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Helicobacter pylori inhibits phagocytosis by professional phagocytes involving type IV secretion components

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Summary

Gastric infections by *Helicobacter pylori* are characteristically associated with an intense inflammation and infiltration of mainly polymorphonuclear lymphocytes (PMNs) and monocytes. The inflammatory response by infiltrated immune cells appears to be a primary cause of the damage to surface epithelial layers and may eventually result in gastritis, peptic ulcer, gastric cancer and/or MALT-associated gastric lymphoma. Our analysis of the interaction between *H. pylori* and PMNs and monocytes revealed that *H. pylori* inhibits its own uptake by these professional phagocytes. To some degree, this effect resembles antiphagocytosis by *Yersinia enterocolitica*. Increasing numbers of bacteria associated per cell are more efficient at blocking their own engulfment. In *H. pylori*, bacterial protein synthesis is necessary to block phagocytic uptake, as shown by the time and concentration dependence of the bacteriostatic protein synthesis inhibitor chloramphenicol. Furthermore, *H. pylori* appears broadly to inhibit the phagocytic function of monocytes and PMNs, as infection with *H. pylori* abrogates the phagocytes' ability to engulf latex beads or adherent *Neisseria gonorrhoeae* cells. This antiphagocytic phenotype depends on distinct virulence (*vir*) genes, such as *virB7* and *virB11*, encoding core components of a putative type IV secretion apparatus. Our data indicate that *H. pylori* exhibits an antiphagocytic activity that may play an essential role in the immune escape of this persistent pathogen.

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Introduction

Helicobacter pylori is a spiral, Gram-negative bacterial pathogen discovered in stomach biopsies from patients with chronic gastritis (Marshall and Warren, 1983). It specifically colonizes the gastric epithelium of humans and other primates, and there is a strong association between active chronic gastritis, duodenitis, gastric or duodenal ulcers and *H. pylori* infection (Wallace, 1991; Tytgat, 1995). Indeed, ingestion of the bacteria by human volunteers produces gastric infection and histologically demonstrable gastritis (Morris *et al*., 1987). Eradication of the organism from patients with ulcers results in resolution of the pathology, and reinfection is associated with an elevated chance of disease recurrence (Megraud and Lamouliatte, 1992).

Significant advances have been made in recent years with regard to the virulence determinants expressed by *H. pylori*. However, the exact mechanisms of pathogenesis and immune evasion remain unclear. A specific immune response is induced during *H. pylori* infection, as demonstrated by the high titres of *H. pylori* antibodies (Dent *et al*., 1988). Despite this, no *H. pylori* eradication or disease recovery is observed (Fiocca *et al*., 1994; Crabtree, 1996), suggesting that antibodies are not sufficient to mediate protection. The presence of a 'pathogenicity island' (PAI) within the bacterial genome is associated with an increased virulence of *H. pylori* strains (Rappuoli *et al*., 1998). Type I *H. pylori* strains express a highly immunogenic protein of unknown function called CagA that is encoded within the pathogenicity island, and the VacA cytotoxin that induces vacuole formation in epithelial cells (de Bernard *et al*., 1997). Other genes encode a putative type IV secretion apparatus with homology to well-known virulence genes from *Agrobacterium tumefaciens*, *Bordetella pertussis*, *Legionella pneumophila and* other pathogens, such as homologues of VirB4, VirB7, VirB9, VirB10, VirB11 or VirD4 proteins (Covacci *et al*., 1999). In contrast, type II strains do not possess the pathogenicity island and, therefore, do not express CagA. They also show no cytotoxic activity *in vitro*, despite the fact that the v*acA* gene may be present (Ghira *et al*., 1995). Other putative virulence determinants that have been identified include those that are thought to be important for survival in the viscous and acidic environment of the gastric mucus

(Lee, 1994). Motility mediated by flagella and the ability to hydrolyse urea are both considered particularly important in this regard (Cover *et al*., 1991). However, the direct contribution of each of these factors to *H. pylori* disease is not known.

An increased number of T lymphocytes, macrophages and polymorphonuclear leucocytes (PMNs) are evident in histological sections of gastric mucosa from patients with *H. pylori* infections (Kazi *et al*., 1989). Tissue damage caused by *H. pylori* infections appears to result from the constant inflammatory reaction in response to this persistent gastric infection (Mai *et al*., 1991), and the severity of mucosal injury that results is directly correlated with the extent of neutrophil infiltration (Kozol *et al*., 1991; Yoshida *et al*., 1993). *H. pylori* type I strains appear to produce and release substances that induce a heightened inflammatory response (Blaser, 1992). Consistent with this, recent studies have shown that sonicates or extracts of *H. pylori* possess chemotactic activity for various inflammatory cells, including neutrophils and monocytes (Mai *et al*., 1991; Craig *et al*., 1992; Nielsen and Andersen, 1992a). Moreover, a *H. pylori* neutrophil-activating protein (HP-NAP) has been identified (Evans *et al*., 1995). *H. pylori* activation of neutrophils is evident as a chemotactic response, as an oxidative burst response and as an increase in adhesion to endothelial cells (Nielsen and Andersen, 1992b; Rautelin *et al*., 1993; Yoshida *et al*., 1993).

Despite the recruitment of inflammatory cells to loci of *H. pylori* infection, the bacteria do persist, thus implying that they are able to evade the mucosal immune response (Zevering *et al*., 1999). Other bacterial pathogens have evolved strategies to deal with inflammation by influencing their interaction with professional phagocytes. For example, a hallmark of gonorrhoea is a urethral or cervical exudate consisting primarily of PMNs containing intracellular- and extracellular-associated *N. gonorrhoeae*. This interaction is mediated by the gonococcal Opa adhesin binding to phagocytic CD66 receptors (Kupsch *et al*., 1993; Dehio *et al*., 1998). Opa–CD66 interactions lead to the rapid internalization of bound bacteria and an increased oxidative response, making the benefit to gonococci uncertain. The fact that gonococcal strains express multiple CD66-specific *opa* genes (Gray-Owen *et al*., 1997) does, however, suggest that adherence to these receptors is an essential function. Contact between pathogenic bacteria and professional phagocytes does not, however, necessarily lead to bacterial engulfment. Some bacteria, including *Yersinia enterocolitica*, instead possess the mammalian cell contact-inducible ability to secrete potent modulators of host cell function directly into the target cell's cytoplasm (Lee, 1997). This process, known as type III protein secretion, is able effectively to block phagocytosis by monocytes and PMNs (Lian *et al*., 1987), allowing the pathogen to survive on the surface of these otherwise potently bactericidal cells.

In this study, we investigated the interaction between multiple *H. pylori* strains and primary PMNs and monocytes isolated from the blood of various human donors. The adherence and phagocytosis of bacteria were assessed by confocal laser scanning microscopy and both transmission and scanning electron microscopy. We found that *H. pylori* effectively inhibits its own uptake by both of these professional phagocytes and that this effect was dependent on the presence of several potential *H. pylori* virulence genes. This effect was independent of the donor's blood type and increased with increasing numbers of bacteria bound per cell. It is an active process controlled by the *H. pylori*, as viable bacteria were engulfed when their protein synthesis was inhibited by chloramphenicol. This remarkable process also blocks the engulfment of inert particles and other bacteria that are normally phagocytosed, suggesting that *H. pylori* imposes a wide-reaching inhibitory effect on the phagocytic process.

Results

H. pylori *resists phagocytosis by human phagocytes*

Our preliminary infection experiments, aimed at characterizing the interaction between *H. pylori* 26695 and freshly isolated human phagocytes, demonstrated that the bacteria associated with monocytes and neutrophils were almost exclusively extracellular. In order to assess whether this lack of phagocytosis was special for *H. pylori*, subsequent infection experiments also included other bacterial species for which the interaction with human phagocytes was better characterized. For these studies, the quantification of adherence and bacterial uptake was performed using immunofluorescence staining for confocal laser microscopic analysis. *H. pylori* grown either in liquid culture or on S plates for 1–4 days showed similar rates of adherence and uptake (not shown). *H. pylori* adhered to monocytes (4.4 bacteria cell^{-1}; Fig. 1B) and PMNs (4.5 bacteria cell^{-1}; Fig. 1D) at a level much higher than the non-virulent laboratory strain *Escherichia coli* HB101 (0.15 and 0.1 bacteria cell^{-1} for monocytes and PMNs respectively). Yet, in the case of both phagocytic cell types, a much higher percentage of associated *E. coli* HB101 was intracellular (53% of infected monocytes and 59% of infected PMNs contained intracellular bacteria) than was seen for *H. pylori* (11% of monocytes and 12% of PMNs contained intracellular bacteria; Fig. 1A and C). Consistent with its well-characterized opsonin–independent interactions with phagocytic cells (Dehio *et al*., 1998), *N. gonorrhoeae* strain N303 expressing the heparan sulphate receptor-specific

