrifuge (Shandon Co.), fixed with methanol, stained with acridine orange (10 mg/ml in distilled water), and examined under the oil immersion objective of a fluorescence microscope. Re-

sults were expressed as the percentage of PMN with ingested *C. pylori*.

Immunofluorescence

A suspension of whole formalised *C. pylori* cells was prepared by adding five drops of a 40% formaldehyde solution to 20 ml of bacterial suspension. The mixture was left at room temperature for 30 min and then centrifuged at 3000g for 5 min, and washed twice in saline. The cells were finally resuspended in HBSS to give an OD₅₄₀ of 0.2.

To each well of a PTFE-coated slide (Hendley Ltd, Loughton, Essex) 0.02 ml of formalised *C. pylori* suspension was applied. After drying in air and fixing in 70% alcohol, 0.02 ml of serum or other source of opsonin was added to each well except the last two cells which contained 0.02 ml of HBSS instead and acted as negative controls. The slide was placed in a moist chamber for 30 min at room temperature and then washed twice in PBS to remove any excess or unattached opsonin. To each well on the top row on the slide, 0.02 ml of fluorescein isothiocyanate (FITC)-labelled rabbit anti-human IgG serum (Dako Ltd, High Wycombe, Bucks), diluted 10-fold in PBS, was added. Similarly, FITC-labelled anti C₃ serum was added to each well on the bottom row. The slide was then left in the moist chamber for 30 min, washed twice in PBS, air-dried and examined with the oil immersion objective of a fluorescence microscope. The observed fluorescence was scored on an arbitrary scale of -, +, ++, +++, and +++, representing the degrees of observed fluorescence as nil, weak, moderate, strong, and very strong respectively.

Results

We first compared the chemiluminescence response, phagocytic uptake, and surface IgG and C₃ immunofluorescence seen with (1) unopsonised organisms, and (2) with organisms opsonised with (a) pooled normal human serum, (b) serum from patients from whom *C. pylori* had been cultured, and (c) serum from patients who were culture negative (fig. 1). With unopsonised organisms there was very little phagocytic uptake and no surface IgG or C₃ immunofluorescence detectable. In the presence of opsonising serum, high values were obtained for all four parameters. All sera tested gave similar results. In particular, there was no significant difference between the opsonic activity of serum from patients who were culture positive and those who were culture negative for *C. pylori*.

We also tested ten *C. pylori* isolates against ten different sera but found no significant variation in either opsonic or binding activity.

C. pylori isolated from different sites in the upper gastrointestinal tract were opsonised with serum.

Fig. 1. Opsonic activity against *C. pylori* of pooled normal human serum and of serum from each of 20 *C. pylori* culture positive and 15 culture negative patients measured by: ▨ chemiluminescence (mV); ■ percentage number of PMN with ingested organisms; ▤ IgG immunofluorescence (IF); ▩ C₃ immunofluorescence (IF). Values expressed as mean for each population group. Bars represent 1 SD. No significant difference ($p > 0.05$) was found between culture positive and culture negative patients with respect to opsonophagocytosis determined by: chemiluminescence and microscopical phagocytic assay (t test); and IgG and C₃ immunofluorescence (Man-Whitney U test).

Experiments were performed in duplicate for each patient. The average value obtained was used to calculate the mean (with 1 SD shown in brackets), for each site of isolation of *C. pylori* (table). No significant difference ($p > 0.05$, analysis of variance) was seen in opsonophagocytosis of *C. pylori*, measured by chemiluminescence and microscopic phagocytic assay, with respect to the site of isolation of the organism in the upper gastrointestinal tract.

The main serum opsonins are IgG, IgM and C₃b. Serum from an agammaglobulinaemic patient opsonised *C. pylori* as well as did normal human serum despite total lack of IgG binding (fig. 2). In contrast, heat-inactivated serum was a poor opsonin. However, its activity could be restored with fresh agammaglobulinaemic serum, thus demonstrating the importance of complement in opsonophagocytosis of *C. pylori*. Opsonisation occurred in the presence of MgEGTA indicating that the alternative pathway of complement activation alone was sufficient to mediate this effect (fig. 3).

We found that absorption of serum with *C. pylori*

did not affect its opsonic activity although it did eliminate surface IgG immunofluorescence (fig. 3). This, coupled with the data obtained with agammaglobulinaemic serum, indicated no role for antibody.

Table. Effect of site of isolation on opsonophagocytosis of *C. pylori**

Site of isolation	Number of patients studied	Chemiluminescence value (SD) in mV	Percentage number (SD) of PMN with ingested organisms
Gastric antrum	14	173 (16)	76 (8)
Duodenum	6	168 (19)	76 (8)
Oesophagus	4	174 (20)	78 (10)

* Experiments were done in duplicate for each patient; average values (SD) are given for each site of isolation (see text).

Fig. 2. Opsonic activity against *C. pylori* of agammaglobulinaemic serum (AS), heat-inactivated serum (HS), and of HS+AS, demonstrating the importance of complement in opsonophagocytosis of *C. pylori*: ▨ chemiluminescence (mV); ■ percentage number of PMN with ingested organisms; ▤ IgG immunofluorescence (IF); ▩ C₃ immunofluorescence (IF).