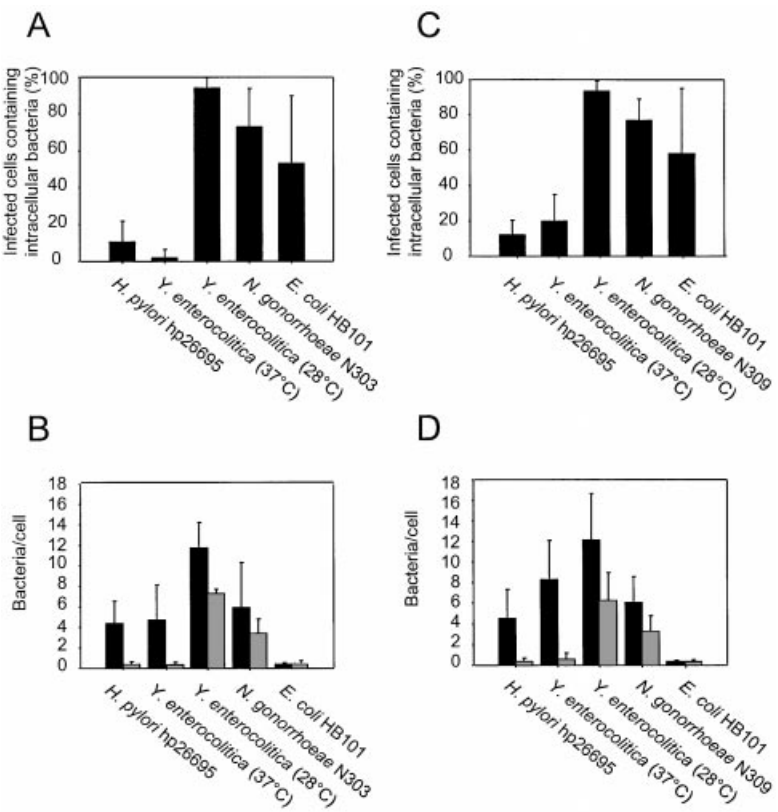

Fig. 1. Bacterial association with human phagocytes. *H. pylori*, *Y. enterocolitica*, *N. gonorrhoeae and E. coli* were incubated for 1.5 h at 37°C with human monocytes (A and B) or PMNs (C and D). After washing, samples were immunostained fluorescently for confocal laser scanning microscopic analysis. The average number of intracellular and total bacteria associated with each randomly counted phagocytic cell was determined (black shading: total associated bacteria, grey shading: intracellular bacteria) (B and D), as was the percentage of infected cells containing at least one intracellular bacterium (% infected cells containing intracellular bacteria) (A and C). Results are the mean of 15 independent experiments. *H. pylori* hp26695; *Y. enterocolitica* serotype 0:9 grown either at 37°C to induce antiphagocytic properties or at 28°C to allow phagocytosis; *N. gonorrhoeae* N303 expressing the heparan sulphate proteoglycan receptor-specific Opa_{50} protein (used for monocyte infections); N309 expressing the CD66 receptor-specific Opa_{52} protein (used for PMN infections); and *E. coli* HB101.

Opa_{50} adhesin or strain N309 expressing the CD66 receptor-specific Opa_{52} protein were found to bind efficiently to monocytes (5.9 bacteria $cell^{-1}$; Fig. 1B) and PMNs (4.7 bacteria $cell^{-1}$; Fig. 1D) respectively. In each case, gonococcal adherence correlated with a high level of bacterial engulfment, i.e. 73% of N303-infected monocytes and 73% of N309-infected PMNs contained intracellular bacteria (Fig. 1A and C). In contrast, when grown at 37°C, *Y. enterocolitica* strongly adhered to the human phagocytes (4.7 and 8.3 bacteria $cell^{-1}$ for monocytes and PMNs respectively) but was not internalized (Fig. 1A–D), clearly demonstrating its previously described antiphagocytic properties (Lian *et al*., 1987). As expected, *Y. enterocolitica* grown at 28°C, which do not express a type III protein secretion function, did not show any antiphagocytic activity (94% of infected monocytes and 93% of infected PMNs contained intracellular bacteria; Fig. 1A and C). These results were confirmed by transmission electron microscopy (TEM) analysis (data not shown) and by gentamicin plating experiments, in which we found the percentage of intracellular bacteria in monocytes to be 7% for *H. pylori* and 0.1% for *Y. enterocolitica* (data not shown). This is consistent with 5% and 0.2% that we obtained using the confocal laser scanning microscopic approach (Fig. 1B). The ability of *H. pylori* to block phagocytosis is an active process (see below). Therefore, the slightly higher rate of phagocytosis obtained for *H. pylori* relative to *Y. enterocolitica* grown at 37°C may relate to the fact that *H. pylori* viability is significantly lower than that of *Y. enterocolitica* in the tissue culture medium used in these experiments (not shown). Together, these data indicate that *H. pylori* binds efficiently but is not efficiently engulfed by human phagocytic cells.

To eliminate the possibility that cytotoxic effects of *H. pylori* could lead to antiphagocytosis, the viability of both PMNs and monocytes was tested using a trypan blue exclusion test. The phagocytes' viability was greater than 95% before infection, and no significant change in membrane permeability was observed during the 1.5 h infection period with any of the bacterial strains used (not shown). The cells were still able to produce an oxidative response (Ramarao *et al*., 2000), further confirming their viability.

H. pylori *antiphagocytic mechanism is not strain specific*

To determine whether this antiphagocytic effect was restricted to *H. pylori* 26695, a panel of different type I strains was used to infect monocytes (Fig. 2). None of the human isolates tested (i.e. hp26695, P12, G27 or P1) were efficiently internalized, with the percentage of

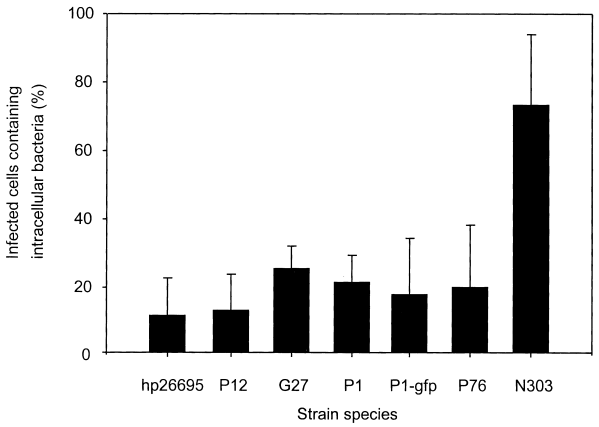

Fig. 2. *H. pylori* interactions with human phagocytes. Human monocytes were infected with different strains of *H. pylori* for 1.5 h at 37°C. After washing, samples were immunostained fluorescently for confocal laser scanning microscopic analysis. The percentage of infected cells containing at least one intracellular bacterium was determined. Results are the mean of four independent experiments. hp26695, P12, G27 and P1, clinical type I *H. pylori* isolates; P76, mouse-adapted *H. pylori* type I strain; P1-gfp, green fluorescent protein-expressing derivative of P1; *N. gonorrhoeae* expressing the heparan sulphate proteoglycan receptor-specific Opa_{50} protein.

infected cells containing at least one intracellular bacterium ranging between 11% and 21%. The mouse-adapted strain P76 was also poorly phagocytosed (20% of infected cells containing one or more intracellular bacteria).

In order to eliminate the possibility that internalized bacteria were being overlooked because the epitopes recognized by the anti-*H. pylori* serum used for immunofluorescence staining were destroyed during ingestion and processing of the bacteria within the phagosome, a *H. pylori* strain expressing the green fluorescent protein (P1-*gfp*) was used. The percentages of infected cells containing at least one intracellular bacterium were indistinguishable, being 21% and 17% for P1 stained using the standard protocol (see *Experimental procedures*) and P1-*gfp* respectively (Fig. 2). The level of interaction was also similar, with 3.7 bacteria associated and 0.6 intracellular bacteria per monocyte for P1 and 3.1 bacteria associated and 0.6 intracellular bacteria per cell with P1-*gfp* (data not shown). The fluorescent labelling of *H. pylori*, *N. gonorrhoeae*, *Escherichia coli and Y. enterocolitica* with TAMRA before infection also showed results that were consistent with those obtained using our standard immunofluorescence staining, further validating the quantification techniques used throughout this study (data not shown).

Human blood group antigen type does not influence H. pylori *adherence and uptake*

Previously, blood group antigens have been reported to influence *H. pylori* binding to epithelial cells (Niv *et al.*, 1996) and disease outcome (Mentis *et al.*, 1991). In order to determine whether the blood group antigens affect the professional phagocyte's ability to engulf *H. pylori*, infection experiments were performed in parallel using primary PMNs and monocytes isolated from donors belonging to different blood groups. In each case, the uptake of *H. pylori* was low, with the percentage of infected cells containing at least one intracellular bacterium (Fig. 3A) and the proportion of bacteria that were intracellular (Fig. 3B) both remaining below 20%. Indistinguishable results were also obtained when the wild-type *H. pylori* strains P1, P12 and G27 were used (data not shown), indicating that the lack of influence of blood group antigens was not strain specific.

Bacterial protein synthesis is necessary for H. pylori *inhibition of phagocytosis*

We found that *H. pylori* that had been killed (e.g. by treatment with gentamicin) before incubation with

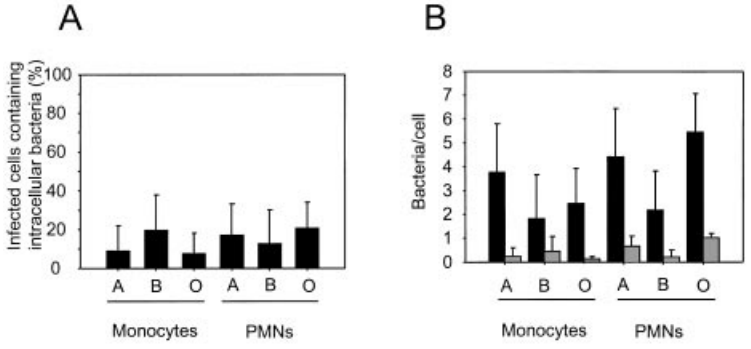

Fig. 3. Effect of blood group antigen expression on *H. pylori* interactions with human phagocytes. Monocytes and PMNs freshly isolated from donors expressing different blood group antigens were infected with *H. pylori* hp26695 for 1.5 h at 37°C. After washing, samples were immunostained fluorescently for confocal laser scanning microscopic analysis. The percentage of infected cells containing at least one intracellular bacterium was determined (A), as were the total number (black shading) and intracellular (grey shading) bacteria associated with randomly counted phagocytic cells (B). Results are the mean of two independent experiments, each performed in triplicate.

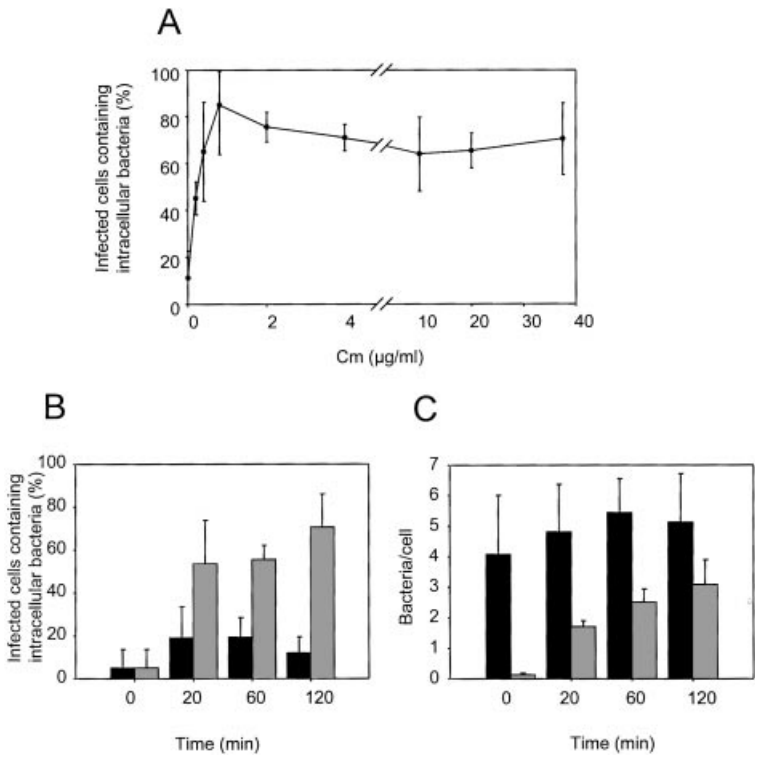

Fig. 4. Effect of bacterial protein synthesis inhibition on *H. pylori* interaction with monocytes. *H. pylori* hp26695 was treated for various times with the indicated concentrations of chloramphenicol to inhibit bacterial protein synthesis. Bacteria were then washed three times in PBSA buffer before being used to infect monocytes for 1.5 h at 37°C. After washing, samples were immunostained fluorescently for confocal laser scanning microscopic analysis.
A. Bacteria were treated with the indicated concentrations of chloramphenicol for 2 h. The percentage of infected cells containing at least one intracellular bacterium was determined. Results are the mean of three independent experiments, each performed in duplicate.
B. Bacteria were treated with 37.5 μg ml^{-1} chloramphenicol (grey shading) for the indicated times or used to infect cells without antibiotic treatment (black shading). The percentage of infected cells containing at least one intracellular bacterium was determined.
C. For treated bacteria, the number of intracellular (grey shading) and total (black shading) bacteria associated with randomly counted phagocytic cells was also determined. Results are the mean of three independent experiments.

phagocytes were efficiently phagocytosed (data not shown), suggesting that the inhibition of phagocytosis was an active process. In order to determine whether the antiphagocytic effect required protein synthesis, *H. pylori* was treated with the bacteriostatic protein synthesis inhibitor chloramphenicol before infection. In contrast to pretreatment with gentamicin, 2 h incubation with 37.5 μg ml^{-1} chloramphenicol had no obvious effect on bacterial viability, as determined by dilution plating onto S plates after antibiotic treatment (data not shown). *H. pylori* was therefore treated for 2 h with chloramphenicol concentrations ranging from 0 to 37.5 μg ml^{-1}. After treatment, the bacteria were incubated with monocytes, and the percentage of infected cells containing at least one intracellular bacterium was determined (Fig. 4A). The percentage of cells containing intracellular bacteria increased drastically in a concentration-dependent manner. For higher concentrations, this reached a plateau of approximately 70–80% of infected cells containing at least one intracellular bacterium versus 11% in the absence of chloramphenicol treatment. Similar results were obtained when PMNs were used instead (data not shown). This effect is time dependent, as increasing durations of chloramphenicol treatment before infection resulted in a concomitant increase in both the percentage of infected cells containing intracellular bacteria (Fig. 4B) and the proportion of associated bacteria that are intracellular (Fig. 4C) compared with untreated controls.

As an independent control, the effect of chloramphenicol treatment on *Y. enterocolitica* grown at 37°C was also assessed. Consistent with what has been reported previously for gentamicin treatment (Fallman *et al.*, 1995), the percentage of infected monocytes containing at least one intracellular bacterium increased from 2% in the absence of chloramphenicol treatment to 65% after antibiotic treatment for 2 h (data not shown). Protein synthesis is therefore clearly required before or in the early stages of contact with the phagocytic cells for *H. pylori* to block its phagocytic uptake. These results were confirmed by TEM in which the number of antibiotic-treated bacteria that were intracellular was significantly higher than in the case of untreated bacteria (data not shown).

In order to compare the effect of chloramphenicol treatment on the rate of bacterial binding to the phagocytes, parallel samples, incubated in either the presence or the absence of chloramphenicol, were fixed and washed at various time points after infection (Fig. 5). Irrespective of antibiotic treatment, the number of associated bacteria increased from less than one at time point zero to five or six bacteria cell^{-1} 90 min after infection. The number of untreated bacteria ingested per cell remained low and constant (less than 0.2 cell^{-1}) throughout the experiment; however, chloramphenicol treatment resulted in a 14-fold increase in intracellular bacteria per cell (compare Fig. 5B and C). The effect of chloramphenicol treatment over time was also evident by

the fact that the percentage of cells infected with at least one intracellular bacterium remained very low (< 20%) for untreated bacteria but increased up to approximately 70% upon chloramphenicol treatment (Fig. 5A).

Dose dependency of the antiphagocytic mechanism

In order to determine whether the number of bacteria bound per cell influenced the ability of phagocytes to engulf *H. pylori*, freshly isolated human monocytes were infected at various bacteria:cell ratios. The number of cell-associated bacteria increased with the multiplicity of infection (MOI), with one bacterium cell^{-1} at an MOI of 1 up to 5.9 bacteria cell^{-1} at an MOI of 1000 (Fig. 6A). In contrast, the percentage of cell-associated bacteria that were intracellular decreased with increasing MOI (data not shown), and the percentage of infected cells that contained at least one intracellular bacterium decreased from 50% at an MOI of 1 to 10% at an MOI of 100 (Fig. 6B). Similar results were obtained when using PMNs, with the percentage of infected cells containing at least one intracellular bacterium decreasing from 33% at an MOI of 1 down to 8% at an MOI of 1000 (data not shown). These results were almost identical to those obtained for *Y. enterocolitica* grown at 37°C (Fig. 6A and B): a similar increase in adherence (Fig. 6A) and a similar decrease in infected cells containing intracellular bacteria (Fig. 6B) were observed, from 50% at an MOI of 1 to 7% at an MOI of 1000. To establish the MOI at which *H. pylori* starts inhibiting phagocytosis, *H. pylori* were treated with bacteriostatic concentrations of chloramphenicol, and monocytes were infected at different MOIs. At an MOI of 1, almost 40% of infected cells contained intracellular bacteria, a percentage similar to untreated *H. pylori*. However, at an MOI of 2, this percentage was already twice as high as for untreated bacteria (60% versus 32%). For increasing MOIs, this difference became even more pronounced (Fig. 6B) for the same adherence (Fig. 6A), showing that the ability of *H. pylori* to evade phagocytosis is therefore a bacterium-driven process that has a cumulative effect, with increasing numbers of associated bacteria to a single cell being more efficient at impeding their own internalization.