Fig. 3. Opsonic activity of sera chelated with MgEGTA and of absorbed sera against *C. pylori*: ▨ chemiluminescence (mV); ■ percentage number of PMN with ingested organisms; ▤ IgG immunofluorescence (IF); ▩ C₃ immunofluorescence (IF).

In each case, results were the same whether pooled normal human serum or serum from campylobacter culture-positive or culture-negative symptomatic patients was used.

Discussion

The significance of *C. pylori* in the aetiology of gastritis, duodenitis, and peptic ulcer remains uncertain. The main controversy centres on whether the demonstration of *C. pylori* in mucosal biopsy specimens in these diseases represents a true pathological process or merely reflects colonisation by an organism taking advantage of conditions that favour its growth and multiplication. Findings that support a causal association include: strong correlation with histologically confirmed inflammation; eradication of the organism with specific antimicrobial therapy, correlating with histological, endoscopic and clinical improvement (Langenberg *et al.*, 1985; McNulty *et al.*, 1986); fulfilment of Koch's postulates at least in one case (Marshall *et al.*, 1985); and the demonstration of both a systemic and a local immune response to the organism. Elevated and specific antibody production is often a hallmark of a host response to a microbial pathogen in many infections. Opsonisation is one of the main protective functions of antibody. Complement may be required for optimal opsonic activity.

The aim of our study was to demonstrate whether the serum antibodies against *C. pylori* are opsonic for the organism. Our results indicate that human serum has strong opsonic activity for *C. pylori*, irrespective of overt infection with the organism. The opsonic activity depended on the alternative pathway of complement activation. Components of the cell wall, such as lipopolysaccharide, of gram-negative bacteria are known to be powerful activators of the alternative complement pathway. During this activation many potent bioactive and chemotactic substances are released. These may be responsible for the underlying intense inflammation seen in mucosal biopsy specimens positive for *C. pylori*. However, even if this is the true mechanism, it clearly fails to eradicate the organism.

Various methods, including complement fixation tests, passive haemagglutination, and ELISA, have been used to detect *C. pylori*-specific antibodies in serum. The prevalence of these antibodies is highest in patients with both organisms and histologically confirmed gastritis and peptic ulcer in biopsy specimens (Jones *et al.*, 1984). Antibodies to *C. pylori* are also detectable in "normal" healthy individuals. In a recent survey (Jones *et al.*, 1986),

12% of a random sample of the population aged 21–30 years had antibody to *C. pylori* in their serum. The prevalence of antibody increased with age.

We found that serum antibodies were not important for opsonophagocytosis of *C. pylori*. They are unlikely to be protective in infection caused by this organism. This may help to explain why colonisation with *C. pylori* tends to be a persistent problem, and why relapse of infection is common even after apparent successful eradication of the organism with specific antimicrobial therapy (Tytgat *et al.*, 1986). A possible reason for the lack of opsonic activity of serum antibodies against *C. pylori* is that the antigenic determinants which may stimulate a protective immune response in the host may be located deep inside the cell wall of the bacterium and thus may be inaccessible to host antibodies.

The presence of *C. pylori* has been documented in the gastric mucosa, duodenum (Phillips *et al.*, 1984; Coelho *et al.*, 1987), and the oesophagus (L.G.V. Coelho *et al.*, unpublished observations). Our results indicate that the site of isolation of the organism does not appear to be important in determining opsonophagocytosis *in vitro*. Testing serum from culture-positive patients, individually, against the homologous and heterologous *C. pylori* isolates showed no difference in opsonophagocytosis. These observations are consistent with the finding that opsonophagocytosis of *C. pylori* is complement-dependent, but not antibody-dependent.

C. pylori-specific IgA, together with low levels of IgM, has been demonstrated in a proportion of patients with gastritis (Rathbone *et al.*, 1986). Immunoperoxidase studies have shown IgG-, IgA-, and IgM-coated *C. pylori* in tissue sections (Wyatt *et al.*, 1986). Organisms deep within the gastric pits appeared to be uncoated, suggesting that they may be protected in this site from host antibody. We have not examined the effect of IgA. This class of antibody does not function as an opsonin and may even inhibit the opsonic activity of IgG and IgM antibodies. If IgA has any role in defence against *C. pylori* this is likely to be linked to its effects on bacterial adherence. In contrast to our results Pruul *et al.* (1987) found that phagocytic killing of *C. pylori* required both specific antibody and an intact complement pathway. The methods used to examine opsonophagocytosis in the two studies were different. We used functional assays to measure phagocytic uptake of bacteria, whereas Pruul *et al.* employed a bactericidal assay. However, a bacterium may be ingested by a phagocytic cell but not killed. Growth and viability of bacteria in serum

may also be affected by mechanisms other than opsonophagocytosis, e.g., serum lysis and agglutination. Thus, the results of the two studies cannot be compared directly.

Although we did not find antibodies to be necessary for opsonophagocytosis, the demonstration of *C. pylori*-specific antibodies is of diagnostic importance and is being used to study the epide-

miology of *C. pylori* infection (Morris *et al.*, 1986). It may also be useful in monitoring responses to specific antimicrobial therapy directed against *C. pylori*.

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