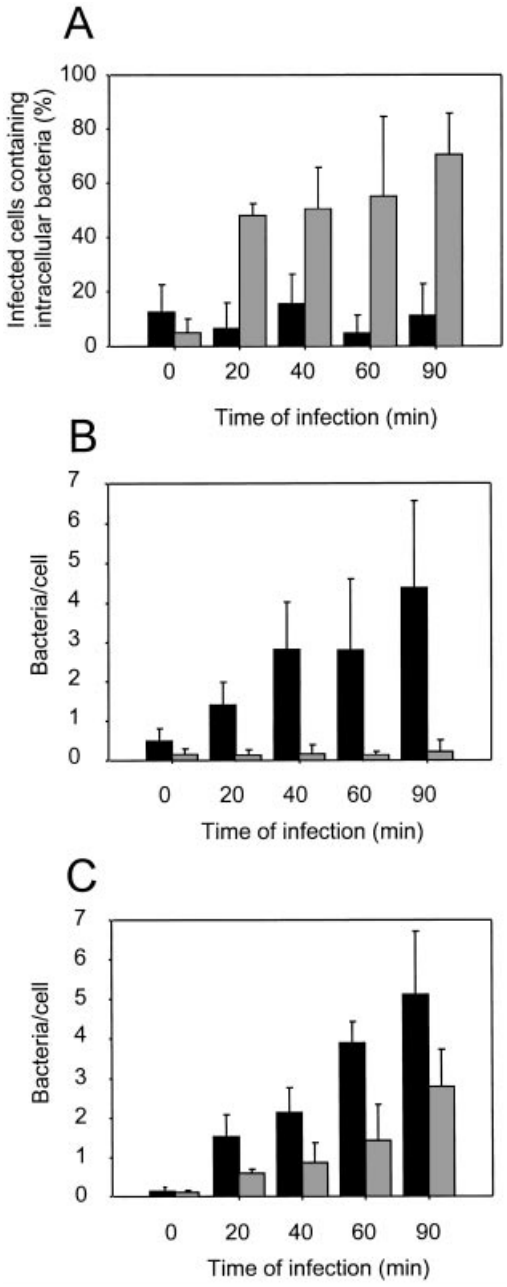

Fig. 5. Time course of *H. pylori* hp26695 association with phagocytes. *H. pylori* hp26695 were incubated for 2 h at 37°C in RPMI medium alone or in medium containing 37.5 μg ml^{-1} chloramphenicol, as indicated. The bacteria were then washed and used to infect monocytes. The infection was stopped by fixing in 3.7% paraformaldehyde at different time points, and samples were then washed and immunostained fluorescently for confocal laser scanning microscopic analysis. The number of intracellular and total bacteria associated with randomly counted phagocytic cells was determined (B and C), as was the percentage of infected cells containing at least one intracellular bacterium (A). Results are the mean of four independent experiments.
A. Percentage of infected cells containing at least one intracellular bacterium for chloramphenicol-treated (grey shading) and untreated (black shading) bacteria.
B. Number of bacteria associated per cell for untreated bacteria (total: black shading; intracellular: grey shading).
C. Number of bacteria associated per cell for chloramphenicol-treated bacteria (total: black shading; intracellular: grey shading).

H. pylori *broadly inhibits the phagocytic activity of human monocytes and PMNs*

The inability of phagocytes to engulf *H. pylori* could potentially result either from the bacteria's ability to bind to

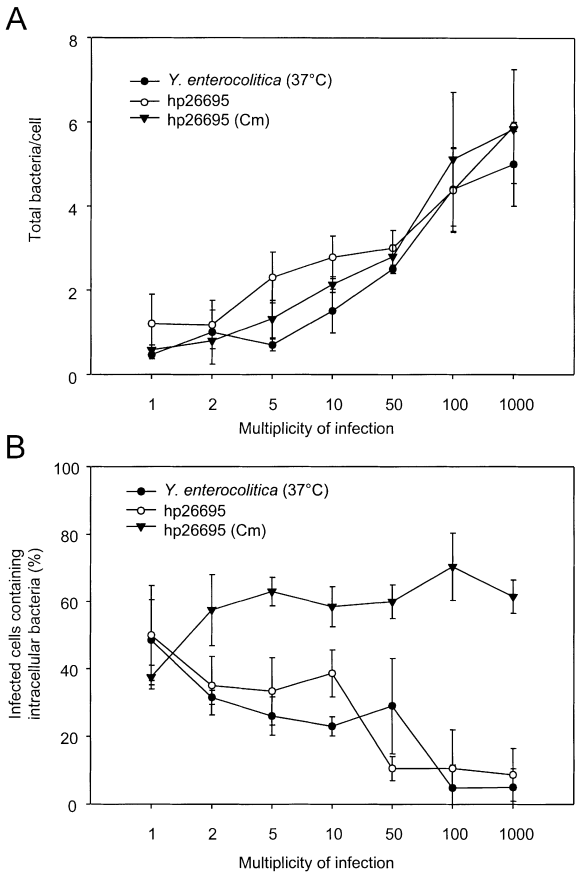

Fig. 6. Effect of *H. pylori* infecting dose on phagocytosis. Monocytes were infected for 1.5 h at 37°C with *H. pylori* strain hp26695 untreated (white circles) or treated with 37.5 μg ml^{-1} Cm for 2 h at 37°C (black triangles) or with *Y. enterocolitica* grown at 37°C (black circles), at the indicated MOI. After washing, samples were immunostained fluorescently for confocal laser scanning microscopic analysis. The total number of cell-associated bacteria (A) and the percentage of infected cells containing at least one intracellular bacterium (B) were determined. Results are the mean of three independent experiments, each performed in duplicate.

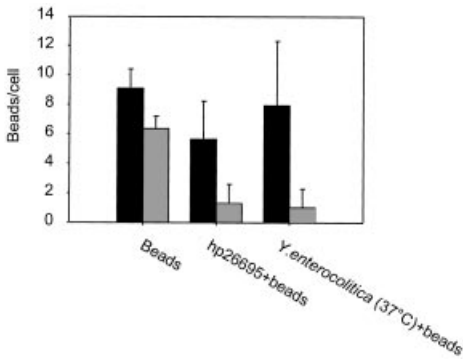

Fig. 7. *H. pylori* inhibition of latex bead phagocytosis. Monocytes were incubated with either latex beads alone or infected with either *H. pylori* or *Y. enterocolitica* for 1 h before the addition of latex beads. The uptake of latex beads was determined using confocal laser scanning microscopy. The results are the mean of three independent experiments, each performed in duplicate. The number of intracellular (grey shading) and total associated (black shading) beads per cell was determined.

a cell surface receptor that cannot be internalized or from a more general inhibition of phagocytic function. As the internalization of latex beads has previously been used as a general model of phagocytosis by professional phagocytes (Dunn and Tyrer, 1981), we assessed the effect of infecting these cells with either *H. pylori* or *Y. enterocolitica* for 1 h before the addition of fluorescent latex beads. The percentage of intracellular beads decreased dramatically after a 1 h preinfection with *H. pylori*, as evidenced by the fact that the percentage of monocytes that contained at least one intracellular bead decreased from 66% to 35% (data not shown) and that approximately 73% of beads were intracellular in cells that had not previously been infected, yet only 19% or 15% of beads were internalized when the cells were preincubated with *H. pylori* or *Y. enterocolitica* respectively (Fig. 7). As with all other experiments performed in this study, parallel experiments were also performed with PMNs, and the results were indistinguishable from those seen with monocytes (data not shown).

We next tested the ability of *H. pylori* to block the opsonin-independent phagocytosis of *N. gonorrhoeae* (Dehio *et al.*, 1998). To do so, cells were first infected with *H. pylori* for various times before the addition of *N. gonorrhoeae*. The reverse experiment, in which cells were first infected by *N. gonorrhoeae* for various times before the addition of *H. pylori*, was also performed. In all cases, the primary infecting bacteria were not removed before the addition of the second bacterial species, resulting in the phagocytes being exposed to both pathogens for the final period of incubation. In each case, the uptake of *N. gonorrhoeae* was quantified by both confocal laser scanning microscopy and TEM, and further characterized with scanning electron microscopy (SEM) (Fig. 8). When phagocytes were infected with *N. gonorrhoeae* alone, a constant increase in bacterial binding was seen to be directly associated with an increase in bacterial internalization, whereas the addition of *H. pylori* at any stage during the infection effectively blocked further gonococcal uptake (data not shown). Consistent with this, the inhibition of *N. gonorrhoeae* uptake occurred when phagocytes were preincubated with *H. pylori*, and the level of inhibition increased along with the duration of phagocytic exposure to *H. pylori* before gonococci were added (Fig. 8A and B). For example, the percentage of infected monocytes containing at least one intracellular *N. gonorrhoeae* N303 decreased from 65% when cells were infected only with N303 to 56% when cells were simultaneously infected with *H. pylori* and N303 to 10% when cells were infected for 1 h with *H. pylori* before inoculation with N303. This significant decrease was evident in both monocytes (Fig. 8A) and PMNs (Fig. 8B),

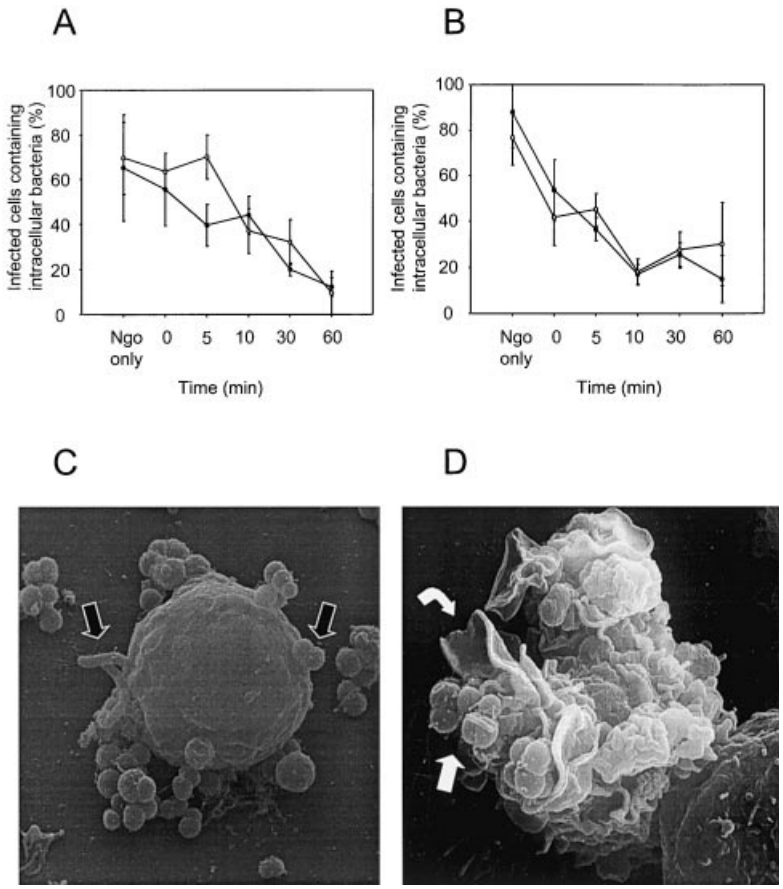

Fig. 8. *H. pylori* inhibits the phagocytosis of *N. gonorrhoeae* expressing both the heparan sulphate proteoglycan (Opa_{50})- and the CD66-specific (Opa_{52}) adhesin. PMNs and monocytes were infected with hp26695 for the indicated times before the addition of *N. gonorrhoeae* N303 (Opa_{50}) or N309 (Opa_{52}) for 1 h at 37°C. Samples were then washed and either immunostained fluorescently for confocal laser scanning microscopic analysis or prepared for electron microscopy. The percentage of infected cells containing at least one intracellular *N. gonorrhoeae* was determined. Results are the mean of five independent experiments.
A. Infection of monocytes: times indicate the period of phagocyte exposure to *H. pylori* before the addition of *N. gonorrhoeae* (open circles, N309; filled circles, N303).
B. Infection of PMNs: times indicate the period of phagocyte exposure to *H. pylori* before the addition of *N. gonorrhoeae* (open circles, N309; filled circles, N303).
C. Scanning EM picture of PMNs infected with hp26695 for 1 h, followed by infection with N309.
D. Scanning EM picture of PMNs infected with N309 for 1 h. White curved arrow indicates the lamella produced by the activated phagocyte; white arrow indicates that bacteria have been taken up by the cell; black arrows indicate bacteria at the surface of the cell.

and occurred when gonococci expressing either the heparan sulphate proteoglycan-specific Opa_{50} adhesin (N303) or the CD66 receptor-specific Opa_{52} (N309) were used. However, the *N. gonorrhoeae* adherence to phagocytic cells was not affected (data not shown). In addition to the confocal microscopy-based assays, these results were also confirmed by TEM (not shown) and SEM (Fig. 8C and D).

As a control, parallel experiments were also performed in which the influence of *Y. enterocolitica*'s well-described antiphagocytic activity (Lian *et al.*, 1987; Fallman *et al.*, 1995) was assessed on *N. gonorrhoeae* uptake. Consistent with what we saw for *H. pylori*, preincubation of the phagocytes with *Y. enterocolitica* grown at 37°C resulted in only 6% of infected cells containing intracellular gonococci (data not shown). Thus, *H. pylori* and *Y. enterocolitica* can each effectively block gonococcal internalization by professional phagocytes.

H. pylori *uses a type IV secretion system to inhibit phagocytosis*

To elucidate further the mechanism by which *H. pylori* inhibits phagocytosis in phagocytic cells, a series of isogenic mutants was used to assess the role of previously described virulence factors on the antiphagocytic properties of *H. pylori* (Fig. 9). Strain P11 was derived from P1 and has no urease activity, as it lacks both enzymatic subunits (UreA and UreB) (Kahrs *et al.*, 1995). The uptake of this mutant was similar to that from the wild type (i.e. 27% and 21% of infected monocytes contained intracellular bacteria respectively). Similar results were obtained with mutants lacking VacA (P14) (Schmitt and Haas, 1994), CagA (P211), CagI (P203) (Censini *et al.*, 1996) or CagF (P204) (Censini *et al.*, 1996) (Fig. 9), indicating that none of these putative virulence factors is responsible for the reduced level of phagocytosis of this organism.

To examine the possibility that genes of the hypothetical type IV secretion apparatus of *H. pylori* are involved in the process of inhibiting phagocytosis, we constructed additional isogenic *H. pylori* mutants including homologues of the *virB7* and *virB11* genes. These two proteins are absolutely essential core components in the assembly of the well-known type IV transporter system in *A. tumefaciens* (Christie, 1997). As shown in Fig. 9, the wild-type P1 and the CagA mutant inhibited their own uptake, whereas the *virB7* and *virB11* mutants were

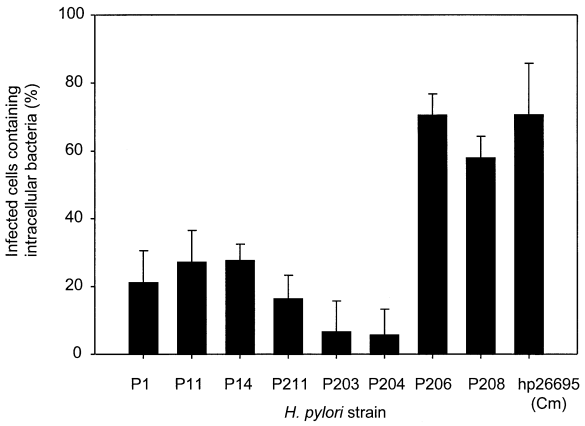

Fig. 9. Human monocytes infected with isogenic *H. pylori* mutants. Human monocytes were infected with different strains of *H. pylori* for 1.5 h at 37°C. After washing, samples were immunostained fluorescently for confocal laser scanning microscopic analysis. The percentage of infected cells containing at least one intracellular bacterium was determined. Results are the mean of three independent experiments. P1, wild-type strain; P11, urease-deficient mutant derived from P1; P14, VacA-deficient mutant derived from P12; P203 and P204, CagI and CagF mutants, respectively, derived from G27; P211, CagA mutants derived from P1; P206 and P208, VirB7 and VirB11 mutants, respectively, derived from P1; hpCm, *H. pylori* hp26695 treated with 37.5 μg ml^{-1} Cm for 2 h at 37°C before infection.

internalized in significantly greater numbers (70% and 60% of infected cells contained intracellular bacteria respectively). The fact that the level of internalization of these mutants was similar to that of chloramphenicol-treated *H. pylori* (Fig. 9) suggests that the activity of a type IV transporter is essential in preventing phagocytosis. Again, these effects were not exclusive to monocytes, as the results obtained when PMNs were infected by each of the strains described in this section (data not shown) did not differ significantly from that obtained with monocytes (Fig. 9).

To confirm our data further, we used TEM to monitor the phagocytosis of several strains. P1, mutants of the *virB7*, *virB11 and cagA* genes as well as P12 and its congenic mutant lacking the PAI of *H. pylori* were incubated with monocytes for 1 h at 37°C. Ultrathin sections of cells showing associated bacteria (Fig. 10B and C) were counted, and the number of counted sections showing at least one intracellular bacterium was determined (Fig. 10A). Consistent with the results obtained by laser confocal microscopy (Figs 2 and 9), the wild-type strains P1 and P12 as well as the *cagA* mutant exhibited an impaired uptake by monocytes revealing at least one intracellular bacterium in 22–35% of the bacteria-associated sections (Fig. 10). The

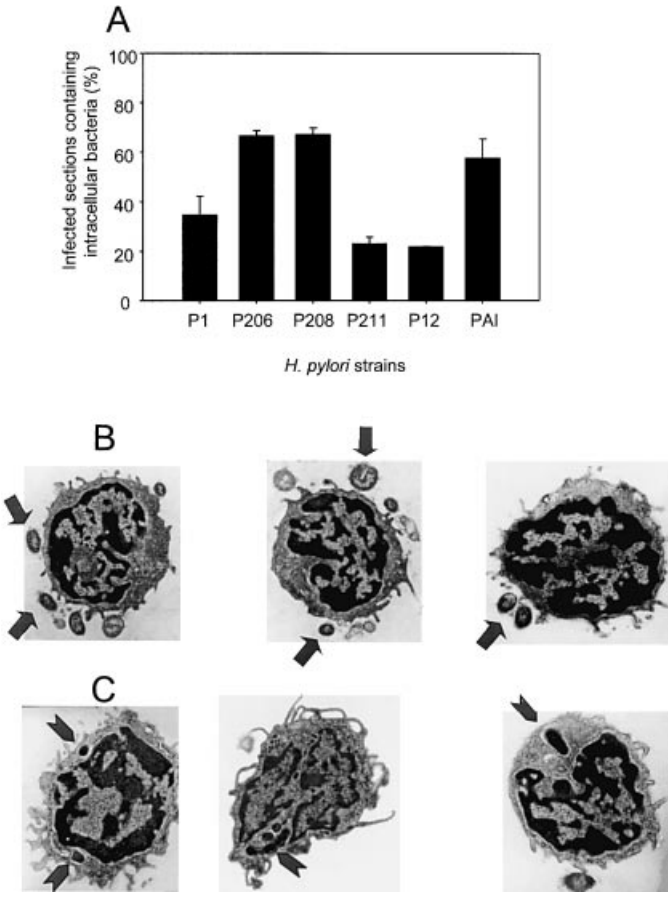

Fig. 10. Human monocytes infected with congenic *H. pylori* mutants. Human monocytes were infected with different strains of *H. pylori* for 1 h at 37°C. After washing, samples were prepared for TEM analysis.
A. The percentage of infected cell sections revealing at least one intracellular bacterium was determined. Results are the mean of two independent experiments. P1, wild-type strain; P206, P208 and P211 are *virB7*, *virB11 and cagA* mutants, respectively, derived from P1; P12, wild-type strain; PAI, PAI deletion mutant derived from P12.
B. TEM picture of monocytes infected with P12.
C. TEM picture of monocytes infected with the PAI mutant.
Arrows show extracellular P12 bacteria and arrow heads show intracellular PAI bacteria.

uptake of the *virB7* and *virB11* mutants was significantly increased, i.e. 67% of sections showing intracellular bacteria. Furthermore, the mutant lacking the PAI of *H. pylori* was also unable to inhibit its uptake by monocytes, i.e. 58% of sections contained intracellular bacteria. These data corroborate the role of distinct type IV secretion components rather than that of the intact PAI in the antiphagocytic mechanism of *H. pylori*.

Discussion

Professional phagocytes, including PMNs and mononuclear cells (monocytes and macrophages), constitute a first line of host defence against confrontation with bacterial pathogens. This interaction typically results in bacterial engulfment and destruction, and may cause the phagocytic release of cytokines in order to attract and stimulate other immune cells (Roitt *et al*., 1993; Rabinovitch, 1995). The ability of *H. pylori* actively to inhibit its phagocytosis by professional phagocytes thus provides a critical clue as to the means by which this organism may colonize and persist to cause chronic infection and, eventually, gastrointestinal disease. In the present study**,** we observed that *H. pylori* broadly inhibits the phagocytic functions of professional phagocytes by a mechanism involving a type IV secretion machinery.

First, the *H. pylori* inhibition of phagocytosis appears to be an active process, as bacteria killed by treatment with gentamicin are engulfed (data not shown). *De novo* protein synthesis by the bacterium is also required, as the inhibition of its protein synthesis by chloramphenicol treatment results in bacterial uptake. In contrast to what has previously been reported with gastric cells (Su *et al*., 1998), *H. pylori* adherence to phagocytic cells was itself independent of new protein synthesis. This implies that a different adherence mechanism functions to mediate phagocytic and epithelial cell binding. However, this question must be explored further.

It has been shown previously that haemagglutinins may mediate *H. pylori* adhesion to PMNs, with the surface haemagglutinin complex possibly disturbing their ingestion (Chmiela *et al*., 1994). Our preliminary results do, however, suggest that the antiphagocytic function described here is independent of the adhesin that *H. pylori* uses for binding. For example, we have not seen a difference in the proportion of bacteria that get phagocytosed when using strains whose binding can be inhibited by heparin versus those that appear instead to adhere via sialic acid-containing receptors (our unpublished observations). Our current findings also demonstrate that the blood group type of donors had little, if any, effect on binding or phagocytosis by any of the strains used, despite the fact that the Lewis b antigen that is reported to be the receptor on epithelial cells for *H. pylori*'s BabA adhesin (Boren *et al*., 1993; Ilver *et al*., 1998) is obscured by the blood group A and B antigens (Niv *et al*., 1996). Several groups have reported previously that opsonization of *H. pylori* by antibodies and/or complement may be necessary for ingestion and killing by phagocytic cells (Bernatowska *et al*., 1989; Andersen and Gaarsle, 1992; Andersen *et al*., 1992; McKinlay *et al*., 1993; Pruul *et al*., 1987). We are currently investigating the influence of immune and non-immune serum on the antiphagocytic function of *H. pylori*.

In epithelial cells, it has already been shown that there are at least three distinct cellular responses to *H. pylori* attachment. First, there is a cytoskeleton response to binding of *H. pylori* characterized by a rearrangement of actin, α-actinin and talin upon *H. pylori* binding to AGS cells *in vitro* (Segal, 1997). Secondly, type I strains (but not type II strains) mediate tyrosine phosphorylation of a 145 kDa host cell protein as well as other minor host cell proteins adjacent to the site of bacterial adherence. This may contribute to the observed formation of pedestals beneath the site of *H. pylori* attachment on epithelial cells (Segal *et al*., 1997) and, probably, phagocytic cells (data not shown), although no causal relationship has yet been determined. A similar effacement of microvilli and cup/pedestal formation has been shown to be associated with the phosphorylation of bacterial and host proteins after infection by bacteria such as *Yersinia* and enteropathogenic *E. coli* (EPEC) (Kenny *et al*., 1997; Lee, 1997; Goosney *et al*., 1999). Finally, type I strains induce interleukin (IL)-8 synthesis by a mechanism that is independent of tyrosine phosphorylation, thus implying that at least two separate signal transduction pathways are induced in response to *H. pylori* attachment (Segal *et al*., 1997). Whether any or all of these responses also occur after *H. pylori* binding to professional phagocytes remains to be determined. It would also be interesting to ascertain whether the bacteria actively inhibit their own entry into epithelial cells. *H. pylori* bind to, but are not engulfed by, T84 cells, a differentiated and polarized human cell line, morphologically resembling human gastric surface cells and able to assemble a brush border (Corthesy-Theulaz *et al*., 1996). Whether this reflects a passive process resulting from bacterial binding to a receptor that is not engulfed or instead requires the active inhibition of phagocytosis by *H. pylori* remains to be determined.

We found that the MOI significantly influences the efficiency of *H. pylori* engulfment by phagocytes. The very low MOI used by Andersen *et al*. (1993) could explain the presence of some bacteria inside the PMNs. However, the fact that they only presented qualitative results makes it difficult to compare their data with our current results. This phenomenon has also been observed for the antiphagocytic function of EPEC, with the percentage of

intracellular bacteria decreasing from 50% to 25% as the infectious dose increases (Goosney *et al*., 1999). Control experiments with *Y. enterocolitica*, a known paradigm of antiphagocytosis (Lian *et al*., 1987), revealed a remarkably similar behaviour to that of *H. pylori*. The observed effect may have important implications for the infection process, as a low-dose infection and/or disseminated organisms could presumably be cleared while bacteria present within a dense focus of infection would be protected. The dependence on bacterial load suggests that the bacterial mediator of this antiphagocytic function must have a dose-dependent inhibitory effect on the host cellular signalling events that would normally function in bacterial uptake. This implies that the inhibition does not result from the simple binding of *H. pylori* to a receptor that is not internalized as, in that case, the effects would presumably not be dose dependent. Such a model is consistent with the fact that *H. pylori* binding also impairs the phagocyte's capacity to ingest other bacteria or particles. Indeed, we found that the normally efficient engulfment of latex beads and the *N. gonorrhoeae* invasion mediated by either its heparan sulphate proteoglycan or CD66 receptors are all prevented by *H. pylori* infection. This antiphagocytic activity increased with the time of exposure to *H. pylori* before the addition of either latex beads or gonococci. These data imply that *H. pylori* affects a signalling pathway that is generally important for phagocytosis, as at least three independent receptor-mediated phagocytosis processes are inhibited.

We found that mutants in CagA, CagI, CagF and VacA were not affected in their rate of uptake by monocytes or PMNs, suggesting that at least these proteins have no function in the antiphagocytic process. Therefore, the fact that CagA is translocated into epithelial cells by the type IV secretion system of *H. pylori* (Segal *et al*., 1999; Backert *et al*., 2000; Odenbreit *et al*., 2000; Stein *et al*., 2000) seems to play no role in the antiphagocytic process in professional phagocytes. A previous study proposed that the *H. pylori* urease may affect phagocytic uptake of this bacterium (Makristathis *et al*., 1998). The urease-negative mutant used in this study (P11) was, however, indistinguishable from the wild-type parental strain P1 with respect to its uptake by either phagocyte tested, thus indicating that urease did not influence bacterial phagocytosis. Unfortunately, the previous study did not provide quantitative data and was based on comparisons of bound bacteria after infections performed in the presence and absence of cytochalasin D, which disrupts the actin cytoskeleton (Makristathis *et al*., 1998). In similar experiments, we have found that cytochalasin D reduces *H. pylori* binding to monocytes and PMNs, suggesting that the decrease in total bacteria could result from a diminution of adherence and not from differences in their uptake.

Although *H. pylori*'s ability to inhibit its phagocytosis and that of other particles (i.e. latex beads or *N. gonorrhoeae*) described in this study is superficially similar to that reported previously for *Yersinia* and EPEC (Lian *et al*., 1987; Fallman *et al*., 1995; Goosney *et al*., 1999), the mechanism is probably different. *Yersinia* and EPEC express the host cell contact-inducible type III secretion apparatus that is able to inject bacterial proteins directly into the host cell cytoplasm (Lian *et al*., 1987; Visser *et al*., 1995; Ruckdeschel *et al*., 1996; Lee, 1997). In the case of *Yersinia*, this includes YopH, a highly active phosphatase, which blocks bacterial phagocytosis by inactivating host cell signalling processes that are controlled by tyrosine phosphorylation (Lian *et al*., 1987; Rosqvist *et al*., 1988). The availability of the *H. pylori* genome sequence (Tomb *et al*., 1997) has allowed the search for homologues of the type III secretion apparatus, yet no such proteins are apparent. Interestingly, sequence homologies were found between several proteins encoded in the CAG pathogenicity island and those that function in the 'type IV' secretion by *A. tumefaciens* (Christie, 1997; Backert *et al*., 1998). As homologues of this secretory system are involved in the translocation of bacterial proteins and DNA (Christie, 1997), a model based upon the antiphagocytic factor(s) being translocated into the host phagocytes by type IV secretion was tempting. The presence of such a transport system is also consistent with the fact that some of these homologues are found to stimulate the induction of IL-8 secretion in gastric epithelial cells, an activity that also probably contributes to chronic inflammation (Segal *et al*., 1994). We therefore assessed the role of essential core components of this potential type IV secretion system. Very interestingly, we could show that knock-out mutants for *virB7* and *virB11* genes were unable to inhibit phagocytosis, supporting our hypothesis that a functional type IV secretion apparatus in *H. pylori* is involved in the antiphagocytic phenotype of this bacterium. In the well-studied type IV secretion system model of *A. tumefaciens*, VirB7 is a lipoprotein, which, after the formation of a heterodimer with VirB9, is postulated to play a role in stabilizing other VirB proteins by stabilizing intermolecular disulphide bridges, leading to the assembly of a functional transporter. Similarly, VirB11, which exhibits ATPase activity, is essential in the assembly of the transporter (Christie, 1997). Most of the VirB proteins are highly similar to the Ptl proteins of *B. pertussis*, proteins that are responsible for the export of proteins across the bacterial envelope (Christie, 1997).

Taken together, this study provided some crucial insights into the mechanism of *H. pylori* pathogenesis. Previous work on this important pathogen has focused primarily on the virulence determinants that allow *H. pylori* to colonize and survive in its niche within the gastric mucosa. Yet the reason for this bacteria's ability to persist

despite the constant inflammation that occurs has remained elusive. Here, we demonstrate that *H. pylori* possesses an active antiphagocytic activity involving *de novo* protein synthesis by the bacterium and a type IV secretion system. This antiphagocytic process appears to act at the level of a general pathway that is involved in phagocytosis via several distinct cellular receptors, as preinfection of PMNs or monocytes with *H. pylori* blocks the subsequent engulfment of either latex beads or *N. gonorrhoeae* expressing its phagocyte-binding Opa adhesins. The ability to evade engulfment by professional phagocytes presumably contributes to the pathogen's ability to establish a chronic infection despite the strong inflammatory reaction that occurs within the gastric mucosa. We identified part of the mechanism involved in this inhibitory process. However, the effector, which could presumably be targeted into the eukaryotic cells through this type IV secretion machinery, remains to be characterized. As it is probably the persistence of *H. pylori* infections that ultimately leads to its contribution to the onset of gastric damage and carcinoma, the identification of these factors and further characterization of this process is obviously of great interest.

Experimental procedures

Bacterial strains and growth conditions

H. pylori 26695 strain hp26695, kindly provided by D. Berg, was used throughout this study. This type I strain was originally isolated in the UK from a patient with gastritis and expresses the vacuolating cytotoxin and elicits immune and inflammatory responses. Its genome sequence is available at http://www.tigr.org with the accession number AE000511(Tomb *et al*., 1997), and it contains both a single contiguous PAI region and the more toxigenic S1a/m1 type cytotoxin (Pan *et al*., 1998). *H. pylori* strain P76 is a spontaneous streptomycin-resistant strain derived from P49, which is a mouse-adapted strain originally provided by H. Kleanthous of OraVax, Cambridge, MA, USA (Gomez-Duarte *et al*., 1998). P12 is a clinical isolate obtained from a patient with a duodenal ulcer at the University of Hamburg, Germany (Schmitt *et al*., 1994). G27 is also a clinical isolate obtained from the Hospitals of Grosseto and Siena, Italy (Censini *et al*., 1996). *H. pylori* strain P1 is a clinical isolate from a patient with non-ulcer dyspepsia and was obtained from the University of Amsterdam (Corthesy-Theulaz *et al*., 1996). Strain P1-*gfp*, provided by R. Haas (Munich, Germany), is a P1 recombinant strain expressing the green fluorescent protein (GFP). The urease-negative strain P11, a derivative of P1generated by transposon shuttle mutagenesis using the TnMax5 mini-transposon, has been described previously (Kahrs *et al*., 1995), as have the isogenic mutants of P12 and G27 that were used to assess the roles of VacA (P14, *vacA*$^-$), CagI (P203, c*agI*$^-$) and CagF (P204, c*agF*$^-$) in the antiphagocytic mechanism (Censini *et al*., 1996). These strains were kindly provided by R. Haas (P14) and A. Covacci (P203, P204; Siena, Italy). We also constructed isogenic P1 knock-out mutants by the insertion of a chloramphenicol resistance gene cassette (*cat*, 1 kb *Bam*HI–*Bgl*II fragment of plasmid pTnMax1) in cloned *virB7* (P206, *virB7*), *virB11* (P208, *virB11*) *and cagA* (P211, *cagA*) genes according to the protocols described by Haas *et al*. (1993). Briefly, the genes of interest were amplified by polymerase chain reaction (PCR) using primers based on the sequences of the genes of *H. pylori* strains 26695 and J99 in the AstraZeneca *H. pylori* genome database (http://www.astra-boston.com/hpylori/). The PCR products were cloned in the pGEM-T vector (Promega). After mutagenesis by insertion of the *cat* resistance cassette in the *vir* genes, 3–5 μg of supercoiled plasmid DNA was added to approximately 1×10^{-8} bacteria ml^{-1}. After incubation for 6 h, chloramphenicol-resistant transformants were obtained by incubating them for 4–5 days on agar plates containing 4–6 mg l^{-1} chloramphenicol according to a standard protocol (Haas *et al*., 1993). Correct integration of the *cat* cassette into the chromosome by homologous recombination was confirmed by PCR. A mutant from P12 lacking the PAI (PAI) was also tested, which was obtained from R. Haas and has been described elsewhere (Wessler *et al*., 2000).

All *H. pylori* strains were grown on horse blood agar plates supplemented with vancomycin (10 μg l^{-1}), nystatin (1 μg l^{-1}) and trimethoprim (5 μg l^{-1}) (S plates). S plates were incubated at 37°C in an anaerobic jar containing a microaerobic gas mix (5% O_2, 10% CO_2, 85% N_2) for 3 days. Plates were typically subcultured once after thawing from frozen stocks before use in experiments 2 days later. For liquid culture, single colonies were used instead to inoculate brain–heart infusion (BHI) medium (Difco Laboratories) supplemented with 10% FCS and then incubated in a sealed jar containing microaerobic gas mix with shaking for 1–2 days before use.

N. gonorrhoeae strains N303 and N309 are pilus-negative mutants that constitutively express the heparan sulphate receptor-specific Opa_{50} and CD66 receptor-specific Opa_{52} protein adhesins respectively (Gray-Owen *et al*., 1997). Daily subculture of both *N. gonorrhoeae* strains to fresh GC agar was carried out using a binocular microscope to select only those variants that expressed the single desired Opa protein. *E. coli* strain HB101 is a standard recombinant strain (Boyer and Roulland-Dussoix, 1969) and was grown overnight on LB agar plates before use in infection experiments. The wild-type *Y. enterocolitica* serotype 0:9 strain used in these studies was kindly provided by J. Heesemann (Munich, Germany), and its antiphagocytic and other properties have been described previously (Heesemann *et al*., 1986). *Y. enterocolitica* was grown on LB agar plates overnight, either at 37°C to induce full expression of the virulence plasmid-encoded antiphagocytic genes or at 28°C to allow bacterial phagocytosis (Lee, 1997).

PMN and monocyte isolation

Peripheral venous blood from healthy donors was collected into citrate-containing tubes. Monocytes and PMNs were isolated by standard Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech). The mononuclear and polymorphonuclear cell fractions were collected separately and washed twice with phosphate-buffered saline

(PBS). Monocytes were separated from contaminating lymphocytes by incubation with glass coverslips at 37°C for 1 h. The coverslips were then washed to remove the non-adherent lymphocytes, and the attached monocytes that remained were covered with RPMI-1640 medium before bacterial infection. Erythrocytes contaminating the PMN fraction were removed by hypotonic lysis, and PMNs were then resuspended in PBS with 0.1% BSA (PBSA) before being allowed to adhere to glass coverslips for 30 min at 37°C in 5% CO_2 (Tomita *et al.*, 1997). PBSA was then replaced by RPMI-1640 medium before infection. The density and viability of phagocytic suspensions were assessed by counting cells in a haemocytometer and by trypan blue staining respectively. The viability of both PMNs and monocytes was $>95\%$ before infection, and no significant change in membrane permeability was observed during the 1.5 h infection period with any of the bacterial strains used.

Bacterial infection experiments

Bacteria were resuspended from agar plates using cotton swabs, diluted in PBSA to obtain a final concentration of 2.5×10^8 bacteria ml^{-1} and then added to cells on glass coverslips to obtain a bacteria to cell ratio of 100:1, unless specified otherwise. Quantification of intracellular and extracellular bacteria associated per cell was performed by immunofluorescence staining and confocal laser scanning microscopy, as outlined below. Where indicated, the bacterial suspensions were pretreated before the infection by incubation with either chloramphenicol (Cm) (0–37.5 $\mu g\ ml^{-1}$) or gentamicin (0–200 $\mu g\ ml^{-1}$) for 2 h at 37°C, or as otherwise specified. In these cases, the bacteria were then washed three times in PBSA before use in infection experiments. In co-infection experiments, cells were infected with *H. pylori* or *Y. enterocolitica* for various times (5, 10, 30 or 60 min) before the addition of either *N. gonorrhoeae* or fluorescein isothiocyanate (FITC)-labelled latex beads (Fluoresbrite Carboxy YG 2.0 micron Microspheres; Polysciences), and the infection was then continued for 1 h before washing and fixing the cells for immunofluorescence staining. Alternatively, cells were infected first with *N. gonorrhoeae* for various times followed by the infection with *H. pylori*. As a control for the phagocytosis assays, infections were also performed in the presence of 5 $\mu g\ ml^{-1}$ cytochalasin D in order to block bacterial uptake, thus excluding intracellular bacteria.

Immunofluorescence staining and confocal laser scanning microscopic analysis

The preparation of samples for confocal laser scanning analysis was based upon that described previously by Hauck and Meyer (1997). After washing with PBS, the infected cells were fixed onto glass coverslips overnight at 4°C in PBS containing 3.7% paraformaldehyde. After three washes with PBS, fixed cells were then incubated in PBS containing 10% fetal calf serum (PBS–FCS) for 5 min in order to block non-specific binding sites. To stain intracellular and extracellular bacteria differentially, the coverslips were incubated with rabbit anti-*N. gonorrhoeae* MS11 antiserum diluted 1:100 in PBS–FCS, rabbit anti-*H. pylori* antiserum (NatuTec) diluted 1:20 in PBS–FCS or rabbit anti-*E. coli* antiserum diluted 1:100 in PBS–FCS for 1 h at room temperature. Samples were then washed three times using PBS and incubated in the dark with FITC-conjugated goat anti-rabbit antibody (Sigma ImmunoChemicals) diluted 1:100 in PBS–FCS for 45 min. After incubation, samples were washed and incubated with PBS containing 0.1% Triton X-100 for 15 min to permeabilize the cells, and then washed three times in PBS. The samples were blocked again and incubated with rabbit anti-*N. gonorrhoeae* MS11 antiserum, rabbit anti-*H. pylori* antiserum or rabbit anti-*E. coli* antiserum, as outlined above, containing a 1:40 dilution of mouse monoclonal anti-H-lamp-1 (clone H4A3 obtained from DSHB, University of Iowa) for 1 h. After three washes, samples were incubated in the dark with a mixture containing 1:100 dilutions of both Cy5-conjugated goat anti-rabbit and Texas red (TR)-conjugated goat anti-mouse antibodies (Sigma ImmunoChemicals) for 45 min. When GFP-expressing bacteria were used, extracellular bacteria were stained using a cy5-conjugated antibody instead of a FITC-conjugated antibody. For experiments using *Y. enterocolitica*, bacteria were stained using TAMRA (Molecular Probes) for 30 min at room temperature and then washed three times in PBSA before infection. When this staining protocol was performed on *H. pylori* and *N. gonorrhoeae*, no significant difference in the adherence or uptake was evident compared with that seen when using the other staining methods tested. For FITC-conjugated latex beads, co-localization with H-lamp-containing phagosomes was used to differentiate between extracellular and intracellular beads. After a final washing step, coverslips were mounted in glycerol medium (Sigma ImmunoChemicals), sealed with nail polish and viewed with a Leica TCS 4D confocal laser scanning microscope (Leica Lasertechnik) equipped with an argon–krypton mixed gas laser. Images were taken serially from the top to the bottom of each cell, using excitation and emission filters as appropriate for the fluorescent dyes employed.

To quantify bacterial association and internalization, 50–100 phagocytic cells in the fixed samples were selected randomly, and the average number of extracellular-associated (stained green and blue) and intracellular-associated (blue only) bacteria were determined by screening from the top to the bottom of each cell (i.e. three-dimensional analysis). The co-localization of intracellular bacteria with the phagosomes stained in red (H-lamp1) helped us to localize phagocytic cells and to confirm extracellular versus intracellular localization of the bacteria. Among the infected phagocytes (i.e. those associated with at least one cell-associated bacterium, at least 50 infected cells counted/sample), the percentage of cells containing at least one intracellular bacterium was also calculated. As specified in the figure legends, several data sets were collected for each experiment.

Sample preparation for transmission and scanning electron microscopy

For TEM, formaldehyde-fixed infected cells were post-fixed for 1 h in 1% osmium tetroxide in PBS (OsO_4; Science Services) and then incubated for 1 h in 1% uranyl acetate. Samples were dehydrated through a graded series of ethanol

and embedded in Durcupan acm (Fluka). Samples were cut with a Diatome diamond knife using an ultramicrotome (LKB), and ultrathin sections were stained with 2.5% uranyl acetate and lead citrate. Electron microscopy was carried out using a Zeiss EM 109 electron microscope at 80 kV.

For SEM, PFA-fixed infected cells were post-fixed in OsO_4, dehydrated in ethanol and critical point dried from CO_2. The samples were sputter coated with 1 nm chromium and then examined at 10 kV accelerating voltage in a Hitachi S-800 field emission scanning microscope equipped with a detector for back-scattered electrons (BSEs) of the YAG type.

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